

Testing multiple hypotheses for the maintenance of male homosexual copulatory behaviour in flour beetles

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Abstract

Diverse animal groups exhibit homosexual interactions, yet the evolutionary maintenance of such behaviours remains enigmatic as they do not directly increase reproductive success by generating progeny. Here, we use *Tribolium castaneum* flour beetles, which exhibit frequent male homosexual copulations, to empirically test several hypotheses for the maintenance of such behaviours: (1) establishing social dominance; (2) practice for future heterosexual encounters; and (3) indirect sperm translocation. We found no evidence that *Tribolium* males use homosexual copulations either to establish dominance or to practice behaviours that increase their subsequent heterosexual reproductive performance. Our results provide limited support for the hypothesis of indirect sperm translocation: when males from two genetic strains mated with females immediately following a homosexual copulation, females produced progeny sired not only by the directly mating male, but also by that male's homosexual partner. However, this phenomenon was detected in only 7% of homosexual pairs, and in each case such indirectly sired progeny accounted for < 0.5% of females' total progeny. Direct observations indicated that mounting males often released spermatophores during homosexual copulations. These observations suggest that homosexual copulations may be a behavioural mechanism that allows males to expel older, potentially low-quality sperm. Additional work is needed to test this new hypothesis, and to determine whether sperm release during homosexual copulations occurs in other groups.

Introduction

Homosexual behavioural interactions have been documented in wild populations of numerous animals, including many birds, reptiles, mammals, insects and spiders (Bagemihl, 1999; Sommer & Vasey, 2006). For example, male homosexual mountings occur in over 100 insect species (reviewed in Thornhill & Alcock, 1983; Iguchi, 1996; Bagemihl, 1999; Leal *et al.*, 1998; Serrano *et al.*, 2000; Switzer *et al.*, 2004; Reinhardt *et al.*, 2007).

Proximate genetic, neural and hormonal causes of homosexual behaviours have been identified for some model organisms (e.g. Adkins-Regan, 1998; Svetec *et al.*, 2005; Yamamoto, 2007). However, only a few empirical studies have examined possible explanations for the evolutionary maintenance of homosexual behaviours (but see Harari *et al.*, 2000; Sommer & Vasey, 2006).

In spite of their common occurrence, the potential costs and benefits of male homosexual behaviours remain poorly understood. Homosexual behaviours are generally assumed to be costly because they reduce opportunities for heterosexual matings and associated progeny production. Several hypotheses have been proposed for the apparent paradox of homosexual behaviours (Thornhill & Alcock, 1983; Bagemihl, 1999; Harari *et al.*, 2000; Sommer & Vasey, 2006). Male homosexual behaviours might provide a net fitness benefit if: (1) such behaviours help establish social

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dominance, leading to higher reproductive success; (2) males gain practice that enhances their subsequent heterosexual reproductive performance; or (3) homosexual copulations allow males to indirectly transfer their own sperm to females through a male intermediary. Alternatively, even in the absence of a net fitness benefit homosexual behaviours might be maintained if: (4) males either cannot distinguish between males and females, or do not distinguish because the cost of lost mating opportunities is low; or (5) such behaviours are genetically correlated with a positively selected trait, such as high sexual activity.

Thus, although anecdotal reports of homosexual behaviours are common and various hypotheses have been proposed, few studies have systematically tested alternative hypotheses for their evolutionary maintenance. Here, we report such studies conducted on *Tribolium* flour beetles, which provide an excellent model system for studying the potential fitness consequences of homosexual behaviours. *Tribolium* flour beetles are cosmopolitan stored grain pests that have long been popular for studies of population ecology and sexual selection, and their natural environment can easily be recreated in the laboratory (reviewed by Park, 1948; Sokoloff, 1974; King & Dawson, 1972; Fedina & Lewis, 2008; Pai & Bernasconi, 2008). Male homosexual copulations (see Fig. 1) are frequent in *Tribolium* spp., and this behaviour occurs in both same-sex and mixed-

sex groups (Wool, 1967; Graur & Wool, 1982, Serrano *et al.*, 1991; Castro *et al.*, 1994; Serrano *et al.*, 2000). These male–male copulations closely resemble heterosexual copulations (detailed descriptions of the latter are provided by Sokoloff, 1974; Bloch Qazi, 2003; Fedina & Lewis, 2008). *Tribolium* male homosexual copulations involve a male dorsally mounting another male, extending its genitalia, and engaging in leg-rubbing behaviour.

Some important aspects of male homosexual behaviour in *Tribolium castaneum* have been characterized in previous studies. Two studies demonstrated a genetic component to male homosexual behaviour: Serrano *et al.* (1991) used a diallele cross among inbred lines, whereas Castro *et al.* (1994) found a response to artificial selection for increased homosexual behaviour. The latter study also tested whether homosexual behaviour is maintained through a positive genetic correlation with overall mating rate, but found no evidence that homosexual copulation rates correlated with overall sexual activity. An earlier study by Spratt (1980) found a marked reduction in adult longevity for males kept in all-male groups (under these conditions males frequently engage in homosexual copulations, Wool, 1967; Spratt, 1980; Serrano *et al.*, 2000): in all-male groups, male longevity was significantly shortened (median of 15 weeks) compared with both males kept alone (median of 50 weeks) or males kept in mixed-sex groups (median of 40 weeks). She also noted that males kept in same-sex groups often developed a hardened, white accretion at the posterior end of their abdomen, and speculated that this accretion might be responsible for reduced longevity by blocking the external opening of the digestive tract. She further speculated that the accretion represented flour mixed with seminal fluid deposited during homosexual copulations, but presented no evidence to support this idea.

Tribolium are sexually dimorphic, as males possess conspicuous setiferous glands located on the femurs of their prothoracic legs (Faustini *et al.*, 1981; Faustini & Halstead, 1982). Mate attraction appears to rely primarily on volatile chemical signals produced by these and other glands (reviewed by Fedina & Lewis, 2008). *Tribolium* adults mate frequently throughout a lifespan of 1–2 years (Sokoloff, 1974). During heterosexual copulations, *T. castaneum* males transfer sperm to females inside a spermatophore produced by two pairs of accessory glands (Bloch Qazi *et al.*, 1996; Fedina, 2007). Previous researchers have suggested that *Tribolium* homosexual copulations represent a failure of sex recognition (Castro *et al.*, 1994), as suggested for homosexual copulations in other insects (Harari *et al.*, 2000). However, this seems unlikely based on marked chemical and morphological differences between the sexes in *Tribolium*. In addition, *T. castaneum* males have been shown to be capable of distinguishing between virgin and previously mated females, as they preferentially contact and mate with virgin females (Graur & Wool, 1982; Lewis & Iannini, 1995). *Tribolium castaneum* males also discriminate



Fig. 1 Homosexual copulation between *Tribolium castaneum* males: mounting male (above) exhibits typical heterosexual copulatory behaviour, extending its genitalia downward and forward to contact the posterior end of the mounted male (below), and rubbing its mid- and hind-legs along the sides of the mounted beetle. Scale bar = 1 mm.

against mating females with whom they have previously mated in favour of females that had previously mated with other males (Arnaud & Haubruge, 1999). Therefore, it seems unlikely that lack of either production or perception components of sexual recognition accounts for homosexual behaviour in these beetles.

In this study, we used *Tribolium castaneum* to experimentally test three adaptive hypotheses for the maintenance of male homosexual copulatory behaviour: social dominance, practice and indirect sperm translocation. In addition, we observed male pairs during homosexual copulations to test the hypothesis that the accretions described by Spratt (1980) are formed by male seminal products, and to determine whether such accretions are formed differentially on the mounting or mounted males. We further examined the effect of these accretions, previously linked to reduced longevity, on males' reproductive performance. Finally, based on our observations showing that mounting males do expel seminal products during homosexual copulations, we hypothesized that homosexual copulations might be a mechanism for males to discard old sperm. We tested one prediction of this novel explanation for homosexual behaviour, which is that previously isolated males should be more likely to initiate homosexual copulations compared with males kept in same-sex groups.

Methods

Beetle maintenance and general methods

All beetles were sexed as pupae and housed in a dark incubator at 29 °C and 70% humidity. Adults were provided with excess flour, and kept either individually or in groups depending on the experimental design (we used a standardized density of one beetle per 2 g flour). Mating observations were conducted at 29–32 °C in plastic arenas with scratched bottoms or filter paper to provide traction. Following previous studies (Lewis & Iannini, 1995), we defined heterosexual copulations as a male dorsally mounting a female in the correct orientation, extending its aedeagus and inserting it into the female's genital opening; during copulation males rapidly rub their legs against the sides of the female's body interspersed with periods of inactivity (Bloch Qazi, 2003). Behaviours exhibited during male homosexual copulations were remarkably similar (see online Supporting information for a video illustrating these behaviours); these are defined throughout this paper as a male dorsally mounting another male in the correct orientation, extending its aedeagus downward and forward to contact the posterior abdomen of the other male, and rapidly rubbing its legs along the sides of the mounted male's body. The duration of such male homosexual copulations ranged from 0.2 to 11 min.

Male heterosexual copulation rate was measured by providing males with a succession of individual virgin

females. Not all copulations result in successful insemination (Bloch Qazi *et al.*, 1996); so, following Lewis (2004), we measured male insemination success (proportion of copulations leading to insemination) by isolating each mated female for 2 weeks and scoring insemination based on the presence or absence of larvae that would develop only if insemination had occurred.

Experimental beetles came from two *T. castaneum* genetic strains differing in adult body colour: a wild-type strain (+/+) homozygous for a chestnut body allele, and Chicago black (b/b) which is homozygous for an autosomal, semidominant black body colour allele. Following established methods (Schlager, 1960; Lewis & Austad, 1990; Lewis, 2004; Fedina & Lewis, 2006), we used the body colour phenotype of adult progeny to distinguish between progeny sired by either black or wild-type males.

Data were analysed using SPSS 16.0, (SPSS Inc., Chicago IL, USA) after verifying assumptions of statistical tests.

Hypothesis 1: social dominance

Although *Tribolium* beetles exhibit no overt intermale agonistic behaviours that might be associated with establishing social dominance (Sokoloff, 1974), we hypothesized that males might establish dominance by copulating with other males. If males use homosexual behaviour to establish dominance, we predicted that certain males would consistently mount other males, and that dominant males might show a size advantage over subordinate males. In addition, this hypothesis predicts that dominant mounting males would show higher reproductive success in their subsequent heterosexual encounters. Such reproductive benefits might occur either immediately by enhancing dominant males' success in a competitive mating situation, or through longer term effects of dominance status (perhaps hormonally mediated, e.g. Kravitz, 2000) on mating and insemination success.

Virgin males were marked individually with paint dots and randomly assigned to male–male pairs ($n = 26$ pairs). Pairs were observed continuously for 1 h, during which we recorded the number of times each male mounted or was mounted. In a subset of these pairs, one of the two males mounted more frequently; for these pairs we designated as 'mounting' those males that mounted at least twice often as the other male, whereas the remaining male was designated as the 'mounted' male. This subset of males was kept together in pairs (in 1 g of flour) for an additional 23 h, then isolated for 24 h before weighing males and comparing their heterosexual reproductive success. Each male was allowed to mate with five successive virgin females; if males did not mate with a given female within 5 min, she was replaced with another virgin female. Male body mass, number of copulations and insemination success were compared between pairs of mounting and mounted males using paired *t*-tests.

We conducted a separate experiment to test whether the mounting males from homosexual pairs were more successful than mounted males when they competed directly for access to females. Virgin males were randomly assigned to pairs ($n = 16$ pairs, each with one marked male) and observed until a homosexual copulation occurred. After the mounting male dismounted, a single female was placed in the arena and we recorded which male successfully mated. We used an exact binomial test to determine if homosexually mounting and mounted males were equally likely to mate when they competed for access to a single female.

Hypothesis 2: practice for subsequent heterosexual encounters

Homosexual copulations might provide behavioural practice that would increase a male's reproductive success in subsequent heterosexual encounters. To examine this hypothesis, we compared the mating rate and the insemination success of males that had been previously isolated vs. males that had previously been kept in groups (thus engaging in frequent homosexual copulations). Wild-type males were randomly assigned as pupae to be kept either isolated ($n = 20$ males) or in groups (two groups, nine males each). At 1–2 weeks post-eclosion, mating rate (number of matings 15 min^{-1}) was measured by offering each male a sequence of single virgin black females. After each mating, females were removed and replaced with a new female; also, if no mating occurred within 5 min, the female was replaced with a new female. Each mated female was kept individually to determine insemination success (proportion of copulations leading to insemination) as described above. To determine whether homosexual copulations might provide a practice advantage, we used separate variance t -tests to examine the prediction that males kept in groups would have higher mating rates and insemination success compared with isolated males.

We also examined whether isolated males differed from group males in their paternity share when mating with previously mated females. Again, wild-type males were randomly assigned as pupae to be kept either isolated or in a group for 1 week. Each focal male was allowed to copulate once with a black female that had been premated with a black male for 24 h and then isolated for 24 h (the presence of progeny from this intermating vial was used to confirm successful insemination by the first male). After mating with the focal (wild-type) male, each female was allowed to lay eggs for 3 days (oviposition period 1), then transferred to a new container for an additional 7 days (oviposition period 2). All progeny were reared to adults, and second-male paternity share was calculated as the proportion of progeny sired by the wild-type focal male. Only females with confirmed insemination by both males were included in the data analysis; this resulted in final sample sizes of 25 isolated males and 17

group males. Because these data were not normally distributed, we used nonparametric Mann–Whitney tests at each oviposition period to compare paternity share of group males with isolated males.

Hypothesis 3: indirect sperm translocation

Males might engage in homosexual behaviour to transfer their sperm indirectly to females through a male intermediary. Carayon (1974) described transfer of male sperm through traumatic insemination during homosexual copulations in the anthocorid bug, *Xylocoris maculipennis*: sperm from the mounting male apparently migrated into the seminal vesicles of the mounted male. However, it is unknown whether any sperm thus passed to this male intermediary is later transferred to females during subsequent copulations by the mounted male, or whether such indirect sperm transfer occurs during homosexual copulations in any other insects. A related but distinct phenomenon was studied by Haubruge *et al.* (1999), who reported evidence that mating *T. castaneum* males removed sperm from previously mated females, and that some of this sperm was subsequently transferred when they mated with a different female. These authors proposed that such translocation through a female intermediary could be facilitated by sperm becoming trapped under spines on the male aedeagus. Thus, it is possible that similar mechanisms could result in indirect sperm translocation by males engaging in homosexual copulations.

