Sexual conflict in the Bean Weevil, *Callosobruchus maculatus*

Helen Sarah Crudgington

A thesis submitted for the
Degree of Doctor of Philosophy
in the
Department of Animal and Plant Sciences
University of Sheffield

April 2001

all is fair when love is war

Acknowledgements

The day I arrived in Sheffield for my PhD interview, not only was the weather sunny but so were all the smiling faces I met in SC lab - this was good enough for me (along with all the sound scientific reasons of course) and Up North I went. Although that sunny day was more or less a one off, I've thoroughly enjoyed my time in Sheffield, and this is entirely due to the wonderful people I've met here.

Without the support of many people in the department, I wouldn't have made it this far - my appreciation goes to Sue, Lisa, Linda and Cath in the office, Jane next door, Darren, Andy, Hazel and Dave for technical support and Phil, whose constant amusement at my space-age anti-allergy get-up has kept me amused too.

Along the way, I've shared good times in the 197, the Interval and even less exciting drinkeries with the one and only Dr. Batty, with Mara and Robert - great movie-going companions, with Fi, Mark, Martin, Matt, Andy Man - the early bird-clubbers, Nick, Emma, Tomasso, Steve, Barry, Vicki, Karl and Anita - a fellow founder member of the now colossal APS ballet appreciation society. Much-welcomed late arrivals were Dave, Petra, Allen, Debs, Tom, Andy, Nancy, Lisa and Rupert - whose love of champers has introduced me to a lifestyle that I wouldn't mind becoming accustomed to, and Ali, Mel and Adam - sweethearts all three.

For making me so welcome in Sheffield my heartfelt thanks go to early Sclabbers - Jill Thompson - commissar for high quality plonk and decent late-night conversation, Jonny Bean (AKA Ryder) - always understanding and real, the great Dr. Al Stutter who I thank for many of the useful things I've managed to pick up about this science-lark, Alex Córdoba-Aguilar - 'no women no cry', Mr Andy Barnes - a wealth of knowledge and good chats about science and politics, and Davey Blakey, who always had a smile - I miss them all. Welcome passers through the lab were Piadad and Paco, Simon, and the

wonderful José Andrés whose penchant for chutney has kept our friendship going despite the distance. More recently there's John Thompson - the master emasculator and saver of all lab disasters, Sophie Armitage - who always has a kind word at the ready, and Jens Rolff - whose already proved so good to have around. Lastly, thanks to Mike Siva-Jothy who saved me from a fate worse than death - primatology - for that, his support and enthusiasm throughout this project, and many other things - I'll always be grateful.

Although I can never thank them enough, the last mention must be of Nelli and Trace, Naomi, my Dad (who gave me a passion for science), Alison and the rest of the family, Sharon, Brendan, Marie-Claire, Cath, Rick and Mads for putting up with me and being there when I needed them most - cheers everyone.

Abstract

Sexual conflict in the Bean Weevil, *Callosobruchus maculatus*

Helen Sarah Crudgington

This study used the bean weevil, *Callosobruchus maculatus*, to examine the functions, causal relationships and life-history consequences of aspects of reproductive morphology and behaviour within the framework of sexual conflict theory. In Chapter 2, the gross genital anatomy of male and female *C. maculatus* are described. Sharp cuticular spines on the male intromittent organ unfurl within the female genital tract during copulation. Chapter 3 established a causal relationship between the male intromittent and damage to the female genital tract. The extent of genital damage varied among once-mated females and increased with each additional copulation. Chapter 4 investigated the function of female mate-kicking behaviour, whereby females kick their mates during copulation. Females prevented from kicking had longer copulations and more genital damage than females permitted to kick, suggesting that mate-kicking has evolved as a counter-adaptation to ameliorate the associated costs. The effect of mate-kicking on two female post-copulatory traits linked to both male and female fitness were examined in Chapter 5. No measurable effects due to mate-kicking were found on either the immediate oviposition rate or the remating interval of females following an initial copulation. These findings indicate that males do not appear to induce favourable changes in female post-copulatory behaviour through the imposition of longer copulations or increased genital damage. In Chapter 6, the effect of varying exposure to males on female fitness traits was examined. With oviposition rates partially standardised across mating frequencies, remated females had shorter lifespans than once-mated females, but the relationship between copulation frequency and lifespan was not linear. With standardised gregarious living conditions, female reproductive output increased with greater opportunities to mate. Thus, despite the apparent longevity cost

associated with copulation, females elevate their fitness through multiple mating and increased exposure to males. The potential for non-mating exposure to males to reduce female fitness traits was also demonstrated. Finally, Chapter 7 assesses the evidence for a conflict between the sexes in *C. maculatus* over male damaging tactics.

Contents

Chapter 1: Sexual selection and sexual conflict theory

1.1 Sexual selection: a historical perspective

1.1.1 Darwin's theory of pre-copulatory sexual selection

Darwin proposed his theory of sexual selection (Darwin 1871) in order to account for the existence of conspicuous or elaborate traits most often displayed by male animals. He recognised that extravagant features such as the peacock's tail were unlikely to arise by natural selection (see Darwin 1859) because of their obvious hindrance to survival. He therefore reasoned that such encumbrances must be of use in an objective other than survival, namely the acquisition of mates:

> "Sexual selection ... depends, not on a struggle for existence, but on a struggle between the males for possession of the females; the result is not death to the unsuccessful competitor, but few or no offspring" (p. 88).

Darwin (1871) proposed two mechanisms by which sexual selection could operate. Firstly, intra-sexual selection which encompasses competition between members of one sex (usually males) for access to individuals of the other sex, and secondly, inter-sexual

selection which involves preference by members of one sex (usually females) for particular mating partners. Thus, sexual selection favours traits conferring competitive ability or attractiveness via the selective agents of sexual rivals and mating partners (see Andersson 1994). Studies of this fundamental evolutionary process are concerned with sexual competition and the advantages that some individuals have over others within this broad arena.

There are two related aspects of Darwin's (1871) ideas which were to have lasting implications for the development of sexual selection theory. Like most people of his time, he assumed that females were sexually monogamous, at least according to writings intended for public consumption (Birkhead 2000b). A corollary of the assumption of female monogamy is that sexual selection will operate prior to, but not beyond, insemination because at this point all opportunities for competition and choice must cease. Evolutionary biologists now recognise that these beliefs are unfounded. However, Darwin's ideas were undoubtedly constrained by the social mores of the Victorian era (Birkhead 2000b). The misapprehension that females were largely passive participants in sexual interactions prevailed and consequently, reproduction was generally considered a cooperative affair with activities such as courtship interpreted in terms of strengthening the pair bond. It is no coincidence that the paradigm shift that finally dispelled the myth of female sexual passivity coincided with the rise of feminist ideology a century later (e.g. Greer 1970; Millett 1971; Dworkin 1974). Darwin's (1871) theory of sexual selection provides a powerful explanation for the existence of many puzzling features of

the natural world, and yet impediments to the progress of the field were inherent to his ideas.

1.1.2 Anisogamy and the evolution of sex differences

Nearly 80 years after Darwin formulated his theory of sexual selection, Bateman (1948) provided the basis for its operation through male-male competition and female choice. Using *Drosophila melanogaster*, he demonstrated that the reproductive success of males was determined by the number of females inseminated (Bateman 1948). In contrast, he found that female reproductive success was constrained by the number of eggs produced, and was augmented little, if at all, by additional copulations (Bateman 1948). The root of this asymmetry in the fitness consequences of polygamy, and many other sex differences, is anisogamy. Anisogamy is the stable dimorphism of gamete sizes, which is believed to have arisen from two opposing selective forces acting on generalist gametes (Parker *et al.* 1972; Alexander and Borgia 1979). The resultant disruptive selection favoured some that specialised in successful fusion with other gametes, and others that specialised in zygote survival. These specialisms correspond to the numerous motile microgametes produced by males and the fewer, less mobile macrogametes produced by females. In polygamous species with no parental care, these divergent gametic strategies represent an imbalance in the relative pre-zygotic investment of males and females (Trivers 1972). This produces a significant difference in the absolute reproductive potential of the sexes, with males generally having the capacity to parent many more offspring than females (Bateman 1948). Females, or more precisely their eggs, then become the limiting resource over which males must compete, whereas males become

subject to mate choice decisions by females (see Andersson 1994). Consequently males show greater variation in reproductive success than females, and since success is achieved at the expense of others, males are subject to more intense selection pressure than females (Bateman 1948).

In 1966 Williams (1966) suggested that the intensity of sexual selection experienced by each sex was determined, not just by their relative expenditure on gametes, but also by the relative investment of resources used for raising offspring. Both Bateman's (1948) and William's (1966) ideas on gametic and parental sex differences led Trivers (1972) to propose that the concept of parental investment be extended to include the total resource expenditure required for raising offspring at the expense of the parent's ability to invest in other offspring. Therefore, the direction of sexual selection is governed by the total amount of parental investment made by each sex in relation to the other (Trivers 1972). Support for this hypothesis is provided by mating systems in which the usual sex roles are reversed as these illustrate how relative parental investment, rather than sex, determines which sex competes and which chooses (Andersson 1994).

1.1.3 Post-copulatory sexual selection

Although Darwin (1871) conceptualised sexual selection solely in terms of pre-copulatory interactions, Parker (1970a) realised that gaining copulations provides no guarantee of paternity because competition between males can continue after insemination in the form of sperm competition. The occurrence of sperm competition in internally fertilising animals is contingent on two female traits. First, females must

copulate with more than one male within a single reproductive cycle, and second, they must have the capacity to store viable sperm. It is now known that multiple mating by females to different males is a widespread phenomenon, with monogamy being the exception rather than the rule (see Ridley 1988; Arnqvist and Nilsson 2000). The potential for females to store viable sperm is also common although abilities vary enormously among taxa (Birkhead and Møller 1998). When combined, the propensities of females for mating multiply and storing sperm exert a major influence on the evolution of male reproductive strategies. More specifically, male reproductive behaviour, morphology and physiology are subject to two opposing selective forces when sperm compete for fertilisations (Parker 1970a). Selection simultaneously favours adaptations that pre-empt the sperm of rival males already stored by a female (sperm offence), and adaptations that prevent pre-emption of self-sperm by later-mating males (sperm defence) (Parker 1970a). Both traits lessen the severity of sperm competition by reducing the temporal and or spatial overlap of ejaculates within the female reproductive tract. Since its inception in 1970, sperm competition theory has stimulated an abundance of theoretical and empirical research, much of it concentrating on inter-specific sperm precedence patterns and the mechanisms by which the sperm of some males out-compete those of others (see reviews by Thornhill and Alcock 1983; Smith 1984; Birkhead and Møller 1998).

More recently, Thornhill (1983) and Eberhard (1991; 1996; Eberhard and Cordero 1995) have proposed that discrimination by females can also continue after insemination if the storage and utilisation, and therefore the outcome of sperm competition, is mediated by

female factors (e.g. Edvardsson and Arnqvist 2000). So-called post-copulatory, or *cryptic*, female choice has been the focus of much debate with arguments centring on how to define and demonstrate the process, and its selective strength relative to that of sperm competition (e.g. Birkhead 1998; Birkhead 2000a; Kempenaers *et al.* 2000; Pitnick and Brown 2000). Since variation in potential reproductive rates generally places males under greater selection pressure than females (Bateman 1948; Parker 1984), post-copulatory choice may be less important in determining sperm usage than sperm competition. Nevertheless, it may be more difficult for males to determine fertilisation events because inseminated sperm is subject to control by female morphology and physiology (Walker 1980). Brown *et al.* (1997) suggested two scenarios where post-copulatory female choice may be particularly likely to evolve. Firstly, where opportunities for pre-copulatory choice are restricted, such as in species with forced or coerced copulations (e.g. Pizzari and Birkhead 2000). Secondly, where mates provide nutrients but the quality of a male in terms of his nutrient-giving ability and attributes as a sire do not necessarily coincide. In this case, post-copulatory sperm choice would enable a female to acquire the optimum nutrient donation from one male whilst choosing another to father her offspring (Brown *et al.* 1997). Post-copulatory sexual selection, whether via sperm competition or cryptic female choice, will tend to exaggerate the non-random nature of male reproductive success established by pre-copulatory mechanisms.

1.2 Sexual conflict

1.2.1 The origins of sexual conflict theory

Sexual conflict can be defined as the evolutionary disputes that arise between males and females as a result of their divergent reproductive interests. Foundations for the development of sexual conflict theory were laid by Bateman (1948) and Trivers (1972), in combination with Hamilton (1964). Hamilton (1964) challenged the traditional group selectionist view that reproduction occurs for "the good of the species" (Williams 1966) by proposing that organisms act for essentially selfish reasons in order increase their genetic representation in future generations. His revolutionary ideas on kin selection provided a powerful new framework within which to reinterpret the ways animals behaved, especially in the context of reproduction. Soon after, Parker (1970a) produced his seminal study of the evolutionary consequences of sperm competition in insects where he pointed out that:

> "The female cannot be regarded as an inert environment in and around which this form of adaptation evolves" (p. 559).

He went on to argue that male traits that prove disadvantageous to females would induce the evolution of counteractive female measures. In doing so, Parker (1970a) highlighted the potential for male adaptations to sperm competition to generate antagonistic coevolution between the sexes.

1.2.2 Antagonistic coevolution between the sexes

Although males and females depend on one another for the successful fusion of their gametes, the extent of their shared interests may end there. This is because the sexes frequently express different fitness optima for the various components of reproduction (e.g. Alexander and Borgia 1979; Brown *et al.* 1997). These disparate interests lead to battles over reproductive events in which each sex seeks the optimum outcome - in other words, to gain the most fitness benefits whilst paying the least costs (Parker 1979; Parker 1984; Lessells 1999). When a mechanism used to control reproduction undermines the fitness of the opposite sex, conflict arises. The losing sex is then predicted to retaliate with counter-adaptations that function to negate, or at least to ameliorate, the detrimental effects of the antagonistic trait (Parker 1970a, Parker 1979).

Sexual dissonance can occur at any stage of reproduction, from the beginning of pair formation to the completion of parenting (Brown *et al.* 1997). For instance, it can arise over choice of mating partner, whether mating takes place, mating duration and frequency, sperm usage and control of fertilisation and parental investment (see reviews by Westneat and Sargent 1996; Choe and Crespi 1997; Birkhead and Møller 1998). Moreover, many of the prerequisites for sexual conflict are apparent, not only in dioecious animals, but also in hermaphrodites (Michiels and Newman 1998). Clutton-Brock and Parker (1995b) explored three types of mechanism that could be used to gain control of copulation from the opposing sex (also see Brown *et al.* 1997):

Force

Force involves the removal of control in order to achieve copulation (Clutton-Brock and Parker 1995b; Brown *et al.* 1997). The benefits of control to the opponent are lost but additional costs are not necessarily imposed. An example of forced copulation is traumatic insemination in the beg bug, *Cimex lectularius*. This occurs when the male's intromittent organ pierces the female's body wall and inseminates directly into her body cavity (Hinton 1964) thereby imposing a survival cost on her (Stutt and Siva-Jothy in press). Mathematical models of force involve games in which each sex behaves independently of the costs paid by their opponent (Parker 1979). Over evolutionary time, such games can escalate into arms races consisting of cycles of reciprocal antagonistic coevolution which impose considerable costs on both sexes (Clutton-Brock and Parker 1995b). The outcomes of arms races may be unpredictable and resolutions are not necessarily derived (Parker 1979).

Harassment

Harassment involves the use of persistent courtship to induce compliance with copulation attempts (Clutton-Brock and Parker 1995b). Control is therefore gained by imposing costs on the opposing sex (Brown *et al.* 1997). Male northern elephant seals, for example, are relatively large and females that refuse repeated copulation attempts risk being crushed (Le Boeuf and Mesnick 1990; Mesnick and Le Boeuf 1991). Mathematical models of harassment employ game theory to show that the costs to each opponent of further persistence increase until they exceed the costs of conceding (Higashi and Yamamura 1994). A so-called asymmetric war of attrition may emerge

from such coercive interactions whereby the costs to each sex increase with each investment by their opponent (Parker 1979; Hammerstein and Parker 1982). Such a contest occurs in water-striders when males subject sexually reluctant females to repeated mating attempts (Rowe *et al.* 1994; Arnqvist and Rowe 1995; Arnqvist 1997).

Intimidation

Intimidation involves the use of physical punishment to gain cooperation with future mating attempts (Clutton-Brock and Parker 1995a; Clutton-Brock and Parker 1995b). Intimidation is temporarily spiteful (Hamilton 1970) because dispensing punishment is costly for the aggressor but the benefits of the strategy are not realised immediately. Such behaviour is necessarily restricted to highly social species which possess relatively advanced capacities for individual recognition, memory and learning (Clutton-Brock *et al.* 1992; Smuts and Smuts 1993; Clutton-Brock and Parker 1995b). For example, intimidation is seen in common chimpanzees, *Pan troglodytes*, when males repeatedly attack females during consort formation until a more proximate position to the assailant is adopted and copulations are more readily achieved (Goodall 1986).

Which sex prevails in coercive encounters is contingent on the ratios of two features of each sex: (1) the value of winning (i.e. the fitness differential between mating and not mating), and (2) the costs of winning (i.e. the expenditure on 'armaments') (Parker 1979; Parker 1984; Clutton-Brock and Parker 1995b).

1.2.3 Sperm competition and the potential for sexual conflict

Insects, in particular, are predisposed to high levels of sperm competition because females often mate multiply and store sperm effectively. The resulting battles between males to ensure fertilisation success can, in turn, generate intense struggles between males and females over sperm storage and utilisation (Parker 1970a; Stockley 1997; Simmons and Siva-Jothy 1998). However, Brown *et al.* (1997) suggested that the common tendency for a last male mating advantage in insect sperm contests could indicate some confluence of interests, with females gaining control of paternity through mating order effects and males benefiting from sperm displacement. Indeed, both sexes may benefit from male adaptations to sperm competition (Parker 1984). Prolonged copulations and mate-guarding, for instance, can reduce the frequency at which females are harassed (Stockley 1997). However, such traits can also run counter to the interests of females, thereby establishing the conditions for antagonistic coevolution (Parker 1984; Stockley 1997; Simmons and Siva-Jothy 1998).

A popular model for the study of sperm competition mechanisms and their consequences for females is the fruitfly, *D. melanogaster*. During mating in this species, males transfer accessory gland proteins to females in their ejaculates (Baumann 1974). Once inside the female, these compounds incapacitate the sperm of rival males, modulate storage of sperm, and induce increases in the oviposition rate and remating interval of the female, all of which serve the male's sperm competitive interests (Chen *et al.* 1988; Harshman and Prout 1994; Clark *et al.* 1995; Wolfner 1997; Price *et al.* 1999; Chapman *et al.* 2000; Heifetz *et al.* 2000). As a toxic side-effect of their sperm competitive function,

these compounds reduce the survival probability of females (Chapman *et al.* 1995) and under some circumstances, their reproductive output (Chapman and Partridge 1996). Rice (1996) demonstrated that male seminal substances form the basis of an antagonistic coevolutionary arms race between the sexes in *D. melanogaster*. In his experiment, the female lineage was held still in evolutionary terms, and the male lineage was allowed to evolve freely, without being constrained by counter-measures in the females. Males from the evolved lineage not only faired better in sperm competition with males from stock lines, but also imposed greater life-history costs on females due to elevated mating rates and increased toxicity of the seminal compounds transferred (Rice 1996). This ingenious study provided important evidence for how antagonistic traits could trigger cycles of adaptation and counter-adaptation as each sex strives to retain/regain control of reproductive events.

2.3.4 Sexual conflict as fuel for evolution

Over the last decade or two, sexual conflict theory has had a profound effect on our understanding of animal mating and it is now clear that sexual antagonism is a potent driving force for the evolution of reproductive strategies in males and females (e.g. Clutton-Brock and Parker 1995b; Choe and Crespi 1997; Stockley 1997; Lessells 1999). These developments led Holland and Rice (1998) to propose a model for the evolution of exaggerated male traits that is based on sexual conflict and female resistance to 'seduction' by such traits. In this "chase-away" model of sexual selection, initiation for the evolution of a male display trait is provided by the pre-existing sensory biases of females (Ryan 1990). The intense attraction for males possessing the display trait

induces females to mate at sub-optimal rates, times or locations. Costs resulting from these superfluous matings select for female *resistance to*, rather than *preference for*, the male trait. Males then predicted to respond with increasingly exaggerated traits in order to overcome female opposition to mating (Holland and Rice 1998).

In addition to sexual selection, sexual conflict has been implicated in the major evolutionary processes of reproductive isolation and speciation (Parker and Partridge 1998; Arnqvist *et al.* 2000; Gavrilets 2000 but see Tregenza *et al.* 2000). The mounting importance of sexual antagonism to our understanding of reproduction shadows the increased prominence that conflict theory has gained in explanations of a diverse range of evolutionary phenomena. Conflicting interests may not only pervade relationships between the sexes, but also those between parents and offspring (Trivers 1974), and the workers and queens of social insect societies (Trivers and Hare 1976). More obvious are the conflicts underpinning the inter-specific interactions of predators and prey (Endler 1986), and hosts and parasites (Poulin 1998).

1.3 The study species

Callosobruchus maculatus (F.) (Coleoptera: Bruchidae) is a cosmopolitan pest of stored leguminous seed crops (Southgate *et al.* 1957; Southgate 1978; Southgate 1979) (see Figure 1.3.1). It inhabits tropical and subtropical regions and has attained a pan-global distribution as a result of human agricultural and economic practices. Culturing and

maintaining populations in the laboratory is straightforward, as is distinguishing between the sexes and obtaining virgin individuals (see Chapter 2). The practical advantages of working with this insect, combined with the relative ease of replicating its natural habitat in the laboratory, have made it a valuable model for examining both applied and theoretical questions. Its economic importance as a pest of human grain stores has led to its widespread use in pest control research. However, it also features extensively in other empirical fields including the study of larval competition (Colegrave 1995) and female oviposition strategies (Wilson 1989). Furthermore, since females mate multiply and exhibit considerable sperm storage capacities, this beetle is particularly amenable to investigations of sperm competition (Eady 1991b). In combination, its numerous attributes make *C. maculatus* an excellent model organism for studying many aspects of sexual selection. In particular, *C. maculatus* exhibits three copulatory traits that indicate its potential as a model for the investigation of sexual conflict:

(1) the intromittent organ of males bears sharp spines (Eady 1991b),
(2) females kick their mates during copulation (Qi and Burkholder 1982; Rup 1986), and
(3) mated females exhibit ruptured genital tracts (Tufton 1993).

Taken together, these genital and copulatory traits point towards a potential conflict over the imposition of genital damage on females by males.

1.3.1 Life-cycle

Gravid females attach their eggs to the surface of a range of host seeds. Within approximately five days, the developing larvae become visible within their translucent egg-shells (van der Meer 1979). Two or three days later, the larvae burrow through the testa into the cotyledon of the seed, filling the chorion with excavated cotyledon as they do so. In the case of the black-eyed bean host, *Vigna unguiculata* (L.) Walp, the translucent egg turns white during the excavation process. Larvae exhibit exploitation competition (Smith and Lessells 1985) as they continue to feed just beneath the surface of the testa, passing through three instars before pupating at around 24 days old. At this stage, the pre-emergent adult is clearly visible through a thin 'window' of testa (Bellows 1982). A day or two later, adults emerge from the seed through a hole cut in the testa. Development time is affected by a range of environmental factors including ambient temperature and humidity (Giga and Smith 1983). At 28°C and 35% r.h. adults emerge within approximately 26 days of egg-laying. Feeding opportunities are rarely available to adults (Larson and Fisher 1938), so nutrient reserves for this life-history stage are fixed at eclosion. Mated adults are relatively short-lived, with lifespan ranging from 1 – 3 weeks at 28°C.

1.4 Thesis aims and outline

This thesis has used a model insect system to examine the functions, causal relationships and life-history consequences of aspects of reproductive morphology and behaviour within the framework of sexual conflict theory.

Chapter 1: Provides a historical perspective of sexual selection theory and considers the evolutionary significance of reproductive conflict between the sexes.

Chapter 2: Describes the gross genital morphology of male and female *C. maculatus*, thereby providing the background necessary for interpreting findings presented in subsequent chapters.

Chapter 3: Investigates the causal relationship between male genital morphology and the ruptured genital tracts of mated females. The extent of genital damage in individual females is quantified and potential determinants of this trait are investigated. The relationship between female copulation frequency and the degree of genital injury sustained is also quantified.

Chapter 4: Investigates the function of mate-kicking behaviour by females during copulation, in particular, the effect of experimental removal of mate-kicking on copulation duration and the degree of genital damage sustained by females.

Chapter 5: Examines the effect of experimental removal of mate-kicking on the immediate oviposition rates and remating intervals of females: these two post-copulatory traits have important consequences for male fitness.

Chapter 6: Examines the impact of varying mating frequencies and exposure to males on two female fitness traits: lifespan and reproductive output. The potential life-history consequences for female fitness traits of non-mating exposure to males are also examined.

Chapter 7: Considers the evidence in favour of a conflict between the sexes in *C. maculatus* over genital injury by males. Avenues for further research of the role of sexual conflict in shaping the reproductive biology of this insect are explored.

Appendix 1: Investigates the consequences for female lifespan of permanent confinement with males and tests methods for controlling costs associated with both male harassment and egg production.

Appendix 2: Determines the rates at which intact and emasculated males sexually harass non-virgin females.

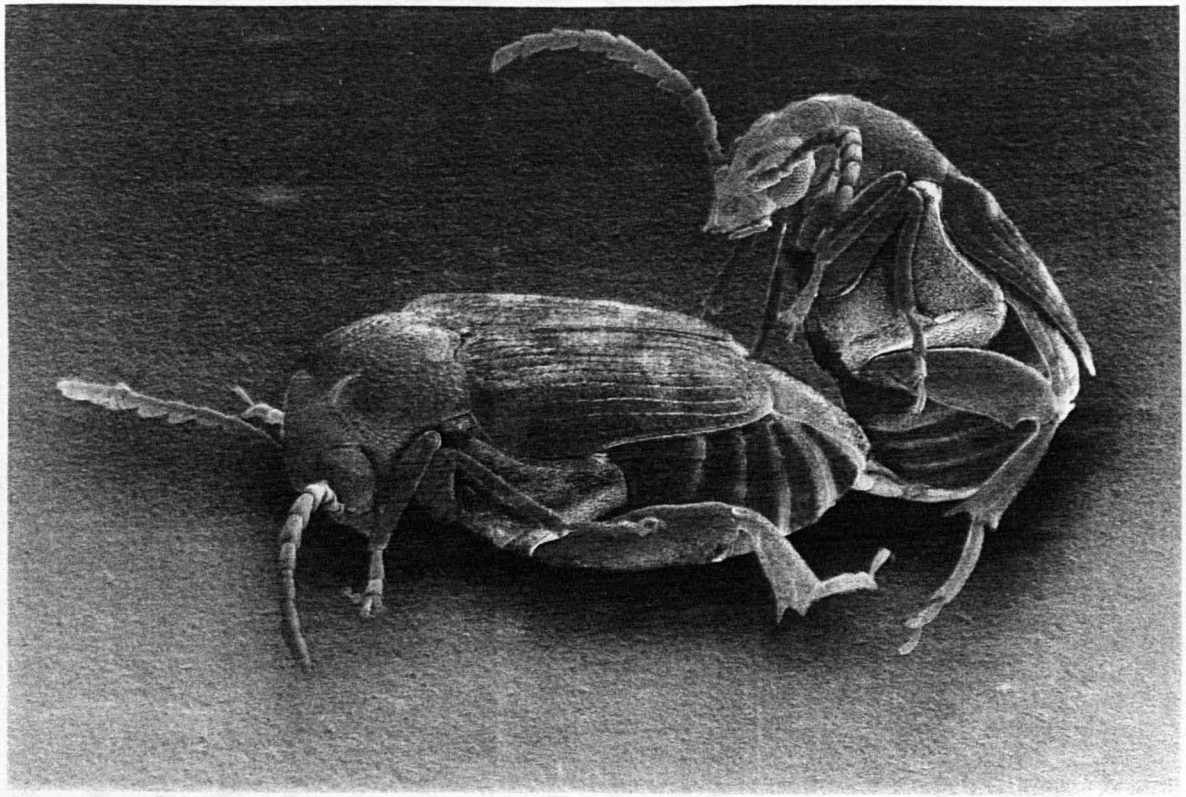


Figure 1.3.1 A pair of copulating *C. maculatus* with the male situated on the female's dorsum. Mean elytron length: male ~ 1.7 mm, female ~1.9 mm.

Chapter 2: Genital morphology in *Callosobruchus maculatus*

2.1 Introduction

The genital morphology of internally fertilising animals, particularly invertebrates, has long been used by taxonomists to distinguish between species. This is because male genitalia typically display a spectacular level of morphological diversity across animal taxa (for review see Eberhard 1985). Even closely related species frequently show remarkable differences in genitalic characteristics, despite being virtually identical in other anatomical respects. Several explanations have been proposed to account for this phenomenon. Eberhard (1985) proposed that rapid and divergent genitalic evolution is due to post-copulatory sexual selection favouring males with genitalic traits that confer improved fertilisation success. By arguing that the evolution of primary, as well as secondary, sexual traits can be influenced by non-random fertilisation success among males (e.g. Tadler 1999), this hypothesis abandoned the division that Darwin (1871) established between primary and secondary sexual traits. Individual differences in genitalic features may benefit males via the mechanisms of sperm competition, female choice or sexual conflict. The idea that sexual selection can account for male genitalic diversity is supported by the finding that polyandrous species, which are likely to be the

subject of more intense sexual selection, display greater elaboration of genitalia than monandrous species (Arnqvist 1998).

Waage (1979a) was the first to show that particular morphological characteristics of the male intromittent organ can arise by sexual selection. He revealed that prior to insemination in the damselfly, *Calopteryx maculata*, the male used his aedeagus to remove previously inseminated sperm from the female's sperm storage organs. Spines on the head of the aedeagus remove sperm from the bursa copulatrix, whilst spermathecal sperm was scooped out by structures projecting from the distal tip of the aedeagus (Waage 1979a). By displacing the sperm of rivals, the male avoided sperm competition (at least until the female remated) thereby increasing the probability that he would sire a greater proportion of the female's eggs (Parker 1970a). Thus, a dual-function for the aedeagus in *Calopteryx maculata* was demonstrated since the intromittent organ was not only responsible for delivering gametes to the female, but also for biasing the fertilisation success of competing males.

In general, the bewildering array of male genitalic forms seen in internally fertilising animals may reflect a diverse range of functions. Studies of genital morphology may elucidate the way in which complex genitalic structures operate during copulation and the mechanisms by which males gain enhanced fertilisation success.

2.1.1 Sperm transfer and precedence in *C. maculatus*

During copulation in *C. maculatus*, males transfer sperm via a spermatophore (Ouedraogo 1978 in Eady 1991b) which is assembled and deposited in the bursa copulatrix within the female's genital tract. A proportion of sperm contained within the spermatophore is then transported via the spermathecal duct to the area of sperm storage (Eady 1991b). Females have a single sperm storage organ, the comma shaped spermatheca, in which a chitinous surface structure ensures a fixed storage capacity (Eady 1991b). Although approximately 46,000 sperm are inseminated, less than 15% of this quantity are required to fill the spermatheca to capacity, the remainder being rapidly degraded in the bursa (Eady 1991b; Eady 1995). When inseminations were separated by 24 hours, the second male to mate fertilised the majority (approximately 83%) of the female's next clutch of eggs (Eady 1991a). This pattern of high last male sperm precedence is maintained over at least three inseminations (Eady and Tubman 1996).

2.2 Aim

The aim of this chapter is to describe the gross morphology of male and female genitalia in *C. maculatus*, thereby providing an anatomical framework within which the results of subsequent chapters may be understood.

2.3 General materials and methods

2.3.1 Maintenance of stock cultures

A single strain of *C. maculatus*, originating from Brazil in 1974 and cultured at the University of Sheffield since 1984, was used throughout this study. Stock cultures were maintained on black-eyed beans, *Vigna unguiculata* (L.) Walp, in transparent plastic boxes (273 x 152 x 102mm) containing two gauze-covered ventilation holes (60mm diameter). Stocks were kept at a constant environment of 27±2°C and 35% r.h. with a 16:8 hrs light:dark photoperiod.

New generations of beetles were obtained on a weekly basis by placing approximately 300 CO_2-anaesthetized adults from the previous generation on approximately 1000 host beans. Mating and oviposition occurred freely until the adults died (usually within 2 weeks), at which point they were removed and the eggs allowed to develop and hatch, producing a new generation. Since this culturing method leads to the establishment of genetically separate lines, individuals from more than one generation were used to parent subsequent generations, thereby permitting a degree of gene flow and preventing the genetic divergence of stock lines. Nevertheless, to minimise genetic and temporal variation, only beetles originating from a single generation were used for each investigation.

2.3.2 Obtaining experimental animals

To ensure the availability throughout the study of virgin animals of known maximum age, emergent individuals were isolated and prevented from mating. Thus, single host beans laden with pre-emergent beetles were placed in 2ml plastic tubes (eppendorfs) containing several ventilation holes. The tubes were checked at regular intervals and any recently emerged virgin adults were collected and maintained singularly in eppendorfs until required for experimental use (usually 18-36 hrs later). Inspection of the host beans often revealed that more than one individual had emerged from the same bean since the previous inspection. In this case, any beetles found with individuals of the opposite sex were discarded. Groups of two or more individuals of the same sex were retained. Animals were sexed by their sexually dimorphic elytral markings (Southgate *et al.* 1957). Unless otherwise stated, copulations throughout the study involved randomly allocated virgin animals and took place under artificial white light, at 25±2°C in plastic petri dishes (3cm diameter) lined with filter paper.

2.3.3 Measuring body size

Optimas® (v. 6.1) image analysis software was used to measure the left elytron length of experimental animals following death. Elytron length was used as a standard measure of body size since this trait is highly correlated with body weight at emergence (Wilson 1989). On occasion, samples were inadvertently destroyed during the measuring procedure.

2.3.4 Investigating genital morphology

Frozen samples were defrosted for 1 hr in distilled water at room temperature before dissection began. Dissections of genitalia were performed under distilled water using a stereo microscope (Leica MZ8) and were photographed with a compound microscope (Leitz Diaplan) set up for photomicrography.

2.3.5 Statistical methods

Statistical analyses for the entire study were performed using Statview 5.0 for the Macintosh computer with the exception of a Fisher's exact test, which was calculated by hand. Data were analysed using parametric tests unless assumptions of these tests were violated, in which case the appropriate non-parametric tests were used. All means are presented ± one standard error with sample sizes (n). Statistical methods and tables were obtained from Sokal and Rohlf (1997) and Rohlf and Sokal (1995) respectively.

2.4 Chapter materials and methods

2.4.1 Examination of female genital morphology

The reproductive organs of females (aged 18-24 hrs) (n = 10) were removed, and the genital tract was opened in an anterior-posterior direction with the aid of fine bow-spring scissors. This allowed examination of its internal structure. The gross morphology of each genital tract was drawn and photographed.

2.4.2 Examination of male genital morphology

The reproductive organs of males (aged 18-24 hrs) (n = 10) were dissected and the aedeagus was everted to allow examination. The gross morphology of the aedeagus was drawn and photographed.

2.4.3 Examination of the *in situ* genitalia of copulating pairs

Females (n = 10) were mated once and 2 mins after the onset of copulation the pairs were snap-frozen in liquid nitrogen to preserve the relative positions of the male and female genitalia. Samples were stored at -80°C for subsequent examination. Prior to dissection, pairs were placed in 80% ethanol cooled to -20°C in order to dehydrate the sample and so maintain the inflated state of the male aedeagus within the female genital tract. The *in situ* genitalia of copulating pairs were then removed from the body of each female, and examined and photographed. All animals were aged 18-24 hrs.

2.5 Results

2.5.1 Female genital morphology

Examination of the female genital tract revealed a non-sclerotised endocuticlar vagina (see Figure. 2.5.1). Located at the anterior end of the vagina is a membranous sac-like bursa copulatrix (hereafter, the bursa) (n = 10). Positioned in the ventral region of the bursa, and embedded in the endocuticule are two transparent, membranous ellipsoid sac-like structures: the cuticular discs. Located equidistantly between, and slightly dorsal to,

the cuticular discs, is a sclerotised structure bearing three small, posteriorly directed chitinous teeth. Posterior to these teeth, and lying on either side of the ventral mid-line are two endocuticular structures: the lateral pads. The spermatheca is small, comma shaped, constructed from sclerotised cuticle and is attached to the dorsum of the vagina by an endocuticular duct.

2.5.2 Male genital morphology

Examination of the male intromittent organ showed that it consists of a sclerotised shaft, the aedeagus, which everts prior to copulation ($n = 10$). The aedeagus is bordered by two sclerotised cuticular parameres (see Figure 2.5.2. and 2.5.3). The distal tip of the aedeagus bears a spherical membranous sac which is only inflated at the last stage of aedeagal extension (see Figure 2.5.4). Embedded in the anterior portion of this sac, and clearly visible before it is inflated are numerous, sharp, sclerotised spines. The bases of these spines form a complex interlocking arrangement. As the aedeagus is everted, the interlocking basal arrangement appears to allow the spines to unfurl radially.

2.5.3 The *in situ* genitalia of copulating pairs

Examination of the *in situ* genitalia of copulating pairs ($n = 10$) indicated that during copulation the cuticular spines on the male genitalia were everted in the region of the female's vagina containing the lateral pads (see Figure 2.5.5). Once intromission is achieved the membranous sac extends from within the aedeagus to fill the bursa (Figure 2.5.5). The parameres did not enter the female genital tract but remained clearly visible outside.

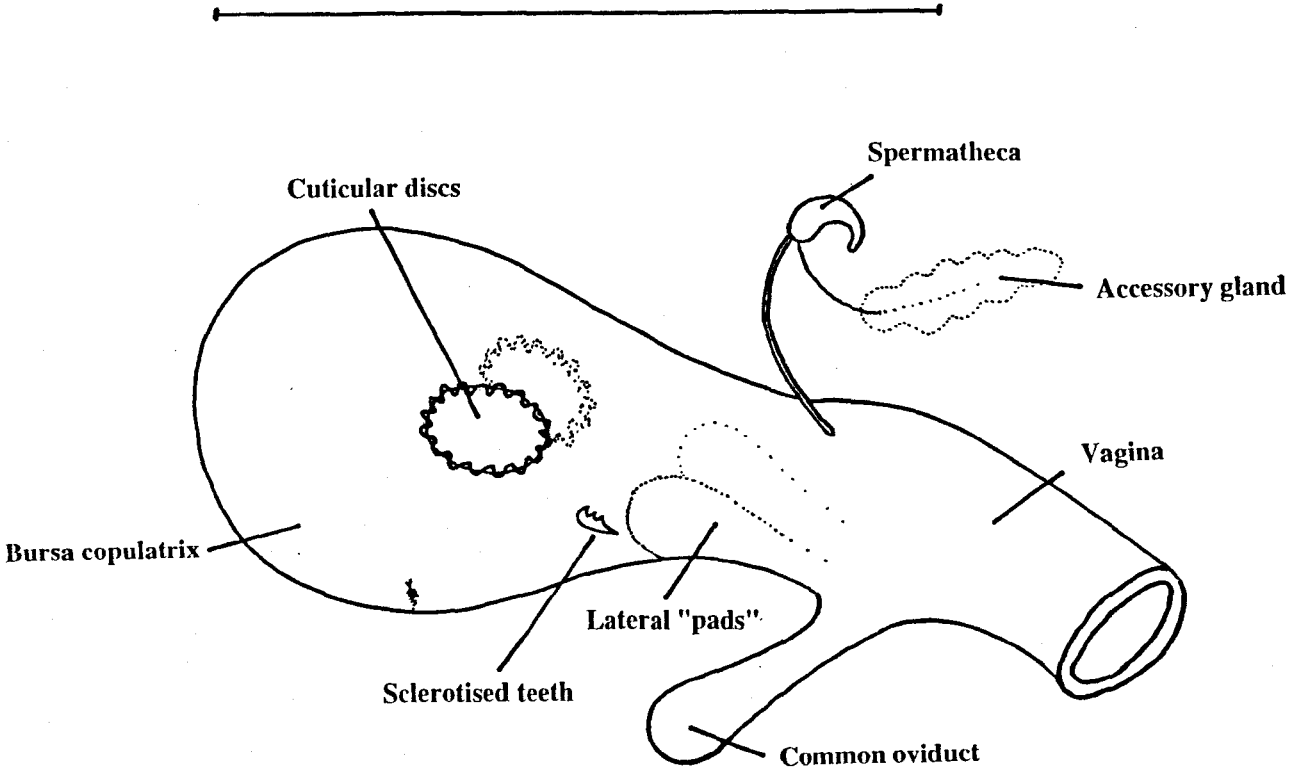


Figure 2.5.1 Stylised representation of the female genital tract showing the major anatomical features. Scale bar ~ 1 mm. Top of page is dorsal.

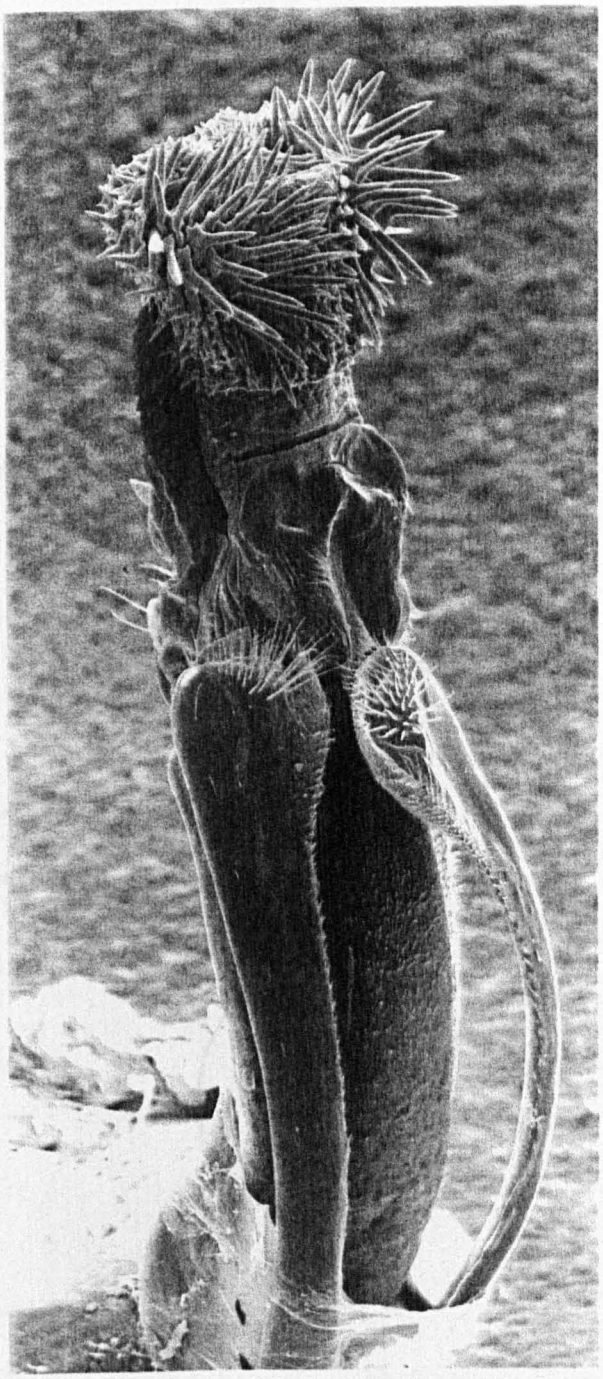


Figure 2.5.2 SEM of the intromittent organ (aedeagus) of male *C. maculatus* (Andrew Syred, Microscopix). The aedeagus is approximately 1 mm long.

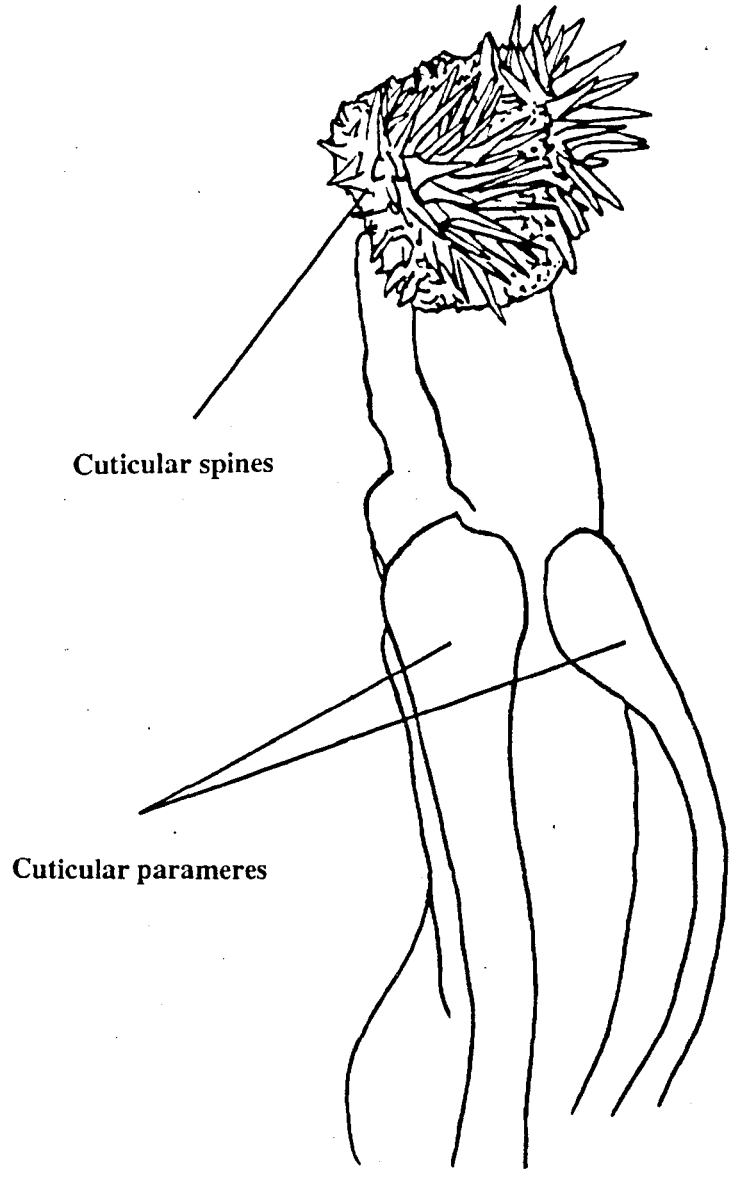


Figure 2.5.3 Stylised representation of the aedeagus showing the major anatomical features. The aedeagus is approximately 1 mm long.

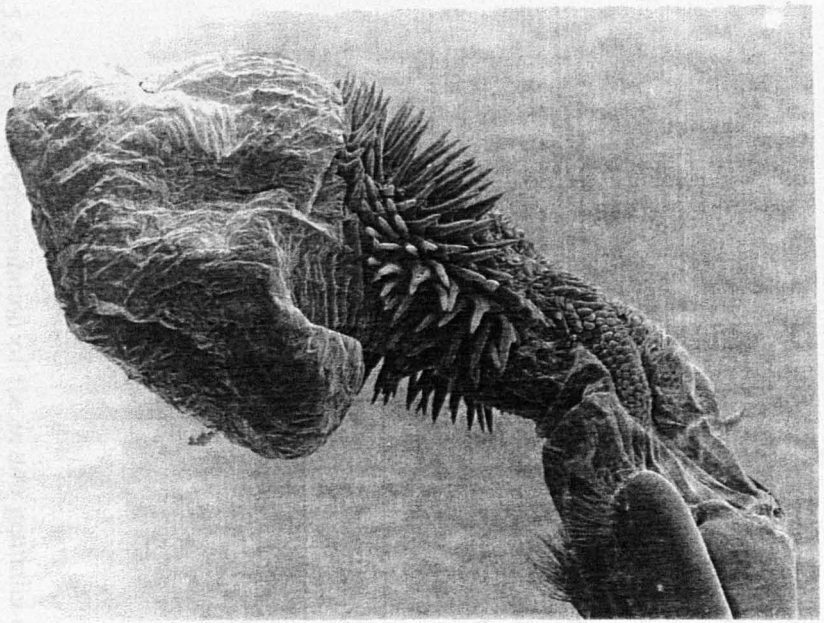


Figure 2.5.4 SEM of the aedeagus of a copulating male *C. maculatus* showing the inflated distal aedeagal sac (Andrew Syred, Microscopix).

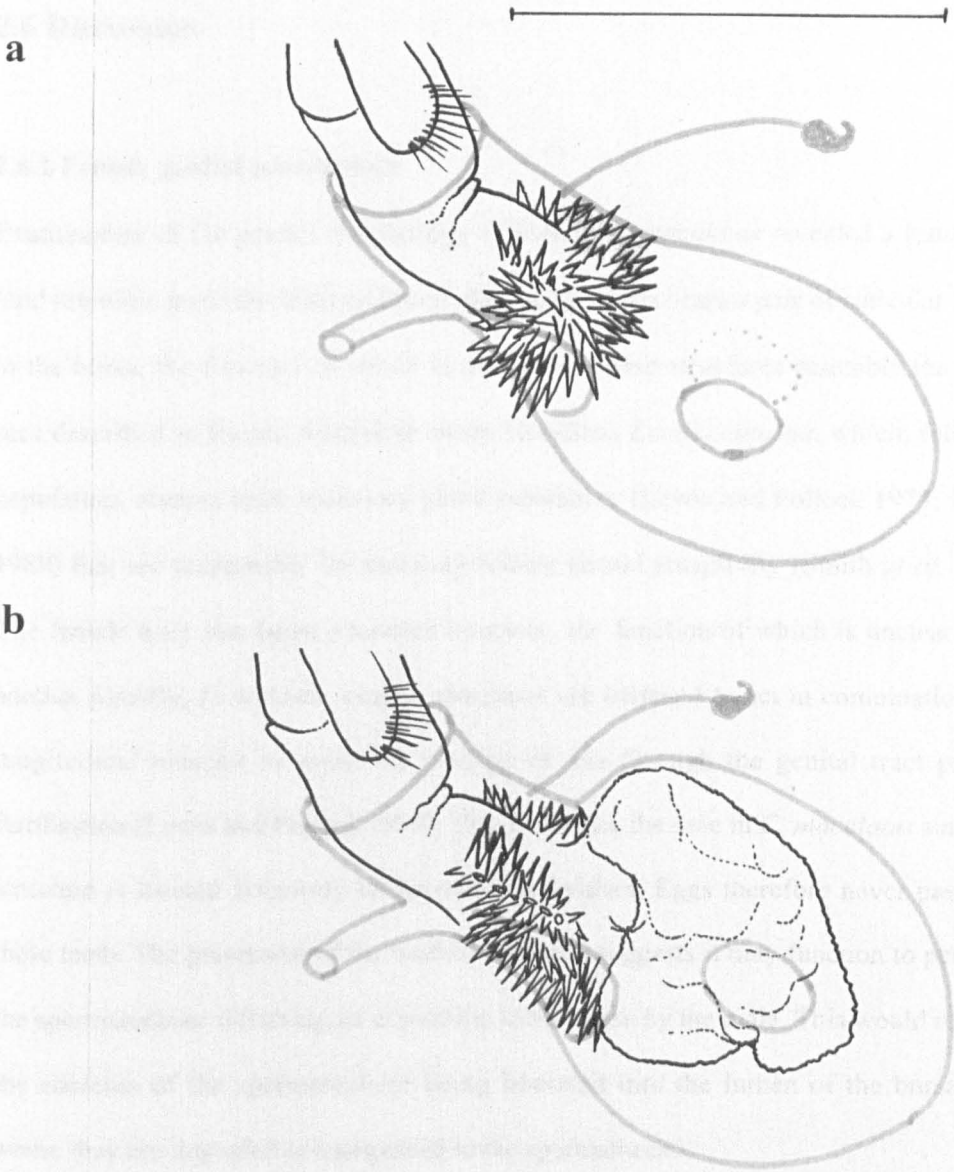


Figure 2.5.5 Stylised representation of the *in situ* genitalia of a copulating pair of *C. maculatus* showing the major anatomical traits and their relative positions (female genitalia in blue). (a) The disposition of the aedeagus on insertion. (b) Note that as the distal sac inflates, the spines unfurl and move outwards in the region of the lateral pads (see Fig. 2.5.1). The bursa copulatrix receives the distal sac and the spermatophore. Top of page is dorsal. Scale bar ~ 1mm.

2.6 Discussion

2.6.1 Female genital morphology

Examination of the genital morphology of female *C. maculatus* revealed a transparent (and therefore probably thin) endocuticular lining which bears a pair of cuticular "discs" in the bursa, the function of which is unknown. These structures resemble the lateral sacs described in female Australian sheep blowflies, *Lucilia cuprina*, which, following copulation, contain male accessory gland substances (Lewis and Pollock 1975; Merritt 1989) that are responsible for reducing female sexual receptivity (Smith *et al.* 1989). The female tract also bears a toothed structure, the function of which is unclear but in another blowfly, *L. sericata*, similar structures are believed to act in combination with longitudinal muscles to assist the passage of ova through the genital tract prior to fertilisation (Lewis and Pollock 1975). This cannot be the case in *C. maculatus* since this structure is located anteriorly to the common oviduct. Eggs therefore never pass over these teeth. The placement of the toothed structure suggests it may function to perforate the spermatophore following its deposition in the bursa by the male. This would result in the contents of the spermatophore being liberated into the lumen of the bursa from where they are degraded or transported to the spermatheca.

2.6.2 Male genital morphology

Examination of the genitalia of male *C. maculatus* revealed a complex intromittent organ. The two sclerotised parameres flank the lateral edges of the phallus and may provide support or guidance for the everted aedeagus as it is introduced into the female.

These structures do not enter the female during copulation. The distal tip of the aedeagus is covered in sharp, radially positioned spines, the red-brown colour of which is a diagnostic characteristic of melanised cuticle indicative of a rigid structure (see Figure 2.5.2) (Chapman 1998). Dissections of mating pairs revealed that during copulation, the aedeagal spines are unfurled within the central region of the female genital tract bearing the lateral pads. The membranous sac that extends from the tip of the aedeagus is inflated within the lumen of the bursa, and may be involved in the process of spermatophore formation and deposition. In general, the disposition and nature of the spines suggests several possible functions for the male intromittent organ in addition to sperm transfer.

Direct sperm removal

In some insects, male genital appendages such as barbs and spines operate directly to remove previously inseminated sperm belonging to rival males from the reproductive tract of mated females (Waage 1979; see review by Simmons and Siva-Jothy 1998). However, spined aedeagi do not necessarily indicate a sperm removal function (Simmons and Siva-Jothy 1998). Indeed in *C. maculatus*, the aedeagal spines are not long enough or in the right position to penetrate the female's primary site of sperm storage, the spermatheca (see Figure 2.5.5). Furthermore, Eady (1994a) has eliminated this potential role of the aedeagal spines. He examined the aedeagi of recently mated males and failed to detect the presence of sperm (Eady 1994a). If the aedeagal spines were involved in direct sperm removal, sperm would be predicted to adhere to the spines as they exit the female genital tract (Eady 1994a).

Maintaining genital contact during copulation

Since the aedeagal spines unfurl during copulation they may function to maintain genital contact by improving the anchorage of the male. Such an adaptation could arise in the context of either sexual conflict or intra-sexual selection.

Firstly, in the context of sexual conflict the sharp spines may enable the male to restrain the female once copulation had commenced. Males of several insect species possess specialised structures for securing reluctant females. Examples include the notal organ of scorpionflies (Thornhill 1980), the abdominal processes of water-striders (Arnqvist 1989b; Arnqvist 1997); the abdominal cerci of bush crickets (von Helversen and von Helversen 1991) and the gin-trap device of sagebush crickets (Sakaluk *et al.* 1995). The ability to prevent a female abandoning copulation may guarantee a successful insemination for the mating male.

Secondly, in the context of male-male competition, the aedeagal spines may reduce the probability that the mating male will be dislodged by rival males. Support for this hypothesis is provided by the common observation that although mating pairs of *C. maculatus* receive repeated harassment from non-mating males attempting to copulate with the female, harassing males never appear to succeed in usurping the mating male, at least under laboratory conditions (pers. obs.). Males able to thwart take-over attempts by rivals gain by avoiding sperm competition (Parker 1970a and 1984).

The role of the large inflatable sac is far from clear, but this structure is common in other beetles that do not possess aedeagal spines but which maintain strong genital contact during copulation (e.g. Carabids and Scarabids (Siva-Jothy; pers. comm.)).

The potential functions of the aedeagal spines outlined above are not mutually exclusive and selective advantages from the evolution and maintenance of these structures in one context may be positively reinforced by fitness benefits derived in the other. Whatever function the spines fulfil, the examination of male and female genitalia as well as mating pairs revealed that (a) the spines are hard and sharp and (b) unfurl in a region of the female's genitalia which appears to consist of thin endocuticle. The potential for these spines to cause injury to females during copulation is investigated in the next chapter.

2.7 Summary

In this chapter I have provided a qualitative description of the male and female genitalia of *C. maculatus* and their relationship to one another during copulation.

Chapter 3: Genital damage in females and the potential for sexual conflict

3.1 Introduction

A variety of hazards are encountered by internally fertilising organisms when they mate with conspecifics (Daly 1978). For example, courting or copulating pairs may be exposed to higher rates of predation, parasitism and cannibalism than single individuals (Thornhill and Alcock 1983; Arnqvist 1989a; Magnhagen 1990; Fairburn 1993; Rowe 1994; Hurst *et al.* 1995; Jackson and Pollard 1997; Thrall *et al.* 2000). One hazard of mating encountered predominantly by females is the risk of physical injury from mates. For instance, sexual harassment, in addition to imposing energetic and foraging costs (Watson 1998; Magurran and Seghers 1994), can result in the injury and death of females attempting to avoid persistent courtship (Ewer 1973; McComb and Clutton-Brock 1994; Clutton-Brock and Parker 1995b; Reale *et al.* 1996; also see review by Clutton-Brock and Parker 1995b). For example, female sea otters, *Enhydra lutris*, sometimes drown during attempts by males to restrain them by the nose during copulation (Mestrel 1994 in Clutton-Brock and Parker 1995b). Females may also suffer during struggles between males for control of territories or mates. In the yellow dungfly, *Scatophaga stercoraria*, for instance, mating competition between multiple males can

result in females being suffocated (Parker 1970b; Clutton-Brock and Parker 1995b); for an example in ducks see McKinney *et al.* 1983). Males may also exhibit aggression towards their mates after copulation. Male rove beetles, *Leistotrophus versicolor*, are reported to attack females with which they have recently mated in attempts to drive them from areas where they are likely to encounter and mate with rival males (Alcock and Forsyth 1988).

In general, males that physically attack or impose injuries on females during courtship or mating may elevate their reproductive success if aggressive acts increase the likelihood that females will comply with copulation attempts, remain in copula until successful sperm transfer has occurred, or advance male reproductive interests in other ways (Thornhill and Alcock 1983; Johnstone and Keller 2000).

3.1.1 Genital injury in mated females

Mated female *Callosobruchus maculatus* exhibit ruptured genital tracts following copulation (see Chapter 1; Tufton 1993). However, injury to the genitalia of mated females does not appear to be a phenomenon unique to *C. maculatus*. The genital tracts of mated female blister beetles, *Lytta nuttali*, for example, contain sclerotised patches that evidently arise from spines on the male genitalia that become embedded in the female's vaginal walls during copulation (Gerber *et al.* 1971). Likewise, injuries from the sharp, paired barbs on the intromittent organs of the blowfly, *Lucilia serricata*, are thought to produce "brown granules" or scars in the bursal wall of the female (Lewis and Pollock 1975). Similar injuries, believed to be caused by male genitalic structures, are

reported in several other insects including the beetle, *Lytta vulnerata* (Gupta 1966), the bush-cricket *Metaplastes ornatus* (von Helversen and von Helversen 1991) and some *Macrodactylus* beetles (Eberhard 1993). Furthermore, genital damage in mated females is not restricted to insects. During genital separation in guppies, *Poecilia reticulata*, females sustain injuries as the hooks and spines on the male gonopodia tear the tissue surrounding the female genitalia and cause bleeding (Kadow 1954). The blue shark, *Prionace glauca*, provides another example as the reproductive tracts of recently mated females contain wounds which develop into deep purple scars (Pratt 1979).

Despite its occurrence in a range of animal taxa direct evidence for, and quantitative analysis of, genital wounding is lacking. Furthermore, interpretation of genital injury from the evolutionary perspective of both males and females is largely absent from reports of this phenomenon. Clearly there is considerable potential for sexual conflict to be generated by genital wounding and this aspect of the phenomenon has been largely neglected.

3.1.2 Copulation in *C. maculatus*

The major behavioural components of mating in *C. maculatus* have been described by Rup (1986). Pre-copulatory courtship is minimal and, prior to mounting females from the rear, males evert the aedeagus and show antennating behaviour (the distal tips of the antennae are used to rapidly and repeatedly drum the dorsal thoracic surface of the female) (Rup 1986). Antennating continues after genital union is achieved until a minute or so after the onset of copulation, when males begin rocking back and forth, slowly and

rhythmically. Approximately two thirds of the way through copulation females begin kicking their mates with their hind legs (Qi and Burkholder 1982). According to Rup (1986), the termination of copulation is initiated by female kicking and the increased locomotor behaviour of females during which they drag their mates behind them. Genital separation is sometimes preceded by the male adopting a quadrapedal stance which leaves the pair facing in opposite directions in the so-called "end-to-end" position Rup (1986).

3.2 Aims

The aims of this chapter are to:

1) Describe behavioural components of copulation and quantify the duration of female kicking behaviour and copulation in *C. maculatus*.

2) Quantify the extent of genital damage sustained by individual females.

3) Identify morphological and behavioural correlates of the extent of genital damage sustained.

4) Determine whether the aedeagal spines damage the female genital tract during copulation.

5) Examine the relationship between copulation frequency and the extent of genital damage sustained.

3.3 Materials and methods

3.3.1 Behavioural components of copulation and body size

Pairs of beetles (n = 41) were mated and the copulatory behaviour of both sexes throughout was recorded (all animals were aged 18-24 hrs). The durations of female kicking behaviour and copulation were also recorded. Copulations were terminated naturally. Male and female body size was then measured as in section 2.3.3, after which the males were stored at -20 C for subsequent dissection whilst the females were retained for further investigation (see section 3.3.2).

3.3.2 Comparison of the genital tracts of virgin and mated females

Following copulation, the females (n = 41) from section 3.3.1 were housed individually for 16 hrs before being stored at -20°C. (Preliminary investigations indicated that after 16 hrs the damage to the female genital tract had been repaired. In general, the terminal stage of cuticular wound repair involves a process of melanisation which produces the typical red/brown colour of damaged tissue (e.g. Lackie *et al.* 1985). The appearance of red/brown tissue in a cuticular structure is therefore diagnostic of wound repair.) In order to allow comparison of the genital tracts of mated and virgin females, 32 virgin females (aged 18-24 hrs) were sacrificed and stored at -20°C for subsequent dissection. Following defrosting, each female's genital tract was removed, dissected and the endocuticular surface photographed as described in Chapter 2 (see section 2.3.4).

3.3.3 Genital damage assay

The amount of repaired genital damage was assayed in the following way: From the micrograph of each genital tract, every patch of damage was traced onto an acetate sheet using a fine, black, permanent maker pen (see Figure 3.4.2). Optimas® (v. 6.1) image analysis software was then used to capture (Pulnix CCD camera), digitise, and quantify the area of each patch. The patch areas were recorded and summed to give the *total area of genital damage* sustained by each female. This procedure was repeated for a randomly selected subset of females (n = 10) in order to assess the repeatability of the assay. Finally, Optimas® (v. 6.1) image analysis software was used to generate the *number of punctures* in each genital tract.

The replicated measures of the total area of genital damage in mated females were highly repeatable ($r = 0.9868$, $F_{1,9} = 150.827$, $p = 0.0001$) (Lessells and Boag 1987).

3.3.4 Potential behavioural and morphological correlates of genital damage

Potential correlates of the extent of genital damage incurred by individual females were examined in order to identify any behavioural or morphological determinants of this trait. It is reasonable to expect that one or more of the following traits might influence the extent of damage sustained:

- time until the onset of female mate-kicking behaviour (see section 3.3.1)
- duration of copulation (see section 3.3.1)
- female body size (see section 3.3.1)

- male body size (see section 3.3.1)
- length of longest male paramere (see section 3.3.5)

In addition, the relationships among the traits listed above were examined in order to identify correlates of both the duration of female kicking behaviour and copulation.

3.3.5 Measurements of male aedeagal traits and female genital punctures

Following copulation, the males (n = 41) from section 3.3.1 were dissected and each aedeagus removed as described in Chapter 2 (section 2.3.4). As there was considerable intra-male variation in spine length it was not possible to gain a repeatable measure of maximum or mean spine length for individual males. Instead, I elected to use *paramere length* as an index of male aedeagus size. Hence, the pair of parameres was separated from each aedeagus and photographed (see Figure 3.4.3). From each photograph, the longest of the two was measured, recorded and used in the investigation of the potential behavioural and morphological correlates of genital damage (see section 3.3.4).

In order to examine whether the aedeagal spines caused the female genital damage, a comparison was made between *mean basal spine diameter* and *mean genital puncture diameter*. A randomly selected sub-set of males (n = 10) from section 3.3.1 was used in this investigation. The aedeagal spines of males were photographed (see Figure 3.4.5) and the basal diameter of 5 spines measured from each micrograph: thus, each datum is the mean of 5 basal spine diameters. Given that longer spines have a higher probability of penetrating the endocuticle of the female genital tract, only the basal diameters of

relatively long spines were measured. Optimas® (v. 6.1) image analysis software was used to generate a measure of *mean genital puncture diameter* for a randomly selected sub-set of females (n = 23) from section 3.3.1.

3.3.6 Examination of the *in situ* genitalia of copulating pairs

Photographs of the *in situ* genitalia of copulating pairs (n = 10) were generated using the methods described in section 2.5.3. Pairs were snap-frozen 5 mins after the onset of copulation.

3.3.7 Effect of copulation frequency on genital damage

Females (n = 32) were allocated to one of three treatment groups. *Single-mated* females were mated once, *Double-mated* females were remated (2 days after their initial mating) and *Triple-mated* females were remated twice (2 and 4 days after their initial mating). One female allocated to the *Triple-mated* group was excluded from the experiment because she failed to mate for a third time within 30 mins of being confined with a male. Following their final copulation, and prior to storage at -20°C, all females were housed individually for 16 hrs to allow repair of any genital damage (see section 3.3.2). Between matings, females were housed individually in plastic petri-dishes (90mm diameter) and provided with 25 black-eyed beans each day as sites for oviposition. All animals were aged 24-36 hrs at the time of first mating.

3.3.8 Statistical analysis

Data from the investigation of genital damage in mated and virgin females were analysed with a Fisher's exact test whilst data regarding the behavioural and morphological traits were analysed using Spearman's rank correlations and a sequential Bonferroni correction (coefficients and p values were corrected for ties). To permit the use of parametric analysis, data from the genital damage and mating frequency experiment were square-root transformed to correct for a correlation between the treatment means and variances.

3.4 Results

3.4.1 Behavioural components of copulation

Conspicuous kicking behaviour (females used their hind legs to kick the posterior region of the male abdomen) was exhibited by all females (n = 41) both before and during copulation.

Pre- and early copulation

Prior to copulation females rejected male mating attempts (100%) by avoiding and kicking the courting male (100%) (with sufficient force to propel the male backwards) (Throughout the study, I observed both virgin and mated females to reject initial mating attempts, although subsequent attempts by the same male were usually successful in virgin females and females that mated 1-3 days previously). Once intromission occurred,

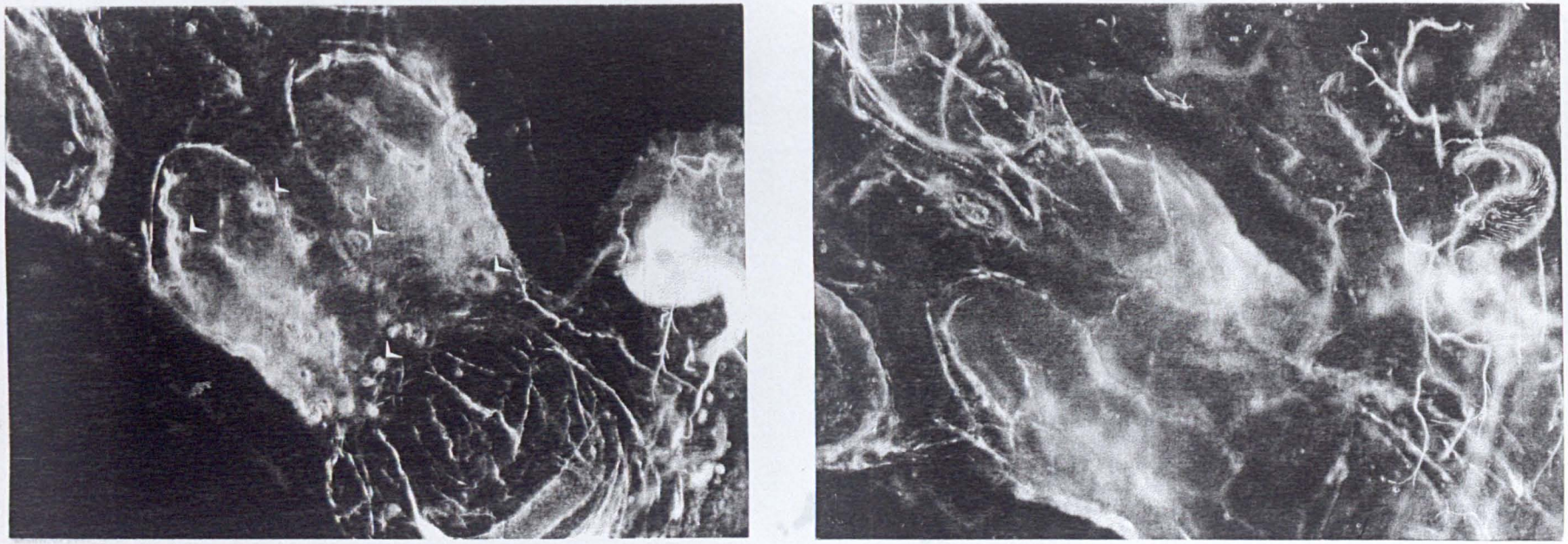
females remained motionless (100%) whilst males continued the rhythmical anntennating behaviour initiated prior to genital union.

Late copulation and genital separation

About two thirds of the way through copulation females (100%), began a vigorous bout of kicking which continued until genital separation occurred (mean time until kicking began = 7.6±3.2 mins, $n = 41$ and mean duration of kicking = 3.8±2.2 mins, $n = 41$). Hereafter, I refer to this behaviour as *mate-kicking*. During this phase, kicking was interspersed with pushing movements of the female's hind legs, which also targeted the male's abdomen. Both the kicking and pushing movements have the appearance of exerting considerable force against the male. In addition, females (100%) became more active: flexing and depressing their posterior abdominal segments ventrally, and walking and dragging their mates across the substrate behind them. At this point, males (100%) exhibited no overt attempt to maintain contact with females other than that afforded by the genitalia. The 'end-to-end' position was achieved immediately prior to genital separation in some copulations (60%). The mean copulation duration of virgin pairs was 11.4±2.9 mins ($n = 41$).

3.4.2 Comparison of the genital tracts of virgin and mated females

Comparison of the genital tracts of once-mated and virgin females revealed the presence of irregularly shaped patches of damaged endocuticle in the genital tracts of mated females ($n = 38$) and no damage in the tracts of virgin females ($n = 32$) (Fisher's exact test: $p = 8.0482 \times 10^{-19}$) (see Figure 3.4.1). All mated females exhibited damage to their



a b

Figure 3.4.1 Dark-field micrographs of the genital tracts of (a) once-mated and (b) virgin female *C. maculatus*. The arrows indicate patches of repaired damage in the endocuticular lining of the genital tract of the mated female 16 hrs after copulation. The genital tract of the virgin female shows no damage.

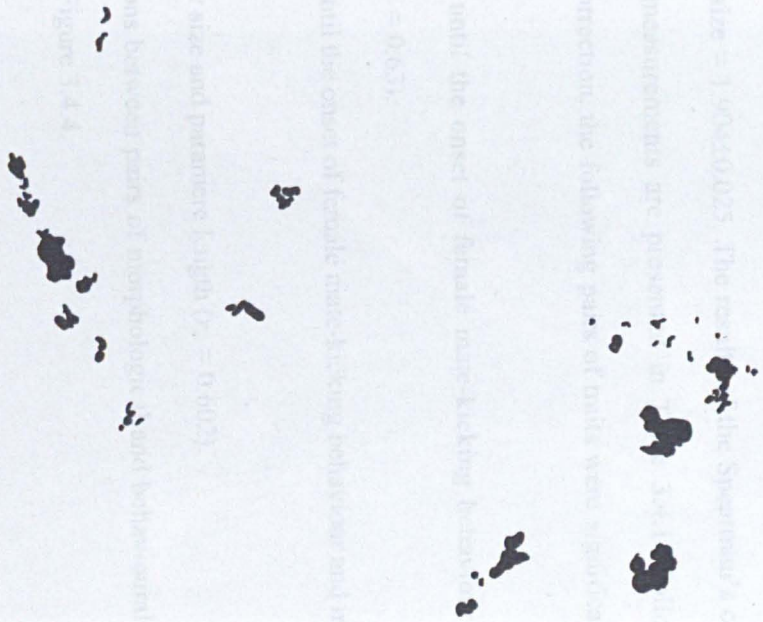


Figure 3.4.2 A tracing of the genital punctures of a mated female, used for assaying the total area of genital damage (see section 3.3.3).

genital tracts although the extent of injuries varied considerably (mean total area of genital damage: once-mated females = 125.01±13.57 μm^2, n = 38 and virgin females = 0±0 μm^2, n = 32; mean number of genital punctures in once-mated females = 27.02±2.50, n = 38 and virgin females = 0±0, n = 32).

3.4.3 Potential behavioural and morphological correlates of genital damage

Measures of the remaining morphological traits are as follows: mean aedeagal paramere length = 631.38±4.81 μm, n = 37, mean male body size = 1.675±0.021 mm, and mean female body size = 1.904±0.025. The results of the Spearman's correlation analysis of all the trait measurements are presented in Table 3.4.1. Following the sequential Bonferroni correction, the following pairs of traits were significantly correlated at $p <$ 0.05:

(1) the time until the onset of female mate-kicking behaviour and the duration of copulation (r_s = 0.63),

(2) the time until the onset of female mate-kicking behaviour and male body size (r_s = -0.5), and

(3) male body size and paramere length (r_s = 0.602).

Six correlations between pairs of morphological and behavioural traits are graphically presented in Figure 3.4.4.

3.4.4 Size comparison of male aedeagal spines and female genital punctures

There was no significant difference between the basal diameter of the aedeagal spines and the diameter of the female genital punctures (t-test: t_{31} = -1.408, p = 0.1692; mean

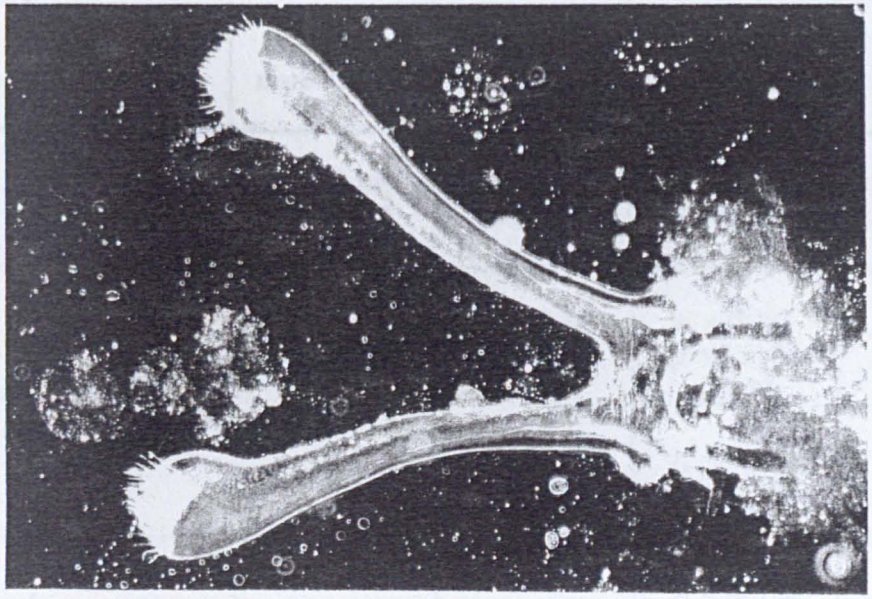


Figure 3.4.3 Dark-field micrograph of a pair of male aedeagal parameres. Approximate paramere length = 600 µm.

Table 3.4.1 Results of the Spearman's correlation analysis. Correlations significant at $p < 0.05$ after the sequential Bonferonni correction are denoted by *. Coefficients are accompanied by values of p and n.

Trait	Genital damage area	Paramere length	Time until mate-kicking	Copulation duration	Female body size	Male body size
Genital damage area	___	$r_s = 0.045$ $p = 0.7939$ $n = 37$	$r_s = -0.109$ $p = 0.5123$ $n = 37$	$r_s = -0.065$ $p = 0.6956$ $n = 37$	$r_s = 0.021$ $p = 0.9004$ $n = 37$	$r_s = 0.103$ $p = 0.5374$ $n = 37$
Paramere length		___	$r_s = -0.363$ $p = 0.0292$ $n = 37$	$r_s = -0.29$ $p = 0.0819$ $n = 37$	$r_s = 0.261$ $p = 0.1176$ $n = 37$	$r_s = 0.602$* $p = 0.0003$ $n = 37$
Time until mate-kicking			___	$r_s = 0.63$* $p < 0.0001$ $n = 41$	$r_s = -0.011$ $p = 0.9466$ $n = 41$	$r_s = -0.5$* $p = 0.0016$ $n = 41$
Copulation duration				___	$r_s = -0.188$ $p = 0.2354$ $n = 41$	$r_s = -0.358$ $p = 0.0237$ $n = 41$
Female body size					___	$r_s = -0.034$ $p = 0.8279$ $n = 41$

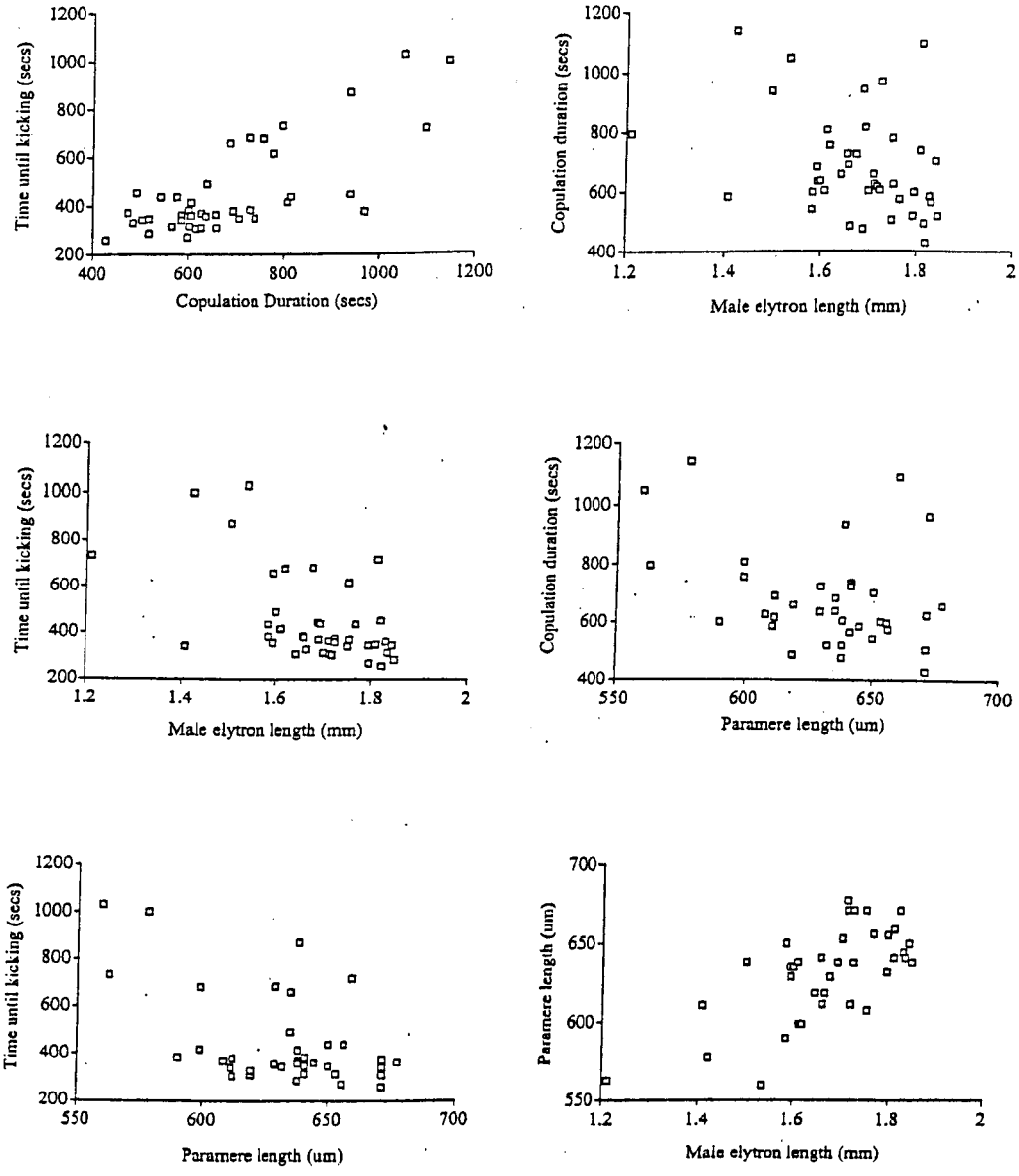


Figure 3.4.4 Relationships between pairs of the morphological and behavioural traits examined.

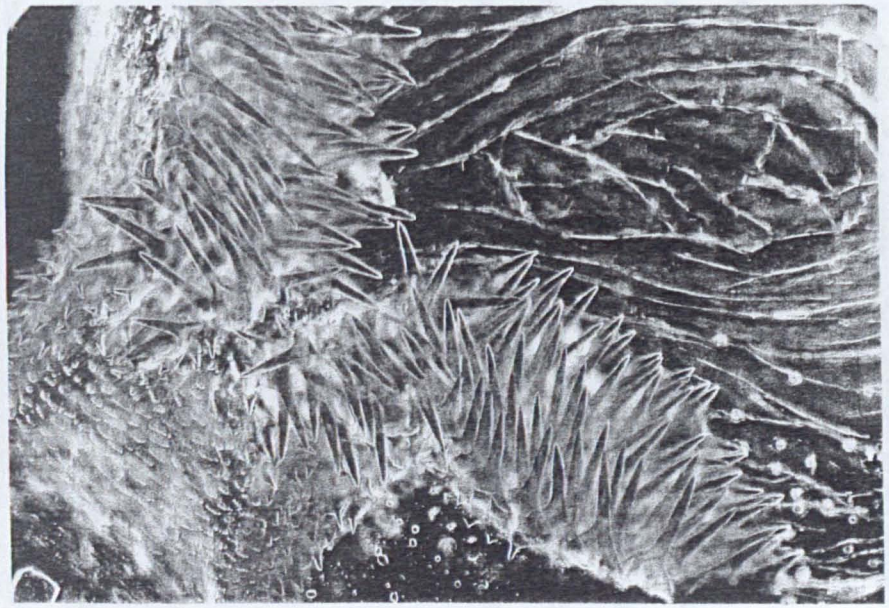


Figure 3.4.5 Dark-field micrograph of the cuticular male aedeagal spines. Mean basal spine diameter ~ 12 µm.

basal spine diameter = 12.86±3.53 μm, n = 10 and mean puncture diameter = 10.80±0.92 μm, n = 23).

3.4.5 The *in situ* genitalia of copulating pairs

Examination of the *in situ* genitalia of copulating pairs (n = 10) revealed that, during copulation, the aedeagal spines punctured the endocuticular lining of the female genital tract and, in so doing, also penetrated the epithelia of the genital tract (see Figure 3.4.6).

3.4.6 Copulation frequency and genital damage

Copulation frequency had a significant positive effect on the degree of genital damage sustained by females (ANOVA: $F_{2,28}$ = 5.077, p = 0.0131; mean total area of genital damage: *Single-mated* females = 65.47±16.68 μm^2, n = 10; *Double-mated* females = 117.88±22.94 μm^2, n = 12; and *Triple-mated* females = 178.86±38.17 μm^2, n = 9) (see Figure 3.4.7).

3.5 Discussion

The results of this chapter show that the dimensions of the punctures in the genital tracts of mated female *C. maculatus* are consistent with them being made by the sclerotised spines of the male aedeagus (see section 3.4.4). Additional indirect support for this causal relationship is provided by the temporal and spatial congruence of the aedeagal spines and genital punctures demonstrated in Chapter 2 (see Figure 2.5.5). Further to

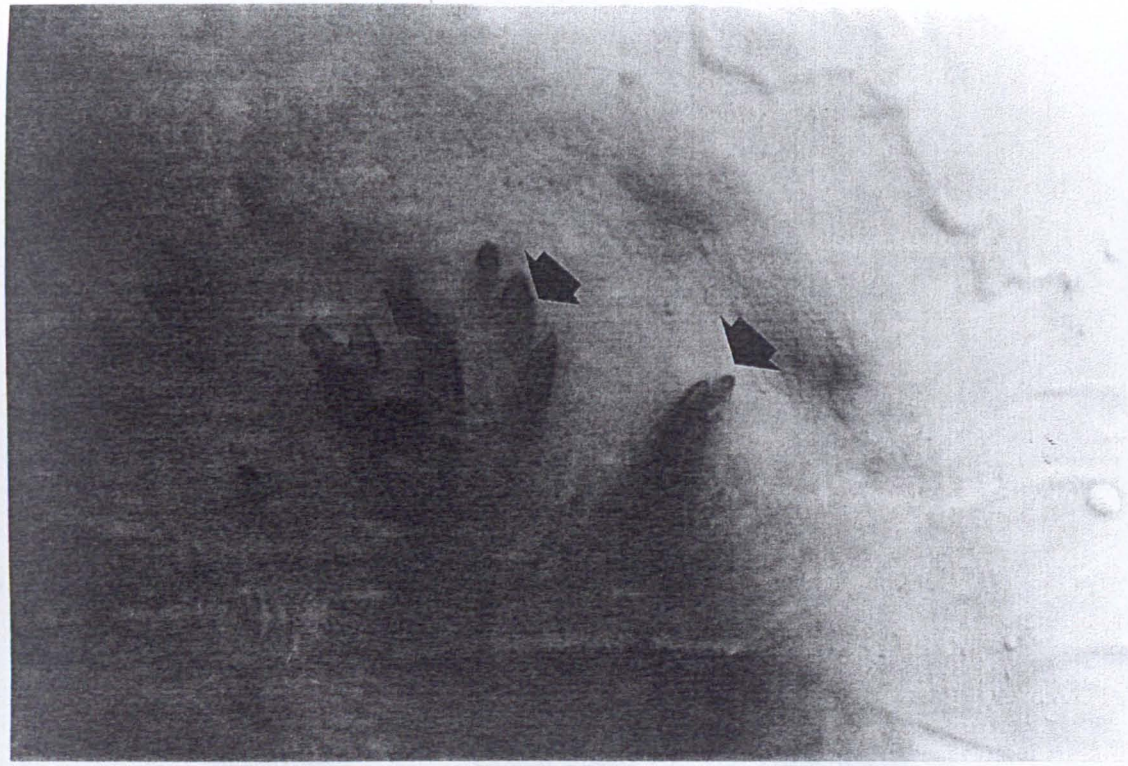


Figure 3.4.6 Normansky micrograph of the *in situ* genitalia of a copulating pair of *C. maculatus* snap-frozen in liquid nitrogen. The arrows indicate the tips of aedeagal spines that have penetrated the cuticular lining of the female's genital tract in the region of the lateral pads.

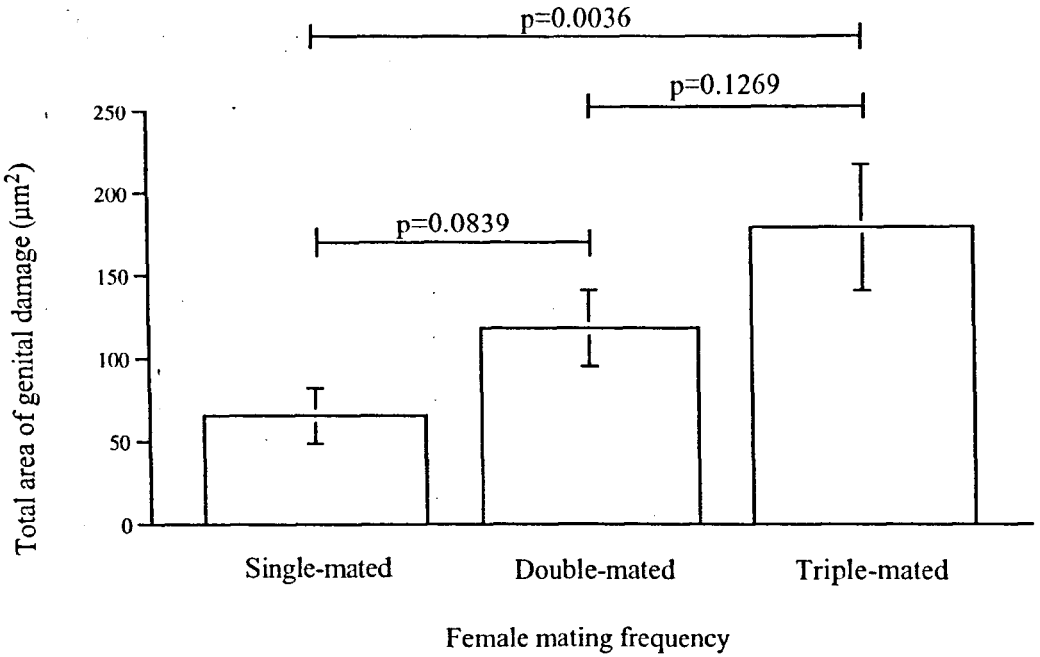


Figure 3.4.7 Copulation frequency and genital damage in females. The extent of genital damage increases with each additional copulation.

this, examination of the *in situ* genitalia of copulating beetles provided direct qualitative evidence that the aedeagal spines punctured the endocuticular lining of the female genital tract during copulation (see Figure 3.4.5) (Crudgington and Siva-Jothy 2000). Within a day of copulation the genital injuries are evident as red-brown patches (see Figure 3.4.1), the colour of which is typical of repaired tissue (Chapman 1998).

Why males inflict injuries on their mates is unclear. The imposition of genital damage by males may be part of a strategy to manipulate female post-copulatory behaviour to their advantage, a hypothesis which is indirectly tested in Chapter 5 (see Johnstone and Keller 2000). Alternatively, the aedeagal spines and the damage they cause may facilitate haemocoelic deposition of male seminal substances in the way proposed by Lewis and Pollock (1975) for the blowfly, *Lucilia sericata*. Males of this species transfer seminal compounds that are thought to reduce a female's propensity for remating (Lewis and Pollock 1975). Lewis and Pollock (1975) suggest that the action of barbs on the male aedeagus is analogous to that of a hypodermic syringe. By puncturing the female genital tract, the barbs may permit the injection of substances into the female haemocoel. Direct haemocoelic deposition of these compounds may accelerate and/or intensify the inhibitory effect on female behaviour, thereby increasing the benefit to males (Lewis and Pollock 1975). However, it is also possible that the damage sustained by female *C. maculatus* is simply an incidental, rather that the primary, consequence of male genitalic morphology. For example, the spines may accidentally puncture the female's genital tract if they function to they maintain genital contact during copulation (see Chapter 2, section 2.6.2).

3.5.1 Potential costs to females of genital damage

Whatever selection pressures led to the evolution of the aedeagal spines and currently contribute to their maintenance, the injuries resulting from their action during copulation may generate a range of physiological costs with potentially important consequences for female fitness.

Costs of infection and associated immune responses

Genital damage is likely to expose females to an increased risk of infection. In insects, selection to reduce the risk of invasion by pathogens has contributed to the evolution of aspects of the cuticle, a structure which functions as a mechanical barrier against the external world (Chapman 1998). Any injury to the endocuticle of the female genital tract will increase the risk of invasion by potentially harmful pathogens. This may heighten the vulnerability of females to infections such as sexually transmitted diseases, which are known to generate fitness costs in some taxa, including insects (Hurst *et al.* 1995; Lockhart *et al.* 1996). Several proximate costs may arise from an increase in infection rates and the associated immune responses in insects. For instance, cuticular wounding can cause a substantial increase in the number of haemocytes circulating in the haemolymph (Nayar and Knight 1995), recruitment of which is likely to carry some cost. The production of phenoloxidase, a key component in immune system activation, may also be expensive since its precursor requires the environmentally-limited essential amino acids, phenylalanine and/or tyrosine (Nappi and Vass 1993). Other potential costs of raising an immune response relate to autoimmunity and the effects of cytotoxic reactive oxygen species that accumulate when the immune system is triggered (Nappi

and Vass 1993). Furthermore, several insect studies have demonstrated phenotypic trade-offs between investment in immune function and other life-history traits, including reproduction (Siva-Jothy *et al.* 1998) and foraging (Konig and Schmid-Hempel 1995). Genital damage therefore has important potential consequences for immune system investment and the risk of pathogenesis in females. Ultimately, the future reproductive potential (see e.g. Stearns 1992) of females could be threatened if genital damage increases the probability of infection-related mortality.

Costs of repair

Another potential proximate cost to females of genital damage is the allocation of resources to the process of repair following copulation. When the cuticular barrier is breached, haemocytes target the site of injury to plug the wound (Lackie 1988). This is followed by deposition of melanin, a pigment which confers a brown colour on repaired tissue and which functions to fortify the wound more permanently (Gillespie *et al.* 1997). Since time, energy and metabolic compounds are likely to be invested in repair processes (Chapman 1998), genital damage may cause the diversion of finite reserves from critical activities such as somatic maintenance and reproduction.

Route for the transfer of costly male seminal proteins

As mentioned earlier, males of some insects transfer physiologically active seminal compounds to females during copulation (Chen 1984). For instance, the seminal proteins of male *D. melanogaster* mediate female oviposition and remating behaviour to the benefit of males (Baumann 1974; Chapman *et al.* 1995; Clark *et al.* 1995; Prout and

Clark 2000). Providing that male *C. maculatus* transfer seminal proteins with functions resembling those reported in other taxa, the aedeagal spines and genital damage may operate in the way suggested for blowflies (Lewis and Pollock 1975), by providing males with a rapid route to the female haemolymph. Females may then be at a selective disadvantage if the seminal compounds manipulate their post-copulatory behaviour in non-adaptive ways. Moreover, the seminal compounds of *D. melanogaster* (Fowler and Partridge 1989; Chapman *et al.* 1995) have been shown to be toxic and to have negative effects on female survival. If male *C. maculatus* use the genital punctures to deliver similarly toxic substances directly to the haemolymph of their mates, any negative consequences for female fitness traits may arise more rapidly and/or be of greater severity.

3.5.2 Variation in extent of genital injuries

The results of this chapter demonstrated considerable variation among females in the extent of genital damage following a single copulation. The precise weight of costs paid by individuals due to such injuries is therefore also likely to differ. In addition, the amount of genital damage sustained increased with copulation frequency (maximum of three copulations). Given that costs associated with copulation can induce females to mate less frequently (Stockley 1997), the cumulative costs of genital damage resulting from successive copulations may constrain female mating frequencies. The strength of such a constraint is likely to depend on the quality and quantity of the benefits of remating and their power to offset the costs of genital damage. Nonetheless, providing the costs arising from genital damage are dose-dependant, this trait has the potential to

generate a conflict of interests between the sexes over mating frequency (see e.g. Stutt and Siva-Jothy in press).

3.5.3 Copulatory behaviour, morphological traits and genital damage

Since once-mated females differ markedly in the degree of damage exhibited, correlates of this variation were sought through examination of a range of morphological and behavioural traits. However, no correlations were found between the extent of genital damage in individual females and any of the traits measured (copulation duration, time until mate-kicking begins, male aedeagus size and male and female body size; see section 3.4.3). These traits are therefore unlikely to exert a measurable influence on the extent of injuries sustained. However, male body size and paramere length were positively correlated indicating that male body size can be used as an index of at least one male genital trait.

Towards the end of copulation, females direct vigorous kicks towards their mates (see section 3.4.1), the function of which is investigated in Chapter 4. The time that females began mate-kicking behaviour was negatively correlated with male body size. Females may begin kicking earlier when copulating with relatively large males if larger males complete transfer of the spermatophore faster than smaller males. Behavioural observations indicate that mate-kicking is often accompanied by the female dragging the male around behind her as she traverses the substrate. Mate-dragging by females is also seen prior to genital separation in the midge, *Culicoides melleus* (Linley and Hinds 1975) and in the scorpionfly, *Panorpa vulgaris,* where males employ force to prolong

copulation duration (Thornhill and Sauer 1991). It is possible that in all these cases, mate-dragging behaviour may signal a lack of cooperation in females to continue with the copulation.

3.5.4 Genital damage and the potential for sexual conflict

The potentially deleterious effects of genital damage on female *C. maculatus* are varied. In addition to a range of physiological costs, it may generate life-history costs due to increased risk of death from infection and dehydration. Given that the injuries are cumulative with respect to copulation frequency, the costs may be amplified over a lifetime. If any cost or combination of costs associated with genital damage is sufficient to threaten female fitness, a conflict of reproductive interests will emerge between the sexes (Parker 1979; Parker 1984; Clutton-Brock and Parker 1995b). Indeed, Brown *et al.* (1997) listed the imposition of genital injury, along with traumatic insemination and forced copulation, as an example of a forceful act. In general, force may be used gain power over aspects of reproduction by removing control from the opposite sex (Clutton-Brock and Parker 1995b; Brown *et al.* 1997). Antagonistic traits, including those applying force, are predicted to produce evolutionary responses in the losing sex that function to offset their detrimental effects (e.g. see Arnqvist and Rowe 1995). Thus, morphological or physiological mechanisms for counteracting any costs of genital damage may have arisen in female *C. maculatus*. Alternatively, or in combination, females may have responded with behavioural strategies to reduce the frequency of copulation and/or the length of time spent in copula (Stockley 1997).

3.6 Summary

In this chapter I have demonstrated that the sclerotised spines on the male intromittent organ of *C. maculatus* punctured and caused damage to the endocuticlar lining of the female genital tract during copulation. An assay was developed and used to show that (a) the degree of damage sustained varied considerably among single-mated females, and (b) that it increased with copulation frequency. No morphological or behavioural correlates of the extent of damage were found. The potential for genital injuries to impose costs on females, and therefore to generate a conflict between the sexes, was discussed.

Chapter 4: The function of mate-kicking in copulating females

4.1 Introduction

Instances of males 'stimulating' females during copulation are widespread and well documented (Eberhard 1991). So-called copulatory courtship (Eberhard 1991) is thought to result from post-copulatory sexual selection on males to influence female reproductive events in ways that increase male fertilisation success (Otronen and Siva-Jothy 1991; Edvardsson and Arnqvist 2000). In contrast, behaviours in females that resemble, at least superficially, copulatory courtship by males, are infrequently documented (Eberhard 1994; Rodriguez 1998). The paucity of reports of this type of female behaviour may reflect its genuine rarity in the natural world. Indeed, Eberhard (1994) reported that instances of females touching or stimulating males during copulation could be identified in only 5% of 132 insect and spider species examined. Such acts vary in the degree of apparent coercion towards males, and include brushing, massaging, vibrating, pushing and kicking by the female (Eberhard 1994). These behaviours may serve no adaptive function and may simply represent reflex responses by females to the physical phenomenon of copulation. Alternatively, females may use them to influence the behaviour of males during copulation to their advantage.

Unfortunately, investigations of their functions are scarce. In an anecdotal report, Ridshill Smith (1970) suggested that female wasps, *Hemithynnus hyalintus*, stroked the abdomen of males during copulation in order to induce the regurgitation of nectar droplets on which females depend for a source of carbohydrate (Ridshill Smith 1970). Copulating female lygaedid bugs, *Ozophora baranowskii*, on the other hand, gently tap the abdomen and genitalia of their mates with their hind legs (Rodriguez 1998). Rodriguez (1998) quantified this trait and found that the incidence of tapping did not correlate with the size of any of the male morphological traits examined, or with female reproductive output. However, when females tapped their mates at high frequencies, copulations were shorter and sperm failed to be transferred (Rodriguez 1998). One explanation for these findings is that tapping signals the female's lack of receptivity, to which males respond by failing to transfer an ejaculate.

4.1.1 Kicking by females during copulation

A copulatory trait with more apparent force than the tapping of female lygaedid bugs occurs in female *Callosobruchus maculatus*. This trait, which I have called mate-kicking, occurs towards the end of mating when females kick and push their mates with their hind legs (see Chapter 3, section 3.4.1). In general, kicking by females is often associated with pre-copulatory interactions during which unreceptive females resist costly male mating attempts (Thornhill 1980; Smith *et al.* 1989; Ringo, 1996). In this context, there is a lack of confluence between the reproductive interests of the sexes and so females use force to potentially regain control of mating decisions (see Clutton-Brock and Parker 1995b; Arnqvist 1997). This association between female kicking and sexual

conflict raises the possibility that kicking by females *during* copulation is similarly antagonistic and denotes the presence of a conflict of interests between males and females. Copulating female midges, *Culicoides melleus*, kick their partners (Linley and Adams 1975). In this species, kicking occurs immediately after the initiation of copulation and is exhibited only by non-virgin females. The response to this trait from males depends on their mating status. Whereas non-virgin males release a kicking female early, virgin males proceed with the copulation (Linley and Mook 1975). To investigate the function of kicking, Linley and Hinds (1975) removed the hind limbs of females and found that an absence of kicking was associated with the transfer of greater quantities of sperm. This result led the investigators to conclude that early-copulatory kicking by non-virgin female *Culicoides melleus* signals a lack of sexual receptivity to which non-virgin males respond semi-adaptively by inseminating fewer sperm (1975). Interestingly, Linley and Adams (1975) suggested that the kicking behaviour arose by modification of the grooming response to allow females to rid themselves of an "irritation".

Using the methodology adopted by Linley and Hinds (Linley and Hinds 1975), this chapter investigates the function of kicking in copulating female *C. maculatus*. Researchers have argued that in both *C. maculatus* (Rup 1986) and the congeneric species *C. subbinotatus* (Mbata *et al.* 1997), copulation is ended by female kicking behaviour (a similar suggestion has been made for the function of late-copulatory kicking in female *Culicoides melleus* (Linley and Adams 1972)). So, by kicking their mates, female *C. maculatus* may control when copulation is terminated and so determine

the duration of copulation. I also considered whether mate-kicking was associated with the genital injuries sustained by females during copulation (see Chapter 3). Any costs generated by genital damage will favour females that reduce the extent of their injuries and mate-kicking may the mechanism by which this is achieved.

4.2 Aims

In this chapter, I investigate the function of mate-kicking during copulation by female *C. maculatus*. I aim to determine the effect of preventing mate-kicking behaviour on two aspects of copulation:

(1) the duration of copulation,

(2) the extent of genital tract damage and the number of genital punctures sustained by mated females.

4.3 Materials and methods

4.3.1 Mate-kicking and copulation duration

Females (n = 72) (aged 24-36 hrs) were assigned to one of three experimental groups: *Non-Kicking* (treatment) females, *Kicking* (procedural control) females and *Control* (unmanipulated) females. In order to prevent mate-kicking behaviour, *Non-Kicking* females were immobilised on ice and their metathoracic limbs were removed using a

dissecting microscope (Leica MZ8) and bowspring scissors. As a procedural control for this manipulation, *Kicking* females had one prothoracic limb and the diagonally opposite mesothoracic limb ablated. All limbs were removed adjacent to the coxa. On recovery from the chilling process, all females resumed locomotory activity and none showed any overt behaviour directly associated with the removal of their limbs. *Control* females were not subject to chilling and were left intact. Females from all three groups were then mated once (to males aged 24-36 hrs) and the duration of each copulation was recorded.

4.3.2 Mate-kicking, genital damage and genital punctures

Females (n = 40) (aged 24-36 hrs) were allocated to one of two experimental groups: *Non-Kicking* (treatment) females or *Kicking* (procedural control) females. For reasons explained in the results section (section 4.4.1) only one control group, *Kicking* females, was used in this experiment. *Non-Kicking* and *Kicking* females were generated as in section 4.3.1 and mated once (to males aged 24-36 hrs). Prior to dissection of their genital tracts, females were left for 16 hrs after copulation to allow any genital damage to be repaired (see section 3.3.2). The total area of repaired damage and the number of genital punctures in the tract of each female was quantified using the methods described in section 3.3.3.

4.3.3 Statistical analysis

Data on copulation duration and genital damage area were square-root transformed to correct for a correlation between the treatment means and variances before being analysed with parametric tests. Data on genital puncture number were log-transformed to correct for non-normality and analysed using a parametric test.

4.4 Results

4.4.1 Mate-kicking and copulation duration

The ability of females to kick their mates during copulation had a significant effect on the duration of copulation (ANOVA: $F_{2,69} = 13.084$, $p < 0.0001$; mean copulation duration: *Non-kicking* females = 15.5±1.3 mins, $n = 24$; *Kicking* females = 8.8±0.7 mins, $n = 24$; and non-manipulated control females = 10.8±1.0 mins, $n = 24$) (see Figure 4.4.1). Fishers PLSD multiple comparison test indicated that copulations involving females from the two control groups did not differ significantly in duration ($p = 0.1373$). For this reason, only the procedural control group of *Kicking* females was used in the mate-kicking and genital damage experiment (see section 4.3.2). The copulation durations of both control groups differed significantly from that of the treatment group (Fishers PLSD test: $p < 0.001$).

All copulations involving females that were prevented from mate-kicking (i.e. *Non-kicking* females) appeared to be terminated by the male. In these instances males used

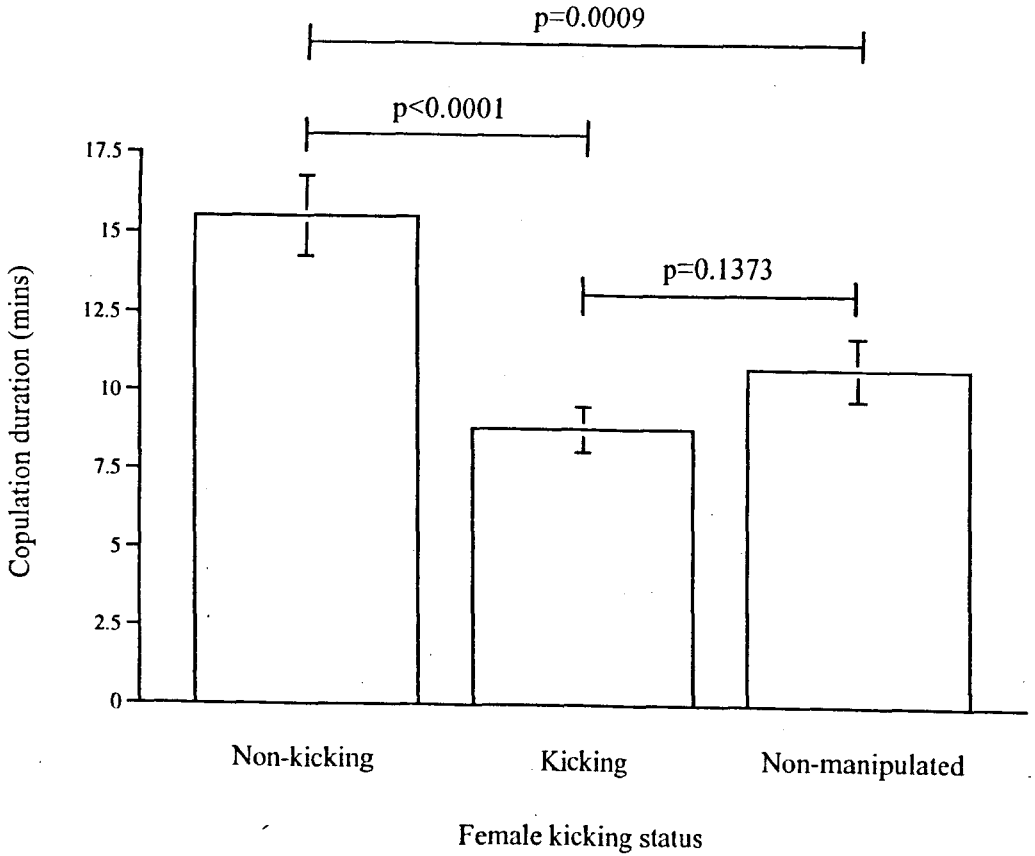


Figure 4.4.1 The effect of mate-kicking ability of the duration of copulation. Copulations involving females that were unable to kick their mates were longer than those involving females that were able to kick their mates.

their hind limbs to repeatedly kick and push their mates until genital separation occurred. Despite the absence of their hind legs, females were active during this phase: flexing and depressing their posterior abdominal segments ventrally, and dragging their mates across the substrate in a similar way to intact females (100%).

4.4.2 Mate-kicking, genital damage and genital punctures

The ability of females to kick their mates had a significant effect on the total area of genital damage sustained during copulation (t-test: $t_{38} = -2.393$, $p = 0.0218$). Females that were prevented from mate-kicking had significantly more damage than females permitted to kick (mean total area of genital damage: *Non-kicking* (treatment) females = 346.86±66.27 μm^2, $n = 20$; and *Kicking* (procedural control) females = 187.26±39.50 μm^2, $n = 20$) (see Figure 4.4.2).

Mate-kicking ability also had a significant effect on the number of genital punctures incurred (t-test: $t_{38} = -2.314$, $p = 0.0262$). Females that were prevented from mate-kicking had significantly more genital punctures than females permitted to kick (mean number of genital punctures: *Non-kicking* (treatment) females = 57.65±4.30, $n = 20$; and *Kicking* (procedural control) females = 42.25±4.11, $n = 20$) (see Figure 4.4.3).

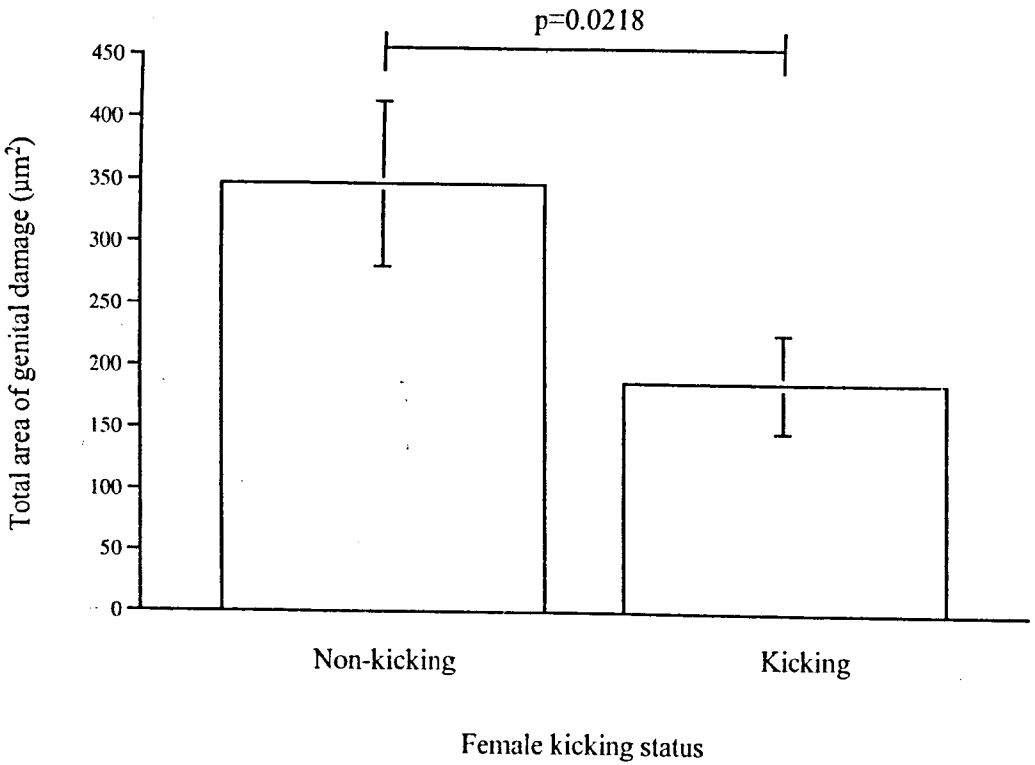


Figure 4.4.2 The effect of mate-kicking ability of the extent of genital damage. Females that were unable to kick their mates sustained more damage to their genital tracts than females that were able to their mates.

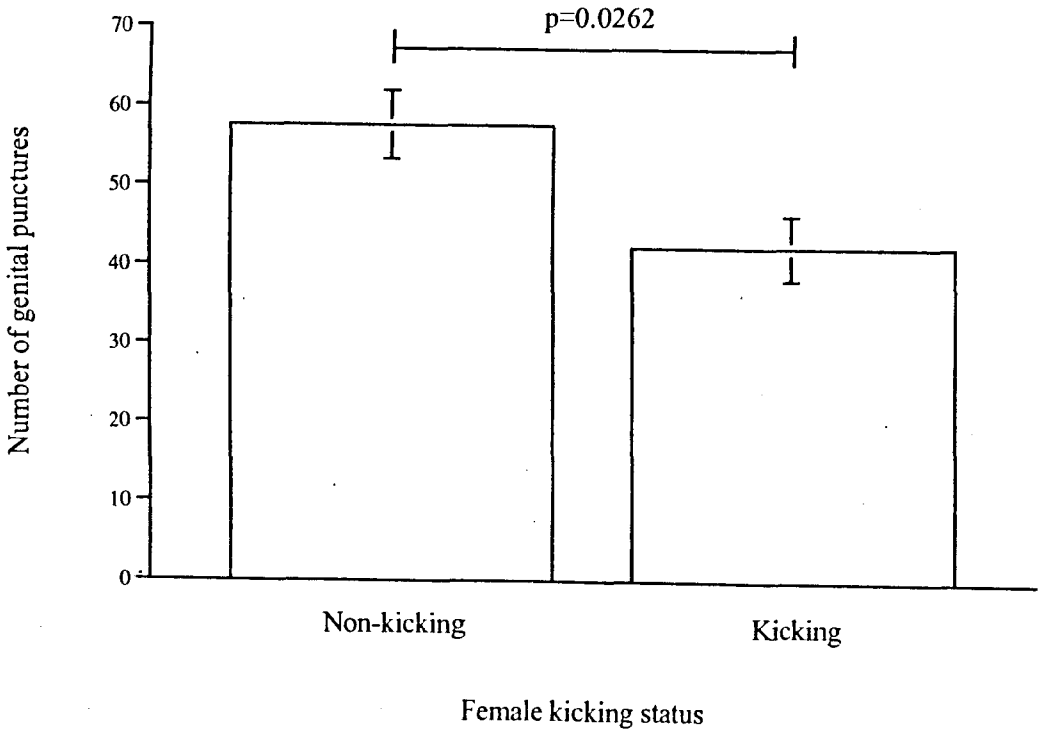


Figure 4.4.3 The effect of mate-kicking ability of the number of genital punctures. Females that were unable to kick their mates incurred more punctures to their genital tracts than females that were able to their mates.

4.5 Discussion

The results of this chapter show that when female *C. maculatus* were unable to engage in mate-kicking behaviour copulations were longer than those involving females that were able to kick. