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FEMALE MATING BEHAVIOR IN THE BEETLE *TENEBRIO MOLITOR*: POLYANDRY AND PARASITE-MEDIATED SEXUAL SELECTION

DISSERTATION

Presented in Partial Fulfillment of the Requirements for the Degree Doctor of Philosophy in the Graduate School of The Ohio State University

By

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ABSTRACT

Female grain beetles, *Tenebrio molitor*, mate many times over their lives, suggesting that they may not be choosy when selecting mates. Grain beetles are hosts for the tapeworm, *Hymenolepis diminuta*, and models of parasite-mediated sexual selection predict that females benefit by selecting non-infected mates. We explored the consequences of multiple mating to females experimentally, and then examined whether polyandrous females showed any mate choice based on the infection status of males.

Females that mate with more than one male may derive both and genetic and non-genetic benefits. In our laboratory experiments, singly mated females produced 44.8 % fewer offspring than females that copulated multiply with the same male, but females that mated with four different males produced 32 % more offspring than females that mated four times with the same male. The results suggest both material and genetic benefits that favor females that delay or reduce oviposition until they have copulated with multiple males.

The mechanism responsible for the effects remain unclear, as we failed to find evidence that male-derived nutrients benefited food-deprived females, and genetic incompatibility was not detected in rates of egg hatchability. Sexual characters may reveal infection by parasites. We measured female preference for odors produced by infected or uninfected male beetles. Beetles did not discriminate against odors from
infected beetles of the same sex, but females were less attracted to odors from parasitized males.

Females that mated with highly infected males produced fewer offspring than females mated to uninfected males. These results are consistent with models of parasite-mediated sexual selection. We examined the effect of parasitism on paternity using RAPD markers when females mate multiply. Infected males experienced a 29% reduction in sperm precedence compared to non-infected males.

Finally, we examined the effects of infection on mate choice and polyandry in a field-caught strain of beetle. Given a choice, females copulated more often with non-infected than infected males, and these females were more likely to remain monogamous than females presented with two non-infected males. The results demonstrate that, despite the benefits of polyandry, females express both precopulatory and postcopulatory mate choice.
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CHAPTER 1
INTRODUCTION

Darwin (1871) described exaggerated male traits in species as benefiting their bearer by increasing his relative mating success. These traits (elaborate coloring, weapons, or ornaments) are believed to be favored by both the advantages they provide males in their competition over females as well as by female preference. Since Darwin’s original treatise on the subject, many models emerged that have attempted to explain the evolution of elaborate sexual traits and female preferences.

Exactly why elaborate sexual displays evolved remains a much speculated and debated question. The major explanations for the evolution of female mating preferences based on male ornamentation are (Andersson 1994): 1) Fisherian self-reinforcing selection; 2) indicator mechanisms; 3) selection for species recognition; 4) direct phenotypic benefits to choosy females; 5) sensory bias; and 6) mating synchronization.

Fisher (1958) proposed that if female preference for a particular male trait becomes common, males with the trait will experience an increase in their mating success. Sons of females that prefer the particular trait will also gain in mating success if the male trait is heritable. Therefore, a positive feedback loop is established. Female preference and the male trait would become associated, and the frequency of each trait (male trait and female preference) would increase. Under this scenario of “runaway selection”, female
choice would select for continually more exaggerated forms of the male trait until natural selection limits further exaggeration.

In explaining the Fisherian runaway process (above), I began with the female preference trait already common in the population. Some process must be responsible for starting this sequence of events. Fisher (1958) suggested that the male trait may initially provide fitness benefits through natural selection. For example, if slightly larger fins on a male fish result in higher fitness through greater swimming speed, then females with a preference for larger fins would be at an advantage since their offspring would express the larger fin trait (assuming the trait is heritable). Alternatively, preexisting sensory bias may explain how female preference could initially be common. For example, in the arctiid moth, *Utetheisa ornatrix*, the male emits a pheromone that is derived from host plant alkaloids (Conner et al. 1981). Females will only mate after being stimulated with this chemical (Conner et al. 1981). This male signal may have taken advantage of a preexisting female attraction to host plant odors (Andersson 1994). Therefore, in the case of sensory bias, female preference or attraction will be widespread at the initial development of the sexual ornament.

Indicator models assume that male ornaments indicate male fitness (Williams 1966). High fitness is not the result of the ornament or sexual trait itself. For example, a healthy bird may be better able to produce brightly colored feathers than a sick rival, so feather color indicates the relative health of potential mates. Here I include both genetic and direct phenotypic benefits models under the broad category of indicator mechanisms. Genetic or “good genes” models predict that the ornament is a good indicator of the
heritable fitness in the signaler. Female preference for such traits would be advantageous because a female's offspring would benefit from the inherited traits associated with higher fitness. Good genes models have shown that an indicator mechanism can lead to the fixation of a male trait given that female preference for the trait is above a threshold value (Maynard Smith 1985; Kirkpatrick 1986; Pomianski 1987, 1988). It is believed that the Fisherian (or other) process would need to be invoked in order to have the female preference trait reach the threshold value necessary for the indicator models to take effect (Andersson 1994).

Male ornaments may also act as indicators of a male's ability to provide parental effort or sperm. This type of indicator mechanism occurs when direct phenotypic benefits to females are advertised by the male trait (Heywood 1989; Hoelzer 1989). Fitness does not need to be heritable since the benefit is measured in the parental effort or fecundity of the signaler.

Two other explanations for the development of sexual traits and female preferences are species isolation and mating stimulation. Species-specific sexual traits and female preferences would be advantageous because this would reduce or eliminate the chance of an individual making a costly mistake by mating with a member of a different species. Species isolation, alone, seems inadequate to explain the development of some extreme sexual traits. Lastly, Marshall (1936) suggested that male traits might stimulate females so that mating occurs during the optimum time for fertilization. He suggested that bright colors in birds function after pair formation to stimulate ovulation in females,
thereby synchronizing reproductive cycles between mates. Sexual selection could still act in such a scenario if females were more likely to pair with brighter males, or if females left dull males for brighter ones (Andersson 1994).

Trivers (1972) reasoned that relative parental investment determines the amount of sexual selection that is to be expected in a species. The sex that invests less into offspring production should compete for mates, while the sex that provides the greater parental investment is expected to show greater discrimination in mate choice. This should be the case because the sex that invests less will be able to invest in offspring more often, and the result will be that the operational sex ratio will be biased towards the sex that invests less per offspring (Bateman 1948; Williams 1966). If the sexes invest equally in offspring, female and male mate choice are expected to be similar in strength. Support for these predictions is found in bushcrickets (Orthoptera: Tettigoniidae). Simmons and Gwynne (1993) demonstrated that the reproductive role of each sex in bushcrickets is determined by food availability. When food is limiting, female parental investment becomes limited so that male parental investment (in the form of a nuptial gift) equals or surpasses that of the female. Under conditions of limited food resources, females compete for males and males express mate choice (Gwynne and Simmons 1990). This reversal of sex roles does not occur, however, when food is more abundant.

Although some form of cooperation is required in most species, mating can be viewed as a conflict of interests between males and females in their attempt to be best represented in future generations. Parker (1979) refers to this conflict as an "asymmetry
of aims [that] may ultimately be a much more important determinant of the evolutionary outcome than selection intensity, though the result must depend on the interactions between the two”.

Typically, females are expected to show mate choice, while males are expected to mate readily with as many females as possible (Andersson, 1994). However, recent evidence has revealed that multiple mating by females is common, and that females often accrue benefits from mating with more than one male (Thornhill and Alcock, 1983; Gowaty, 1985; Birkhead and Moller 1993, 1998; Gray 1997). When selection favors multiple mating by females, sexual selection may act in both precopulatory mate choice and in behaviors and mechanisms that occur postcopulation that have the potential to influence fertilization success of a male’s sperm.

Benefits of Polyandry

Traditionally, the expected mating strategies for females have been viewed as being quite distinct from those of males. Bateman (1948) demonstrated with Drosophila sp. that male, but not female, reproductive success is tightly correlated with mating success. However, molecular tools that examine paternity in field and lab populations have yielded evidence that females, once believed to be monogamous, actually mate with multiple males (see Birkhead and Møller 1998). Several hypotheses have been proposed which provide adaptive explanations for multiple mating by females. These benefits fall into two main categories, nongenetic (material) or genetic.

Among the nongenetic benefits, the most obvious is fertilization assurance (走向-
1980). If some males or copulations fail to provide a female with healthy sperm, then monogamous females risk the possibility of complete reproductive failure. In some species, males provide spermatophores or nuptial gifts that contain substantial nutrients that females use in egg production or somatic maintenance (e.g., Forsberg and Wiklund 1989; Gwynne 1984). In water striders, mating benefits females by reducing harassment from other males (Rubenstein 1984, Arnqvist 1989). Ejaculates may contain compounds that stimulate egg maturation and/or oviposition rate (e.g., Destephano et al. 1982). In addition, mating with more than one male may provide a replenishment of sperm if one mating provides insufficient sperm to fertilize all the eggs a female may produce in her lifetime (Thornhill and Alcock 1983).

The genetic benefit hypotheses predict an increase in mean offspring fitness with multiple mates. Unlike some material benefits in which additional resources may be acquired through mating multiply with the same male, genetic benefits result from the acquisition of sperm from two or more males (Tregenza and Wedell 1998). Polyandry (defined here as mating with more than one male) may be advantageous by increasing the genetic diversity of a female’s offspring (Hamilton 1987; Brown 1997). This appears to be the case for bumblebees where higher within-colony genetic variation leads to lower levels of parasites (Liresh and Schmid-Hempel 1998). Other hypotheses have focused on the potential for sperm competition or the filtering of sperm within females, either actively or passively, to bias fertilizations towards high quality males or males that are simply better genetic partners for a given female. If sperm competitiveness and offspring quality
are correlated, then females may increase their fitness by mating multiply (Madsen et al. 1992; Watson 1998). Additionally, sperm competitiveness may be heritable, resulting in polyandrous females producing sons that produce more competitive sperm than sperm produced by sons of monandrous females (Keller and Reeve 1995).

Another set of genetic hypotheses focuses on how multiple mating increases the diversity of sperm within a female’s reproductive tract, enabling the screening of sperm based on its genetic compatibility with the genotype of the egg (Zeh and Zeh 1997a,b). Gamete incompatibility as a result of inbreeding (Stockley et al. 1993) or other genetic factors (Zeh and Zeh 1997a,b) may be reduced or avoided completely by mating with multiple males. Support for these hypotheses comes from studies that have found biased paternity towards more genetically compatible mates (Olsson et al. 1996; Wirtz 1997; but see Stockley 1997) and increased survivorship of embryos produced by females that mated with more than one male (Madsen et al. 1992; Zeh 1997; Tregenza and Wedell 1998; Newcomer et al. 1999).

**Parasite-mediated sexual selection**

In Fisherian runaway selection, the attractive traits signal nothing about the potential quality of the mate (Zuk 1992). However, in “good genes” models, individuals with the best secondary sexual characters also have the best genes for survival and reproduction. This could occur if the secondary sex characters are costly handicaps (Zahavi 1975) and males (most often) are signaling their ability to survive despite having such costly ornamentation or displays. The ornaments would demand relatively less cost
for truly genetically superior individuals. Another possibility for good genes selection (or perhaps an extension of the handicap principle) is that elaborate displays or characters reveal an individual's resistance to parasites (Hamilton and Zuk 1982).

According to the Hamilton and Zuk (1982) hypothesis, elaborate displays and secondary sex characters evolved because they are indicators of resistance to parasites. The choosy sex will gain an increase in fitness by mating with disease-resistant individuals that passing resistance to their offspring. Thus, resistance to infection must be heritable. Hamilton and Zuk suggested that genetic cycling in coadaptation between the host and parasite maintains variability in genetic resistance. This constant variation prevents fixation of “good” genes that would eliminate the fitness information contained in elaborate secondary characters.

Two predictions were presented in Hamilton and Zuk’s hypothesis: (1) an interspecific positive correlation will be found between degree of sexual showiness and parasite load and (2) an intraspecific negative correlation between parasite load and mate choice (and thus mating success).

The majority of interspecific tests have been performed on avian taxa. This is probably due to the vast amount of information available on bird behavior, ecology, and parasite levels, as compared to other taxa (Zuk, 1992). Originally, Hamilton and Zuk (1982) ranked bird species independently with respect to parasite loads, plumage brightness, and male song. Positive correlations were found between parasite load and three characteristics: male brightness, female brightness, and male song complexity. This
study has been criticized for the non-quantitative measurement of brightness and the lack of control of possible confounding factors such as phylogeny and species ecology (Read 1990; Zuk 1992). Some researchers have attempted to control for these variables with mixed results (Read, 1990). For example, in two approaches that examined showiness and parasite load, Read (1987) found that, after controlling for taxonomy and some ecological factors, the positive correlation was maintained in European and North American birds. However, Read and Harvey (1989) found that the correlation became nonsignificant once phylogeny was controlled. Significant positive and nonsignificant correlations have been found for the relationship between sexual dichromatism and parasite load in fish (reviewed in Read, 1990).

Zuk (1991) found that neotropical, resident bird species do have a positive correlation between brightness and parasite load, whereas no significant correlation exists for neotropical migrant species. Her hypothesis is that continuous coadaptation cycles between the host and the same parasites are more likely to be found in resident species than migratory species.

Read (1990) stated that the null hypothesis (showiness and parasites not correlated) cannot falsify the hypothesis since many post hoc explanations can be made. Investigating the wrong parasite taxa, subjective measurement of host brightness or showiness, and differences in techniques determining parasite load among the various species, are all possibilities. Consistent, multi-taxa, positive correlations for the interspecies prediction are necessary to provide a significant evaluation of the theory.
Finally, the most difficult problem is one of "which came first". If Fisherian choice for arbitrary characters has caused the proliferation of these costly displays or ornaments, perhaps the species with these characters are more susceptible to parasites (Read, 1990). This could occur by resources being directed toward secondary sex characters at the expense of immune system function. The difficulty of determining the true cause of any positive correlations between showiness and parasite load seems insurmountable.

Many intraspecific tests have also been conducted and, as with interspecific tests, results have shown mixed support. Taxa tested have been more diverse than those in interspecific tests (insects, fish, amphibians, reptiles, birds), although birds still have received the majority of attention (Read 1990; Zuk 1992; Hamilton and Poulin 1997). Intraspecific assumptions of the Hamilton and Zuk hypothesis include (1) resistance to infection is heritable, (2) host fitness is decreased by parasites, (3) resistant males are preferred over less resistant males in mate choice, and (4) expression of secondary characteristics is negatively correlated with parasite load (Read 1990, Zuk 1992). Experiments have supported one or several of these assumptions. However, very few studies have tested all of the assumptions. One thoroughly studied species is the barn swallow (Hirundo rustica). The mite, Ornithonyssus bursa, negatively affects male fitness and tail length, and females prefer less parasitized males and males with the longest tails (Møller 1991). Cross fostering experiments appear to indicate that parasite resistance is heritable (Møller 1990). Additionally, support for all of the major predictions and assumptions has been found in jungle fowl (Zuk et al. 1990) and guppies (Kennedy et al. 1990).
However, several studies have not supported the correlation between parasite level and the quality of sex characters. A prediction of this hypothesis is that parasite infection should disproportionately affect secondary sexual characteristics over non-sexual characters. This would be the case if sexual characteristics are the primary signal females (usually) use to judge health of males (Zuk, et. al. 1990). Zuk et al. (1990) found that male red jungle fowl (*Gallus gallus*) that were infected as chicks with an intestinal nematode differed from uninfected control males in ornamental characteristics (comb length, tail length, and comb color, etc.) to a greater extent than non-sexual characteristics (body size, tarsus length, etc). Females chose control males more often than infected males. Among the studies that appear to support the hypothesis, many are problematic. In some, the parasites tested (e.g. lice and mites) have been ones that could be transmitted horizontally (directly between two conspecifics). These studies do not support “good genes” models exclusively because an alternative hypothesis for this observation is that individuals avoid infected conspecifics and reduce their chances of contracting the infection themselves. Also, other studies have focused on birds in which biparental care is known. The choosy sex in these cases may be choosing healthy males because they provide better parental care, not better genes. Parasite/host models should be selected carefully to rule out these alternative hypotheses. Studies of this kind are few in the current literature.

One very thorough study by Rosenqvist and Johansson (1995) of a pipefish, *Syngnathus typhle*, examined the effects of a physical display produced directly by a
trematode parasite, *Cryptocotyle* spp. The parasites produce black spots in the skin of the pipefish. Rosenqvist and Johansson found that male pipefish (that are solely responsible for the care of offspring) were more attracted to non-parasitized females than infected (spotty) females. This discrimination appears to have a sexual basis since males did not avoid infected males. In this study, parental care and infection avoidance were eliminated as possible reasons for mate choice. The results seem supportive of good genes selection exclusively; however, this is not the case. Rosenqvist and Johansson found that heavily parasitized females produced fewer eggs than their less infected counterparts. Since copulation in this species is conspicuous and may attract predators, selection should cause males to choose to mate as few times as necessary to maximize their offspring production. Therefore, males would directly benefit from mating with less infected females since they could fertilize more eggs per mating effort.