We conducted an experiment using males from different genetic strains to examine whether *T. castaneum* males engaging in homosexual copulations might indirectly inseminate females through a male intermediary (Fig. 2). Randomly selected, virgin wild-type males were paired with randomly selected, virgin black males and these male pairs were allowed to copulate once ($n = 86$ pairs; both strains were represented approximately equally as mounting males). Immediately after each male homosexual copulation, males were separated and then both the mounting and mounted males were isolated to copulate once with a virgin black female (total of 172 matings). These mated females were isolated to oviposit with weekly transfers to new containers for 2 weeks. To control for any progeny that might arise due to back/forward mutations between the black and wild-type alleles, we also mated single black or wild-type males with virgin black females ($n = 65$ matings), and collected eggs from each female as above. We scored body colour phenotypes (as b/b or b/+) of all adult progeny from matings of the mounting, mounted and control males (27 180 total progeny were scored). To detect progeny fertilized by any sperm translocated during the initial homosexual copulation, we used the presence of progeny showing unexpected body colour phenotypes based on the known genotype of the directly mating males.

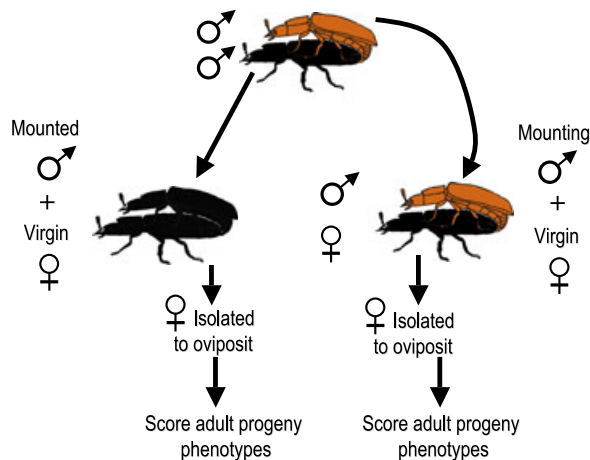


Fig. 2 Experimental design used to test the indirect sperm translocation hypothesis. After homosexual copulations between virgin males from two genetic strains ($n = 86$ pairs), mounting males (here shown as b/b for illustrative purposes only) and mounted (shown here as b/b) males were each allowed to copulate with a virgin b/b female. Females were isolated to oviposit for 2 weeks and adult progeny phenotypes were scored after 45 days. Indirect sperm translocation was detected as the presence of progeny indirectly sired by the mating male's homosexual partner.

Accretion formation and effects on reproductive performance

We observed male–male pairs to determine how the accretions previously shown to be associated with all-male groups (Spratt, 1980) are formed. Pairs of virgin, wild-type males ($n = 14$ pairs) were introduced into a short length of glass tubing 4 mm wide, and observed under $30\times$ magnification until homosexual copulations occurred or 1 h elapsed. For the 12 pairs that engaged in homosexual copulations, we noted whether and where the mounting male deposited any material.

Tribolium accretions begin to appear in all-male groups after several weeks, yet it is not known whether the accretion is formed on the mounting male, the mounted male or both. We hypothesized that mounting males might cause accretions to form on, and thus decrease longevity of, other males thereby incapacitating their reproductive rivals. To examine this hypothesis, we observed 16 male pairs until copulation occurred, after which both the mounting and mounted males ($n = 16$ of each) were placed individually in 2 g of flour. Males were checked for accretion formation after 15 days, and we used an exact binomial test to evaluate the null hypothesis that mounting and mounted males were equally likely to show accretions.

We also examined the effect of male accretions on heterosexual reproductive performance, specifically male mating behaviour and insemination success. To obtain males with and without accretions, we kept 30 virgin

wild-type males together in groups with flour for 3–5 months. Mating behaviour was measured over 10 min for males with accretions (minimum size 0.25 mm^2) and without accretions: each male was placed with five wild-type virgin females, and the number of male-initiated contacts and copulations was recorded. Each time a mating occurred, the mated female was removed and replaced with a new virgin female. Each male's insemination rate (number of inseminations 10 min^{-1}) as well as insemination success was measured as described above. During these mating observations, some males' accretions fell off; so, these males were assigned to a third treatment group. We used ANOVA to examine differences among these three male groups in their total number of contacts and copulations 10 min^{-1} , as well as in the number of copulations per contact (data were checked for homoscedasticity and normality). Because neither insemination rate nor insemination success met normality assumptions, we used nonparametric Kruskal–Wallis tests to compare these aspects of reproductive performance among male groups.

Because our observations indicated that mounting males do release seminal products during homosexual copulations, we hypothesized that homosexual copulations might be a mechanism that allows males to get rid of old, and potentially damaged, sperm (Reinhardt, 2007). One prediction of this novel hypothesis is that males kept isolated should be more likely to initiate homosexual copulations than males kept in groups, presumably because the former lacked any previous opportunities to discard sperm. To test this prediction, we used beetles that had been kept for 3–5 months either in same-sex groups of 20–30 males or isolated males (beetles were marked with small paint dots to distinguish these two treatments). We observed pairs ($n = 24$) interacting in 1.5-cm^2 arenas, and during 10 min observations we measured the number and duration of homosexual mountings by each male.

Results

Hypothesis 1: social dominance

In 32.1% of the 26 male pairs, males frequently switched roles between mounting and being mounted during 1 h observations, such that no clear designation could be made. In the remaining pairs, one male mounted more frequently; however, there was no significant difference in body mass between these mounting males (mean \pm SE $2.1 \pm 0.05 \text{ mg}$) and the males they mounted ($2.2 \pm 0.08 \text{ mg}$; paired $t = -0.710$, 17 d.f., $P = 0.487$). Moreover, when tested following 24 h in isolation, mounting and mounted males did not differ in either the number of heterosexual copulations they achieved (Fig. 3a; paired $t = 1.455$, 18 d.f., $P = 0.163$) or their insemination success (Fig. 3b; paired $t = 1.396$, 18 d.f., $P = 0.180$). Similarly, no reproductive advantage was evident when

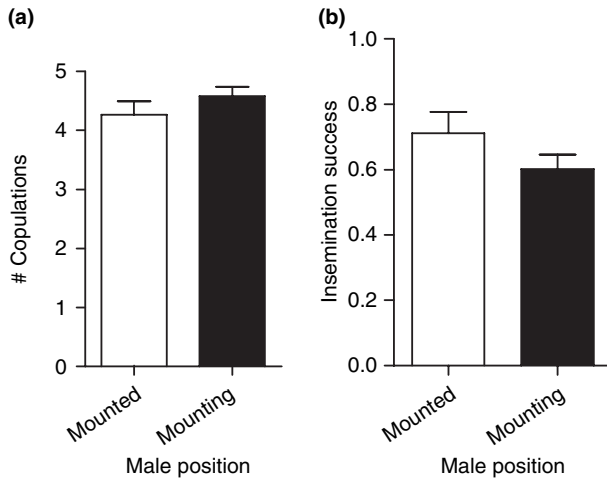


Fig. 3 Heterosexual reproductive performance of *Tribolium castaneum* males as a function of male position during prior homosexual encounters. Each male's heterosexual reproductive performance was measured 24 h after homosexual encounters as (a) the number of heterosexual copulations (of five females offered) and (b) insemination success (proportion of copulations that resulted in insemination). Mean + SE are shown for $n = 19$ mounted males (open bars) and $n = 19$ mounting males (solid bars).

such males were tested in mate competition for a single female; mounting (nine males) and mounted males (seven) were equally likely to copulate with females ($n = 16$ pairs, exact binomial test $P = 0.402$).

Hypothesis 2: practice for subsequent heterosexual encounters

When males that had been kept in all-male groups (thus engaging in homosexual copulations) were compared with same-aged males that had been isolated, group males had significantly lower copulation rates (Fig. 4a: separate variance $t = 2.623$, 36 d.f., $P = 0.013$), contrary to the predictions of the practice hypothesis. However, males did not differ in their insemination success (Fig. 4b: separate variance $t = 0.437$, 36 d.f., $P = 0.665$) or in their last-male paternity share when they mated with previously mated females (Fig. 5, oviposition days 1–3: Mann–Whitney $U = 175$, $P = 0.336$; oviposition days 4–10: Mann–Whitney $U = 191.5$, $P = 0.590$).

Hypothesis 3: indirect sperm translocation

To detect indirect sperm translocation during homosexual copulations, immediately after a homosexual copulation between males from two different genetic strains, we allowed the mounting males and the mounted males each to mate with a virgin female. By inspecting body colour phenotypes of all adult progeny resulting from these subsequent heterosexual matings, we could detect

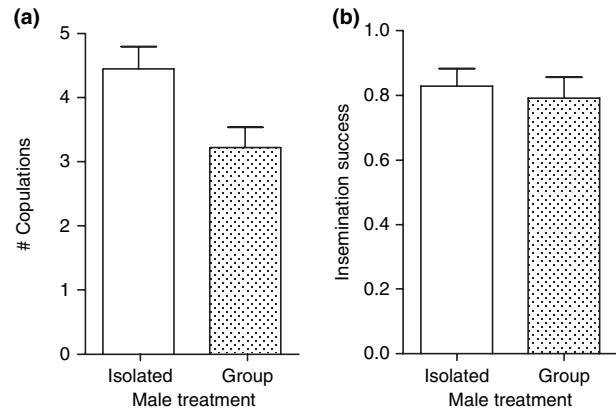


Fig. 4 Heterosexual reproductive performance measured immediately after *Tribolium castaneum* males were kept in isolation (open bars, $n = 20$) or in groups (shaded bars, $n = 18$), measured as (a) the number of heterosexual copulations per 15 min and (b) insemination success (proportion of copulations resulting in successful insemination). Mean + SE are shown.

whether sperm translocation between males had occurred during the initial homosexual copulation. In six of 86 pairs (7%), females produced progeny with body colour phenotypes indicating that they were sired indirectly by their mate's previous homosexual partner. In three of these cases the indirect sire was the homosexually mounting male, whereas in the remaining three cases the indirect sire was the mounted male. In each case only a single progeny was indirectly sired, representing ~0.5% of each females' total progeny. None of the 65 control matings produced any unexpected progeny (of 11 765 total progeny scored), indicating a low probability that these represent back mutations from black to wild-type or vice versa.

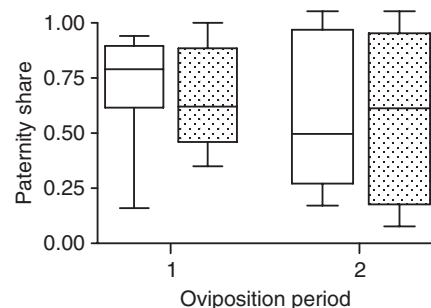


Fig. 5 Boxplots for paternity share (P_2 = proportion of progeny sired by focal second male) of *Tribolium castaneum* males that were either kept in groups (shaded boxes, $n = 17$) or isolated (open boxes, $n = 25$) for 1 week prior to testing. Because P_2 changes over time, data are presented separately for oviposition period 1 (1–3 days after the second mating) and 2 (days 4–10).

Accretion formation and effects on heterosexual reproductive performance

In 8 out of 12 male–male pairs (67%) observed under 30× magnification during homosexual copulations, mounting males released spermatophores (Fig. 6) that became attached to either the mounted male, the mounting male or some other surface. When homosexual copulations take place in flour, the sticky surface of these spermatophores is likely to become coated with flour and develop into the accretions that form on males kept in same-sex groups (Spratt, 1980). To determine which males developed accretions, we allowed 16 pairs of males to engage in homosexual copulations, and then moved each mounting male and mounted male individually into flour. After 15 days, substantial flour-covered accretions were found on six mounting males and on one mounted male. Although not significantly different than expected by chance (exact binomial test, $P = 0.063$), this indicated a trend toward a greater likelihood for mounting males to develop such accretions, opposite of what would be predicted if males initiated homosexual copulations to incapacitate their reproductive rivals.

To examine the effects of accretions on males' heterosexual reproductive performance, males were kept in same-sex groups until many of them had developed accretions (3–5 months). Mating behaviour and insemination success was compared for males with accretions, males without accretions and males whose accretions got dislodged during the experiment (Table 1). Males in these groups did not differ in their mean number of male-initiated contacts (ANOVA $F_{2,23} = 0.599$, $P = 0.558$), number of copulations (ANOVA $F_{2,23} = 1.188$, $P = 0.323$) or insemination success (Kruskal–Wallis $H = 4.613$, $P = 0.100$). However, when the proportion of contacts that led to successful copulation was compared, male groups differed significantly (Fig. 7: ANOVA $F_{2,23} = 3.693$, $P = 0.041$); males with accretions showed reduced ability to successfully copulate with the females they contacted, and this was significantly reduced relative to males whose accretions were dislodged during the experiment (Bonferroni $t = 2.598$, $P = 0.016$). In addition, the number of inseminations achieved by males was significantly different between groups (Table 1: Kruskal–Wallis $H = 9.103$,

Table 1 Effects of accretions resulting from *Tribolium castaneum* male homosexual copulations on male reproductive fitness, measured as the number of male-initiated contacts, number of copulations and number of inseminations during 10-min observations and male insemination success (proportion of copulations that led to successful insemination).

	Accretion	No accretion	Accretion dislodged
No. of contacts	12.0 (± 1.2)	11.4 (± 1.3)	9.6 (± 4.3)
No. of copulations	3.4 (± 0.6)	4.3 (± 0.5)	4.8 (± 2.1)
No. of inseminations	0.6 (± 0.2)	1.3 (± 0.4)	2.6 (± 1.2)
Insemination success	0.3 (± 0.1)	0.3 (± 0.1)	0.5 (± 0.2)

Mean \pm SE based on 10 males with accretions, 11 males without accretions and five males whose accretions were dislodged during the experiment are shown.

$P = 0.011$); males with accretions achieved significantly fewer inseminations 10 min^{-1} compared with males whose accretions were dislodged (Dunn's test, $P < 0.01$).

We found that after males had been kept isolated, they were much more likely to initiate homosexual copulations when paired with males that previously had been kept in same-sex groups (and thus had engaged in homosexual copulations). In such pairs, isolated males were the mounting individual in 95.2% of all observed homosexual copulations (80 of 84 homosexual copulations), which was significantly greater than a random expectation of 0.5 ($z = 8.3$, $P < 0.0001$). Isolated males also had significantly higher mounting rates (mean difference 3.2 ± 0.5 mounts per 10 min, paired $t = 6.87$, 23 d.f., $P < 0.0001$), and remained mounted for significantly longer durations (mean difference 5.1 ± 0.5 min, paired $t = 9.24$, 23 d.f., $P < 0.0001$) compared with males previously kept in groups.

