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An integrative view of sexual selection in *Tribolium* flour beetles

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ABSTRACT

Sexual selection is a major force driving the evolution of diverse reproductive traits. This evolutionary process is based on individual reproductive advantages that arise either through intrasexual competition or through intersexual choice and conflict. While classical studies of sexual selection focused mainly on differences in male mating success, more recent work has focused on the differences in paternity share that may arise through sperm competition or cryptic female choice whenever females mate with multiple males. Thus, an integrative view of sexual selection needs to encompass processes that occur not only before copulation (pre-mating), but also during copulation (peri-mating), as well as after copulation (post-mating), all of which can generate differences in reproductive success. By encompassing mechanisms of sexual selection across all of these sequential reproductive stages this review takes an integrative approach to sexual selection in *Tribolium* flour beetles (Coleoptera: Tenebrionidae), a particularly well-studied and economically important model organism.

Tribolium flour beetles colonize patchily distributed grain stores, and juvenile and adult stages share the same food resources. Adults are highly promiscuous and female reproduction is distributed across an adult lifespan lasting approximately 1 year. While *Tribolium* males produce an aggregation pheromone that attracts both sexes, there appears to be little pre-mating discrimination among potential mates by either sex. However, recent work has revealed several peri-mating and post-mating mechanisms that determine how offspring paternity is apportioned among a female's mates. During mating, *Tribolium* females reject spermatophore transfer and limit sperm numbers transferred by males with low phenotypic quality. Although there is some conflicting evidence, male copulatory leg-rubbing appears to be associated with overcoming female resistance to insemination and does not influence a male's subsequent paternity share.

Evidence suggests that *Tribolium* beetles have several possible post-mating mechanisms that they may use to bias paternity. Male sperm precedence has been extensively studied in *Tribolium* spp. and the related *Tenebrio molitor*, and several factors influencing male paternity share among a female's progeny have been identified. These include oviposition time, inter-mating interval, male strain/genotype, the mating regimen of a male's mother, male starvation, and tapeworm infection. Females exert muscular control over sperm storage, although there is no evidence to date that females use this to differentiate among mates. Females could also influence offspring paternity by re-mating with additional males, and *T. castaneum* females more readily accept spermatophores when they are re-mating with more attractive males. Additional work is needed to examine the possible roles played by both male and female accessory gland products in determining male paternity share.

Sexual selection during pre-mating episodes may be reinforced or counteracted by peri- and post-copulatory selection, and antagonistic coevolution between the sexes may be played out across reproductive stages. In *Tribolium*, males' olfactory attractiveness is positively correlated with both insemination success and paternity share, suggesting consistent selection across different reproductive stages. Similar studies across sequential reproductive stages are needed in other taxa to provide a more integrative view of sexual selection.

Key words: sperm competition, cryptic female choice, pheromones, sperm precedence, paternity bias, intersexual selection, intrasexual selection.

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I. INTRODUCTION

Sexual selection is a major force driving the evolution of diverse behavioural, morphological, anatomical, and physiological traits. This evolutionary process is based on reproductive advantages that arise either through intrasexual competition or through interactions between the sexes (e.g. choice, coercion, sensory exploitation). For nearly 150 years after Darwin (1859) first coined the term, studies of sexual selection focused primarily on traits leading to increased mating success gained through either competitive ability or attractiveness to the opposite sex (Darwin, 1871; Clutton-Brock & Parker, 1992; Andersson, 1994). More recently, however, studies using molecular tools for paternity determination in diverse taxa have revealed that female polyandry is a common feature of animal mating systems (Smith, 1984; Birkhead & Moller, 1998; Simmons, 2001; Birkhead & Pizzari, 2002). Whenever females mate with multiple males, the processes that take place during copulation (perimating, or pericopulatory), as well as after copulation (postmating, or postcopulatory), can produce differences in males' paternity success (also termed sperm precedence or paternity share). Paternity success refers to the proportion of a female's progeny sired by each mating male. Thus, the same sexual selection mechanisms of intrasexual competition, intersexual choice, and intersexual conflict that can cause differences in mating success can also cause differences in male paternity share. In analogy to male-male competition for access to mates, sperm competition was originally defined as "the competition between the sperm from two or more males for the fertilization of

a given set of ova" (Parker, 1970); this includes any male-mediated peri- and postmating processes that bias paternity. Sperm competition among several mating males or their sperm for access to fertilizations has subsequently been demonstrated in numerous animals (Smith, 1984; Birkhead & Moller, 1992, 1998; Simmons, 2001). In addition, since with internal fertilization these later stages of sexual selection occur within the female's body, females can also influence male paternity success. Such nonrandom paternity biasing by females during and after mating has been termed cryptic female choice (reviewed in Eberhard, 1996; Simmons, 2001).

Until recently, however, these various pre-, peri- and postmating sexual selection processes have mainly been studied in isolation. Many studies have examined how mate choice and intrasexual competition lead to differential mating success, while others have focused exclusively on specific mechanisms generating post-mating paternity bias. As a result of this polarization, we currently lack a comprehensive view of how various traits and behaviours influence reproductive success. In addition, mate assessment and choice during sequential reproductive stages may rely on signals in different sensory modalities (Candolin, 2003): for example, male olfactory signals may be used for long-distance mate attraction, while tactile cues may determine actual mate choice, and male copulatory behaviours may determine paternity success. Use of such sequential cues may allow females to correct possible mate choice errors when choosing sires. There may also be tradeoffs between different reproductive episodes such that some individuals are successful at mate choice/competition, while others are

more successful during pericopulatory and postcopulatory stages. In addition, because males and females usually differ in their optimal reproductive strategies, intersexual conflict can lead to sexually antagonistic coevolution (Rice & Holland, 1997; Arnqvist & Rowe, 2005; Tregenza, Wedell & Chapman, 2006), which may potentially occur across different episodes of selection. Thus, it is important to consider factors influencing not only mating success but also paternity success, in order to develop a comprehensive understanding of sexual selection in the many taxa where females mate with multiple males.

This review aims to apply this integrated view of sexual selection to understanding the evolution of sexual behaviour, anatomy, and physiology, using *Tribolium* flour beetles (Coleoptera: Tenebrionidae) as a model organism. Flour beetles are distributed worldwide, and several of the approximately 26 described species are economically important pests of cereals and grains stored by humans (Sokoloff, 1974; Levinson & Levinson, 1995). These insects have long been used as a model system for studies of population ecology (e.g. Park, 1948; Mertz, 1969; King & Dawson, 1972), evolution (Stevens, 1989; Wade, 1991), genetics (reviewed in Sokoloff, 1974, 1977; Brown, Denell & Beeman, 2003) and mathematical ecology (e.g. Constantino *et al.*, 1995). Finally, considerable knowledge of *T. castaneum* mating behaviour and post-copulatory sexual selection has accumulated over the past two decades, yet a comprehensive review of these processes is lacking. For comparison, we also consider aspects of sexual selection in another tenebrionid, the mealworm beetle, *Tenebrio molitor*.

The purpose of this review is to provide a critical integrative view of various sexual selection processes in *Tribolium* flour beetles, in hope that this framework can then be applied to studying sexual selection in other insects with polyandrous mating systems. In particular, we aim to (1) summarize behavioural, physiological, and morphological traits subject to sexual selection during three sequential stages of reproduction: precopulatory, pericopulatory, and postcopulatory, (2) investigate how these sequential stages might interact with each other, (3) explore how sexual selection is influenced by organismal natural history, and (4) suggest additional studies that may further our understanding of sexual selection.

II. LIFE HISTORY

(1) Habitat and food

Many insects that are now pests of stored products are thought originally to have consumed dried fruits, seeds cached by birds or rodents, or scavenged in nests of birds, rodents, spiders and other insects (Levinson & Levinson, 1995). Ancestral *Tribolium* habitats may have included decomposing wood; extant species have been found under bark feeding on decaying organic matter, fungi and bacteria (Hinton, 1942; Pray & Goodnight, 1995). When ancient Egyptians started stockpiling grain to safeguard against periodic famines around 4.5 thousand years ago, many

insects took advantage of this rich resource. Some attributes that may have facilitated movement into this newly created niche include tolerance for low water availability, polyphagy, and reproduction in constant low light environments (Levinson & Levinson, 1995). *Tribolium* flour beetles have been a human commensal associated with stored food products since at least 2000 B.C. As a result of human commerce and transportation, *Tribolium* spp. are now found worldwide, mainly inhabiting stored grain and cereal products, but also nuts, dried fruit, spices, chocolate, and yeast (Pray & Goodnight, 1995).

(2) Life-history attributes

Organismal life histories and mating systems are intimately connected to sexual selection processes that affect the evolution of reproductive traits. Most information concerning *Tribolium* life-history traits and behaviours has been gathered from cultures kept in the laboratory for many generations, and these may differ from natural populations (White, 1984). Life-history traits for *T. castaneum*, a commonly studied species, are summarized below; other *Tribolium* flour beetles may deviate quantitatively from this pattern, but their life histories are fundamentally similar (Dawson, 1964; reviewed in Sokoloff, 1974; see also Imura, 1987; Bernasconi *et al.*, 2006). Adults are relatively long-lived beetles with reproductive lifespans of 0.5-1 year (Nilsson, Fricke & Arnqvist, 2002; reviewed in Sokoloff, 1974). *T. castaneum* males become sexually mature two days post-eclosion, while females, which can be fertilized as early as 3 h after eclosion, do not begin laying eggs until four days later (Dawson, 1964). Virgin females isolated from males will lay unfertilized eggs (reviewed in Sokoloff, 1974), each weighing only about 2% of female body mass (Bernasconi *et al.*, 2006). Mated *T. castaneum* females lay on average 10 eggs per day for 3-6 months, after which oviposition rates decline; female lifetime fecundity ranges between about 500-1500 eggs (Sokoloff, 1974; Sonleitner, 1978; Bernasconi *et al.*, 2006). Developmental period for *T. castaneum* in optimal conditions is about five days for eggs, 20 days for larvae, and seven days for pupae (reviewed in Sokoloff, 1974).

Many *Tribolium* life-history traits show considerable phenotypic plasticity in response to environmental variation. Abiotic conditions such as temperature, humidity, or food, influence flour beetles as they do many other insects (reviewed in Sokoloff, 1974). For instance, as in other income breeders (Stearns, 1992), *Tribolium* female fecundity depends on resource nutritional quality (reviewed in Sokoloff, 1974). However, unlike most insects, flour beetles live in a food resource that is shared by adult and juvenile life-history stages. Because of this, *Tribolium* populations exhibit density fluctuations that affect profoundly both their abiotic and biotic environments. As population density increases, the flour medium that beetles occupy loses its nutritional quality and accumulates waste products and benzoquinones, which are defensive compounds produced by adults. Even slight conditioning of the medium decreases the oviposition rate of *Tribolium* females (reviewed in

Sokoloff, 1974), with fecundity dropping over 50% in conditioned compared to fresh flour (Park, 1936; Prus, 1961; Sonleitner & Guthrie, 1991). Another life-history trait affected by population density is larval developmental period, which is extended at higher densities: both conditioning of the media and tactile stimulation were found to mediate this effect (Park, 1935; Sokoloff, 1974; Matsumura & Yoshida, 1988; Blanco, Kotaki & Nakakita, 1990). High population densities also cause increased pre-adult mortality due primarily to cannibalism by active stages (adults and larvae) on inactive stages (eggs and pupae); depending on conditions, larvae and adults can cannibalize up to 99% of eggs and pupae (reviewed in Sokoloff, 1974). Finally, increasing density and conditioning of the medium induce dispersal behaviour in *Tribolium* flour beetles (reviewed in Sokoloff, 1974; Ziegler, 1978; Zyromska-Rudzka, 1966a,b).

Both the ancestral and more recent stored grain habitats of flour beetles occur in discrete, often widely separated habitat patches. *Tribolium* life-history traits have likely been shaped by cycles of new colonization events followed by rapid population growth, alternating with longer periods of population stabilization or decline (Dawson, 1964; Mertz, 1969). These beetles exhibit several life-history characteristics typical of good colonizers, including high intrinsic population growth rate, fast maturation, rapid larval development, and good dispersal ability. Their atypically long adult lifespan and prolonged, evenly distributed reproduction may represent a “bet-hedging” strategy (Stearns, 1992) that enables them to cope with fluctuating conditions encountered during colonization-overexploitation cycles, and especially with high juvenile mortality. Because *Tribolium* generation time is short relative to adult lifespan, individuals are likely to encounter a range of conditions associated with different colonization stages. Thus, phenotypic plasticity apparent in several life-history traits, including oviposition rate, developmental time, and dispersal ability, may represent adaptation to density-dependent environmental fluctuations. In addition to phenotypic plasticity, *Tribolium* life-history characteristics such as pupal mass, developmental period, and cannibalism rate have been shown to have a genetic component (reviewed in Sokoloff, 1977; Lavie & Ritte, 1980; Stevens, 1994).

In contrast to *Tribolium* spp., *Tenebrio molitor* is characterized by larger body size, shorter adult lifespan, much lower female fecundity, and lower tolerance to high densities (Cotton & George, 1929; Dick, 1937); such life-history differences make mealworm beetles an interesting comparison to flour beetles.

III. MATING SYSTEMS

(1) Characteristics of mating systems

Mating systems of *Tribolium* spp. are highly polygamous, with both sexes mating frequently throughout their adult lives (Park, 1933; Sokoloff, 1974). As flour beetles move through their food medium, they excavate tunnels in which

matings generally take place. In stored product facilities flour beetles tend to aggregate rather than distribute themselves randomly (Sokoloff, 1974; Subramanyam & Harein, 1990; Trematerra & Sciarretta, 2004), but natural densities and mating rates are unknown. Mating rates have been measured by observing beetles interacting in open arenas, and several studies indicate that males engage in copulations every 2–3 min (Shrode, 1960; Haubruge *et al.*, 1999; Lewis, 2004). However, these studies may overestimate natural mating rates because they used virgin or previously isolated males having unlimited access to virgin females for short periods of time. Wool (1967), who also noted high mating frequencies in *T. castaneum*, found that mating rates were reduced two-to-threefold in groups of recently mated beetles compared to virgins. Females in *T. castaneum* also mated with up to 12 different males per hour (Pai, Feil & Yan, 2007; Pai & Yan, 2003b). Given that females are able to lay over 700 fertile eggs over a span of 4 months following only a single mating (Bloch Qazi, Herbeck & Lewis, 1996), there must be some net benefit to frequent mating besides sperm replenishment.

(2) Costs and benefits of female polyandry

Polyandry (mating with multiple males) could provide females with an opportunity to obtain direct benefits from several males, and/or to obtain indirect genetic benefits by allowing sperm competition and exercising cryptic female choice. Polyandry may also lead to antagonistic coevolution between the sexes, and thus result in net costs to females (Rice, 1998; Arnqvist & Rowe 2005; Pai & Bernasconi, 2007).

Several studies in flour beetles have identified direct benefits of female polyandry, which increased female fecundity (Park, 1933 for *T. confusum*; Lewis & Austad, 1994 for *T. castaneum*) as well as the probability of successful insemination (Pai, Bennett & Yan, 2005). The same direct benefits of female polyandry have been identified for *Tenebrio molitor*, where the increase in female reproductive output has been suggested to arise from putative oviposition-boosting substances in male ejaculates (Gerber, 1967; Drnevich *et al.*, 2001; Worden & Parker, 2001). In these mealworm beetles, it appears unlikely that females gain nutritional benefits from multiple matings, since no interaction has been found between the effects of mating treatment (single *versus* multiple) and female nutrition (poor *versus* rich) on offspring numbers (Worden & Parker, 2001). Considering their similarities in reproductive biology, *Tribolium* females are also unlikely to draw any nutritional benefits from male spermatophores, especially considering that undigested spermatophores are expelled by these females soon after mating (Bloch Qazi *et al.*, 1996; Fedina, 2007). Females also mate repeatedly with the same male, and some of the observed direct benefits of polyandry may simply reflect higher ejaculate quantities. In *T. castaneum*, repetitive mating with the same male increased the lifetime offspring production of females (Nilsson *et al.*, 2002).

Apart from direct benefits, several indirect genetic benefits of polyandry have been detected for flour beetles.

These include higher larval viability of offspring (Pai *et al.*, 2005; but see Pai & Yan, 2003*b*), higher insemination success of sons (Pai & Yan, 2002*b*), and higher egg viability for F₁ offspring (Pai & Yan, 2002*b*). A study examining sperm offence and defence abilities of sons from polyandrous *versus* monandrous lines found that sons from polyandrous mothers had higher sperm offence abilities in the F₁ generation, but these differences disappeared in later generations (Bernasconi & Keller 2001). Costs of polyandry have also been identified; in particular, the fitness of daughters (Pai & Yan, 2002*b*; Pai *et al.*, 2007) and sperm defence capacity of sons (Bernasconi & Keller, 2001) were lower for polyandrous females.

Since flour beetles are known to exhibit inbreeding depression (Gaur & Rao, 1997; Pray & Goodnight, 1995; Wade, Shuster & Stevens, 1996; Wool & Mendlinger, 1981), cryptic female choice following matings with several different males may help females to avoid inbreeding. Even without paternity biasing mechanisms, it was proposed that polyandrous females might benefit through reduced inbreeding in their grand-offspring when they disperse into new habitat patches (Cornell & Tregenza 2007). Polyandry may also help reduce negative fitness effects of interspecific matings. Several studies have explored conspecific *versus* heterospecific sperm precedence in *Tribolium* spp. In crosses between *T. castaneum* and *T. freemani*, females produced near-normal numbers of sterile offspring (Wade *et al.*, 1994). However, when *T. castaneum* females mated with both a conspecific and a heterospecific male, they produced predominantly (over 99%) conspecific offspring (Robinson, Johnson & Wade, 1994; Fricke & Arnqvist, 2004). Post-copulatory, prezygotic mechanisms appear to be responsible for maintaining such conditional reproductive isolation between these *Tribolium* species.

IV. PRE-MATING SEXUAL SELECTION

In this section we describe specific traits and behaviours affecting mate-finding and acquisition in flour beetles. Since different factors may be involved in long-range mate attraction and short-range (including contact) mate assessment and choice, we consider these stages separately.

(1) Long-range attraction and mate localization

Many taxa rely on visual, acoustic, or chemical signals to attract mates over long distances. Because *Tribolium* inhabit grain storage areas with dim or no light, it is unlikely that visual signals play any major role in mate attraction or assessment in this group. In addition, no evidence has been found for *T. confusum* that either sex produces any acoustic signals (Wojcik, 1969). Rather, long-range aggregation and mate attraction in flour beetles appear to be based entirely on chemical signals.

In several *Tribolium* species, adult males produce a highly volatile pheromone, 4,8-dimethyldecanal (DMD), which is attractive to both sexes; this compound has been isolated

from *T. castaneum*, *T. confusum*, *T. freemani* and *T. madens* (Arnaud & Haubruge, 2002; Suzuki, 1980; Suzuki, Nakakita & Kuwahara, 1987; Suzuki & Sugawara, 1979). Although DMD is commonly referred to as an aggregation pheromone, it may have a dual function as a sex attractant for females (Levinson & Levinson, 1995). Synthetic DMD elicited greater behavioural and electroantennogram responses from female compared to male virgins in both *T. castaneum* and *T. confusum* (Levinson & Mori, 1983), although in a different study no sex difference was found in either response when mated *T. confusum* were tested (Verheggen *et al.*, 2007). In yet another study, synthetic DMD was more attractive to males than to females of *T. castaneum* (Obeng-Ofori & Coaker, 1990).

Tribolium flour beetles show strong sexual dimorphism in the form of setiferous glands located on the prothoracic femora of males (absent in females) in *T. castaneum*, *T. destructor*, *T. freemani*, *T. anaphe* and *T. madens*, and on all six femora of males in *T. confusum* and *T. alpine* (Faustini & Halstead, 1982). These glands produce a waxy secretion that accumulates on the gland surface as males age, and which is strongly attractive to both females and males in olfactometer tests (Faustini, Burkholder & Laub, 1981; Bloch Qazi, Boake & Lewis, 1998*b*; Olsson *et al.*, 2006). Although these glands were originally thought to be the major site of DMD production (Hinton, 1942; Faustini *et al.*, 1981; Faustini & Halstead, 1982; Faustini, Post & Burkholder, 1982*a*; Faustini, Rowe & Burkholder, 1982*b*; Levinson & Levinson, 1995), males lacking these gland-bearing legs still produced normal DMD quantities in *T. castaneum* (Bloch Qazi *et al.*, 1998*b*) and were equally attractive to females in both *T. castaneum* and *T. confusum* (Bloch Qazi *et al.*, 1998*b*; Olsson *et al.*, 2006). These results suggest that other male glands are involved in producing DMD, and that additional work is needed to characterize secretions of the sexually dimorphic setiferous glands in *Tribolium* spp.

The potential exists for *Tribolium* females to use chemical signals to assess potential mates. Significant variation among males in DMD production has been observed (Bloch Qazi *et al.*, 1998*b*), as well as within-male variation over time (Hussain *et al.*, 1994). Starved *T. castaneum* males showed reduced DMD production (Hussain *et al.*, 1994), which suggests that females could use DMD as a condition-dependent indicator of male quality. It has also been proposed that individual differences in DMD stereoisomeric composition could be responsible for differential male attractiveness (Levinson & Mori, 1983). Additionally, males may emit other volatile compounds that could influence their long-range attractiveness to females. Thus, along with repellent benzoquinones, a number of volatile and non-volatile hydrocarbons have been isolated from flour beetles; some studies have suggested that these compounds act as repellents (Suzuki, Huynh & Muto, 1975), while others found them to induce copulatory activities in males (Keville & Kannowski, 1975). Verheggen *et al.* (2007) identified several benzoquinones and hydrocarbons that elicited increased physiological (electroantennogram) and behavioural (olfactometer assay) responses in males and females of *T. confusum*, suggesting that the chemical communication

system of *Tribolium* may be more complex than previously thought.

Several laboratory studies have used behavioural bioassays to measure the degree to which the composite chemical signals of individual *Tribolium castaneum* males attract groups of females in closed pitfall arenas (Boake & Wade, 1984; Boake, 1985, 1986; Bloch Qazi *et al.*, 1998b; Wang, 1992; Lewis & Austad, 1994; Pai & Yan, 2003a). In these studies, male olfactory cues were collected from live males onto filter paper disks, so these stimuli reflect a time-integrated, composite male profile, which likely contains many compounds. Boake (1986) found that the long-range olfactory attractiveness of individual *T. castaneum* males was not correlated with the reproductive performance of their progeny, suggesting that these chemical signals are not used as a basis for adaptive female choice. Furthermore, females did not differentiate between filter paper disks exposed to males infected with intestinal parasites and disks from uninfected males (Pai & Yan, 2003a). Males that were more attractive based on long-range olfactory signals did gain higher paternity success when they subsequently mated with previously mated females (Lewis & Austad, 1994). However, taken together these studies do not provide convincing evidence for females using long-range chemical signals to discriminate among potential mates.

Circumstantial and contradictory evidence exists for female sex pheromones in flour beetles. Early olfactometer results found that the response of *T. castaneum* males to virgin female extracts depended on what time of day females were extracted and whether females had been irradiated (Abdu *et al.*, 1985; Abdel-Kader, Abdu & Hussien, 1987). Another study identified a possible female sex pheromone in *T. castaneum* (Rangaswamy & Sasikala, 1990), but these results were not confirmed (Subbaraman, Mithran & Mamdapur, 1991). Male *T. castaneum* have been shown to discriminate between virgin and previously mated females, as they more often contacted and copulated with virgins when given equal access to both types of females (Arnaud & Haubruge, 1999; Lewis & Iannini, 1995). In addition to discriminating against mated females, males also discriminated against females with whom they had previously mated in favour of females that had mated with other males (Arnaud & Haubruge, 1999). These results raise the possibility that, as has been suggested for *Tenebrio molitor* beetles (Happ 1969), males might transfer anti-aphrodisiac substances, perhaps from their setiferous glands, to females.

In *Tribolium confusum*, evidence from early behavioural assays suggested that both sexes produce pheromones (Ryan & O'Ceallachain, 1976; O'Ceallachain & Ryan, 1977; Hughes, 1982). In these studies male aggregation pheromone attracted both sexes, and female sex pheromone attracted only males; interestingly, males showed a greater response to male volatiles than to female volatiles. By contrast, a recent behavioural study found no evidence for a female-produced sex pheromone in *T. confusum*, and male volatiles attracted only females (Olsson *et al.* 2006). Such contradictions may be due either to methodological differences (i.e. using live beetles *versus* extracts of bodies as the pheromone source), and/or to differences in the behavioural assays used.

In general, some caution should be exercised when interpreting results of these and similar bioassays for several reasons. First, *Tribolium* flour beetles release benzoquinones when disturbed (Roth & Howland, 1941; Alexander & Barton, 1943), and female response is likely to reflect both attraction to male-released pheromones and repellency of these benzoquinones; the latter may depend on how beetles are handled. In addition, most bioassay studies have used virgin beetles to measure the attractiveness of olfactory cues, but virgins may be more motivated to mate and therefore less discriminating than mated beetles (Gabor & Halliday, 1997; Jennions & Petrie, 2000). Finally, the importance of long-range olfactory cues in attracting mates is likely to depend on population density. Under low densities following patch colonization, it is reasonable to infer that males capable of attracting more females to their vicinity will gain higher mating success. However, as population density increases within a patch or under laboratory conditions, it may be difficult for females to locate a specific pheromone source because males are highly mobile and use other beetles' tunnels (Hagstrum & Smittle, 1980). In addition, high beetle densities result in decreased DMD production by males (Hussain *et al.*, 1994). Thus, in high-density populations male-female encounters may essentially be random, with potential female choice restricted to later stages that involve close-range pre-mating assessment, perimating, or post-mating choice. Therefore, it remains to be determined whether the long-range olfactory attractiveness of *Tribolium* males is actually correlated with mating success in natural populations.

Thus, the chemical signals active in *Tribolium* long-range mate attraction and possibly assessment are complex, and appear to involve several compounds that may work in opposition or synergistically. Future studies should focus on testing field populations, as the high densities typical of laboratory populations may decrease selection on both the production and perception components of chemical recognition signals.

(2) Close-range assessment of potential mates

Once males and females reach close proximity, further assessment and choice among potential mates may occur based on different cues, including contact chemicals such as cuticular hydrocarbons (Howard & Blomquist, 2005), physical cues such as body size and shape, or courtship behaviours. In flour beetles, there is no evidence for any direct male-male competition or male courtship behaviours before mating (Sokoloff, 1974). Once contact is made, a male may briefly inspect the female's head or abdomen, but often mounts immediately. In *T. castaneum* a male attempts copulation by dorsally mounting the female, while rapidly rubbing his legs along the sides of the female's body and extruding his aedeagus (Wojcik, 1969). During such attempts, females appear to be able to exercise mate choice by allowing or refusing male intromission (Wojcik, 1969), or by dislodging a mounted male (Pai & Yan, 2003b). Only about half of all male copulation attempts in *T. castaneum* lead to successful intromission, independent of female mating status (Lewis & Iannini, 1995). However, information

is currently lacking on any male traits that affect the likelihood of successful intromission.

A puzzling feature of *Tribolium* mating systems is male homosexual copulations, as such lack of discrimination seems likely to negatively impact male fitness. Male homosexual copulations are quite common in flour beetles, occurring at frequencies reflecting sex ratios (Wool, 1967; Castro, Toro & Lopez-Fanjul, 1994; Serrano *et al.*, 2000), and often resulting in spermatophore expulsions on the outside of the male body (Spratt, 1980; Fedina, 2007). In addition, interspecific copulations and sperm transfer commonly occur between several species in flour beetles (Wade *et al.* 1994; Graur & Wool, 1985; Fricke & Arnqvist, 2004), further suggesting that males lack discriminatory abilities. On the other hand, this seemingly maladaptive behaviour may reflect low costs of male ejaculates, and/or the necessity to dump old spermatophores in order to manufacture fresh ones.

While information on close-range assessment in flour beetles is scarce, several studies in another tenebrionid, *Tenebrio molitor*, showed that close-range chemical cues are important for both sexes. In this species, both males and females produce volatile sex pheromones that attract the opposite sex (August, 1971; Bryning, Chambers & Wakefield, 2005; Happ, 1969). In addition to sex pheromone, females produce copulation-releasing pheromone, a non-volatile compound that induces males contacting it to attempt copulation even with inanimate objects (Happ, 1969; August, 1971; Tanaka *et al.*, 1986; Bryning *et al.*, 2005). Infection with parasites appears to affect pheromone production by both sexes. When *T. molitor* males were offered glass rods coated with extracts from females infected with parasitic tapeworms, their copulatory response decreased by 32% compared to extracts from uninfected females (Hurd & Parry, 1991). *Tenebrio molitor* females also were preferentially attracted by filter paper discs from uninfected compared to infected males (Worden, Parker & Pappas, 2000; Worden & Parker, 2005), and from males with higher immunocompetence (Rantala *et al.*, 2002). Females mating with more attractive males in these studies produced more offspring (Worden & Parker, 2001) and had increased longevity (Vainikka *et al.*, 2006), suggesting that they may benefit directly from choosing males in better phenotypic condition. This apparently greater reliance on pheromones for pre-mating discrimination may possibly be due to life-history differences between *Tenebrio molitor* and *Tribolium* spp.

V. PERI-MATING SEXUAL SELECTION

Eberhard (1991, 1996) pointed out that behavioural interactions during copulation have the potential to influence a male's subsequent paternity success by affecting the processes of sperm transfer, storage, and use. This may involve perimating sperm competition mechanisms (e.g. male manipulation of rival ejaculates, or adjustments to a male's own ejaculate) as well as cryptic female choice mechanisms that can potentially mediate or alter male manipulations (Eberhard, 1996; Simmons, 2001). Because

such processes are likely to be influenced by both sexes, it is often difficult to partition male *versus* female influences during copulation. However, this can be accomplished through experimental manipulations of female control, or through statistical partitioning of male *versus* female genotype effects achieved by crossing different strains.

Here we review evidence from flour beetles that behavioural interactions during copulation can alter the likelihood of successful insemination, spermatophore placement, and the amount of sperm transferred and stored by females. Because these processes take place within female reproductive tracts, understanding peri-mating selection requires knowledge of male and female reproductive anatomy, summarized below.

(1) Reproductive anatomy

During copulation (Fig. 1) *Tribolium* males transfer to the female's bursa copulatrix a spermatophore produced by two pairs of male accessory glands: short bean-shaped glands and long tubular glands. Male spermatophore production and histochemistry have been particularly well described in the related *Tenebrio molitor* mealworm beetles, where an initially invaginated spermatophore everts to release sperm inside the female bursa (Gadzama & Happ, 1974; Dailey, Gadzama & Happ, 1980; Grimnes, Bricker & Happ, 1986; Shinbo, Yaginuma & Happ, 1987; Yaginuma & Happ, 1988; Paesen & Happ, 1995; Paesen, Feng & Happ, 1996). In *T. castaneum*, spermatophores are also transferred to the female as an invaginated tube into which sperm are injected during copulation (Fedina, 2007). Although *T. castaneum* spermatophores (Fig. 2A) are morphologically similar to *Tenebrio molitor* spermatophores (Fig. 2B), the former evert and release sperm more quickly after transfer (Gadzama & Happ, 1974; Bloch Qazi *et al.*, 1996; Fedina, 2007). Such rapid sperm release in *T. castaneum* may represent one strategy for dealing with frequent female remating, while spermatophore inhibition may serve the same purpose in *Tenebrio molitor* (described below).

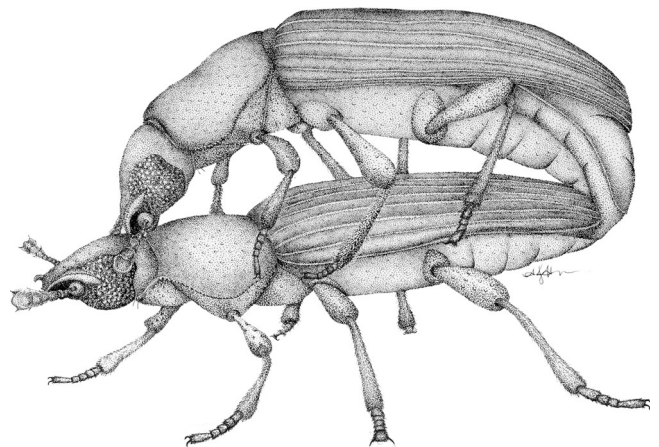


Fig. 1. Copulatory position of *Tribolium castaneum* flour beetles (drawing by April Hobart). Scale bar, 3mm.

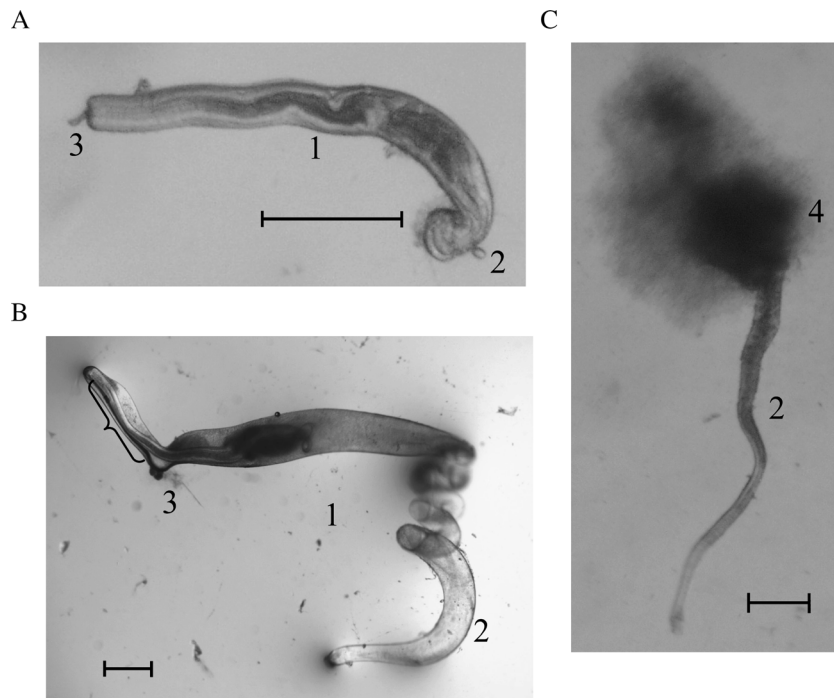


Fig. 2. Structure of male spermatophores in two tenebrionid beetles: (A) *Tribolium castaneum* early-stage, non-everted spermatophore, (B) *Tenebrio molitor* partially everted spermatophore, (C) *Tribolium castaneum* last stage of spermatophore eversion and sperm release. Modified from Fedina (2007). Spermatophore components: 1, anterior part with darker core; 2, posterior tail; 3, cap; 4, sperm sac. Scale bars, 100 µm.

In *T. castaneum*, sperm are initially released from the spermatophore into the female bursa copulatrix. Short-term sperm storage (approximately 1 week) occurs in the anterior bursa, while longer term storage takes place within the female spermatheca (Fig. 3A). This structure consists of several long, narrow and convoluted tubules connected to the anterior bursa through a common duct (Surtees, 1961; Fedina & Lewis, 2004). Spermathecal shape differs widely in other tenebrionids. Thus, in *Tribolium confusum* it consists of a wide, U-shaped sac connected to the bursa copulatrix via a narrow duct (Surtees, 1961; Fig. 3B). In *Tenebrio molitor* (Fig. 3C) and in *Tribolium brevicornis* (Pai & Bernasconi, 2007) the female spermatheca is similar to that of *T. castaneum*, but the tubules are considerably longer, thinner, and more numerous. In *T. castaneum*, a prominent spermathecal accessory gland lies in close proximity to the spermatheca, and this gland releases secretions into the anterior bursa (Fig. 3A). The cellular structure of this spermathecal gland has been studied in both *T. castaneum* (Al-Khalifa, 1981) and *Tenebrio molitor* (Happ & Happ, 1970, 1977). Secretions from the spermathecal gland have been suggested to serve a nutritive function for stored sperm, but this has not been verified experimentally. The close proximity of this gland to the sites of sperm storage and fertilization suggests that it would be worthwhile to investigate its role in female sperm choice.

(2) Copulatory behaviours

In *T. castaneum*, copulation durations are highly variable, and while they typically last from 0.5 to 2 min, some may

last over 30 min (Sokoloff, 1974; Bloch Qazi *et al.*, 1996; Haubruge *et al.*, 1999; Edvardsson & Arnqvist, 2000; Lewis, 2004). During copulation, males in many tenebrionid beetles perform stereotypic movements with their legs and antennae (Wojcik, 1969; Carazo *et al.*, 2004). In *T. castaneum* (Fig. 1), this behaviour consists of bouts during which males rapidly rub their legs against the sides of the female's body interspersed with periods of inactivity (Bloch Qazi, 2003). During copulation, females usually exhibit quiescence behaviour during which they stop moving and often assume a characteristic posture with extended forelegs and raised head (Bloch Qazi, 2003). This female behaviour occurs during spermatophore transfer; when copulations were experimentally interrupted during quiescence, only 40% resulted in spermatophore transfer, compared to 80% when copulations were not interrupted or interrupted after full quiescence (Bloch Qazi, 2003). Female quiescence during copulation may therefore indicate female cooperation in the insemination process.