The pipefish model is unique since the signals (black spots) are produced directly by the parasite and do not (presumably) involve secondary sex characters already present. However, direct benefits are likely in most, if not all, the other host-parasite systems studied to date. According to the Hamilton and Zuk hypothesis, the parasite must have some negative impact on host fitness. Reductions in either sperm or egg production are a likely route for such reductions in host fitness. For example, infection by metacestodes of *Hymenolepis diminuta* have been shown to reduce production of viable eggs in females of two species of beetles, *Tenebrio molitor* and *Tribolium confusum* (Hurd and Arme, 1986; Keymer, 1980). As with other competing hypotheses, it is difficult to show exclusive
support for one or the other. Exclusive support for a direct benefits model would be shown if resistance was not heritable. Conversely, exclusive support for the good genes model could only be produced from a system where viable egg or sperm production was not affected by parasite load (at least not at parasite levels being tested). It seems likely that both direct benefits and resistance benefits to an individual’s offspring are often the consequences of mate choice.

Folstad and Karter (1992) introduced a verbal model by which honest signals of parasite resistance could be maintained. Their model, the immunocompetence handicap, states that elaborate sex characters are regulated by the endocrine system, and hormones stimulating development of these characteristics have a negative feedback on the immune system. According to this hypothesis, male birds with the brightest plumage are best able to cope with the testosterone-induced suppression of their immune system. Males with the good genetic resistance have an extra advantage to fighting off parasitic infection that compensates for the costs (in terms of reduced immune response) of their elaborate displays (Zuk, 1992). Some evidence does support the components of this model and is discussed by Moller and Saino (1994). If this model is correct, then cheating would be unlikely, since the production of elaborate displays would have a higher cost to low quality individuals than high quality ones. It is this unequal ability to pay the cost that would make these displays “honest” (Moller and Saino, 1994).

Finally, infection by parasites has been shown to affect both female mate choice and male mate choice (Rosenqvist and Johansson 1995). However, I have not found an
example of both male and female mate choice being examined in the same species. Interestingly, Hamilton and Zuk (1982) found a correlation between incidence of parasite infection and female brightness as well as male brightness but not with sexual dichromatism. It is possible that sexual selection for health could operate in both sexes simultaneously (Hamilton and Zuk 1982). Recent evidence suggests that male mate choice may be common even in species where females provide the primary parental care (Grant et al., 1995). These possibilities in relation to the Hamilton and Zuk hypothesis need to be explored.

The research presented here examines two important aspects of the mating system of the grain beetle, *Tenebrio molitor*. First, benefits and costs associated with multiple mating are examined in female beetles. Both genetic and nongenetic benefits of polyandry are explored in laboratory beetles by examining the effects of mating multiply to the same male or different males. Next, the effects of the parasite, *Hymenolepis diminuta*, on sexual selection in the beetle, *Tenebrio molitor*, are studied. The parasite/host interaction is divided into three experiments examining the affect of the parasite on male mating success and reproductive success. This host/parasite system is one in which many of the direct benefits for selecting uninfected mates can be eliminated. First, the attractiveness of odors from infected and noninfected male beetles is compared. In addition, the reproductive success of females mated with either a noninfected or infected male is measured. Then the reproductive success of infected and noninfected males is examined under the condition of sperm competition (a typical situation given the mating rates of females). Finally, the
effect of polyandry and precopulatory and postcopulatory female mate choice is explored using the progeny of field-caught beetles.

_Tenebrio molitor_

The yellow mealworm beetle, _Tenebrio molitor_ L. (Coleoptera: Tenebrionidae), is a cosmopolitan pest of stored grain products. The beetle is mostly nocturnal and prefers damp, decaying grain and livestock feed in or near agricultural developments (Cotton 1927, 1956). Although adults spend much of their time on the surface, females lay their eggs buried in foodstuff such as grain (Gerber 1975). In the natural environment, time to hatching is temperature-dependent, ranging from 4 to 19 days (Cotton 1956). The majority of _T. molitor_’s lifecycle is spent in the larval stage, and the duration and number of instars during of this stage is variable. Although larvae often reach their maximum size (about 2.4 cm) in only 3 months, larvae usually overwinter in temperate climates and do not pupate until spring, making the total length of the larval stage 6 to 9 months (Cotton 1927). Larvae migrate towards the surface to pupate and emerge as adult beetles in approximately 1 to 2 weeks (Cotton 1927; Thompson 1995).

Adults of _T. molitor_ are not sexually mature until three to four days after emergence, at which time they typically have copulated at least once (Gerber 1973). In the laboratory, adults live two to four months (pers. obs.), and females oviposit throughout most of their lives (Cotton and St. George 1929). Male and female adults are virtually indistinguishable, but pupae can be readily sexed by examination of caudal papillae on the ventral side of the 7th visible abdominal sternite (Bhattacharya et al. 1970).
The papillae are relatively large and widely diverging structures in females compared to the small, blunt papillae found on males. Shortly after emergence, adults are tan to light brown, but attain a dark brown to black color a few days later. Beetles range in length from 17 to 25 mm (unpub. data). Although *T. molitor* has a complete set of metathoracic wings, laboratory-cultured beetles and beetles caught at agriculture facilities near Columbus, Ohio, are unable to fly or are very poor fliers (pers. obs.).

Both sexes produce pheromones that are attractive to the opposite sex (August 1967, 1971; Happ 1969). Through bioassay techniques, several researchers have examined the production of pheromones in both sexes. Pheromone production peaks at about 4 to 5 days and at 7 to 8 days after adult ecdysis in females and males, respectively (Tschinkel et al. 1967; Menon 1976). This peak roughly coincides with the time of sexual maturation in each sex (Gerber 1975). In females, pheromone production and egg maturation have been shown to be independent of food intake by adults (Menon 1970). The corpora allata (CA) controls pheromone secretion by females (Menon 1970), but apparently not by males (Menon 1976). Removing the CA from adult females significantly reduces pheromone secretion, but this effect can be partially alleviated by the application of juvenile hormone analogues (Menon 1970). The identity of all of the pheromones produced by the sexes remains unknown, but Tanaka et al. (1986) isolated the volatile pheromone produced by females and identified it as 4-methyl-1-nonanol.

*Tenebrio molitor* has a polygynandrous mating system. Both males and females copulate throughout their lifespan (pers. obs.). Copulation stimulates both oocyte
development and oviposition (Mordue 1965; Gerber 1967, 1975). Happ (1969) suggested that male pheromones stimulate increased egg laying rates because he observed virgin females extruding their ovipositors when he exposed them to male odors, but Gerber (1973) discounted the role of pheromones because in his experiments he found that virgin females laid very few eggs even though females were kept in the same room as males (and presumably exposed to volatile male pheromone). This question, however, has not been resolved because neither Gerber (1973) nor Happ (1969) specifically tested for this interaction, and Gerber's (1973) findings do not address whether contact pheromones, which are nonvolatile, affect oviposition rates.

In the lab, females and males will mate several times in a given day, and both sexes remain receptive throughout their lives (unpublished data; pers. comm. J. Drnevich). Under these conditions, precopulatory mate choice by females may be relaxed, but postcopulatory selection (via cryptic female choice or sperm competition) should be intense. Although females may exhibit some preferences when several males are present, females should mate with low to moderate quality males when they are encountered sequentially, as long as the signals received by females meet a critical threshold value (Alexander et al. 1997). This is particularly true if females accrue benefits, either material or genetic, through multiple mating. Either active or passive postcopulatory bias of sperm use would result in disproportionate fertilization success between males. The highly branched morphology of the spermatheca (Drnevich et al. in press), or sperm storage organ, suggests that selective sperm storage is possible.
The cost of producing a spermatophore by male *T. molitor* is certainly not as great as that found in some crickets (30-40% of male body mass, Vahed and Gilbert 1996) or butterflies (15-30% of body mass, Wiklund and Forsberg 1991). By weighing beetles immediately before and after mating, spermatophores mass is estimated to be approximately 0.25% of a male’s body mass (unpublished data). This estimate concurs with a conservative estimate of less than 0.3% of a male’s body weight based on average spermatophore size reported in Gadzama and Happ (1974). Therefore, the quantity of material delivered into a female is small. However, it is possible that some protein component of the spermatophore is particularly limiting in the diet of this species and even small quantities of this substance can significantly increase reproductive success (Gwynne 1988). Gage and Baker (1991) found that males that are placed with other males five minutes prior to copulation deliver significantly more sperm to females than isolated males. This suggests that sperm production may be a limiting factor for males. In fact, males are unreceptive for approximately 30 minutes postcopulation (unpublished data).

One disadvantage with using *T. molitor* as a study organism is that its lifecycle is long compared to some insects. Even in the laboratory environment, one can only expect two generations of beetles per year (Thompson 1995), so *T. molitor* may not be the best organism for multigenerational studies. However, *T. molitor* represents a nearly ideal model system to test whether females benefit by mating multiply even when it does not appear that males produce a substantial nutritive donation. Because both males and females copulate multiply, this system is ideal for experiments where individuals are
assigned to a given number of copulations with the same or different individual(s). In this way, this species represents one extreme in mating behavior; therefore, the effects of multiple mating are expected to be substantial. The fact that copulations are short (< 3 minutes) also makes this system convenient for observing complete copulations and assigning multiple copulations within a single day. *Tenebrio molitor* has adapted to laboratory cultures, and both lab strains and field-caught individuals are easily maintained in the laboratory environment. For these reasons, *T. molitor* provides a very good model for the study of the evolution of mating systems.

*Tenebrio molitor/Hymenolepis diminuta* association

*Tenebrio molitor* serves as an important intermediate host for the rat tapeworm, *Hymenolepis diminuta* (Cestoda). The adult parasite lives in the small intestine of a rat or other suitable mammalian host and produces eggs that are passed out with the host’s feces. Beetles are infected when they ingest the tapeworm eggs, the eggs hatch in the gut, and the immature tapeworms (cysticercoids) develop in the beetle’s haemocoel. The cysticercoids mature in 11 to 14 days postinfection and are infective at that point to a mammalian host that eats the infected beetle (Voge and Heyneman 1957). The cysticercoids are not horizontally or vertically transmissible from beetle to beetle.

When transmission of a parasite from an intermediate host to the definitive host occurs through predation, the effects of the parasite are expected to be more severe in the intermediate host compared to the definitive host (May and Anderson 1983; Ewald 1994). Behavioral or physiological changes in the intermediate host may act to increase predation
rates and, accordingly, parasite transmission rates (Hurd 1990; McCurdy et al. 1999; Levri 1999. Parasites can also benefit if infection results in reduced reproductive effort in the host, thereby freeing up resources for the parasite (Hurd 1998). Infection with cysticercoids has been shown to alter behavior and decrease fecundity in this species of beetle (Hurd and Arme 1986; Hurd and Fogo 1990). Hurd and Fogo (1990) demonstrated that beetles with mature infections (11-12 days postinfection) exhibited decreased activity, photophobic behavior and response to odors collected from beetles of the same sex. These effects were not detected in beetles with immature infections (4-5 days postinfection). Defensive glands from infected beetles also contain less defensive compounds (methylbenzoquinone and m-cresol) than glands from noninfected beetles (Blankespoor et al. 1997). Although both the behavioral changes and the reduction in defensive compounds could be viewed as beneficial to parasite transmission (and parasite manipulation), this interpretation is in doubt. First, in two other intermediate host species, *Tribolium castaneum* and *Tribolium confusum*, infection has been shown to cause significant but opposing effects on behavior between the two species and between different genetic strains within a species (Yan et al. 1994). Because *T. castaneum* and *T. confusum* are very similar in habitat and in their predators, adaptive interpretations of the parasite's effects are problematic. Despite the reduction in defensive compounds produced by infected *T. molitor* (Blankespoor et al. 1997), Webster et al. (2000) failed to find a difference in the predation rates by rats on infected and non-infected beetles in the lab.
Several studies have examined the effects of *H. diminuta* on reproductive physiology and behavior in *T. molitor*. Infection results in a reduced fecundity in females (Hurd and Arne 1986) because a product of the parasite directly inhibits the synthesis of vitellogenin (Hurd 1998; Webb and Hurd 1999). Infection was also shown to alter the production of sex pheromone(s) in female beetles, and infected males displayed decreased sexual response to female pheromone (Hurd and Parry, 1990). Hurd and Parry (1990) placed ethanol washes from female beetles onto glass rods. They found that male mating behavior (rod mounting with extension of genitalia) was significantly reduced in response to infected female wash as compared to control (uninfected) female wash. These findings support the hypothesis that parasites manipulate the allocation of host resources away from reproduction, thereby freeing up resources for the parasite (Hurd 1998). The effect of infection on the attractiveness of female odors also supports one prediction of the Hamilton and Zuk hypothesis: expression of secondary sexual characteristics is negatively correlated with parasite load.

This *Tenebrio molitor* parasite system is a good candidate for testing the Hamilton and Zuk hypothesis. The metacestode stage reduces host fitness and occurs as a chronic infection. Both of these characteristics are thought to be necessary for infection-based sexual selection to occur. If the parasite kills the host quickly or recovery from infection is rapid and complete, the choosy sex may not be able to make an accurate assessment of resistance among individuals (Hamilton and Zuk 1982; Read 1990). Also, *Hymenolepis diminuta* requires two different hosts (a beetle and usually a rodent) for a complete
lifecycle. This fact may slow adaptive changes in the parasite and produce a more even host/parasite coadaptation cycle which is necessary to maintain continued selection pressure for resistance-based mate choice (Hamilton and Zuk, 1982).

Other important advantages of this system eliminate some alternative hypotheses. Because the metacestode stage of the tapeworm cannot be transmitted horizontally, infection avoidance can be eliminated as a factor in mate choice. Additionally, parental care is absent in both sexes, so parenting ability cannot influence a choice for a healthy mate. The ease of rearing both the host and the parasite in the lab makes this system ideal for manipulations that would be quite difficult in other model systems. The fact that females mate with many males suggests that sexual selection may be particularly strong in the postcopulatory arena. If infection affects sex characters of males (odor), then examinations of female mate choice based on infection status need to be examined in precopulatory mate choice and in the behaviors and outcomes associated with postcopulatory female choice.
CHAPTER 2

POLYANDRY IN GRAIN BEETLES LEADS TO GREATER REPRODUCTIVE SUCCESS: MATERIAL AND GENETIC BENEFITS?

ABSTRACT. Females that mate with more than one male may derive both material and genetic benefits, and differentiating between the two benefits is often difficult. We tested for both material and genetic effects associated with multiple mating in the highly promiscuous yellow mealworm beetle, *Tenebrio molitor*. Females that mated four times to the same male laid more eggs and produced more larvae than females that mated only once. Whether copulations occurred on the same day or over several days, the result was an immediate increase in the production of eggs by females. Some females were kept on a restricted diet to test whether nutrients in the spermatophore disproportionately benefited food-deprived females. Although females on poor diets produced fewer and smaller offspring, diet did not significantly affect the proportional benefit of mating treatment on female fecundity. By controlling for male mating history, we were able to separate the effects of mating with different males from the effects of receiving multiple spermatophores from the same male. Females that mated with four different males achieved substantial gains in numbers of eggs produced (32% increase) beyond those of females that mated an identical number of times with the same male. We found no evidence that males allocate fewer sperm to previous mates. Egg hatchability was unaffected by mating behavior, suggesting that genetic incompatibility at that stage is not
responsible for the low reproductive success of females mated with a single male. These results suggest that females may delay or reduce oviposition or be incapable of achieving maximal fecundity until they have gained the material and/or genetic benefits of mating with multiple males.

Females across a wide range of taxa commonly mate with multiple males (Dewsbury 1984; Ridley 1988; Birkhead and Møller 1998) despite the fact that adaptive advantages of multiple mating are not as apparent for females as for males (Williams 1966). Potential costs associated with mating include the risk of disease transmission, increased susceptibility to predators, retaliation or abandonment by social mates, and energetic costs. In addition, females may suffer from harmful chemicals within a male’s ejaculate (Andersson 1994; Chapman 1995). Taken together, the number and magnitude of potential costs to females suggest that we might expect to see them balanced by large gains. Only recently has the relationship between female mating history and fitness begun to be thoroughly investigated in order to better understand this aspect of female mating behavior. Several hypotheses have been proposed which provide adaptive explanations for multiple mating by females. These benefits fall into two main categories, nongenetic (material) or genetic.

Nongenetic benefits include fertilization assurance (Walker 1980), nuptial gifts or ejaculate nutrients (e.g. Gwynne 1984; Oberhauser 1989), ejaculate defensive compounds (Eisner and Meinwald 1987; Dussourd et al. 1988), and sperm replenishment. In some
insect species, spermatophores provide substantial nutrients to females and incur substantial costs to males (e.g., Forsberg and Wiklund 1989; Gwynne 1984). Increased fecundity may result from ejaculate compounds that stimulate egg maturation or oviposition in females (e.g., in crickets, Destephano et al. 1982). In species where there is considerable interaction between males and females after copulation, mating with multiple males may provide the female with benefits if male partners are more cooperative in protecting or rearing offspring, or allowing the female access to their territorial resources (e.g., Agelaius phoeniceus, Gray 1997).

The importance of material benefits, such as ejaculate nutrients, may depend on the resource status of the female. Females that have access to abundant food resources to allocate towards reproductive effort would not be expected to gain as much from ejaculate nutrients as females with fewer resources. In developing a model for the role of male ejaculate nutrients in female reproductive success, Boggs (1990) predicted that as a female’s food consumption increases or becomes more nutritionally complete, the effect of male-donated nutrients should decline. Compared to well-fed females, females on poor diets eat more male-donated nutrients in cockroaches (Schal and Bell 1982) and Drosophila (Steele 1986). Similarly, female bush crickets (Gwynne 1990) and seed beetles (Savalli and Fox 1999) kept on poor diets mated more often than females on richer diets. In crickets, egg size increased with multiple mating when females were kept on a restricted diet (Simmons 1988).