Discussion

Although homosexual behaviours have been described in many animals, few studies have addressed evolutionary aspects of this phenomenon. In this study on *Tribolium* flour beetles, we conducted a series of experiments that allowed us to simultaneously examine multiple hypotheses that have been proposed to explain such apparently paradoxical behaviour.



Fig. 6 Spermatophore release during male homosexual copulations in *Tribolium castaneum* flour beetles: (a) mounting male is releasing a spermatophore from its extended genitalia, (b) a spermatophore deposited on a mounted male after release by the mounting male. Arrows indicate spermatophores, scale bars are 0.5 mm.

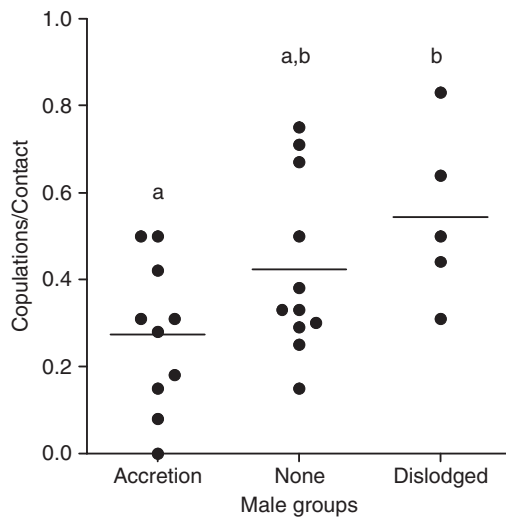


Fig. 7 Differences in the ability of *Tribolium castaneum* males to successfully copulate with females they contacted during 10-min observation periods for three types of males that had been kept in same-sex groups for 3–5 months prior to the experiment. Males either had hardened accretions on their posterior body ($n = 10$), lacked such accretions ($n = 11$), or had accretions that were dislodged during the course of the experiment ($n = 5$). Horizontal lines represent mean values and letters indicate subgroups that differ significantly by Bonferroni multiple comparisons.

Social facilitation and/or dominance hypothesis

Studies on several vertebrates, including flamingos, greylag geese, bottlenose dolphins, rhesus macaques and bonobos (Sommer & Vasey, 2006), have provided evidence that homosexual behaviours can alter social interactions by facilitating alliances or by establishing dominance relationships. However, we found no evidence that *Tribolium* males use homosexual copulations to establish dominance, as paired males frequently switched roles, and for those pairs with more consistent roles we found no differences in body size or reproductive performance between mounting and mounted males tested in either competitive or noncompetitive mating situations. Also counter to the dominance hypothesis, Harari *et al.* (2000) found that small males typically mounted larger males during homosexual copulations in the weevil *Diaprepes abbreviatus*; as females are generally larger, the authors attributed male homosexual copulations to mistakes in sex recognition. Based on the lack of courtship or agonistic interactions in *Tribolium*, other social functions (e.g. social bonding, alliance formation and conflict avoidance) also seem unlikely, although, in the horned beetle *Allomyrina dichotoma*, Iguchi (1996) suggested that male homosexual mountings may inhibit intrasexual aggression.

Practice for future heterosexual encounters hypothesis

We found no evidence to support the idea that homosexual copulations provide *T. castaneum* males with behavioural practice that increases their reproductive success in subsequent heterosexual encounters. When compared with same-age isolated males, males kept in same-sex groups (thus engaging in homosexual copulations) did not show better reproductive performance with females. In fact, males kept in groups had significantly lower mating rates (number of heterosexual copulations per 15 min) compared with isolated males. There were no differences between groups in their insemination success once they copulated with virgin females, or in their paternity share when they copulated with previously mated females. In addition to increased homosexual activity, other factors may alter the behaviour of group males compared with isolated males. For example, it is plausible that males kept in groups might alter their behaviour in response to higher perceived sperm competition risk. However, such an effect seems unlikely because increased sperm competition risk would be expected to increase, rather than decrease, male mating rates (Simmons, 2001).

Indirect sperm translocation hypothesis

Our results provide limited support for the hypothesis of indirect sperm translocation, which proposes that males engaging in homosexual copulations might indirectly transfer sperm to females through a male intermediary. In six instances, females that mated with males immediately following a homosexual copulation produced progeny whose body colour phenotype suggested they were sired not by the directly mating male, but rather by that male's homosexual partner: however, only a single unexpected progeny was observed in each case, representing ~0.5% of each females' total progeny. In addition, the indirect sires were equally likely to be mounted or mounting males, suggesting the possibility of bidirectional sperm transfer. These unexpected progeny are unlikely to represent genotyping mistakes, as +/b heterozygotes are easily distinguished from b/b homozygotes. In addition, unexpected progeny are unlikely to have arisen from mutations, as the observed rates are substantially above the typical mutation rates for phenotypic mutations of 10^{-4} – 10^{-6} per locus per generation (Futuyma, 2005).

How might such indirect sperm translocation happen? Haubruge *et al.* (1999) reported that in heterosexual *T. castaneum* copulations, sperm may be translocated between sequential females, and suggested that sperm trapped under penile spines might be implicated in such indirect fertilization. Although our study focused on the distinct phenomenon of sperm translocation through a male (rather than through a female) intermediary, a

similar mechanism might be responsible. In the hemipteran *X. maculipennis*, Carayon (1974) described male-to-male sperm transfer through traumatic insemination during homosexual copulations, although it is not known whether any such sperm is later transferred to females. Using irradiated males, Harari *et al.* (2000) found no support for indirect sperm translocation in the weevil *D. abbreviatus*. Thus, although our results are not conclusive, it is possible that during homosexual copulations *Tribolium* males exchange small quantities of sperm that get transferred when either male subsequently mates with a female.

Accretion formation and effects on reproductive performance

This study provides additional insight into the causes and consequences of the hardened, white accretions that develop on the posterior abdomen of some *T. castaneum* males when they are kept in same-sex groups (Sokoloff, 1974; Spratt, 1980; this study). Our direct observations of homosexual copulations under high magnification support Spratt's (1980) suggestion that these accretions form when spermatophores released by the mounting male become coated with flour over time. We found that both mounting and mounted males developed flour-covered accretions by ~2 weeks following a single homosexual copulation.

Although Spratt (1980) found reduced longevity for males kept in same-sex groups, the evidence that the accretions themselves might be responsible, perhaps by blocking the anus, was merely correlative. Our results indicate that these accretions do physically interfere with males' mating abilities; males with accretions had difficulty copulating with females they contacted, which resulted in their successfully inseminating fewer females relative to other males. However, we also found that male accretions were often dislodged during males' interactions with females, and so any reproductive or survival handicaps they entail may be temporary.

Possible advantages of sperm dumping

These observations suggest a novel hypothesis for the maintenance of homosexual copulations as a behavioural mechanism that allows males to discard sperm. Reinhardt (2007) reviewed evidence that sperm performance (including motility, fertilizing ability and sperm competitive ability) declines with sperm age, and Reinhardt & Siva-Jothy (2005) found that younger sperm are more likely to be stored by female house crickets, *Acheta domesticus*. Such a reproductive advantage for young sperm could select not only for sperm dumping associated with homosexual copulations, but also for autonomous spermatophore expulsion in male crickets (Kumashiro *et al.*, 2003; Reinhardt & Siva-Jothy, 2005), masturbation and spontaneous sperm emission

in human males (Baker & Bellis, 1993), and numerous other sexual behaviours as suggested by Reinhardt (2007). Sperm dumping may also be selected when males cannot down-regulate their sperm production rates. *Tribolium* beetles have life-history features likely to result in selection for continuous sperm production, as they are income breeders whose gamete production is fuelled by adult nutrient input and adults mate repeatedly during their 1- to 2-year adult lifespan (reviewed by Fedina & Lewis, 2008). When many females are available (e.g. under high population densities), high sperm production rates provide a selective advantage; indeed, *T. castaneum* males can mate with up to seven different females within 15 min (Lewis, 2004). On the flip side, however, these males may be physiologically incapable of down-regulating their sperm production when lower population densities are encountered. Thus, homosexual copulations may provide *T. castaneum* males with a mechanism for discarding sperm. Although of course this idea will require much further testing, this hypothesis is consistent with two observations in the present study: (1) males tested immediately after removal from same-sex groups had lower heterosexual copulation rates compared with isolated males; and (2) previously isolated males were much more likely to mount other males and exhibited higher rates and durations of homosexual copulations relative to males from in same-sex groups.

In summary, homosexual behaviours commonly occur in diverse animal groups, yet such interactions have been poorly studied. In *T. castaneum* beetles we found no evidence that homosexual copulations are used to establish dominance, or to provide behavioural practice that increases males' reproductive success in subsequent heterosexual encounters. We found limited support for the hypothesis of indirect sperm translocation to females through a male intermediary, and this phenomenon deserves further study. Observations of spermatophore release during homosexual copulations led us to propose that homosexual copulations may represent a behavioural mechanism that allows males to discard older, potentially low-quality sperm. Additional work is needed to experimentally test this hypothesis, and to determine whether seminal products might be released during male homosexual interactions in other species. Future research approaches that rely on empirically testing multiple hypotheses should lead to more rapid progress in understanding the apparent paradox of animal homosexual behaviours.

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Supporting information

Additional supporting information may be found in the online version of this article:

Appendix S1 *Tribolium* heterosexual and homosexual copulatory behaviours: this QuickTime movie illustrates homosexual copulations in red flour beetles, and shows a heterosexual copulation for behavioural comparison.

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