Male leg-rubbing may represent copulatory courtship behaviour that could alter the likelihood of females accepting, storing or using a male's sperm. Several studies in *T. castaneum* have tested this hypothesis by examining the relationship between male leg-rubbing behaviours and paternity, but have reached different conclusions. Edvardsson & Arnqvist (2000, 2005) manipulated female perception of male leg-rubbing by partially ablating males' legs, but observed no changes in second-male paternity or female offspring production for manipulated compared to control males. They did, however, detect a positive relationship

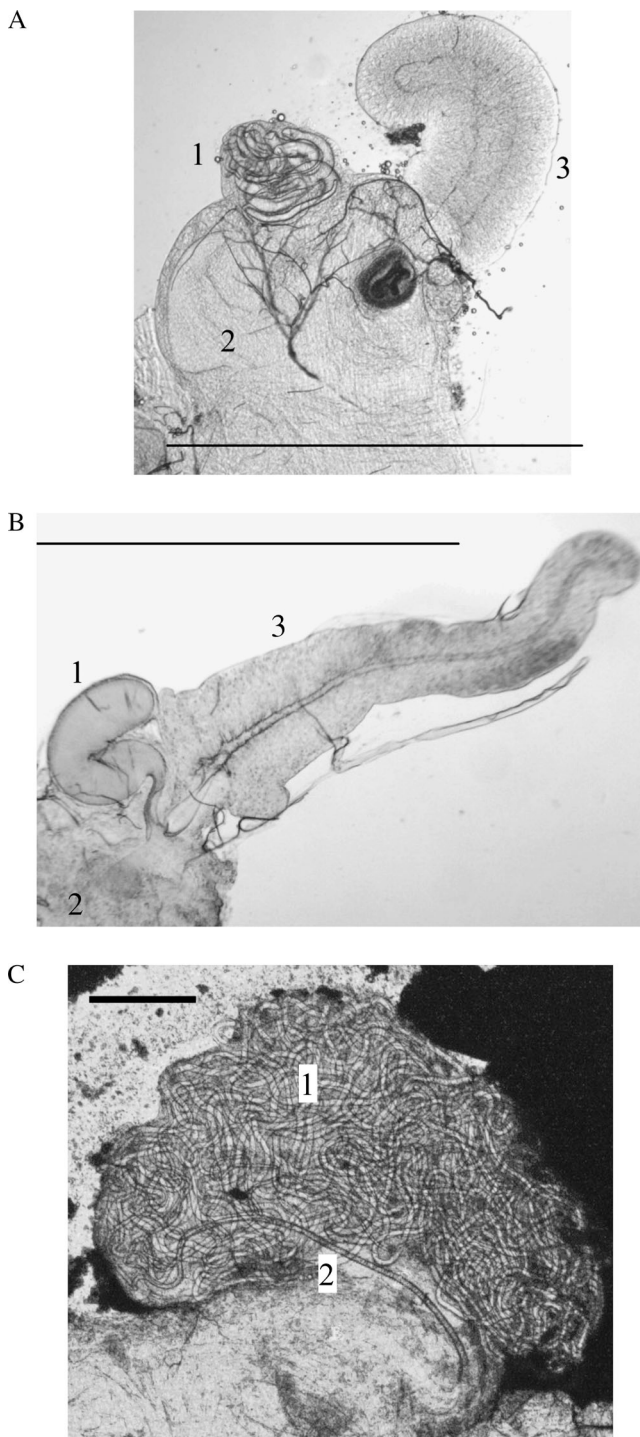


Fig. 3. Female spermathecal structures in three tenebrionid beetles: (A) *Tribolium castaneum*, (B) *T. confusum*, (C) *Tenebrio molitor*. Structures: 1, spermatheca, 2, anterior bursa, 3, spermathecal gland. Scale bars, 0.5 mm.

between leg-rubbing rate (measured as total number of rubbing bouts summed across all legs and divided by copulation duration) and paternity for control (unmanipulated) males. Another study found no relationship between male paternity share and several more detailed measures of

male leg-rubbing behaviour measured during female quiescence (Bloch Qazi, 2003). An additional study measured leg-rubbing rate as defined by Edvardsson & Arnqvist (2000), yet found no relationship with paternity (Fedina & Lewis, 2006). This study also observed a weak positive relationship between male paternity success and percentage of time spent in leg-rubbing; however, the latter was also associated with higher probability of insemination failure. Interestingly, no changes in several measures of male leg-rubbing behaviour were found when male condition was manipulated through starvation (Fedina & Lewis, 2006), rearing density (Edvardsson & Arnqvist, 2006), or induced parasitism (Pai & Yan, 2003a). Finally, no genetic benefits (progeny survival or heritability of leg-rubbing rate for sons) were detected when the progeny of intensely rubbing males were examined (Edvardsson & Arnqvist, 2006). Taken together, the current evidence does not support the hypothesis that *T. castaneum* females use male leg-rubbing behaviours as a signal of male quality to bias paternity.

(3) Spermatophore transfer

In flour beetles, not every observed mating results in spermatophore transfer. Dissections of *T. castaneum* females immediately after copulation demonstrated that only 85% of copulations with virgin females (Bloch Qazi *et al.*, 1996), and 70–90% (depending on male condition) of copulations with previously mated females (Fedina & Lewis, 2006) result in the successful transfer of a spermatophore. Studies have also demonstrated that females play an active role in permitting male insemination (Fedina & Lewis, 2006). These experiments manipulated *T. castaneum* male phenotypic condition by starvation, and found that live females blocked spermatophore transfer by starved males, while insemination success measured in dead females did not differ between starved and fed males.

In addition to successful spermatophore transfer, other processes occurring during copulation have the potential to influence male reproductive success. Such processes include positioning of the male spermatophore within the female's reproductive tract, as well as its placement relative to any previous spermatophores deposited within the female bursa copulatrix. In *T. castaneum*, a male's spermatophore can be deposited in two distinct locations: either in the anterior bursa (close to the spermathecal duct entrance) or in the posterior bursa, which has the disadvantage of being further from the sperm storage site and subject to displacement during oviposition (Fedina & Lewis, 2006). No evidence has been found to date that females use spermatophore positioning to differentiate between males (Fedina & Lewis, 2006). However, the 24 h inter-mating interval used in their study precluded direct interaction between male spermatophores, because first-male spermatophores were expelled by females before their second mating. Therefore, with shorter intervals between matings spermatophore positioning can potentially be another mechanism for cryptic female choice, and this deserves further investigation.

Even following successful transfer, male spermatophores can be expelled before sperm release is completed. If

females are capable of selectively expelling spermatophores after copulation with some mates but not with others, spermatophore expulsion could represent a mechanism for cryptic female choice. Spermatophore expulsion was reported to occur in 33% of *T. castaneum* females within 1 h after a single mating (Bloch Qazi *et al.*, 1996), and spermatophores still containing sperm are frequently expelled by females within a few minutes of mating (Fedina, 2007).

(4) Quantity of sperm transferred

In diverse taxa with polyandrous mating systems, sperm quantity transferred during copulation has been shown to influence male paternity share (Parker, 1970; Eberhard, 1996; Simmons, 2001). It has been estimated that *T. castaneum* males transfer $0.5 - 3.0 \times 10^5$ sperm during a single copulation (Arnaud, Haubruge & Gage, 2001c; Bloch Qazi *et al.*, 1998b, 1996; Fedina & Lewis, 2006); sperm quantities estimated by Edvardsson & Arnqvist (2000) exceed this by an order of magnitude. Number of sperm transferred did not depend on male body mass or copulation duration (Bloch Qazi *et al.*, 1996), but it did increase with longer female quiescence (Bloch Qazi, 2003). When *T. castaneum* males mated with several females in rapid succession, the number of sperm they transferred showed a dramatic threefold decline (Bloch Qazi *et al.*, 1996), suggesting that males may be limited in their effective mating rate. In spite of declining sperm numbers, no decrease was observed across successive matings in male insemination success or short-term (two days) female fecundity; however, rapid sequential matings did reduce males' paternity share when they mated with previously inseminated females (Lewis, 2004). Differences in sperm length and ejaculate size were found between *T. castaneum* males from 14 different populations (Arnaud *et al.*, 2001c), but the functional significance of such variation has not yet been addressed.

Recent work has shown that *T. castaneum* females can exert control over sperm numbers transferred during mating, and use this as a mechanism for cryptic female choice that favours males in better phenotypic condition (Fedina, 2007). In this study, when male phenotypic condition was reduced by starvation, males that were mated with live females transferred 50% fewer sperm than did fed males, but sperm numbers did not differ between starved and fed males mated with dead females.

Independent of physiological limitations on male ejaculate size, male paternity share is also diminished when females re-mate with another male (Lewis & Jutkiewicz, 1998; Lewis *et al.*, 2005). Therefore, male reproductive returns from any transferred sperm will depend not only on sperm numbers, but also on the female's previous and future mating history.

(5) Male manipulation of rival ejaculates

Sperm competition by removal of rival male sperm from the female reproductive tract has been demonstrated for many odonates, but for very few insects from other orders

(Simmons, 2001). In *T. castaneum* (Arnaud, Haubruge & Gage, 2001b) and *T. molitor* (Gage, 1992) males have backward-pointing spines or brush-like structures on their aedeagus. Gage (1992) proposed that these structures are used by *Tenebrio molitor* males to remove sperm transferred to the female reproductive tract by previous mates. This suggestion was based on electronmicroscopy observations showing that sperm is trapped under the aedeagal spines of males mated to previously mated females but not to virgins. However, this conclusion was questioned by Siva-Jothy *et al.* (1996), who found equal quantities of trapped sperm on the aedeagal spines of a female's first and second mates, suggesting that any sperm removed by this mechanism would be a mixture of a male's own as well as any rival male's sperm. In *T. castaneum*, a similar mechanism has been suggested to promote indirect transfer of another male's sperm (Haubruge *et al.*, 1999). Using malathion-resistance as paternity marker, this study reported that when males mated with recently mated females (within 5 min of a previous mating), they sometimes translocated sperm that had been deposited in the females's bursa by previous males to the next female they mated with. In about 25% of the trials, over 50% of the progeny from these second females appeared to be sired by the "proxy" male. Given the apparent magnitude of the fitness advantage from "proxy" fertilizations, replicating these results would be worthwhile and would provide insight into the importance of this potential sperm competition mechanism.

Another unusual mechanism of offensive sperm competition was described for *Tenebrio molitor* mealworm beetles by Drnevich, Hayes & Rutowski (2000), and involves the inhibition of rival male spermatophores within the female reproductive tract. When two males mated with the same female in rapid succession (within 5 min), the first male's spermatophore failed to release sperm, while the second male's spermatophore went through the normal process of spermatophore eversion and sperm release. Such inhibition of a previously transferred spermatophore resulted in complete loss of first-male paternity. However, this phenomenon is unlikely to occur in *T. castaneum*, because sperm are released from the spermatophore much more quickly (Fedina, 2007).

Males may defend their own sperm against competition from other males by prolonging copulation or by engaging in postcopulatory mate-guarding. Such male behaviours prevent females from remating quickly, increasing the likelihood of sperm storage and fertilization by the guarding male. Gage & Baker (1991) reported that after mating, *T. molitor* males stay mounted or in close proximity to their mate for longer when potential competitors are present. No mate-guarding behaviours have been described for flour beetles.

VI. POST-MATING SEXUAL SELECTION

Arthropods are unique in that females store sperm in specialized organs to fertilize their eggs later, sometimes several months after mating. Most arthropod females also

mate with multiple males. These attributes select for traits promoting male sperm competition and cryptic female choice. Several post-mating mechanisms have been proposed (and some confirmed) that result in biased paternity after a female has mated with several males. Among them is female control over sperm storage and usage for fertilization, and male accessory gland substances influencing female physiology and incapacitating rival sperm (reviewed in Eberhard, 1996; Simmons, 2001; Birkhead & Pizzari, 2002).

Major advances in understanding post-mating sexual selection have been attained by using genetic markers to determine paternity of multiple males. Such markers can be either morphological traits (e.g. body colour), physiological traits (e.g. allozymes, insecticide resistance), male sterility induced by irradiation, or molecular markers (Loxdale & Lushai, 1998; Behura, 2006). In this section, we first review paternity (sperm precedence) patterns that have been demonstrated in flour beetles, and the factors affecting these patterns. We then consider evidence for morphological, physiological and behavioural mechanisms of sperm competition and cryptic female choice that might be responsible for generating these paternity patterns in polyandrous females.

(1) Patterns of paternity in polyandrous females

Starting with the pioneering studies of Schlager (1960), patterns of male paternity when females mate with multiple males have been extensively studied in tenebrionid beetles. Indeed, we may know more about how male paternity changes as a function of time since mating and of intermating interval for *Tribolium* beetles than for any other model organism. In this section we briefly review paternity patterns, and discuss how they may be used to provide insight into how males and females influence sperm utilization.

Paternity patterns have been studied most extensively in *T. castaneum* and *T. confusum*, and some studies have also been made in their relative *Tenebrio molitor* (summarized in Table 1). Schlager (1960) demonstrated that when *T. castaneum* females mated sequentially with different males, for approximately one week the resulting progeny were sired nearly exclusively by the last male to mate. Over the next 2–6 weeks, there was a gradual rise in the proportion of progeny sired by earlier mates. Later studies in *T. castaneum* using various paternity markers have confirmed this temporal pattern of sperm precedence (Lewis & Austad, 1990; Yan & Stevens, 1995; Haubruge, Arnaud & Mignon, 1997; Lewis & Jutkiewicz, 1998; Arnaud, Gage & Haubruge, 2001a; Fedina & Lewis, 2004). In a similar sequential mate-replacement study conducted with *T. confusum*, Vardell & Brower (1978) documented a longer initial period of mixed paternity, followed by an increase to 90% last-male paternity that was maintained for approximately 2 weeks. Such contrasting results for *T. confusum* compared to *T. castaneum* may be explained by differences in spermathecal morphology between the two species (Fig. 3).

Significant within-species variation in male paternity success has been documented in *T. castaneum*. Lewis & Austad (1990) found significant differences in second-male paternity success among male pairs, suggesting that some males have a consistent advantage in sperm competition and/or cryptic female choice. In this study, relative body lengths of the two males were found to be correlated with male paternity success, but other studies have found no effect of male body size on paternity success (Lewis & Austad, 1994; Bernasconi & Keller, 2001). Pai & Yan (2002a) and Nilsson, Fricke & Arnqvist (2003) quantified the effects of male and female genotypes (strains) on both sperm defence (measured as P_1 , the proportion of progeny sired by the first of two mating males) and sperm offence (measured as P_2 , the proportion of progeny sired by the second of two mating males). This study showed that males from some wild-type strains had better sperm offence ability than males from other strains. In addition, a significant male strain by female strain interaction was documented for both P_2 (Nilsson *et al.*, 2003) and P_1 (Pai & Yan, 2002a), providing suggestive evidence for cryptic female choice in *T. castaneum*.

Some variation in the paternity patterns observed among studies is likely due to using different mating regimens (Table 1); some studies allowed each male only a single copulation, while in other studies males and females were housed together for some duration. The latter design does not control for the number of copulations achieved by each male; in these studies last-male paternity is often higher, perhaps due to multiple ejaculates being transferred by the second male. Intermating interval is another factor known to influence sperm precedence. Longer intervals (>24 h) between matings resulted in higher last-male sperm precedence in *T. castaneum* (Arnaud *et al.*, 2001a), presumably due to fewer sperm from previous males being present in the female reproductive tract, with consequently greater relative representation by new ejaculates. With shorter intermating intervals (<1 h), sperm storage from the previous mating is not yet complete, so presumably sperm mixing within the female bursa occurs, resulting in lower last-male paternity share.

Finally, some concern has been raised regarding whether experimental studies of paternity success that use only two males are relevant for natural populations, since females are likely to mate with multiple males. Paternity patterns were similar for *T. castaneum* females mated to either two or three successive males (Lewis & Jutkiewicz, 1998; Lewis *et al.*, 2005). These studies both used experimental designs with a single observed mating by each male, and similar results were found when males and females were assigned a fixed period of co-habitation (Arnaud *et al.*, 2001a). Also when *T. castaneum* males were mated once to females that had previously mated with multiple males, their sperm precedence was comparable to that of doubly-mated females (Fedina & Lewis, 2006). Different patterns were, however, observed for *T. molitor*, where males that mated to females previously inseminated by multiple males fathered only small portion of progeny (Table 1). This pattern of sperm use may be due to the relatively large spermathecal volume compared to male ejaculate size in this species. Differences in female

Table 1. Factors influencing paternity patterns (=sperm precedence) in three tenebrionid beetles: *Tribolium castaneum* and *T. confusum* flour beetles, and *Tenebrio molitor* mealworm beetles. Abbreviations: M = male, F = female, SP = sperm precedence (reported as mean \pm S.E.M., or as a range), ~ indicates that SP was estimated from graphs, P₁, P₂, P₃, proportion of a female's progeny sired by 1st, 2nd, or 3rd male when either 2 or 3 males mate with a female, P_{last}, proportion of progeny sired by the last of multiple males that have mated with a female, * indicates that ranges of means are reported, PGI, phosphoglucosomerase.

Experimental design										
Species	Main effect studied in relation to SP	Female strain	Male strains (in mating order)	- Number of matings per M - Inter-mating interval	Days post-mating	Sperm Precedence		Result	Additional effects studied	Source
						Control	Treatment			
<i>Tribolium castaneum</i>	SP	b/b	+ / +, b/b	- Multiple (over 7 days)	0-7	P ₂ ~ 0.87	P ₂ decreased over time	Mating order of M genotypes	Schläger 1960	
	Individual (M pair) variation in SP	+ / +	b/b, + / +	- None - Multiple (24 h)	15-35 0-30	P ₂ ~ 0.62 P ₂ = 0.62 \pm 0.04	M pair effect, genotype effect	M size, number of mounts, copulation duration	Lewis & Austad 1990	
	M olfactory attractiveness	+ / +	+ / +, b/b b/b, + / + + / +, b/b b/b, + / +	- 24 h - Multiple (24 h)	0-30 0-30 0-30 0-9	P ₂ = 0.34 \pm 0.09 P ₂ = 0.69 \pm 0.05 P ₂ = 0.65 \pm 0.06 P ₂ = 0.10 - 1.00	Positive correlation	M size and mass	Lewis & Austad 1994	
	M tapeworm infection	b/b	+ / +, b/b	- 24 h - Multiple (24 h)	0-1	P ₂ ~ 0.98				
	Male starvation	+ / +	3 b/b, + / +	- None - Multiple (24 h), then single	1-2 2-3 0-3	P ₂ ~ 0.93 P ₂ ~ 0.89 P ₂ = 0.84 \pm 0.05				Yan & Stevens 1995
	SP of malathion-resistant males	Asm	PRm (resistant), Asm	- 24 h - Multiple (6 days)	4-11 0-2	P ₂ = 0.51 \pm 0.06 P ₁ ~ 0.03	P ₁ increased with time post-mating	Copulation duration		Fedina & Lewis 2006
	SP when three males mate	b/b	Two males	- None - Single	30-32 100-102 0-2	P ₁ ~ 0.35 P ₁ ~ 0.27 P ₂ = 0.75 \pm 0.04		Two-day egg production by Fs		Haubruge <i>et al.</i> 1997
			b/b, + / +	- 24 h	7-9 14-16	P ₂ = 0.33 \pm 0.07 P ₂ = 0.37 \pm 0.08		Sperm stored in spermathecae of females mated to one, two, or three Ms		Lewis & Jukiewicz 1998
			Three males: b/b, b/b, + / +		0-2 7-9	P ₂ = 0.67 \pm 0.05 P ₃ = 0.26 \pm 0.06				
			Three males (Asm, PRm) in six orders		14-16 0-30	P ₃ = 0.40 \pm 0.07 P ₁ = 0.11 \pm 0.02				
			+ / +, Rap	- None - Single	0-30 0-30 0-20	P ₂ = 0.09 \pm 0.01 P ₃ = 0.79 \pm 0.02 P ₁ = 0.46 \pm 0.07				Arnaud <i>et al.</i> 2001a
			+ / +, Rap, R ^{phd}	- 24 h	0-20 0-20 0-20 0-20	P ₂ = 0.54 \pm 0.07 P ₁ = 0.24 \pm 0.06 P ₂ = 0.19 \pm 0.05 P ₃ = 0.57 \pm 0.07	Second M lost more paternity than first M after third M mating	SP over time (10 x 2 days)		Lewis <i>et al.</i> 2005

	Repeated male copulations with the same female	b/b	b/b, +/+ (x1)	- Single (first M, 1, 2, or 3 (second M)	0-10	$P_2 \sim 0.57 \pm 0.08$	No effect	P_2 declined over sequential M copulations	Lewis 2004
	Male copulatory courtship (leg-rubbing)	+/+	b/b, +/+ (x2) b/b, +/+ (x3) b/b, +/+ (w/ intact or ablated legs)	- Single	0-10 0-10 0-7	$P_2 \sim 0.58 \pm 0.06$ $P_2 \sim 0.56 \pm 0.12$ $P_2 = 0.49 \pm 0.07$	$P_2 = 0.47 \pm 0.07$	Copulation duration by first and second Ms	Edvardsson & Arqvist 2000
	Inter-mating interval	+/+	b/b, +/+ Three b/b, +/+	- 24 h - Multiple (24 h) - Multiple (24 h), then single - 24 h - Multiple (48 h)	0-10 0-3	(intact Ms) Not reported $P_2 = 0.84 \pm 0.05$	(ablated legs) Not reported	Female quiescence Copulation duration	Bloch Qazi 2003 Fedina & Lewis 2006
<i>PTribolium castaneum</i>	Inter-mating interval	Asm	Asm, PRm, or PRm, Asm	- 24 h - Multiple (48 h)	4-11	$P_2 = 0.51 \pm 0.06$	Higher P_2 for longer intermating intervals	SP dynamics over time (days 0-10, 10-20, 20-30)	Bernasconi <i>et al.</i> 2006
	Arnaud <i>et al.</i> 2001a				0-30 0-30 0-30	$P_2 = 0.69 \pm 0.02$ $P_2 = 0.82 \pm 0.02$ $P_2 = 0.95 \pm 0.01$	No effect of intermating interval	Spermathecal morphology	Bernasconi <i>et al.</i> 2006
	SP of sons of polyandrous /monandrous Fs	+/+	+/+, b/b	- None - 24 h - 14 days - Single	0-14 0-14 from 0-4 to 0-3	$P_1 = 0.27 \pm 0.10$ $P_1 = 0.40 \pm 0.11$ $P_2 = 0.91 - 0.96^*$	Higher P_2 for + + Ms of polyandrous mothers	Offspring number, M size, M genotype order	Bernasconi & Keller 2001
	Effect of M & F strain/genotype, and M X F interaction	+/+	b/b, +/+, or +/+, b/b	- 24 h - Multiple (24 h)	0-7	$P_1 = 0.31 - 0.35^*$ (polyandrous) $P_2 \sim 0.53 - 0.60^*$	Higher P_1 intra-strain		Pai & Yan 2002a
	Phylogenetic distance between M & F strain	(three strains) +/+	(three + + strains) b/b, +/+	- None - Single	0-7 0-14	$P_1 \sim 0.38 - 0.70^*$ (intra-strain) $P_2 = 0.32 - 0.62^*$	F affected P_1 & P_2 ; F X M affected P_2	Copulation duration	Nilsson <i>et al.</i> 2003
	F muscular control (CO ₂ anaesthesia)	(three strains) +/+	(three + + strains) bb, +/+	- 24 h - Single	0-14	(intra-strain) $P_2 \sim 0.69 \pm 0.11^*$	Lower SP with greater phylogenetic distance		Fricke & Arqvist, 2004
	Time & M/F genotype order & combination	(single strain) +/+	+/+, bb (five + + strains) +/+, b/b	- 24h - Single	0-14 0-3	$P_1 \sim 0.32 \pm 0.10^*$ (intra-strain) $P_2 = 0.46 \pm 0.04$	CO ₂ - treated F had slower decline in P_2 over time	Spermathecal morphology	Fedina & Lewis 2004
<i>Tribolium confusum</i>		+/+	+/+, b/b	- 30 min (in air or CO ₂) - Multiple (7 days)	12-15 27-30 0-3	$P_2 = 0.32 \pm 0.04$ $P_2 = 0.15 \pm 0.04$ $P_2 \sim 0.74$	No decrease in P_2 over time		Vardell & Brower 1978
		b/b	b/b, +/+	- None	11-14 0-3 11-14 0-3 11-14 0-3 11-14	$P_2 \sim 0.66$ $P_2 \sim 0.85$ $P_2 \sim 0.79$ $P_2 \sim 0.96$ $P_2 \sim 0.90$ $P_2 \sim 0.94$ $P_2 \sim 0.94$			

Table 1. (cont.)

Species	Experimental design					Sperm Precedence	Result	Additional effects studied	Source
	Main effect studied in relation to SP	Female strain	Male strains (in mating order)	- Number of matings per M - Inter-mating interval	Days post-mating				
<i>Tenebrio molitor</i>	Time, remating interval		Two males sterile and normal in four combinations	- Single	0-4	$P_2 \sim 0.89$	No effect	Frequency and degree of sperm removal by male aedeagus	Sivasubthy <i>et al.</i> 1996
				- <10min - 24 h	12-16 0-4 12-16	$P_2 \sim 0.48$ $P_2 \sim 0.92$ $P_2 \sim 0.19$			
	Remating interval	SS, FF	SS, FF, or	- Single			Complete SP by second M when intermating intervals <5 min		Drnevich <i>et al.</i> 2000
M immune investment (cuticular darkness)			FF, SS (PGI allozymes)	- < 5 min - 15-20 min - 4 h - 24 h - Single	0-16 0-16 0-16 0-16 0-2	$P_2 \sim 0.91 \pm 0.06$ $P_2 \sim 0.63 \pm 0.09$ $P_2 \sim 0.65 \pm 0.07$ $P_2 \sim 0.68 \pm 0.08$ $P_2 \sim 0.50 \pm 0.12$			
		SS, FF	SS and FF:				Dark Ms lost SP over time; light M did not	Egg number compared between high & low-immunity (light/dark) Ms	Drnevich <i>et al.</i> 2002
			dark, light, or light, dark	- 20 min	7-9	$P_2 \sim 0.51 \pm 0.12$	$P_2 \sim 0.55 \pm 0.11$		
Number of mates and inter-mating interval		SS, FF	Three males (SS & FF in all possible combinations)	- Single	0-2	(light second M) $P_1 \sim 0$	Similar last-M SP when two or three Ms mate; SP lower when many Ms mate	SP over time (up to 17 days); egg number and hatch rate over time	Drnevich 2003
				- <5 min	0-2	$P_2 \sim 0$ $P_3 > 0.96$			
			SS, FF, or FF, SS	- Single - 15-25 min - Multiple (6 days), then single	0-2 0-2 0-2 0-2	$P_1 \sim 0.18 \pm 0.05$ $P_2 \sim 0.29 \pm 0.07$ $P_3 \sim 0.61 \pm 0.07$			
			- 6 h	7-9	$P_{last} = 0.16 \pm 0.03$				

spermathecal structure can influence sperm utilization patterns, as reviewed in the next sections.

(2) Sperm storage

Sperm movement from the site of deposition in the bursa copulatrix into the female spermatheca, where sperm are stored, is an important process because females use stored sperm to fertilize their eggs for long periods after mating. In *T. castaneum* females can use such stored sperm for fertilizations for over four months following a single mating (Bloch Qazi *et al.*, 1996). In *T. castaneum*, sperm are released from the spermatophore almost immediately after transfer, and begin to move into the spermatheca often even before copulation ends (Bloch Qazi *et al.* 1996). Sperm storage is largely complete within 1 h, by which time the spermatheca contains 4,000–5,000 sperm (Bloch Qazi *et al.*, 1996). This represents only about 4% of total sperm transferred, and another 10% remain in the anterior bursa. Remnants of the spermatophore and remaining sperm (approximately 85%) are generally expelled from the female bursa soon after mating (Bloch Qazi *et al.*, 1996; Fedina, 2007). Bloch Qazi, Aprille & Lewis (1998a) investigated whether females play an active role in moving sperm into storage by comparing sperm quantities in control females and in females that were anaesthetized (to reduce contractions of their reproductive tract musculature). At 30 min post-mating, three- to 11-fold fewer sperm were stored in the spermathecae of anaesthetized females. Anaesthesia did not directly affect sperm motility (Bloch Qazi, Aprille & Lewis, 1998a) or subsequent long-term survival of stored sperm (Fedina & Lewis, 2004). Reduced sperm storage was shown to decrease a male's subsequent paternity share when in competition with another male (Fedina & Lewis, 2004). While these studies have demonstrated that females can actively control the movement of sperm into storage, they did not show that females can selectively bias paternity by differentially storing sperm of particular males. The only indirect support for female selective sperm storage based on male quality is that females mated to starved compared to fed males stored fewer sperm relative to the total number transferred by these males (Fedina & Lewis, 2006).

(3) Female spermathecal morphology

The shape of female sperm storage organs influences sperm displacement and the dynamics of fertilization. Also, their volume relative to male ejaculate size is important because it may affect the likelihood that previously stored sperm will be displaced when the female remates. For instance, in *T. castaneum* a single spermatophore is large enough to distend the female's bursa (Fedina & Lewis, 2006), while in *T. molitor* the bursa can accommodate up to 12 spermatophores (Drnevich, 2003). In *T. castaneum*, a single copulation generally fills the female spermatheca to about two-thirds of its capacity (Lewis & Jutkiewicz, 1998), while second and further ejaculates result in displacement of previously stored sperm (Lewis & Jutkiewicz, 1998; Lewis *et al.*, 2005); by contrast, in *T. molitor* the spermatheca is not filled to capacity even after three matings (Drnevich, 2003).

Major differences exist between tenebrionid species in their sperm precedence dynamics; in *T. castaneum* last-male sperm precedence is high initially and decreases over time, while in *T. confusum* temporal changes are lacking. These patterns may be explained by female spermathecal shape (Vardell & Brower, 1978); the long, narrow spermathecal tubules of *T. castaneum* are likely to promote initial sperm stratification, while the open, U-shaped spermatheca of *T. confusum* will promote mixing of sperm from multiple males. In *T. castaneum*, stratification of different males' sperm in the female spermatheca was supported by the finding that when a female re-mates with a third male, there is disproportionate loss of paternity by the female's second mate compared to her first mate (Lewis *et al.*, 2005).

Within species, variation in spermathecal morphology and size also has the potential to influence paternity. In *T. castaneum* female spermathecal morphology was highly variable in tubule number, shape (length and width), and positioning (Fedina & Lewis, 2004; Bernasconi *et al.*, 2006). It appears unlikely that these females can partition sperm from different males into different spermathecal tubules since there is no visible evidence of appropriate muscular sphincters (Fedina & Lewis, 2004). However, females may still use strategic sperm expulsion *versus* uptake to manipulate the relative representation of a male's sperm in storage. One study found a negative correlation between second-male paternity success and females' spermathecal volume (Fedina & Lewis, 2004, but see Bernasconi *et al.*, 2006). This negative relationship may reflect greater storage and/or reduced displacement of first-male sperm in females with larger storage organs.

(4) Male accessory gland substances

During copulation males transfer accessory gland substances to females in addition to sperm. In many insects, these substances have been shown to exert a profound influence on female reproductive physiology and behaviour (Eberhard, 1996; Simmons, 2001). For instance, in *Drosophila* spp., male accessory gland proteins increase ovulation rate and suppress female receptivity (Wolfner, 2002). *Tribolium* spp. males possess two pairs of accessory reproductive glands, short (also called bean-, rod-, or pear-shaped) and long (tubular) accessory glands. Histological studies in *T. brevicornis*, *T. freemani*, and *T. anaphe* have identified at least five differentially staining cell types forming spatially distinct regions within short male accessory glands, and one uniform cell type within long glands (Sevener, Dennard & Grimnes, 1992; Roberts & Grimnes, 1994; Novaczewski & Grimnes, 1996). While these studies suggest that male accessory glands produce mostly proteins and some carbohydrate-containing macromolecules, detailed molecular and physiological characterization of these substances is lacking. However, indirect evidence points to the potential effects these male gland substances might have on female physiology. Thus, reciprocal matings of males and females from different *T. castaneum* strains demonstrated the influence of male genotype and male X female genotype interactions on female mating rate, reproductive performance,

and longevity; increased reproductive investment was observed in females mating with heterogamic compared to homogamic males (Attia & Tregenza, 2004; Nilsson *et al.*, 2002). The presence of oviposition stimulants in male ejaculates is supported by the observation that females' lifetime fecundity increased over 10% when female mating frequency with the same male was increased from 0.5 to three times a week (Nilsson *et al.*, 2002). Recent sequencing of the *Tribolium castaneum* genome (Brown *et al.*, 2003; Wang *et al.*, 2007) should greatly facilitate the functional characterization of accessory gland proteins.

Extensive studies have characterized male accessory gland proteins in *Tenebrio molitor*. Several proteins produced by long accessory glands may perform hormone-transport and sperm-capacitation functions, based on their molecular characteristics and sequence similarity with male proteins from *Drosophila melanogaster* (Paesen & Happ, 1994, 1995; Feng & Happ, 1996). In addition, the male bean-shaped accessory glands of *T. molitor* secrete trehalase into the male spermatophore; it serves as a structural component and may also provide sperm nutrition/activation in concert with trehalose found in females (Yaginuma & Happ, 1988; Yaginuma *et al.*, 1996).

(5) Female remating

Female re-mating can be considered as an additional mechanism for post-mating female choice (Eberhard, 1996; Simmons, 2001). Because in *T. castaneum* the last male to mate with a female fertilizes the majority of eggs laid over the next few days, and because mates are generally available, females could potentially manipulate offspring paternity by re-mating with a higher quality male. Nilsson *et al.* (2003) found significant male by female genotype interactions in how long it took *T. castaneum* females to remate depending on the genotype of her previous mate, implying that females may control their re-mating interval in order to bias paternity. In agreement with that, females were more likely to accept a male's spermatophore during remating if they had previously been inseminated by a less attractive male (Fedina & Lewis, 2007).

In *Tenebrio molitor*, females were also more likely to re-mate when they mated first with smaller males, and also re-mated more quickly when their first male was infected with intestinal parasites (Worden & Parker, 2005).

VII. CONCLUSIONS

(1) The life history of *Tribolium* spp. flour beetles is representative of many coleopteran human commensals. Juveniles and adults live together in aggregations competing for the same food resource, and cannibalizing eggs and pupae at high population densities. Adults are long-lived, and females are highly polyandrous and lay eggs continually. These life-history and mating-system features are likely to influence mechanisms of sexual selection across three sequential stages of reproduction: pre-mating, peri-mating, and post-mating.

(2) Long-range attraction in *Tribolium* spp. is mediated by several chemical compounds, but flour beetles do not appear to use long-range chemical signals to discriminate among potential mates before mating. This may reflect the high mate availability associated with high population densities in laboratory cultures or field populations. In the absence of pre-mating discrimination by females, a series of peri-copulatory and post-copulatory mechanisms of paternity biasing has evolved.

(3) During mating, several mechanisms of sexual selection influence paternity in *T. castaneum*. Although inconsistent, most evidence suggests that male copulatory behaviours (leg-rubbing) do not affect paternity. However, females exercise cryptic choice while copulation is in progress by accepting or rejecting male spermatophores, and also by adjusting sperm quantity accepted depending on male phenotypic quality.

(4) Several mechanisms of paternity biasing may also operate after mating in *Tribolium* flour beetles, but more studies are needed to demonstrate this phenomenon clearly. Thus, movement of sperm into storage is mainly under female control, but demonstration that females use this mechanism to favour certain males is lacking. Re-mating by females is another potential mechanism by which females could bias offspring paternity, and *T. castaneum* females are more likely to accept spermatophores when they remate with more attractive males. There is also indirect evidence that substances produced by male accessory glands in *Tribolium* spp. increase female oviposition rate, but this area requires further investigation.

(5) Sexual selection during earlier episodes may be reinforced or counteracted at later reproductive stages, and antagonistic coevolution between the sexes may be played out across reproductive stages. To date only a few studies have measured the relationship among different reproductive episodes in any organism. In *Tribolium castaneum* male olfactory attractiveness is positively correlated with both insemination success and paternity share, suggesting consistent selection across these different reproductive stages. To better understand the evolution of sexual traits, studies in other taxa need to consider sexual selection taking place across sequential reproductive episodes.

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