The genetic benefit hypotheses predict an increase in mean offspring fitness with multiple mates. Unlike some material benefits in which additional resources may be
acquired through multiple mating with the same male, genetic benefits result from the acquisition of sperm from two or more males (Tregenza and Wedell 1998). Polyandry (defined here as mating with more than one male) may be advantageous through increasing the genetic diversity of a female's offspring (Hamilton 1987; Brown 1997; Liersch and Schmid-Hempel 1998). Other hypotheses have focused on the potential for sperm competition or the filtering of sperm within females. If sperm competitiveness and offspring quality are correlated, then females may increase their fitness by mating multiply (Madsen et al. 1992; Watson 1998). Alternatively, multiple matings may increase the diversity of sperm within a female's reproductive tract, enabling the screening of sperm based on its genetic compatibility with the genotype of the egg (Zeh and Zeh 1997 a,b; Madsen et al. 1992; Zeh 1997; Tregenza and Wedell 1998; Newcomer et al. 1999) leading to increased egg hatchability or offspring fitness.

These hypotheses are not mutually exclusive. In fact, it is possible that mating with more than one male will have several effects. For example, if males provide costly nutrients in their ejaculates, it would be highly advantageous for males to develop a mechanism to stimulate females to produce as many young as possible immediately after mating, or else risk losing much of their nutrient "donation" to subsequent male competitors. Likewise, polyandry that is initially driven by genetic benefits would select for males that can manipulate the immediate use of sperm either through stimulating compounds or nutrient gifts. Therefore, studies should take care to examine for multiple effects after one type of benefit has been found. Differentiating between genetic and nongenetic benefits is difficult, but one important distinction is that genetic benefits result
from copulations with two or more different males, whereas many types of direct benefits can be gained by multiple copulations with the same individual. Few studies have attempted to separate the effects of mate number and multiple copulations in a controlled laboratory experiment (but see Tregenza and Wedell 1998; Newcomer et al. 1999).

In this study, we examined the effects of multiple mating on female reproductive success in the grain beetle, *Tenebrio molitor* (Coleoptera, Tenebrionidae). *Tenebrio molitor*, or yellow mealworm beetle, is a cosmopolitan pest of stored grains that can be easily reared in the laboratory. Females and males in the lab will mate readily several times in a single day. Although this beetle has been widely studied, we are not aware of any studies examining whether multiple matings are beneficial to female reproductive success. Second male sperm precedence has been demonstrated in *T. molitor*, but females that mate with two males usually produce offspring with mixed paternity (Siwa-Jothy et al. 1996), suggesting that genetic benefits are possible. In a set of three experiments, we examined the relationship among number of mates, reproductive success and survivorship in females. First, we examined various aspects of female reproductive success when females mated with one, two, or five males. In the second and third experiments, we tested several predictions of material- and genetic-benefit hypotheses to investigate which potential advantages of multiple mating might apply to this species. We measured the magnitude of the interaction of multiple matings with female condition, examining whether females on poor diets gained relatively greater benefits from nutritional components of the spermatophore. By controlling for male mating history, we were able to examine the effects of mating with multiple males (polyandry) beyond the effects associated with
procuring multiple spermatophores from a single male. We also examined larval weight and survivorship to determine whether polyandry affects offspring quality. Instead of focusing on either material or genetic benefits separately, we attempted to examine the entire range of benefits that may be acquired by polyandrous females. We provide evidence from controlled experiments that female beetles gain material and, potentially, genetic benefits and attain the greatest reproductive benefits by mating multiply with different males.

**Maintenance and general methods**

The *T. molitor* used in these studies were obtained from stocks maintained by B.D.W. and Dr. P. Pappas at the Ohio State University (for additional information on maintenance of the beetle stock see Worden et al. 2000). We collected pupae and sexed them by the morphology of the eighth abdominal segment (Bhattacharya et al. 1970). Newly emerged adults were placed into individual 60 x 15 mm petri dishes with wheat bran and potato. Females were marked with a single dot of white correction fluid (Liquid Paper, Gillette Co.) so that we could easily identify the sex of individuals within mating pairs.

Statistical analysis was performed using parametric ANOVAs after normality was confirmed by Shapiro-Wilk test. In the cases where data were proportions (egg hatchability, larval survivorship), the data were arcsine-transformed prior to analysis. All *P* values are two-tailed, and means are provided with ± 1 SE. We performed statistical analyses using SPSS version 9.0. Power analysis was performed by SPSS for observed
differences and calculated for hypothetical differences or effects from Zar (1996).

EXPERIMENT 1: THE EFFECT OF MULTIPLE MATES ON FEMALE LIFETIME REPRODUCTIVE SUCCESS

MATERIALS AND METHODS

Twenty-four hours prior to mating, females were weighed and randomly assigned to a treatment group: one (n = 21), two (n = 20), or five (n = 21) mates. When an adult female was 8 days old, she was placed with a male and allowed to mate. Males used in the experiment were all 8-day-old virgins. Mating between receptive individuals usually occurs shortly after initial contact, and copulation duration ranges from 45 seconds to 2 minutes. If a mating failed to occur within 10 minutes, we replaced the male. If the replacement male also failed to copulate with the female within 10 minutes, the female was removed from the experiment. Mated pairs were separated immediately after copulation was complete (end of intromission), and we allowed one hour to pass between matings so that females that were assigned to the five-matings treatment completed their final copulation 4.5-5.0 hours after their first pairing.

We collected all of the eggs produced by each female on the third day after mating, and then every five days thereafter until the twenty-fourth day after mating. Eggs were placed in wheat bran and kept in a 34-36°C incubator for 14 days, after which the bran was sifted and the hatched larvae were counted. In addition, we counted all larvae, but not eggs, produced by a female from the twenty-fourth day after mating until her death. We weighed 10 randomly chosen larvae that a female produced 4 to 8 days after mating.
If a female produced fewer than 10 larvae during this time, we weighed all the larvae available.

RESULTS

A total of 7 of 62 (11%) females did not produce any offspring, and these non-reproductive females were not distributed evenly between mating treatments: one mate, 5 of 21 (24%); two mates, 2 of 20 (10%); five mates 0 of 21 (0%). A greater proportion of singly-mated females did not produce any offspring than females that mated multiply with either two or five males (2 of 41 multiply-mated females vs. 5 of 21 singly-mated females, Fisher's exact test, $P=0.04$).

We performed a multiple analysis of covariance (MANCOVA) on the total number of larvae produced (lifetime RS), hatchability of eggs, and female survivorship. Mating treatment had a significant effect on the number of larvae produced (Fig. 2.1; $F_{2,58} = 5.5$, $P = 0.006$). Females mated to five males produced significantly more larvae than females mated to one male ($P < 0.01$, Bonferroni post-hoc comparisons), but not significantly more than females mated to two males ($P = 0.87$, Bonferroni post-hoc comparisons). Although females mated to two males produced nearly twice as many offspring as females mated to one male, this difference was not significant ($P = 0.15$, Bonferroni post-hoc comparisons).

Mating treatment had no significant effect on the proportion of eggs hatching (arcsine-transformed proportions $F_{2,58} = 0.4$, $P = 0.65$) or the survivorship of females ($F_{2,58} = 1.4$, $P = 0.27$). Overall, egg hatchability was high; one mate, mean $= 0.82 \pm 0.24$; two mates, mean $= 0.89 \pm 0.08$; five mates, mean $= 0.84 \pm 0.19$. Female mass, which was
included as a covariate in the model, did not have a significant effect on any of the dependent variables (number of offspring, $F_{1,58} = 2.2, P = 0.15$; arcsine-transformed hatchability, $F_{1,58} = 0.8, P = 0.37$; female survivorship, $F_{1,58} = 2.4, P = 0.13$).

To assess whether the effect of mating treatment on egg production varied with time after copulation, we performed a repeated measures ANOVA on egg production for the five collection periods after copulation. Egg production declined with time since mating ($F_{4,235} = 9.9, P < 0.001$), but there was no significant difference in the patterns of egg production between treatments (treatment*time interaction $F_{8,236} = 1.4, P = 0.20$).

Because some females failed to produce larvae during days 4 to 8, data on larval mass was available for only 52 of 62 females. Neither female mass (ANOVA $F_{1,48} = 0.8, P = 0.36$) nor mating treatment (ANOVA $F_{2,48} = 0.9, P = 0.42$) had a significant effect on the average mass of larvae produced 4 to 8 days after mating.

**EXPERIMENT 2: THE EFFECTS OF FEMALE NUTRITIONAL STATUS AND MULTIPLE MATING PARTNERS ON FEMALE REPRODUCTIVE SUCCESS**

**MATERIALS AND METHODS**

Experiment 1 demonstrated that females that mated with five males in a single day produce significantly more offspring than females that mated singly; however, it is not clear whether this increase in reproductive success was a result of multiple copulations or multiple mating partners. Therefore, we performed a second experiment to differentiate between these two possibilities as well as further examine the cause(s) of the effect found in experiment 1. Females were randomly assigned to either a single copulation with one
male \((n = 44)\), four copulations with one male \((n = 43)\), or four copulations with four different males \((n = 43)\). Each female began mating when she was 8 days old, and, unlike in experiment 1, females were allowed to copulate only once per day. One possible confounding factor with this basic design was that females mating with the same male would mate with nonvirgin males on their second through fourth matings, potentially receiving smaller successive ejaculates than females that always mated with a different virgin male. To control for this, we mated the males in the different-males treatment to nonexperimental females prior to copulations with experimental females so that an experimental female’s second, third, and fourth mate had mated on one, two, or three previous occasions, respectively.

Another potential confounding factor in this experiment is the amount of exposure and social interaction with other conspecifics. Females assigned to the multiple copulation treatments had more exposure to males as well as more copulations than females in the one copulation treatment. This could be important if some external stimuli (e.g., pheromone) stimulate females to produce more eggs. Therefore, we included an additional treatment group in which four different males were allowed to interact with each female \((n = 30)\), but only the first male placed with the female was allowed to copulate with her. In this last treatment, the second, third, and fourth males had mated with nonexperimental females five minutes prior to being placed with the experimental female. We used nonvirgin males because males have a roughly 30-minute refractory period between copulations (unpublished data), and this enabled us to permit interaction between the beetles without having to constantly prevent copulation. Each noncopulatory
interaction lasted 10 minutes, approximately the same amount of time that females spent with males that copulated with them.

To examine whether ejaculate nutrients are a major contribution to female reproductive success or survivorship, we manipulated the feeding regime so that some females were on a restricted diet and presumably more deprived of resources necessary for egg production. Approximately one-half of the females \((n = 72)\) in each mating treatment were provided excess wheat bran and water. We supplemented the wheat bran with potato every five days. The rest of the females \((n = 70)\) were provided water *ad libitum*, but were starved for 3 days prior to the beginning of the mating treatments, and then given *ad libitum* wheat bran thereafter. In addition, we supplemented the ‘food-limited’ treatment with potato only every 10 days.

Females were placed into a new dish containing wheat bran every four days up to the nineteenth day into the experiment, and then the female remained in a dish until death. The dishes containing each female’s eggs were kept in a 34-36 °F humid incubator for 14 days, at which time we counted the number of hatched larvae. We only counted eggs for the first time period after all mating treatments were complete (days 5-9), so egg hatchability data are only for this time period. We randomly selected ten larvae produced by each female during days five through nine. These larvae were weighed and then placed into a dish containing excess wheat bran. Larvae were kept in a 34-36 °F humid incubator for 60 days. At this time, we determined survivorship and the mean mass of all surviving larvae.

We performed separate statistical tests for dependent variables that differed in their
respective sample sizes. We performed two-way analysis of variance on the number of larvae produced and female survivorship to examine the effects of mating treatment and feeding treatment. Because some females did not produce any eggs during the four-day period that eggs were collected, we performed separate analyses of variance on egg hatchability and measures of offspring quality.

RESULTS

Mating treatment had a significant effect on the number of offspring that a female produced (Fig. 2.2; ANOVA $F_{3,152} = 16.7, P < 0.001$). Females that interacted with four males but mated only once with one of the males produced $35.0 \pm 6.4$ offspring compared to $36.7 \pm 6.2$ offspring produced by females that mated once with one male without further social interaction ($P > 0.99$, Bonferroni post-hoc comparisons). Because there was no difference in reproductive success between these two groups of females that mated only once, we combined the data for these groups for further comparisons.

Although interaction with males did not affect a female’s fecundity, the number of copulations did. Females that mated with the same male four times and females that mated with four different males produced $29.2 \pm 7.1$ and $49.4 \pm 7.1$ more larvae, respectively, than females that mated once with a single male ($P < 0.001$ for each comparison, Bonferroni post-hoc comparisons). Females that mated with four different mates produced $85.4 \pm 5.5$ larvae compared to $65.2 \pm 5.5$ larvae produced by females mated four times to a single male, a difference that remained significant after Bonferroni correction ($P = 0.04$). The temporal trend in larvae production was similar to that found in experiment 1: larvae production declined as the time since the last mating increased.
Female longevity was not influenced by mating treatment ($F_{2,154} = 0.06, P=0.94$).

Feeding treatment had a significant effect on the production of larvae (ANOVA $F_{1,154} = 5.1, P = 0.03$) but not female survivorship (ANOVA $F_{1,154} = 0.9, P = 0.33$). Females on the unrestricted diet produced more offspring than females on the restricted diet across all mating treatments, but none of the pairwise comparisons among mating treatments was significant ($P > 0.05$, Bonferroni post-hoc comparisons). Since one prediction for nutrient donations from males is that females on poor diets should gain greater advantages from multiple mating than females on richer diets, we examined the interaction between female diet and number of mates. There was no significant interaction between the number of mates and diet on either the number of offspring produced (ANOVA $F_{2,154} = 0.46, P = 0.63$; observed power = 0.12, but power > 0.78 if interaction accounts for 10% of observed variation) or female survivorship (ANOVA $F_{2,154} = 0.06, P = 0.94$; observed power = 0.06, but power > 0.70 if interaction accounts for 10% of observed variation).

As in experiment 1, some females did not produce any offspring: 11 of 44 (25%) females mated only once, 2 of 43 (5%) mated to the same male four times, and 0 of 43 (0%) of females mated to four different males. The difference in the number of non-reproductive females was significant between comparisons of females that mated only once and females that mated multiply to the same male (Fisher's exact test, $P = 0.01$) or to different males (Fisher's exact test, $P < 0.001$). However, the difference in proportion of non-reproductive females did not differ significantly between females that mated multiply
to the same vs. different males (Fisher's exact test, $P = 0.49$). The differences in reproductive success that we found cannot be explained solely on the basis of a difference in the number of non-reproductive females, because the effect of the number of mates on female reproductive success remained significant even after removing non-reproductive females from the analysis (ANOVA, $F_{2,137} = 9.0, P < 0.001$).

Sample sizes for egg hatchability data were reduced to 27 in the single mating treatment, 40 in the 'same male' treatment, and 43 in the 'different males' treatment. Neither mating treatment nor feeding treatment affected egg hatchability (Two-way ANOVA; mating treatment, $F_{3,126} = 0.6, P = 0.62$; feeding treatment, $F_{1,126} = 0.1, P = 0.78$).

We found no evidence that mating treatment affects any of the measures of offspring quality: mass at hatching and at 60 days old (repeated measures ANOVA, $F_{2,102} = 2.0, P = 0.15$); or larval survivorship (arcsine-transformed) to 60 days (ANOVA $F_{2,101} = 0.88, P = 0.42$). However, feeding treatment did affect the mass of larvae (repeated measures ANOVA $F_{1,102} = 10.8, P = 0.001$). Larvae produced by females on a restricted diet weighed $0.81 \pm 0.02$ mg at hatching and $28.38 \pm 0.42$ mg at 60 days post-hatching, compared to $0.89 \pm 0.02$ mg and $30.67 \pm 0.60$ mg for larvae produced by females in the high-quality feeding treatment (t-test; $t_{114} = -2.6, P = 0.01$ and $t_{106} = -3.1, P = 0.002$, respectively). Survivorship of larvae to an age of 60 days was very high ($97.0 \pm 5.9\%$), and, despite the differences in larval mass, it did not differ between feeding treatments (ANOVA on arcsine transformed survivorship $F_{1,101} = 0.01, P = 0.92$).
EXPERIMENT 3: SPERM PRODUCTION BY MALES WHEN MATING WITH NOVEL VS. PREVIOUS MATES

MATERIALS AND METHODS

If males adjust spermatophore content based on past mating history with a female, then this could affect the reproductive success of females mated to the same male compared to females mated to different males. To test for this, we performed a third experiment to determine whether the number of sperm allocated to spermatophores differed between males that mated to a new versus previous mate. Virgin males (n=39) were assigned to mate with the same female twice (n=20) or to two different females (n=19). As in experiment 2, we controlled for mating history by mating the second female in the different-female treatment to a nonexperimental male 24 hours prior to use in this experiment. Therefore, the first female that mated with a male was always virgin, and the second female had always mated once previously. Consistent with experiment 2, we allowed 24 hours to pass between copulations. Immediately after a male’s second copulation ceased, we decapitated the female and carefully dissected her reproductive tract. Spermatophores do not begin to eject their contents until ~7 to 10 minutes after copulation is complete, and the spermatophore changes dramatically after discharge (Gadzama and Happ 1974); therefore, the spermatophore that was just produced was quickly removed from the female’s bursa and examined to verify that it was still intact and full of sperm. The spermatophore was placed on a slide with 20 μL phosphate buffered saline, pH 7.4. After discharge was observed using a dissecting microscope, the solution was transferred into a small tube, mixed, and diluted in a total volume of 100-120 μL of
Tenebrio saline (Butz 1957). Sperm counts of four samples from each spermatophore were obtained by using an Improved Neubauer hemocytometer at X400 magnification.

RESULTS

Of the 39 trials in this experiment, six were removed because the pair did not remate within 15 minutes (3 different-female trials, 3 same-female trials). All spermatophores ($n = 33$) contained sperm, but the number of sperm varied from a low of 1,334 to a high of 234,900 (median = 97,200). A male's previous mating experience with a female did not significantly affect the number of sperm allocated to a spermatophore (same-female = 116,271 ± 16,106, different-female = 118,233 ± 18,984, $t_{32} = -0.08$, $P = 0.94$; observed power = 0.05, and power <0.30 if means differ by 10%).

DISCUSSION

This study provided evidence that, by copulating more than once, female grain beetles increased significantly their reproductive success through increased egg production. Whether matings occurred on the same day (experiment 1) or over four successive days (experiment 2), females that mated with five or four males, respectively, produced nearly twice as many larvae as females that mated only once. This increase in egg production could be explained by either material or genetic benefits.

Material benefits can be gained from multiple ejaculates, regardless of the genotype of the sperm. We were able to test this hypothesis because it predicts that females that mate with the same male four times should produce more offspring than
females mated only once. Our data support this hypothesis since females that mated four times to the same male produced nearly 1.8 times more offspring than females mated only once.

Females may mate with more than one male to negate the risk of mating with an infertile male (fertility assurance hypothesis, Walker 1980). Our data from Experiments 1 and 2 show that females that mate with more than one male are less likely to fail to produce offspring altogether. However, our data do not indicate infertile males, necessarily, because females that mated to the same male four times were less likely to have complete reproductive failure than females that copulated only once. It appears that if fertility assurance is an important selective force to female grain beetles, it is because of 'infertile' copulations, not infertile males. There are two possible explanations for these results: males sometimes either failed to pass spermatophores or passed one with inviable sperm (but this was not supported by sperm count data in experiment 3), or single copulations are occasionally insufficient to stimulate females to produce eggs. Even after removing females that did not reproduce from the analysis, the effect of mating treatment on female reproductive success remained significant. Therefore, the failure of some males to transfer viable sperm is not sufficient to explain the overall relationship between number of copulations and reproductive success.

We also tested for nutritional benefits. One prediction that arises from the nutritional benefits hypothesis is that females on poor diets will gain more from multiple mating than females on richer diets. We did not find support for this hypothesis since the proportional increase in larvae production with increasing copulation number was actually
greater, but not significantly, for females kept on the richer diets. This result was not because the food manipulation failed to alter female reproductive reserves, because females on poor diets produced fewer and smaller offspring than females on richer diets. Male nutrient donations have been shown to increase egg size and/or female survivorship in some arthropods (e.g., Boggs 1990; Andersson 1994), but, in our study, mating treatment did not have any effect on the mass of larvae or female survivorship even when females were deprived of food. However, males may supply a special nutrient that is rare in the environment, and our diet manipulation may not have limited the critical resource (Eberhard 1996).

Sperm replenishment is another possible direct benefit attributable to multiple matings. We did not examine sperm depletion directly; therefore, we cannot exclude it as an important factor in the mating system of *T. molitor*. However, our finding that multiple matings with the same male resulted in an immediate (within first 4 days) 57 percent increase in offspring production compared to females that mated only once indicates that the benefit occurs even when sperm from previous copulations is likely to be present (Fig. 2.3). The assumption that sperm is not particularly limiting soon after mating is supported by the fact that the daily rate of offspring production during days 5 through 9 (2.6 ± 3.0 larvae / day) did not decline significantly from the rate during the first four days (2.7 ± 2.9 larvae / day) in females that mated to only one male (paired samples t-test, \( t_m = 0.27, P = 0.79 \)).

By controlling for male mating history, we were able to examine the effects of polyandry compared to multiple copulations with a single male. Our experiments allowed
us to attribute any observed differences in female fecundity or offspring fitness to the number of mating partners per female. Females that mated to different males produced 31% more offspring on average than females mated multiply to the same male. This difference is very similar to the 32% increase in lifetime reproductive success of female pseudoscorpions, *Cordylochernes scorpioides*, when mated to two different males versus the same male (Newcomer et al. 1999) and the 29% increase in hatching success found in field crickets when females mated to four different males (Tregenza and Wedell 1998). Recently, genetic compatibility of gametes has been discussed as an important factor in female mating strategies (Zeh and Zeh 1997 a,b). In studies where genetic compatibility has been tested carefully, it has been detected by its effect on embryonic survivability (Tregenza and Wedell 1998; Newcomer et al. 1999). However, unlike these studies, in our population of grain beetles, the number of males that mated with a female did not affect egg hatchability. We also failed to find any significant differences in post-hatching measurements: mass at hatching, survivorship, or mass at 60 days.

One explanation for the increased reproductive success of polyandrous females is that males adjust their ejaculates based on past mating experience with a female so that novel females receive more sperm. We did not find any evidence that males adjust their ejaculates when copulating with a previous mate. The proportion of pairs that failed to remate was equivalent between same-female and different-female treatments (3 of 17 and 3 of 16 pairs, respectively), and the number of sperm allocated to females did not differ between mating treatments. Although the power of the test was low, owing largely to the large variance in number of sperm, the observed difference between same-female and
different-female treatments was less than two percent (1,962 sperm). While it is possible that males adjust the amounts of some seminal product other than sperm, these data indicate that the increase in reproductive success among females that mated to different males is not due to differing male strategies in sperm allocation, but is consistent with female plasticity in oviposition rate. If females are responding flexibly to multiple copulations, then the presence of ejaculates from multiple males may trigger increase oviposition in females.

The immediate increase in egg production exhibited by females that mated multiply is consistent with stimulatory ejaculate compounds or other cues. Multiple direct benefits may exist, but the immediate increase in female reproductive success is most likely the result of some signal that results in oviposition by females. Although high thresholds for ejaculate triggering cues could also explain the complete lack of reproduction among 25 percent of singly-mated females, it is equally plausible that multiply-mated females gain the additional benefit of fertility assurance.

Females may delay or suppress oviposition until they have mated several times to increase their chance of obtaining genetic or material benefits. Ejaculate triggering substances or copulation itself could act as the proximate cue stimulating oviposition. Archer and Elgar (1999) found that 65% of female hide beetles that mated only once failed to oviposit, but these females began ovipositing after mating with a second male. Paternity analysis of doubly-mated females revealed that at least 70% of first males transferred viable sperm (Archer and Elgar 1999). This behavior would be advantageous if increased genetic variation among offspring were important (Ridley 1993), or if
increased sperm competition results in offspring that are more competitive (Madsen et al. 1992; Keller and Reeve 1995). We did not find any significant differences between mating treatments in the early life histories of the offspring. However, this does not exclude the possibility that sperm competition or sibling diversity is correlated with offspring fitness in ways we did not measure, at later periods in life, or in more variable environments than we provided in the lab. Female pseudoscorpions (Zeh et al. 1998) and female hide beetles (Archer & Elgar 1999), *Dermestes maculates*, prefer novel males as mates. If *T. molitor* females can detect differences between males, females may increase oviposition after mating with different males. Such behavioral plasticity would explain the observed difference in production of offspring between females that mated multiply to the same versus different males.

Alternatively, the patterns of fecundity enhancement in this study support predictions of interlocus contest evolution (ICE) that result from conflict between the sexes over female reproductive investments (Rice and Holland 1997). Male ejaculate proteins are under selection to have both a defensive and offensive function in the face of sperm competition, and ejaculate compounds may stimulate oviposition in ways that benefit males but are harmful to females (Chapman et al. 1995). However, unlike studies on *Drosophila*, we failed to find an effect of mate number on female survivorship in *T. molitor*.

In conclusion, the results reported in this study demonstrate that female *T. molitor* produce more eggs after copulating with multiple males (polyandry). The benefit of multiple copulations exists for matings that occur within a single day as well as over the
course of four days. Given this finding, it is not surprising that female grain beetles mate frequently and often actively solicit copulations (personal observation). As described by Newcomer et al. (1999), few studies have specifically tested for genetic benefits once material, or direct, benefits have been demonstrated. Not only are material and genetic benefits not mutually exclusive, but we suggest that the two types of advantages may co-occur commonly in many polyandrous species. An initial advantage, either material or genetic, would select for polyandry among females. In turn, polyandry would select for postcopulatory manipulation and competitiveness in both sexes. Polyandry expands mate choice from selection of a suitable mate into the potential ability to select or passively screen gametes (Eberhard 1996). With the exception of two recent studies (Tregenza and Wedell 1998; Newcomer et al. 1999), studies that claim to have demonstrated genetic benefits to polyandry have not experimentally controlled for confounding factors such as male mating history, female quality, or benefits attributable to multiple ejaculates instead of multiple mates. Controlling for these factors, the study reported here has demonstrated that polyandry is a mating strategy that enhances female fecundity in T. molitor.

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Figure 2.1. Lifetime reproductive success of females mated to one, two, or five males in a single day. Asterisk indicates significant difference ($P < 0.05$) between the pairwise comparison indicated. Error bars represent $\pm 1$ SE.
Figure 2.2. Lifetime reproductive success of females mated to one male, to one male with additional social interaction with three other males (1+), four times to the same male, or to four different males. Solid bars indicate females kept on a poor diet, whereas open bars indicate females kept on a richer diet. All pairwise comparisons between mating treatments are significant ($P<0.05$, Bonferroni post hoc comparisons) unless indicated. None of the pairwise comparisons within mating treatments were significant ($P>0.05$, Bonferroni post hoc comparisons). Error bars represent ± 1 SE.
Figure 2.3. Periodicity of larvae production through the first 19 days after a female mated with first mate. Larvae were collected at four-day intervals. Lines represent different mating treatments: solid line = females mated to one male, dotted line (• • •) = females mated to same male, dashed line (— — —) = females mated to different males.
CHAPTER 3

PARASITES REDUCE ATTRACTIVENESS AND REPRODUCTIVE SUCCESS IN
MALE GRAIN BEETLES

ABSTRACT. Sexual characters may reveal the quality of a potential mate, including the
mate’s level of infection with parasites. Females that show preference for males with low
levels of infection or no infection may benefit in several ways. Direct benefits may include
avoidance of infection, acquisition of larger nuptial gifts, or enhancement in fecundity due
to differences in male fertility. Females may also benefit indirectly by producing offspring
that are more resistant to infections. We measured female preference for odours produced
by male grain beetles, *Tenebrio molitor*, that were either infected by a tapeworm,*
*Hymenolepis diminuta*, or uninfected. This parasite is not transmitted directly between
con specifics. Females were attracted to odours of all males, but they were less attracted to
those from parasitized males. To the contrary, females were preferentially attracted to
infected females. Males did not show any biased attraction to odours from infected and
uninfected male beetles. Females that mated with highly infected males produced fewer
offspring than females mated to uninfected males, indicating parasitic infection inflicts
multiple costs to males. These results are consistent with models of parasite-mediated
sexual selection.
Models of sexual selection based on honest indicators suggest that the expression of secondary sex characters is condition dependent (Kodric-Brown & Brown 1984; Andersson 1994), and there is empirical evidence supporting this relationship (e.g. Endler 1980; Clutton-Brock et al. 1982; Zeh & Zeh 1988; Hill 1990; Evans 1997). However, it is not clear what benefits females receive when exercising mate choice based on such condition-dependent traits. Many models (summarized in Andersson 1994) propose that females gain either direct benefits (which would enhance the choosing female’s survivorship, fecundity, or immediate reproductive success), or indirect benefits (which would enhance the genetic quality of the female’s offspring).

Hamilton & Zuk (1982) proposed that host-parasite coadaptation could lead to the exaggeration of sexual characters. According to Hamilton & Zuk’s hypothesis, an individual’s genetic resistance to parasite infection is revealed by the quality or quantity of sexual characters such as colour of plumage or bird song. The Hamilton & Zuk hypothesis can be viewed as a subset of the handicap models for sexual signals (Andersson 1994; Wedekind 1994). In concordance with the handicap principle (Zahavi 1975, 1977), individuals in good health are able to produce more elaborate sex characters than infected individuals because these signals are costly to produce. They predicted that, within a species, parasite load and sexual attractiveness should be negatively correlated. Females that selectively mate with uninfected males may benefit by producing offspring that are more resistant to infection, if resistance is heritable. One potential problem with this hypothesis is that an individual’s health or condition is dependent on many genetic and environmental factors (Read 1990; Wedekind 1994). Therefore, it may be that sexual
characters act as honest indicators of overall quality, but not solely of resistance.

Studies testing the Hamilton & Zuk hypothesis have yielded mixed support, and the data supporting the intraspecific prediction are equivocal (see reviews in Read 1987, 1990; Möller 1990; Zuk 1992; Hamilton & Poulin 1997). An alternative interpretation of preference for uninfected mates is a general avoidance of individuals with contagious infection (Borgia 1986; Borgia & Collis 1989; Able 1996). Male sex characters may reveal the degree of infection with 'associatively transmissible' parasites (Able 1996). If this is the case, females may accrue direct benefits by avoiding costly infections. If the risk of infection is an important selective force in the population and assuming individuals can distinguish between infected and noninfected conspecifics, the rule should be to avoid all infected individuals, regardless of sex. Additionally, females may receive direct benefits by preferentially mating with uninfected males if infected males provide fewer resources such as parental care, nuptial gifts or sperm (Hoelzer 1989; Rosenqvist & Johansson 1995).

In this study, we examined the effects of parasitism on male attractiveness and reproductive success in the grain beetle *Tenebrio molitor* (Coleoptera, Tenebrionidae), a cosmopolitan pest of stored grains. *Tenebrio molitor* lives much of its life as growing larva (1-2 years) before maturing into an adult beetle (Back & Cotton 1922; Day 1989). There is no obvious sexual dimorphism in this species; however, each sex produces distinct pheromones that attract members of the opposite sex (Happ 1969; August 1971; Tanaka et al. 1986). Attraction to mates and the stimulation of copulatory behaviour itself have been linked to pheromones produced by adults (August 1967, 1971; Hurd & Parry 1991).
While there have been many studies examining the effects of parasites on song or morphological traits, few studies have examined the interactions between parasites and pheromones (Penn & Potts 1998). Pheromones should be good indicators of an individual’s current condition because they may change qualitatively and quantitatively over short periods of time. In the mouse, *Mus musculus domesticus*, females find the odours of parasitized males to be aversive (Kavaliers & Colwell 1993, 1995). However, recent work suggests that although the odours of mice infected with influenza are less attractive to conspecifics than those of healthy mice, they are not aversive (Penn et al. 1998). In *T. molitor*, males are less responsive to odour extracts taken from infected females than to extracts from noninfected females (Hurd & Parry 1991). Here we ask whether parasitism affects olfactory attractiveness of male grain beetles.

The tapeworm, *Hymenolepis diminuta*, infects a wide range of intermediate hosts, but adults of *T. molitor* are considered to be the principal host (Rau 1979; Schmidt & Roberts 1989). Beetles are infected by eating tapeworm eggs that are excreted by infected rodents. The metacestode (cysticercoid) develops within the beetle’s haemocoel. Because a rodent host is required for the tapeworm to complete its development, female beetles are not likely to increase their risk of infection by mating with infected males. This is an important advantage of this system because most past studies used host/parasite models where parasite transmission between conspecifics was possible (Able 1996). If adult *T. molitor* follow a general rule of avoiding all sick (and potentially infectious) individuals, then females should show an avoidance of all infected individuals regardless of sex.

We compared male and female attraction to the pheromone obtained from infected
and noninfected beetles of the same sex. Furthermore, we compared a female's attraction
to odours from parasitized and nonparasitized males to test whether females express a
preference for nonparasitized males. To further examine the effects of infection, we
examined the reproductive success of parasitized and nonparasitized males when mated
singly.

METHODS

General Beetle Culture- We collected all individuals used in this study as pupae from 15
to 20 main beetle cultures maintained at populations of approximately 500-1500 larvae
and 50-100 adults each. The beetles originated from an Ohio State University stock
maintained by P.W.P. for many years. These cultures have been supplemented periodically
with beetles from various commercial sources.

We collected pupae at regular intervals for a 4-month period. We determined the
sex of each pupa by examining the developing genitalia on the eighth abdominal segment
(Bhattacharya et al. 1970). The pupae were examined daily, and the healthy eclosed adults
(those with both antennae and no obvious morphological defects) were removed and
placed into individual 60x15 mm petri dishes with an excess of wheat bran and potato. We
assigned individuals a unique number so that the age of each individual was known. Each
sex was housed in a separate incubator (34-36 °C) until used in the experiment.
Hymenolepis diminuta infection - We collected *H. diminuta* eggs from the faeces of infected rats using a standard salt flotation protocol. The eggs were subsequently washed and stored in distilled water at room temperature for up to 7 days prior to their use in the experiment.

Prior to treatment assignment (uninfected or infected), we weighed beetles and selected pairs of males or females of the same age and mass (within 5% body mass) for use in the odour preference trials (see below). We used a randomized block design in which the infected treatment was randomly assigned to one member of a pair. The other beetle in the pair was assigned to the uninfected treatment.

Ten days after eclosion, we starved experimental beetles for 3 days and then allowed them to feed for 24 h on either plain apple scrapings (uninfected) or apple scrapings mixed with *H. diminuta* eggs (infected). The infections were allowed to mature for 14 days before the beetles were used in experiments. We chose infections of this maturity because Hurd & Parry (1991) found no differences in mating behaviour of uninfected male beetles and those infected with *H. diminuta* for 14 days. Therefore, all beetles in this study were virgins and 28 days old at the start of the experiment. After each experiment we dissected all beetles and counted the number of cysticercoids.

**Odour Preference Trials** - Odours from infected and uninfected adult males were collected by placing each male in a petri dish that contained two squares (1 cm²) of filter paper on days 11-14 following ingestion of either tapeworm eggs or apple, respectively, after which time we began the preference trials. Because *H. diminuta* cysticercoids
immediately migrate through the gut into the haemocoel of the host after hatching (Voge & Graiwer 1964), it is unlikely that any parts of the parasite or an entire parasite would have been passed out of the host during the time that odours were collected. To ensure that odours were transferred from male beetles to the filter paper, we performed an initial experiment in which we placed a female \((N=11)\) into a circular arena (described below) containing two squares of filter paper taken from the dish containing an individual male beetle and two squares that had been kept in a plastic dish containing only wheat bran. The procedure for these trials was identical to the procedure described below for the female preference tests involving males of different infection status.

To test female preferences for males based on infection status, we presented odours from the infected/uninfected male pairs, matched by weight and age, to a virgin female. The arena for the female choice trials consisted of a 20 cm diameter glass dish inverted over a 25 cm diameter piece of filter paper. A female beetle \((N=27)\) was placed under a covering \((area = 12.5 \text{ cm}^2)\) in the centre of the circular arena during the 10-min acclimation period preceding each trial. We placed two filter paper squares from the uninfected male’s culture (UM squares) and two squares from the infected male’s culture (IM squares) in positions equidistant from the centre and from each other in an alternating pattern. At the start of the trial we removed the small cover that restricted the female to the centre area and placed the glass dish over the entire arena. Each trial lasted 8 min, during which time the female’s movements were videotaped using red light illumination. We recorded the time a female spent on each paper square.

We also conducted a control experiment to test whether females were attracted to
the odours of infected males. We videotaped each female ($N=12$) was in the arena with two squares of filter paper taken from an infected male's dish, and two squares that had been kept in a dish containing only wheat bran (control).

**Same-sex preference tests** - We conducted two other experiments to determine whether odour preferences are specific to intersexual interactions or represent a general avoidance of infected individuals. These experiments were conducted with an identical protocol as the female preference trials (above) except that the subject and stimulus beetles were of the same sex. In the first experiment, a virgin male ($N=20$) was placed in the arena described above with two squares from an infected male beetle and two from an uninfected male. In another experiment, we tested a female beetle's ($N=20$) response to odour squares from infected and uninfected female beetles.

**Reproductive Success** - To determine the effect of parasite infection on male reproductive success, we counted the number of larvae produced by females mated singly to either an infected or an uninfected male. Under natural conditions both male and female beetles will mate multiply. Here, reproductive success was determined by counting the number of larvae produced by a single male-female mating.

We randomly assigned male beetles ($N=38$) to control ($N=13$) or infected ($N=25$) treatments. All individuals were weighed and then fed either plain apple scrapings (control) or apple scrapings mixed with *H. diminuta* eggs. All beetles were allowed to feed for 24 h and were then placed into a petri dish containing wheat bran. The infections were allowed to mature in the males for 14 days before being evaluated for reproductive
success. We weighed virgin females and then we placed each female with a single male (either infected or uninfected) for 24 h. Following this mating period, each female was removed and placed into fresh bran. Females remained in this culture for 3 days. They were then placed into another petri dish on day 4 and then again on day 7. This allowed for the quantification of larvae produced during three time periods: days 1-3, 4-6 and 7-27 postcopulation. These time increments were chosen because females that mate only once tend to produce one-third to one-half of their eggs during the first week after mating (personal observation). Also, in a pilot study, singly mated females rarely produced offspring after 25 days. After we removed the female, we stored the dishes containing the bran and any offspring in an incubator for 14 days, then sifted through the bran and counted larvae. All viable eggs should have hatched in 4-14 days (Day 1989).

Data Analysis- The winner of an odour preference trial was determined by examining the proportion of time a beetle spent on each type of square. The type of square that was visited for the longest total time was recorded as the winner. All tests were two-tailed unless reported otherwise.

We used multiple regression analysis to test for factors related to male infection intensity. Because relatively few males were highly infected and few females were highly fecund, apparent relationships may have simply been the result of the low likelihood of having a mating between a highly fecund female and a highly infected male. Regression analysis is not appropriate if the relationship between X and Y is not continuous over the entire range of observed values for the variables tested (Garvey et al. 1998). For these
reasons we also analysed our data with a two-dimensional Kolmogorov-Smirnov (2DKS) test which tests whether a bivariate distribution pattern is likely to have arisen from the independent univariate distributions of the two factors (Garvey et al. 1998). If the interaction between the two variables is not continuous over their entire distributions, the 2DKS test gives a threshold level of a factor beyond which the variance of the response variable is constrained (see Garvey et al. 1998 for a full description of the test). The program can be downloaded at www-personal.ksu.edu/~jgarvey/2dks.html.

RESULTS

Odour Preferences - In all 11 trials in which a female was presented with blank filter paper squares and squares taken from an uninfected male’s culture, the female spent more time on the squares from the male culture (Fig. 3.1; Binomial test: $N=11$, $P=0.001$). Females spent a mean of $68.0 \pm 36.8$ s (median = 81s) on squares taken from a male beetle’s culture and $4.8 \pm 5.3$ s (median = 4s) on squares stored in wheat bran.

In the infected versus uninfected male odour trials, infection levels in male beetles ranged from 10 to 606 cysticercoids (median=159, $N=27$). The amount of time an individual female spent on all squares during the 480-s trial ranged from 17 to 431 s. Because the amount of time a female spent on squares varied greatly among trials, we converted the time spent on each type of square into a proportion of the total time spent on all squares. Nine (33%) females spent more time on IM squares and 18 (67%) spent more time on UM squares (binomial test: $N=27$, $P=0.12$). The proportion of time a female spent on IM squares was negatively correlated with the number of cysticercoids that were
present in the male (Fig. 2; $F_{1,23}=13.31$, $R^2=0.35$, $P=0.001$).

We used the 2DKS test to determine whether the relationship between male infection level and female preference was likely to have arisen by chance. Female preference and male infection level were related ($D_{BKS}=0.17$, $N=27$, $P=0.002$). The threshold infection level occurred at 159 cysticercoids (Fig. 2). When the infection level was at or above this level, 13 (87%) females spent more time on UM squares and two females spent more time on IM squares (binomial test: $N=15$, $P=0.007$). Because each trial used two squares from each male, it is possible that the distribution of odour between the squares differed between treatments. We examined the coefficient of variation (CV) for the amount of time females spent on each square. CV did not differ significantly between IM squares (0.88) and UM squares (0.74) for the trials in which males were highly infected (paired t test: $t_{14}=-0.81$, $P=0.43$), nor was there any correlation between CV and the parasite load of the male (Pearson correlation: $R=0.18$, $N=27$, $P=0.37$).

We tested whether this female preference represented an aversion for squares from infected males. In all 12 trials in which a female was presented with 'blank' squares and squares taken from an infected male’s dish, the female spent more time on the squares from the male’s dish (binomial test: $N=12$, $P<0.001$). This result remained significant after removing three trials where the number of parasites in the male was below 158 (binomial test: $N=9$, $P=0.004$). For this restricted set of trials (>158 cysticercoids per male), females spent a mean of $63.0 \pm 17.8$ s (median = 68s) on squares taken from infected male’s dish and $14.1 \pm 6.4$ s (median = 14s) on control squares.
**Same-sex preference tests** - The null hypothesis that male beetles visited squares from infected and uninfected male beetles equally could not be rejected. Twelve males spent more time on squares from infected males and eight males spent more time on squares from uninfected males (binomial test: $N=20$, $P=0.50$). No significant correlation was found between the proportion of time a male was on IM squares and the infection level in the male (Fig. 3a; $F_{1,18}=3.2\times10^{-4}$, $R^2=1.8\times10^{-5}$, $P=0.99$).

More females (75%) expressed a preference for the odours of infected females than for the odours of uninfected females (binomial test: $N=20$, $P=0.04$). Female preference for female odours was correlated positively with the level of infection (Fig.3b). Thus, females spent proportionally more time on squares from infected females as the level of infection within the stimulus female increased (nonlinear regression: $F_{1,18}=18.67$, $R^2=0.51$, $P<0.001$).

**Reproductive Success** - Thirty-three of the original 38 dyads yielded complete results (uninfected: $N=13$; infected: $N=20$). Those that were discarded included two pairs in which the male died before dissection, and three pairs in which the female died before day 27 of egg production.

There was a high level of variation in reproductive success between male/female dyads. One dyad produced only one larva, while the most productive pair produced 145 larvae. None of the control males contained any cysticercoids. Infected males contained from 28 to 990 cysticercoids.

We performed a multiple regression analysis using male infection level, female weight and male weight as predictors of male reproductive success. The number of
cysticercoids in the male and fecundity of the female were negatively correlated ($t=-2.36$, $R=-0.40$, $N=33$, $P=0.025$). There was a trend for larger females to produce more larvae than smaller females ($t=1.82$, $R=0.32$, $N=33$, $P=0.08$), but male weight had no significant correlation with reproductive success ($t=-0.22$, $R=-0.04$, $N=33$, $P=0.83$). Because female mass appeared to be linearly correlated with fecundity, we divided the number of larvae produced by each female by the female’s mass (adjusted reproductive success). Nonlinear regression revealed that the parasite load of the male had a significant effect on adjusted reproductive success ($F_{1,31}=12.74$, $R^2=0.29$, $P=0.001$; Fig. 4).

Two-dimensional Kolmogorov-Smirnov analysis confirmed the negative relationship between male infection level and female fecundity (2DKS test: $D=0.13$, $N=33$, $P=0.005$) and identified the threshold value in male infection at 257 cysticercoids (Fig. 4). This suggests that the number of offspring that females produce may not be constrained by male infection levels below this threshold value. Alternatively, the relationship may be continuous but nonlinear. Females ($N=7$) mated to males with a level of infection at or greater than this threshold value produced $37.0 \pm 20.3$ larvae compared to $63.7 \pm 37.3$ larvae produced by females ($N=13$) mated to uninfected males (one-tailed t test: $t_{16}=1.73$, $P=0.05$).

We divided female fecundity temporally (1-3, 4-6 and 7-27 days after mating) to determine whether the effect of male infection on female fecundity was biased towards early or late egg production. The negative correlation between male infection level and male reproductive success was present for larvae produced on days 7-27 ($F_{1,31}=24.01$, $R^2=0.44$, $P<0.001$), but not for larvae produced on days 1-3 ($F_{1,31}=2.73$, $R^2=0.08$, $P=0.11$).
DISCUSSION

**Odour Preference**- In the present study, *T. molitor* females expressed a preference for the odours of uninfected males, but the strength of this preference depended on the level of infection (Fig. 2). The preference was expressed despite the fact that the metacestode stage cannot be transmitted by association of conspecifics. This suggests that parasite-mediated sexual selection may occur in this species, and that contagion avoidance is not likely to be important in this system. However, females may avoid the odours produced by unhealthy individuals regardless of the exact cause of illness if this behaviour prevents some infections that are transmitted horizontally. A general avoidance of unhealthy individuals is not supported in this study, however, because neither females nor males showed any discrimination against odours produced by infected beetles of the same sex (Fig. 3). Females, in fact, spent more time on squares from highly infected stimulus females. In addition, females were more attracted to odours of infected males than blank squares of filter paper. Together, these results suggest that females expressed a reduced level of attraction but not an aversion to the odours produced by infected males.

Differences in the relative attractiveness of odours of infected males could be the result of a qualitative or quantitative change in pheromone production, the production of some other beetle-derived chemical, or a change in odour caused by a direct product of the parasite. Male *T. molitor* produce volatile pheromone(s) that attracts females (August 61) or 4-6 ($F_{1,31}=3.65$, $R^2=0.11$, $P=0.07$).
1967, 1971; Happ 1969) and a nonvolatile copulatory release pheromone similar to those produced by females (Tanaka et al. 1986). Because female, but not male, beetles showed discrimination in their response to odours from infected and non infected male beetles, it is likely that at least one sex-specific chemical (i.e. male pheromone) was affected by *Hymenolepis* infection. August (1971) has shown that a female’s response to male pheromone is dose dependent. Therefore, a reduction in the amount of pheromone produced by infected males is a possible explanation for the preference patterns reported in this paper.

An alternative explanation is that a product of the parasite itself altered the attractiveness of odours collected from infected males. Although we did not test this hypothesis directly, two of our results suggest that a product of the parasite is unlikely to be the sole mechanism leading to female preferences for odours from uninfected males. First, beetles do not always find the odours of infected conspecifics to be unattractive. To the contrary, females actually spent more time on odours of highly infected females than uninfected females, and we found that males did not express any preference with respect to the intensity of infection of male beetles (Fig. 3). If these findings were a result of a substance produced by the parasite itself, our results suggest that parasites only produced the substance in male beetles and the response to this product was sex-specific. In addition, females showed a preference for the odours of healthy males, but females still found the odours of infected males attractive. This last point is not completely at odds with a mechanism invoking a product of the parasite; however, it does indicate that females did not show an aversive response to odours of infected males.
An alternative explanation for the female preference patterns that we found is that infected males moved less in their dishes, resulting in a patchier distribution of pheromone in their housing. If this were the case, we would predict that a clumped distribution of pheromones should correspond to more variable female responses to the two squares from an infected male than to those from an uninfected male. We found no significant difference in the coefficient of variation between female responses to infected and uninfected male, nor was there a correlation between CV and the level of infection. Therefore, the possibility of reduced mobility of infected beetles is an unlikely explanation of our results.

The result of this parasite-induced reduction in male attractiveness is that females may preferentially mate with noninfected males or males with lower levels of *H. diminuta* infection. While we have not demonstrated a direct correlation between odour preferences and mate choice in females, females do use male odours to locate males (August 1967, 1971; Happ 1969). Therefore, attractiveness of odours and encounter rate of potential mates should be correlated. Preference for odours from uninfected males could benefit females in several ways. The pattern of female preference demonstrated in this study may be advantageous if infected males provide less direct benefits to females than noninfected males (Halliday 1978; Heywood 1989; Hoelzer 1989). Because, in this species, neither sex provides any parental care and male-female associations do not last much longer that copulation itself (personal observation), the only male contribution is a spermatophore.
Infection and Reproductive Success- In addition to reducing the attractiveness of a male's odour to females, infection by *H. diminuta* reduces a male's potential reproductive output. The number of larvae a male sired (adjusted for female mass) was dependent upon the level of infection in the male. This relationship appeared to be nonlinear. Alternatively, the effect of infection may not occur until the level of infection in the male reaches 257 cysticercoids (Fig. 4) so that highly infected males (>257 cysticercoids) sired fewer offspring than uninfected males. There are several possible mechanisms that could lead to this relationship. Perhaps highly infected males did not copulate as often as uninfected males. The sexual activity of each male during the 24-h pairing period was not observed, but all males mated with the female once within the first 10 min of pairing. Infection could also have an impact on the ejaculate itself. In insects, several components of the ejaculate can affect female oviposition rates. Oviposition-stimulating compounds (e.g. in *Drosophila*, Chen et al. 1988; in crickets, Destephano & Brady 1977), nutritional components of the spermatophore that females use for egg production (e.g. in butterflies, Boggs & Gilbert 1979; in bushcrickets, Gwynne 1984), and the number of viable sperm can all affect the number of offspring produced by females. The mechanism(s) responsible for the reduction in male reproductive success cannot be determined from this study. In *T. molitor*, male bean-shaped accessory glands are enlarged in beetles infected with metacestodes (Carver & Hurd 1998). This suggests that accessory gland products may be altered by *H. diminuta* infection. When larvae production was divided temporally, the negative relationship between the level of infection in the male and female fecundity was significant only for larvae produced after the seventh day of egg production. This temporal
Effect is best explained by sperm depletion (through reduced mating activity of the male or reduced number of viable sperm per ejaculate), or a reduction in some vital nutritional component in the ejaculate.

Both males and females of this species mate multiply (Gage 1992; personal observation). Therefore, sperm competition may further elevate the costs of this parasite to the reproductive success of males. The reproductive output of female *T. molitor* is increased by multiple mating (unpublished data) so females may be able to compensate for any reduction in offspring produced from one mating by mating with other males. However, a general strategy of mating only with the healthiest males could still be beneficial to a female’s lifetime reproductive success.

The natural range of infection intensities has not been well studied. We are aware of only one survey of sympatric populations of *T. molitor* and *T. obscurus*, which found cysticercoid frequencies ranging up to 83 cysticercoids in *T. molitor* and 1200 cysticercoids in *T. obscurus* (Rau 1979). Both species have similar diets (Back & Cotton 1922) and become infected in the same manner (ingestion of eggs). In our study, male infection levels were greater than those found in Rau’s survey for *T. molitor*. However, Rau (1979) suggested that a possible explanation for the reduced parasite loads found in *T. molitor* compared with *T. obscurus* is that *T. obscurus* emerges one month earlier than *T. molitor* (Back & Cotton 1922) and, thus, had greater exposure to the parasite at the time the survey was conducted. More field studies of natural populations that examine infection levels throughout the adult’s life span are needed to get realistic estimates of the range in infection intensities.
Our results demonstrate that the odour of male *T. molitor* can indicate infection intensity to female beetles. In addition, infected males suffer reduced reproductive success. There are many possible benefits to females that display a preferential attraction to males with low-level or no infections. Females may be able to increase their own reproductive success by mating preferentially with uninfected males. While direct benefits to a female's reproductive success may exist, indirect benefits are also possible. If resistance to *H. diminuta* is heritable, preferential attraction to mates with low levels of infection would result in offspring with greater resistance to parasites (Hamilton & Zuk 1982). Reduced reproductive potential (or complete castration) may be a common effect of infection in many host-parasite relationships. Reduced fecundity is a common result of infection in invertebrates, and it has been argued that this confers an advantage upon the parasite if host resources are diverted from gamete production and made available to the parasite (Hurd 1998). Therefore, the ability to detect and mate preferentially with uninfected individuals may generally provide direct fitness benefits.

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Figure 3.1. The amount of time each *T. molitor* female (trial) spent on squares taken from dishes containing a conspecific male beetle (●) and squares taken from a dish containing wheat bran only (○).
Figure 3.2. The relationship between infection intensity (number of *H. diminuta* cysticeroids) and the proportion of time a *T. molitor* female spent on squares of filter paper taken from dishes containing an infected male (IM): time on IM squares / (time on IM + time on UM squares). The arrow on the X axis indicates the infection intensity threshold (159 cysticeroids) detected by 2DKS analysis. Regression analysis: Proportion of time spent on IM squares= -0.0011(infection intensity)+0.6572.
Figure 3.3. The relationships between infection intensity (number of *H. diminuta* cysticercoids) and the proportion of time an individual *T. molitor* beetle spent on squares taken from an infected beetle of the same sex out of the total time the beetle spent on all squares in the arena. (a) Relative male preference of male odours. Linear regression: NS. (b) Relative female preference for female odours. Linear regression: Proportion of time spent on squares with odour from infected females $= 0.475 \times$ (infection intensity) + 0.0012.
Figure 3.4. The relationship between infection intensity (number of *H. diminuta* cysticercoids) and adjusted female fecundity (the number of larvae/ female mass). The arrow on the *X* axis indicates the infection intensity threshold (257 cysticercoids) detected by 2DKS analysis. Nonlinear regression analysis ($Y$=$\exp[-b_1 X + b_0]$).
ABSTRACT. Several models predict that parasites have a significant affect on host evolution. The effect of parasites on one important component of fitness for males, sperm competition, has received little attention. We examined the effect of parasitism by *Hymenolepis diminuta* on second-male sperm precedence in the grain beetle *Tenebrio molitor*. Paternity was determined by using RAPD markers for offspring produced by a female that had been placed sequentially with two males matched by age and mass. The first and second males to mate with a female were either: 1) both non-infected; 2) infected (first) and non-infected (second); or 3) non-infected (first) and infected (second). There was second-male sperm precedence when females mated to two non-infected males (controls) and when only the first male of the pair was infected, but not when only the second male was infected. The significance of this effect of parasitism to male reproductive success is discussed in relation to the mating system of *T. molitor*. 
The effect of parasites on host fitness has been proposed to have great importance in several aspects of host evolution (Maynard Smith 1978; Hamilton 1980; Hamilton & Zuk 1982; Moore 1984). Parasites are also under selective pressure by hosts. When transmission of a parasite from an intermediate host to the definitive host occurs through predation, the effects of the parasite are expected to be more severe in the intermediate host compared to the definitive host (May and Anderson 1979; Ewald 1994). Behavioral or physiological changes in the intermediate host may act to increase predation rates and, accordingly, parasite transmission rates (Hurd 1990; McCurdy et al. 1999; Levri 1999; but see Webster et al. 2000). Parasites can also benefit if infection results in reduced reproductive effort in the host, thereby freeing up resources for the parasite (Hurd 1998). Determining the effects of parasites on host fitness is important to our understanding of the evolutionary implications of parasitism (Yan 1997).

Metacestodes of the parasite *Hymenolepis diminuta* affect both the reproductive physiology and behavior of their intermediate host *Tenebrio molitor* (Hurd 1990; Hurd & Fogo 1991, Hurd & Parry 1991; Worden et al. 2000). *Hymenolepis diminuta* has an obligate two-host lifecycle in which the mature tapeworm resides in the gut of its mammalian host (usually a rat), and the parasite’s eggs are passed in the feces of the host. When a grain beetle, *Tenebrio molitor*, ingests the parasite’s eggs, the metacestode stage develops within the beetle’s hemocoel and remains in this immature state until a suitable mammalian host eats the infected beetle.

While much is known about the effects of infection on female *T. molitor*
(Hurd & Parry 1991; Webb & Hurd 1999), the effects of infection on male reproductive physiology are not as clearly understood. Reproductive success of male beetles is correlated negatively with infection intensity, and when given a choice between a non-infected and an infected male, females prefer to mate with non-infected males (Worden et al. 2000; Worden & Parker In review). Infected males have larger bean-shaped accessory glands (Carver & Hurd 1998). Carver et al. (1999) demonstrated that spermatophores produced by infected males contain elevated trehalase activity and protein levels, but the amount of protein transferred to the spermatheca of females was not different from amounts from non-infected males. Therefore, the adaptive significance of these findings is unclear because it is not known what effect, if any, the enhanced levels of protein content have on sperm and sperm uptake (Carver et al. 1999). Although infected males had lower reproductive success than non-infected males when mating to monogamous females (Worden et al. 2000), how infection affects male reproductive success under the situation of sperm competition typical in this species has not been demonstrated.

Due in part to the growing evidence that females of many species mate with multiple males, attention has increasingly focused on the success of a male’s sperm after copulation (Parker 1970; Eberhard 1996; Birkhead & Møller 1998). Although mating order has a major effect on the general patterns of sperm use in insects (Simmons & Siva-Jothy 1998; but see Zeh & Zeh 1994), considerable individual variation in sperm precedence suggests that other factors besides mate order are important (e.g., Lewis & Austad 1990, 1994; Birkhead & Møller 1998).

In this study we examine the effects of infection by *H. diminuta* on sperm
precedence in *T. molitor*. Females that mate with different males have higher fecundity than females that mate singly or multiply to one male (Worden & Parker In press), and the majority of females mate more than once in a 90 minute period (Worden & Parker In review). Therefore, sperm competition is likely to be extremely important component of male reproductive success. If the increased protein content in the spermatophores of infected males is advantageous to sperm viability and storage, then infected males may be more competitive in gaining fertilizations. Alternatively, the negative effects of parasitism that resulted in reduced reproductive success in monogamous pairs of beetles (Worden et al. 2000) may also lead to reduced competitiveness when females are polyandrous. In another tenebrionid beetle, *Tribolium castaneum*, males infected with *H. diminuta* had lower second-male sperm precedence (P2) compared to non-infected males (Yan & Stevens 1995). To our knowledge, this is the only other study that has tested the effects of parasitism on sperm precedence.

**MATERIALS AND METHODS**

Beetles used in this study were obtained from O.S.U. laboratory stocks maintained by P.W. Pappas (described in Worden et al. 2000). We collected pupae every three days over a period of 18 days. We determined the sex of each pupa (Bhattacharya et al. 1970) and isolated each sex into separate petri dishes in separate incubators kept at 34-36 °C. When beetles emerged as adults, we first weighed then placed each one into a 60 x 15 mm petri dish supplied with an excess of wheat bran and pieces of potato. Each individual was assigned a unique number so that the age
of each individual was known.

*Hymenolepis diminuta* infection

We collected *Hymenolepis diminuta* eggs (O.S.U. strain) from the feces of infected rats using a standard salt flotation protocol (Keymer & Anderson 1979). The eggs were subsequently washed and stored in distilled water at room temperature for up to 7 days prior to their use in the experiment.

Males were matched by mass (± 5%) at adult emergence. We assigned one member of the pair (N=20) to the infected treatment and the other to the non-infected treatment by the flip of a coin. Ten days after adult eclosion, we starved experimental male beetles for three days and then allowed them to feed for 24 h on either plain apple scrapings (non-infected treatment) or apple scrapings mixed with *H. diminuta* eggs (infected treatment). Infections were allowed to mature for 14 days so that males in this study were 28 days old when they were used in the mating trials.

We paired another group of males that were all non-infected (control males). Pairs (N=10) of control males were fed plain apple scrapings and were otherwise treated in an identical manner as pairs in the treatment group.

**Experimental matings**

We used a randomized block design to assign females to the three possible mating schemes. A female’s first and second mating partners were either infected then non-infected (N=10), non-infected then infected (N=10), or both non-infected controls (N=10).

Females were also virgins and 28 days old when they were used in the mating trials. At the start of a trial the female and a male were placed together into a dish
containing wheat bran and potato. After 24 hours together, we separated the beetles and placed them into their individual dishes. We provided a two-hour interval after separation before we placed the second male with the female for another 24-hour period.

Females were kept in an incubator for 15 days after the removal of the second male. We placed females into a new dish after the first five days. The dish containing any eggs laid by the female during the first five days was kept in the incubator. Females were removed and decapitated at the end of the trial so that we could extract their DNA. Both dishes from a female containing eggs produced during the first five days or 6-15 days after the last copulation were stored in the incubator for 20-22 days. This length of time provided ample time for all eggs to hatch (unpublished data). We collected offspring during these two time periods because Siva-Jothy et al. (1996) found that second male sperm precedence is high during the first several days after copulation, but then declines to approximately 50% afterward (presumably because sperm are eventually evenly mixed in the spermatheca). Therefore, we expect that if infected males produce less sperm, we would find a bias toward the noninfected male for offspring produced after day five; however, offspring produced early should reflect the typical last male sperm precedence patterns.

**Extraction of DNA and PCR**

In the adults, DNA was extracted from soft tissue removed from the exoskeleton. We decapitated adults then removed a small section of the posterior of the abdomen. Using a blunt needle and syringe, we used phosphate buffered saline (pH 7.4) to flush as much of the internal tissues from the exoskeleton as possible. We
immediately examined the contents of all male beetles under a dissection microscope and counted the number of parasites (cysticercoids) present. Soft tissue was then frozen in liquid nitrogen and ground into a coarse powder. The sample was immediately added to a tube containing 300 µl lysis buffer (100 mM Tris pH 8.0, 100 mM EDTA, 10 mM NaCl, 0.5% SDS; Longmire et al. 1988) and 2 µl Proteinase K (100 µg/ml). For offspring, the entire larva was placed in the lysis buffer/proteinase K mixture, and broken up into small pieces using a pestle. Samples of larval and adult tissue were shaken periodically during the 4 hours of incubation at 65°C. We extracted the samples once with 300 µl of phenol, then with a 25:24:1 solution of phenol:chloroform:isoamyl alcohol, followed by a final extraction with a 24:1 solution of chloroform:isoamyl alcohol. For each step in the extraction, we added the reagent, mixed it well, and spun the sample for 5 minutes at 10,000 r.p.m. in a centrifuge. We quantified the DNA concentration in each sample with a spectrophotometer and adjusted the DNA concentration in each sample to 20 ng/µl by adding autoclaved distilled water.

For each amplification reaction we used 0.1 µl Taq DNA polymerase (5 U/µl), 2.6 µl of 10 µM dNTPs (containing equal proportions of dATP, dCTP, dGTP and dTTP), 2.25 µl MgCl₂ (50 mM), 1.9 µl reaction buffer (containing 200 mM Tris-HCl (pH 8.0) and 500 mM KCl), 1.5 µl primer (10 µM), 1 µl template DNA (20 ng/µl), and 9.6 µl autoclaved distilled water. Each primer used in the reaction was a random 10-nucleotide oligomer (Operon Technologies Inc., Alameda, California, USA). The first cycle of the amplification consisted of 1.5 min at 94°C, 1 min at 37°C, and 0.17 °C/s ramp to 72°C for 3 minutes. The amplification process continued for 37
additional cycles consisting of 1 min at 94°C, 1 min at 37°C, and 0.17 °C/s slope to 72°C for 3 minutes. We stored the amplified product at 4°C for up to 1 day until visualized. For analysis, the amplified products were loaded in a 1.4 % agarose gel in 1 X TBE and run at 110 V for 4-5 hours. We then stained the gels in ethidium bromide for 20 min, destained for 5 minutes in water, and photographed them under UV light.

Paternity Analysis

We screened adults with 60 primers (Operon Technology, Inc. kits OPAB, OPAC, and OPAD). We ran the triad of adults and the offspring with primers that produced at least one band that was found in one adult male but not in the mother or the other adult male (unique parental band). To determine paternity, the number of unique parental bands (UPBs) present in each offspring’s lane was then scored for each primer. We ran at least three separate amplified products for each adult on a gel to verify that bands were repeatable and unique. For paternity assignment we only used UPBs that were repeatable across amplifications. We continued to run samples with different primers until we identified at least two UPB’s per offspring. We calculated the proportion of offspring that were sired by the second male (P2).

Because the P2 values were proportions, we transformed (arcsine square root) the data (Sokal & Rohlf 1995) before using a parametric repeated measures analysis of variance test to examine the effect of time and mate treatment on P2. Otherwise, we used nonparametric statistical tests on the unmanipulated data. To determine if male treatment affected P2, we used the Kruskal-Wallis test using SPSS (Windows version 9.0). P2 values in either of the two treatments were compared to the control
group (neither infected) by using the Dunn test (Dunn 1964; Zar 1996). All tests are two-tailed and means are given ± 1 S.D.

RESULTS

After matings were complete, one or more adults died before DNA extraction in two of the 30 families. Of the remaining 28 females, 25 produced offspring during both time periods when eggs were collected. We removed three additional families from analysis because the male in the infected treatment contained no, or very few (<5) cysticercoids. The number of families analysed was reduced further to a total of 17 because samples of one or more adults would not amplify or samples were lost due to lab accidents. The distribution of treatment groups (first male, second male) among the remaining 17 families was: 5 controls (non-infected, non-infected); 6 treatment A (infected, non-infected); and 6 treatment B (non-infected, infected). Among the remaining families, males in the infection treatment contained 196.2 ± 100.0 cysticercoids (range 95-411).

Females in the 17 families produced 36.3 ± 10.7 offspring (range 21-58) during the 15 days of collection. On average, the 20 offspring sampled for paternity represented 59.7 ± 17.4% of all larvae produced during the period of collection.

Of the 60 Operon Technologies, Inc. primers screened, we selected nine to use in paternity assignments because they produced clear, repeatable, and polymorphic banding patterns (AB-09, AB-12, AB-17, AC-02, AC-06, AC-17, AC-20, AD-02, AD-04). We conducted finer scale screening of these nine primers on the three adults in each trial to optimize the number of UPBs before analyzing the entire family. On average, each primer yielded 11.6 obvious bands per individual, of which 2.0 (17%)
were unique to one male among the trio of adults. To attain at least two UPBs per offspring, we used 2.6 ± 0.7 primers (range 2-4) per family. We did not find any cases where an offspring contained UPBs from both potential fathers.

Overall, the proportion of offspring sired by the second male (P_2) was 0.66 ± 0.25 for offspring produced on days 1-5 after copulation and 0.59 ± 0.20 during days 6-15, but this temporal effect was not significant (repeated measures ANOVA; F_{1,14}=3.0, P=0.10). However, infection treatment of the pair of males did effect P_2 (F_{2,14}=8.5, P < 0.005). Because the effect of infection treatment did not differ significantly between the two time periods (time x treatment interaction; F_{2,14}=1.5, P=0.25), we used overall P_2 values (days 1-15) in subsequent analyses.

Infection status of the males had a significant effect on the overall proportion of offspring sired by the last male (Fig. 1; Kruskal Wallis test; \chi^2=10.2, P<0.01). When neither male partnered to a female was infected, the last male sired 71.0 ± 16.4% (median=65.0%; range 55-95%) of the 20 offspring produced. Among females partnered with an infected male, the second male sired 77.2 ± 12.1% (median=77.5%; range 63-95%) when the first mate was infected and 41.7 ± 13.7% (median=40.0%; range 25-60%) when the last (2^{nd}) mate was infected. We compared P_2 between each of the two treatment groups and the control group in which neither male was infected. Females whose last mates were parasitized had P_2 values that differed significantly from control females (Dunn test for posthoc comparisons; Q=2.25, P<0.05). We did not detect any difference in P_2 values between females whose first mates were parasitized and females in the control group (Dunn test; Q=0.67, P>0.40).
DISCUSSION

In the present study we found that the cestode parasite, *Hymenolepis diminuta*, reduced the fertilization success of sperm from male *Tenebrio molitor* when the female had mated previously. When the second male was infected, $P_z$ was reduced by 29.3% (median difference = 15.0%), compared to the proportion of eggs fertilized by non-infected second-mating males. We did not detect a significant difference in $P_z$ when a female’s first mate was infected. Our finding of second-male sperm precedence for the control group ($P_z=0.71$) is consistent with other studies of *T. molitor* using similar mating intervals ($P_z=0.92$, Siva-Jothy et al. 1996; $P_z=0.68$, Drnevich et al. In press). The reduction in sperm precedence for infected males is likely to have a large effect on a male’s fitness. Because individuals aggregate near larval food supplies (grain) and females mate multiply even within a few hours (Worden & Parker In review), sperm competition between males is likely to be common. These data, taken with the results of other studies examining the effect of the parasite on different aspects of male reproductive success (Hurd & Parry 1991; Worden et al. 2000; Worden & Parker In review), demonstrate that parasitism seriously reduces male reproductive potential.

Several mechanisms could be responsible for the parasite-induced reduction in sperm precedence. Parasites may affect the mating behaviors of males so that they are less likely to copulate with females. Hurd & Parry (1991) provided evidence for this when they demonstrated that infected males are less responsive to the pheromone of females. However, they did not detect differences in male responsiveness when the metacestodes were mature (9-11 d), as they were in this study. It is not known
whether the parasite affects male sperm production, sperm viability, sperm competitiveness, or male remating rates. Infection has been shown to affect protein content and trehalase activity in spermatophores (Carver et al. 1999), but it is not known what impact, if any, this has on sperm or sperm use. It is also possible that females preferentially store or use sperm from non-infected males (cryptic female choice, Eberhard 1996). The importance of these factors on the precedence patterns observed in this paper needs to be investigated.

Previously, we demonstrated that females prefer the odors of non-infected males (Worden et al. 2000), and females bias copulations toward non-infected males (Worden & Parker In review). These findings were consistent with predictions of parasite-mediated sexual selection (Hamilton & Zuk 1982). According to this model, the strength of the correlation between sexual characters (e.g., plumage colors, vigorous displays) and genetic resistance to parasites is dependent on the idea that the parasite negatively affects host fitness. It is now clear that infected male as well as female (see Hurd 1990; Hurd & Parry 1991) grain beetles are negatively affected by *H. diminuta*.

Recent evidence suggests that sperm precedence is influenced by variation in male traits (Lewis & Austad 1990, 1994; Simmons & Parker 1992; Edvardsson & Arnqvist 2000). The impact of parasitism on sperm precedence has received little attention. Yan & Stevens (1995) found that males infected with *H. diminuta* also had reduced second-male sperm precedence in a related beetle, *Tribolium castaneum*. They found that precedence was reduced by 12% five days after copulation. In species like *T. castaneum* and *T. molitor*, where female remating rates are high, the
parasite-induced reduction in sperm precedence will have a significant impact on host fitness.

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Figure 4.1. Second male sperm precedence (± 1 S.D.) for offspring collected on days 1-5 post-copulation (a) or days 6-15 (b). Mating treatments on the x-axis indicate whether the first and second male were non-infected (N-I) or infected (I). An asterisk indicates that the P2 value for the treatment group differed significantly (P<0.05) from P2 value for the control group (Both N-I).
CHAPTER 5

FEMALES PREFER NON-INFECTED MALES AS MATES IN THE GRAIN BEETLE *TENEBRIO MOLITOR*: EVIDENCE IN PRE- AND POSTCOPULATORY BEHAVIORS

ABSTRACT. We examined the effects of infection by the tapeworm *Hymenolepis diminuta* on mate choice in a field-caught strain of grain beetle, *Tenebrio molitor*. If sexual selection is important in this species, the fact that female beetles mate multiply means that both precopulatory and postcopulatory behaviors may be important. When virgin females were allowed to choose between tethered males, one infected and one non-infected, they spent more time near and copulated more often with non-infected males than infected males. During the 90-minute trials, the majority (65%) of control females, presented with two non-infected males, copulated with both males at least once. However, females presented with one infected and one non-infected males were much less likely (30%) to be polyandrous. Among monogamous females, 81% mated exclusively with non-infected males. Within the group of polyandrous females, contact with the second male was made earlier for those that copulated first with the infected male. Mass of males also influenced female remating behavior. Females that first mated with larger males copulated fewer times than females that mated with smaller males. Infected males were not less sexually responsive than non-infected males, so these results are consistent with female mate choice. These results demonstrate that
female beetles prefer non-infected males in both precopulatory and postcopulatory choice.

Females may gain genetic or material benefits by selectively mating with males expressing the most exaggerated, condition-dependent sexual traits (Kodric-Brown and Brown 1984; Andersson 1986; Andersson 1994). In particular, some models emphasize the role of parasites when sexual characters in the host reveal resistance to parasites or immunocompetence (Hamilton and Zuk 1982; Folstad and Karter 1992). Alternatively, indicators of infection may provide choosy females with direct benefits by reducing the risk of infection (Borgia and Collis 1989; Able 1996) or by indicating males that are best able to provide material benefits (Hoelzer 1989; Rosenqvist and Johansson 1995).

Previously, Worden et al. (2000) demonstrated that infection by the rat tapeworm, *Hymenolepis diminuta*, reduces the attractiveness of odors and reproductive success of male grain beetles, *Tenebrio molitor*. Females discriminated against odors from infected males but not infected females, suggesting that females may use the change in odor accompanying infection in mate choice. Because mate choice was not examined directly, the effects of infection on sexual selection in *T. molitor* were not known. Here we examine this question in an environment where we experimentally prevented male-male competition, and limited coercion by males. We also allowed females to mate multiply. This allows us to test female preferences in both precopulatory mate choice and some postcopulatory behaviors.

*Tenebrio molitor* is an important intermediate host for the tapeworm, *H. diminuta* (Rau 1979; Schmidt and Roberts 1989). Because of the complicated lifecycle of the parasite (see Schmidt and Roberts 1989; Worden et al. 2000), females do not
increase their chance of infection by mating with infected males. *Hymenolepis diminuta* has a significant effect on the fitness of its intermediate host (Keymer 1980; Hurd and Arme 1986; Yan 1997; Webb and Hurd 1999; Worden et al. 2000), so selecting non-infected or lightly infected mates could provide material or genetic benefits (if resistance is heritable) to females.

The mating system of *Tenebrio molitor* is polygynandrous. Both males and females are attracted to pheromones produced by the opposite sex (Happ 1969; August 1971), and copulations occur rapidly, lasting less than three minutes (August 1971; personal observation). Females will remate quickly in the lab (personal observation), and females that mate multiply produce more eggs than females that mate only once (Worden and Parker in press). In addition, females that mate to different males produced more eggs than females that mate an equal number of times to the same male (Worden and Parker in press).

Under some conditions, the benefits of mating with multiple partners may outweigh the benefits of carefully choosing the first mate. For example, material benefits may have selected for females on low-quality diets to mate quickly and indiscriminately compared to more discriminant females fed high-quality diets in the tree cricket *Oecanthus nigricornis* (Brown 1997). Several possible genetic benefits (e.g., Zeh and Zeh 1997; Newcomer et al. 1999; Jennions and Petrie 2000) can also select for females to mate with many different males. Therefore, polyandrous females might be expected to relax precopulatory mate choice to gain the benefits of multiple mating. On the other hand, multiple matings by females presents an opportunity for selection to act on postcopulatory mechanisms (e.g., remating frequency, sperm storage,
etc.) for biasing the success of sperm from different males (Thornhill 1983; Eberhard 1996) or for sexual conflict to incite intersexual competition over control of fertilization (Alexander et al. 1997). Certainly, postcopulatory manipulation by females or males may work in concert with precopulatory mate choice (Eberhard 1996). However, females that mate multiply may express preference for certain males in ways that are obscured by protocols examining only the first male with which a female mates.

In the present study we examined female mate choice between an infected and non-infected male. The grain beetles used in this study were the offspring of a semi-natural population. We believe this is the first study examining the behavioral affects of *H. diminuta* on a non-laboratory strain of *T. molitor*. We tested whether females associated and mated more frequently with non-infected males. We allowed females to mate repeatedly during the trials, allowing us to examine mate choice in a situation typical for this species. Because female grain beetles mate multiply, we tested whether a female’s tendency to remate was affected by the infection status of a female’s first mate.

**METHODS**

**The Study Population**

We collected 16 adult and 24 late-stage larval yellow mealworm beetles from an O.S.U. horse stable in Columbus, Ohio, in June 1998. We divided this population in half and placed each group in a 4,480-cm³ container of wheat bran supplemented with potato. Individuals were allowed to breed freely within these containers. We used offspring from these two groups in the behavioral trials. First-generation pupae were collected, sexed (Bhattacharya et al. 1970), and housed individually in 60 × 15 mm Petri dishes. In each trial, the female always originated from a different container than the
male so that female and males were never siblings. For each adult, we had information on its sex, age, and mass at eclosion. Beetles were housed in the same incubator at $34 \pm 2^\circ$C.

**Experimental Protocol**

Male beetles ($N=134$) were matched into pairs by mass (within 5%) and age (within 24 hours) for use in the behavior trials. We randomly divided the paired males into two groups: infection group ($N=46$ pairs) and non-infected control group ($N=21$ pairs). For the infection group we used a randomized block design in which the infected treatment was randomly assigned to one member of a pair. The other beetle in the pair was assigned the non-infected treatment. Males in the non-infected control group were all assigned to the non-infected treatment.

We collected *H. diminuta* eggs from feces of infected rats using a salt flotation protocol. The eggs were subsequently washed and stored in distilled water at room temperature for up to 7 days.

We starved newly emerged adult males for 3 days, and then allowed them to feed for 24 h on either plain apple scrapings (non-infected treatment) or apple scrapings mixed with *H. diminuta* eggs (infected treatment). Although copulatory response to pheromone from female beetles is reduced in 6-7 day-old infected males compared to non-infected males, infection does not appear to reduce copulatory response in older, 12-14 day-old males (Hurd and Parry 1991). Therefore, to reduce the possibility of differences in sexual responsiveness between infected and non-infected males, we allowed infections to mature for 11 days before the beetles were used in the experiments. As a result, all males in this study were virgins and 15 days old during the
behavioral trials. Females were also virgins, but they were tested when they were 7 days old.

Twenty-four hours prior to the start of a behavioral trial, we randomly assigned a female (N=67) to a pair of males. At this time we also attached ~10 cm of black sewing thread to each male by placing each male on ice for 1-2 minutes until they became immobile, and then tying one end of the string around the beetle between its thorax and abdomen. We took care not to tie the string too tightly, and we always tested the ability of a male to walk after tethering. Leg movement did not appear to be hindered by the string. Males were then returned to their Petri dish until the trials the following day.

The female choice arena consisted of an 11.0 X 11.0 cm plexiglass container that was divided at one end by a 15 mm high barrier that extended about one-third of the length of the container (Fig. 1). We placed the two tethered males into each corner on either side of the barrier and attached the other end of the string to the top of the apparatus. Because the string did not hinder leg movement, males were able to walk freely within the area contained by the barrier, but they were not able to travel beyond the imaginary line perpendicular to the arena walls and the tip of the barrier itself. Therefore, males never had physical or visual access to each other, and approximately two-thirds of the arena was not accessible to either male. We placed a female that was chosen at random into the center of the ‘female-only’ portion of the arena and covered her with a 2.5 mm diameter clear plastic dish during a 10-minute acclimation period. At the start of a trial, we removed the covering that was over the female, and videotaped the interactions between the three beetles for 90 minutes under red-light illumination.
Behavioral Data

Videotape of each trial was analyzed, and portions of the arena were assigned into either male's contact area (area where each male had access) or 'no choice' areas (all other areas where neither male had access). We recorded the occurrence and duration of the following behaviors: visitation by the female to either male's contact area, contact between a male and the female that was initiated by the female, contact between a male and the female that was initiated by the male, male mounts on females, and copulations. We differentiated copulations from mounts by the criteria that copulations necessarily consisted of all of the following: extension of the male's genitalia with genital contact, a cessation of movement by the female, and a period while the male is mounted on the female in which the male strokes the female with his antennae and/or prothoracic legs followed by a period when the stroking ceases (personal observation; August, 1971).

The behavioral data were then used to determine a female's overall mating strategy and preferences. We determined the winner among a pair of males by male/female interaction time and number of copulations. The male with which a female spent most of her time was scored as the winner by time. Likewise, the male with which a female copulated most was scored as the winner by copulations. We also compared non-infected control group trials to infection group trials in terms of mating frequency and the occurrence of polyandry versus monandry. Specifically, we examined the effect of infection status of a female's first mate on the tendency for the female to mate with the other male in the trial. Finally, we examined male/female post-mating associations in trials where females mated to both the infected and the non-infected male. When
parametric tests were used, data had been checked for normality (Kolmogorov-Smirnov one-sample test) and homogeneity of variances (Levene's test). We used SPSS 9.0 for Windows for all statistical analyses. Statistics are given as mean ± SD unless stated, and $P$ values are for two-tailed tests.

RESULTS

Samples- Of the 67 trials, we excluded 10 from analysis because one or more males broke free from their tethers, a male climbed up the divider, or a female flipped over and was unable to right herself within a 10-minute period. This left scorable sample sizes of $N=31$ trials for the infection group and $N=20$ trials for the non-infected control group.

Male Behavior- Hurd and Parry (1991) failed to find an effect of infection on the copulatory responses of males aged 12-14 days. We compared male reproductive behaviors between infected and non-infected males within each trial to confirm that infected males did not exhibit a reduction in responsiveness to females. To evaluate the sexual activity of males, we counted the number of contacts between the male and female that were initiated by males and the number of mounts per male. Although the infected male in a pair of males usually initiated fewer contacts and mounts with females (Wilcoxon signed rank tests: $Z=-3.1, N=36, P=0.002$ and $Z=-2.2, N=36, P=0.02$, respectively), this difference was confounded by unequal female visitation rates to each male that resulted in fewer opportunities for infected males to interact with females. Therefore, we divided the contact and mount counts for each male by the amount of time that a female was within a male's contact area, excluding 9 trials in which the female never visited one of the male's contact area. This adjustment revealed that infected
males were not less sexually responsive than non-infected males. The infected male actually made 0.44 contacts/minute (median) compared to only 0.25 contacts/minute by the non-infected male (Wilcoxin signed rank test: Z=-2.3, N=28, P=0.02). Infected and non-infected males did not differ significantly in actual copulation attempts (Wilcoxin signed rank test: Z=-1.0, N=28, P=0.32); infected males mounted females 0.17 times/minute (median) compared to 0.12 times/minute by non-infected males. Infection levels among males ranged from 14 to 597 cysticercoids (mean = 263.2 ± 179.0). None of the non-infected males contained any cysticercoids.

Female Behavior- We examined each female’s interactions with the two males. We then calculated the total amount of time each female was in each male’s contact area. Females spent a total of 2741.3 ± 1473.1 s (50.8 % of total time) in the contact areas of the males. Females spent 1774.4 ± 1488.0 s with the non-infected male compared to only 966.9 ± 1144.0 s with the infected male (one-sample t-test on difference ≠ 0: t=2.2, N=37, P=0.03). Twenty-six of the 37 females (70%) spent more time with the non-infected male than the infected male (binomial test: P=0.02). We then calculated the proportion of time that a female spent in each male’s contact area out of the total time spent in the contact area of both males. There was no correlation between the proportion of time that females spent in the contact area of the infected male and the number of cysticercoids that were present in the male ($F_{1,36}=0.10$, $R^2=0.003$, $P=0.75$).

Females assigned to the infection group trials copulated 1.7 ± 1.1 times during the 90 min trials. Four of 37 (10.8%) females did not copulate with either male, and these females were not included in the determination of the winning male by
Four of the 33 copulating females mated equally with each male, and these females were divided evenly so that both the infected male and non-infected male were scored as winning two additional copulation contests. Seventy percent of copulating females copulated more with the non-infected male than the infected male (binomial test: $N=34$, $P=0.04$). Fourteen of the 33 copulating females (42%) copulated only once, and, of these females, 10 of 14 (71%) mated exclusively with the non-infected male (binomial test: $P=0.18$). Among all monogamous females, 17 of 21 (81%) mated exclusively with the non-infected male. We found no correlation between the number of copulations with the infected male and the number of cysticercoids in the male ($r=0.10$, $P=0.57$).

Even though most females biased copulations toward the non-infected male, 11 of 36 (30%) females mated at least once with both males. We examined the postcopulatory behaviors of these 11 polyandrous females to examine the reactions of females after mating with each type of male. We calculated times for two aspects of female postcopulatory behavior that may be important to male fertilization success: duration of a female’s visit in a male’s contact area after copulation, and the length of time after copulation with one male until the female visits the other male’s contact area. We determined these times by averaging data from all copulations with each male within a trial. Because one of the 11 polyandrous females never left one of the male’s contact areas after copulation, and two of the 11 females never visited the other male after copulation, we removed those trials from the respective analyses. The time after copulation when a female first left the male’s contact area did not differ significantly between infected and non-infected males (paired $t$-test: $t=0.11$, $N=10$, $P=0.92$).
However, females visited the other male three times sooner after copulating with the infected male than after copulating with the non-infected male (paired t-test: \( t = -3.4 \), \( N=9, P=0.01 \); Fig. 2).

The tendency for females to remate with another male is an extremely important postcopulatory behavior affecting a male’s potential reproductive success. Females that had access only to non-infected males (non-infected control group) differed in their mating behavior from females assigned to the infection group. We tabulated the number of males with which each female copulated (0, 1, or 2), and compared these values between trials that contained an infected male and those that did not (Table I). Females in the non-infected control group were more likely to mate with both males than females assigned to the infection group (\( \chi^2 = 7.2, P<0.05 \)). In the control trials, 76% of females that copulated at least twice (and 65% of all females) copulated with both males. Overall, the male treatment group (both males not infected or one infected) had a significant effect on the number of copulations for each female, with females assigned to the infected group mating less frequently (ANOVA: \( F_{1,55}=6.1, P=0.02 \)).

Examining only females that had at least one copulation, we included the mean mass of the males as a covariate in the model and male treatment group as the main factor. Both mean mass of the males (ANCOVA: \( F_{1,48}=4.0, P<0.05 \)) and male treatment group (\( F_{1,48}=7.3, P=0.009 \)) had a significant effect on the number of copulations per female. Among the subset of females that copulated at least once, females in arenas with heavier males copulated fewer times than females presented with small males.

To further investigate the differences in remating between the non-infected control and the infection group, we controlled for mate order effects and examined the
proportion of females that copulated with the other male after her initial copulation. Unfortunately, the experimental design of this study, while good at detecting overall preferences, was not particularly powerful at comparing remating tendencies after mating with infected or non-infected males. Because of the overall bias against copulating with infected males, only 10 of 33 females in the infection group copulated with the infected male first, leading to a small sample size for this group. However, this analysis revealed significant differences in the tendency to remate by females that mated first to each of the three possible classes of males (Table II; $\chi^2 = 8.5$, $P < 0.02$). Females that first copulated with the non-infected male in the infected male trials were less likely to remate with the other male in the trial than females assigned to the control group ($\chi^2 = 7.8$, $P = 0.005$).

**DISCUSSION**

Female *T. molitor* prefer the odors of males that are not infected with *H. diminuta* cysticercoids (Worden et al. 2000). Although females use male odors to locate males (August 1967, 1971; Happ 1969), and preference by females for odors of non-infected individuals is directed only towards males (Worden et al. 2000), it had not been known previously whether females actually select mating partners based on infection status. *This study demonstrates that females prefer non-infected to infected males in duration of association with the males and as actual choice of mates.* We could not attribute this preference to a decrease in reproductive behavior by infected males. In fact, infected males made contacts with nearby females at a greater rate than non-infected males. Although the increase in male contact rate could be caused by infection, we suggest it is more likely a consequence of fewer copulations between females and
infected males. Male grain beetles have ~30-min refractory period after copulation before they can remate, and males do not attempt copulations during this time (personal observation). Because non-infected males copulated more often, they were sexually unreceptive for a greater proportion of time than infected males.

Unlike our previous study showing a negative correlation between attractiveness of male odors and parasite burden (Worden et al. 2000), here we found no relationship between female visitation rate or copulation number with the infected male and the number of cysticercoids present. This difference suggests that females are more sensitive in detecting the cues of infection in actual live beetles than the pheromone bioassay was able to reveal. In fact, trials with males at infection levels below the critical value determined by Worden et al. (2000) (159 cysticercoids) did not differ in copulation preferences demonstrated by females. Because females exposed to odors from infected and non-infected males were never able to copulate with either male in Worden et al. (2000), that study tested female attraction to, and persistence at locating, a potential mating partner. However, the present study examined female mating behavior under very different circumstances, where attraction to a male was only one of several behavioral components ultimately leading to the bias in copulations toward non-infected males.

Females may benefit from biasing copulations toward non-infected males through material gains if parasitized males have poorer quality ejaculates than non-infected competitors. Females in both Tribolium confusum and Tenebrio molitor show marked reductions in fecundity associated with infections with Hymenolepis diminuta (Keymer 1980; Hurd and Arme 1986; Webb and Hurd 1999), and the reproductive
success of a pair of *T. molitor* is negatively correlated to infection intensity in the male (Worden et al. 2000). Therefore, one might predict that individuals should seek mating partners with no or few parasites to maximize reproductive success, particularly when mating is costly (e.g., Rosenqvist and Johansson 1995). The mechanism responsible for the decline in reproductive success of infected males (Worden et al. 2000) is not known. Carver et al. (1999) found that spermatophores from males infected with *H. diminuta* actually contained more protein than spermatophores from non-infected males, although the amount of protein transferred to the female’s spermatheca was not affected by the infection status of the male. Studies on the affects of infection status on sperm number and sperm transfer within the female are needed to better understand the impact of infection on male fitness.

Females may also accrue genetic benefits through mate choice if a male’s resistance or overall quality is heritable (Hamilton and Zuk 1982; Kirkpatrick and Ryan 1991; Maynard Smith 1991). Evidence suggests that immunocompetence and the ability to produce sexual ornaments are tightly linked and mediated by testosterone (e.g., Verhulst et al. 1999; Roulin et al. 2000; Willis and Poulin 2000) in vertebrates. The generality of this relationship in invertebrates has been questioned because invertebrates lack testosterone or a similar hormone (Zuk and McKean 1996; Sheridan et al. 2000). However, a positive correlation between degree of sexual showiness and heritable resistance could still occur through alternative mechanisms.

Female *T. molitor* mate multiply and fecundity is enhanced both by multiple copulations and copulations with multiple individuals (Worden and Parker in press). This leads to tradeoffs between acquiring a mating partner of high quality and gaining
benefits provided by copulations with different males. In the control group, 65% of all females and 70% of females that copulated at least once copulated with both males during the 90 min trial, indicating that polyandry is common even during the short time of the trials. However, females in the infection group that mated to the non-infected male first copulated less frequently, and were less likely to mate with the other (infected) male. Only 26% of these females were polyandrous during the trials. These differences indicate that, despite the benefits associated with multiple mating partners, females do discriminate between potential mates. These data also indicate that females may adjust postcopulatory behavior depending on the quality of her mate. Females visited the other male more quickly after mating with the infected male than after mating with the non-infected male. This difference in postcopulatory exploration and/or attraction to another male may be the proximate cause for the reduction in polyandry by females in the infection group that copulated first with the non-infected male. We also found that the mass of a female’s mate was negatively correlated with remating frequency. We have not specifically examined the importance of male size to female mate choice, but we do know that the number of sperm delivered by males is correlated positively with male mass and length (unpublished data). Therefore, we suspect that large ejaculate volume could inhibit the frequency of female remating.

Several hypotheses could explain the differences in female postcopulatory behavior. First, infected males could differ in their ability to suppress females from remating. Males in some insects produce ejaculate compounds that inhibit female receptivity (e.g., Chen et al. 1988). Second, the difference could represent postcopulatory choice by females for non-infected males. For example, females could
alter their postcopulatory behaviors based on the quality of the prior mate relative to a future mate (e.g., Petrie et al. 1992). Alternatively, this difference could be a passive consequence of reduced attractiveness of infected males. Because odors from infected males are less attractive than odors from non-infected males (Worden et al. 2000), females may simply not detect immediately the infected male after copulating with the non-infected male. To examine these possibilities, further experiments are needed specifically aimed at examining female remating when the prior mate has been manipulated. In addition, tests that determine whether infection affects the material transferred to the female at copulation are needed.

In total, our results demonstrate that wild-caught female *T. molitor* bias copulations against males infected with *H. diminuta* metacestodes. When neither male that was presented to a female was infected (control group), the majority of females mated with both males during the trials. However, females presented with an infected and a non-infected male were more selective in their copulations, supporting the role of precopulatory female mate choice. Postcopulatory behavior of females also differed depending on the infection status of the males in her arena, suggesting that they express postcopulatory choice as well.

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and use of animals.
Table 5.1. Number of females’ copulation partners when presented choice of infected versus non-infected male (Infection Group) or 2 non-infected males (Control Group)

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<th>Treatment</th>
<th>0</th>
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<tr>
<td>Control group</td>
<td>2 (10%)</td>
<td>5 (25%)</td>
<td>13 (65%)</td>
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<tr>
<td>Infection group</td>
<td>4 (11%)</td>
<td>22 (59%)</td>
<td>11 (30%)</td>
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<tr>
<td>First mate</td>
<td>Mate with other male?</td>
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<td></td>
<td><strong>NO</strong></td>
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<td>Non-infected (control group)</td>
<td>5 (29%)</td>
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<td></td>
<td>12 (71%)</td>
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<tr>
<td>Non-infected (infection group)</td>
<td>17 (74%)</td>
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<td></td>
<td>6 (26%)</td>
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<tr>
<td>Infected</td>
<td>4 (40%)</td>
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<td></td>
<td>6 (60%)</td>
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Table 5.2. Number of females that mated with other male in trial after first mating with a non-infected or infected male.
Figure 5.1. Female choice arena.
Figure 5.2. The amount of time after copulating with either an infected or non-infected male before females visited the contact area of the other male in the arena. Error bar represents ± 1 S.E.
CHAPTER 6
CONCLUSIONS

The mating system of *Tenebrio molitor* makes it a valuable model for understanding the effects of parasites on sexual selection under conditions of extreme polyandry. Females in many species mate multiply and we are only beginning to understand the forces driving this behavior. Few studies have explored the possibility that both genetic and material benefits may occur (Newcomer et al. 1999). If it is beneficial for females to mate multiply, then mate choice may often be subtle or occur in the postcopulatory arena. This study examined the effects of a parasite, *Hymenolepis diminuta*, to male reproductive success in precopulatory mate choice and under the conditions of sperm competition typical to *T. molitor*.

The series of multiple mating experiments presented in this work explored the effects of mating multiply to the same male or different males. It was found that females that mate more than once produce more eggs and larvae than females that mate only once. The results suggest that infertile copulations might explain this result in part, but analysis reveals that another mechanism must be operating to explain the full effect. This study also demonstrated that females that are polyandrous (mating with more than one male) produce more offspring than females that mate an equal number of times with the same male. However, there was no indication that genetic incompatibility affected the hatchability of eggs or early growth and survivorship of offspring. None of the multiply
mated females exhibited any cost associated with multiple copulations in terms of their survival or the mass of their offspring. Boggs (1990) predicted that as a female’s food consumption increases or becomes more nutritionally complete, the effect of male-donated nutrients should decline. Female grain beetles kept on food-limited diets produced smaller offspring, but did not benefit from multiple mating any more than females fed ad libitum. The results reported here, taken together with the fact that the spermatophore is relatively small, suggest that there are no nutritional benefits gained from spermatophores.

The results of the multiple mating experiments are consistent with female preference for multiple partners (Archer and Elgar 1999). Females appear to increase offspring production after mating with different males, suggesting a preference for multiple partners. Multiple matings may provide genetic benefits that this work did not examine. For example, offspring of females that mate multiply may be more fit at later stages in development (e.g., pupation), or they may have higher reproductive success as adults (e.g. more competitive sperm, Keller and Reeve 1995). If multiple mating does provide genetic benefits and males are not limiting, then females that delay reproduction until they have mated several times would be favored, as long as the benefits outweigh the costs associated with mating.

The intensity of sexual selection is dependent on the mating system and relative parental effort of each sex (Trivers 1972). A male’s ability to manipulate a female’s use of his sperm should be under great selection pressure in polyandrous systems. Behaviors such as mate guarding (Alcock 1994) and removal of a previous male’s sperm (e.g., Gage 1992; Haubruge et al. 1999) are two examples of such manipulation by which a male may
limit the ability of a female to use another male’s sperm for fertilization. Chemicals in male ejaculates have been discovered which alter female physiology and behavior in many insects (e.g., Destephano and Brady 1977; Chen 1984; Smith et al. 1989, 1990).

Eberhard (1996) suggests that females may also be under selection to resist manipulation by male products. Selection would favor females whose response thresholds are high enough so that only males that produce high quality or large quantities of ejaculate products would trigger a response. The male offspring sired by these successful males would be expected to have higher reproductive success if they inherit the ability to produce high quality ejaculates. The process of runaway (Fisherian) selection for greater quantity or more effective ejaculate compounds would select for males to produce ejaculates that are more and more effective at manipulating sperm use. This scenario seems especially probable for species in which both males and females mate with multiple individuals.

Selection in the scenario just discussed would favor a more hostile female reproductive tract environment for sperm, thus selecting for better quality sperm or more effective ejaculate compounds (Rice and Holland 1997). This selection would act at both the intraejaculate as well as interejaculate level. Natural selection acts on the female reproductive tract to reduce the number of sperm reaching and subsequently penetrating the ovum (Eberhard 1996). This filtering of sperm could reduce the risk of malformed sperm reaching the egg, and it would also reduce the possibility of having multiple aggressive sperm reaching the egg at one time (Eberhard 1996). Entry of too many sperm into the ovum can lead to developmental abnormalities (e.g., Longo 1987). However, in females that mate only once, filtering that is so extreme that it results in an
inadequate number of sperm available to fertilize all of her eggs would be selected against. The amount of sperm that a female receives during her lifespan will increase with degree of polyandry. Males in polyandrous species typically devote more of their total reproductive budget to ejaculate production as a consequence of sperm competition risk (Parker et al. 1996). Therefore, it seems likely that the constraints on selection for harsh (stringent) conditions within the female reproductive tract would be lessened in polyandrous species because possible sperm limitations that might result from this stringent filtering process would be compensated by multiple ejaculates. J. Drnevich has found evidence that, despite the large number of sperm in an ejaculate, sperm use appears to be very inefficient in *Tenebrio molitor* (unpublished data, pers. comm.). If this is the case, then *Tenebrio molitor* fits this model correlating degree of polyandry and stringency in the female reproductive tract.

In a species like *Tenebrio molitor*, where females mate so frequently and multiply mated females produce more offspring, overt mate choice might be relaxed. On the other hand, the opportunity for postcopulatory sexual selection via sperm competition or cryptic female choice is great. In this work, the importance of parasites in mate choice and male reproductive success was studied to examine the impact on both precopulatory and postcopulatory mating success.

Few studies have examined the role of pheromones as indicators of infection (Penn and Potts 1998). This work demonstrates that the attractiveness of male odors is correlated negatively with the intensity of infection by *H. diminuta* metacestodes. Because neither sex discriminated against infected individuals of the same sex, it appears that this preference of odors from noninfected males is likely to be specific to mate
finding. These results fail to support a model of avoidance of unhealthy individuals due to associatively transmissible parasites (Able 1996), because the preference was sex-specific. Females did not have an aversive response to the odors from infected males, but females appeared less attracted to these odors compared to odors from noninfected males.

Experiments were performed exploring the reproductive success of infected males with and without sperm competition. The number of offspring produced by females that mated only with one infected male was negatively correlated with the intensity of infection (number of cysticercoids). This result could mean that infected males delivered less sperm or other ejaculate compounds over the 24-hour period that the male and female were together. An alternative but not mutually exclusive explanation is that females reduce their rate of oviposition after mating to infected males (as appears to occur when females mate only with one male; see Chapter 2). When females mated first to noninfected male and then to an infected male, the proportion of offspring sired by the second (infected) male ($P_2$) was significantly less than the $P_2$ value when females mated to two noninfected males. Therefore, infection exerts a significant cost to male grain beetles in terms of limiting their reproductive success.

Although the odor experiments demonstrated that females have the ability to detect the differences in the odors of infected and noninfected males, a final experiment was necessary to examine whether females from a natural population actually express mate choice based on infection status. It was found that wild females spend more time and mate more often with noninfected wild males compared to infected wild males. Despite the benefits provided by multiple mating, females presented with one infected and one noninfected male were less likely to be polyandrous than females presented with
two noninfected males (30% versus 65%, respectively). The tendency for individuals to aggregate near food resources probably means that males are rarely limiting. Given this, the cost of rejecting a male as a mate is likely to be low. When females did mate with both the noninfected and infected males, a female left the infected male and visited the other male sooner than she did after mating to the noninfected male. This difference in postcopulatory behavior could have a significant impact on male reproductive success because rapid remating by a female can result in complete loss of reproductive success for the first male (Drnevich et al. In press).

Further investigation is necessary to understand the mechanisms responsible for the increased fecundity of polyandrous females and the reduction in sperm precedence found for infected males. Attention needs to be given to the events that occur within the female to complete our understanding of sperm competition and a female’s control over paternity (Simmons and Siva-Jothy 1998). Two findings presented in this work show that the amount of ejaculate material received by females has a significant effect on female behavior. First, females that mate multiply lay more eggs than females that only mate once (Chapter 2). Secondly, females that mate with larger males mate less often than females that mate with smaller ones (Chapter 5), and male mass is correlated with the number of sperm per spermatophore (unpubl. data). Clearly, copulation alone does not insure the success of a male’s sperm. Infection with *H. diminuta* results in decreased mating success in *T. molitor*, but postcopulatory mechanisms may further reduce the reproductive success of infected males. Understanding the sperm use and storage in the female’s complex reproductive tract is likely to provide further understanding of sexual selection in *Tenebrio molitor*. 

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APPENDIX

DNA EXTRACTION PROTOCOL FOR *Tenebrio molitor*

Adult beetles:

1a. Pipet 300 µl of lysis buffer into each autoclaved Eppendorf tube.

2a. Using a small scissors, cut off the head and then the tip of the abdomen from the beetle. Fill a clean syringe with a blunted needle attached with approximately 3 ml of saline. Insert the needle through the opening in the thorax (anterior end). While holding the beetle over a glass dish with one hand, force the saline solution through the body so that the internal tissue is forced out the posterior opening of the beetle.

3a. Use a clean tweezer to pick up the tissue from the glass dish, and place it into the appropriately labeled tube (1a). Discard the exoskeleton.

4a. Make sure that all equipment is cleaned with 70% ethanol between beetles so that contamination between samples does not occur. The syringe should be rinsed several times by forcing distilled water through it.

(Proceed with step 5 below)

Larvae:

1b. Place the larva into an autoclaved Eppendorf tube and label it.

2b. Pipet 300 µl of lysis buffer into each tube.

3b. Using a clean plastic pestle, plunge up and down the tube until the larvae has been squished enough that all its internal contents have been released. Do not grind the pestle because this could break the DNA.

4b. Make sure that the pestle is clean and rinsed in ethanol before using it again on another larvae (see 4a).

All samples:

5. Pipet 30 µl of proteinase K enzyme into each sample and shake tubes well.
6. Place samples in the incubating bath at 65°C for 4-6 hours. Return at least once to shake the samples during the incubation. When samples have incubated for a sufficient period of time, they should be considerably less viscous than the original sample.

7. Once the samples have been incubated, remove them to a vented hood. Add 300 µl of phenol to each tube.

8. Shake each sample vigorously for at least 30 s, or until the phenol is mixed completely into the sample.

9. Put the samples into the centrifuge, making sure that the tubes are balanced, and spin them for 5 minutes at 10,000 r.p.m.

10. When centrifuge is complete, remove the tubes and return them to the hood.

11. Using a disposable plastic transfer pipet, extract the TOP (clearer) portion of the sample and squirt it into a fresh, appropriately labeled, tube.

12. Dump the rest of the sample (darker bottom portion) in to the waste container.

13. After this pure phenol extraction, the samples are ready for one phenol/ CIA extraction.

14. To the tube containing the sample, add 0.15 ml phenol and 0.15 ml CIA.

15. Repeat steps 8-12 above.

16. Now do one final extraction with pure CIA.

17. Add 0.3 ml CIA to each sample, shake vigorously, and centrifuge at 10,000 r.p.m. for 5 minutes.

18. Extract the top layer of solution for each sample (place them into new tubes), and discard the waste.

19. Place each sample into dialysis tubing and three changes (1 hour each) of 2X solution.

20. Store samples in refrigerator.


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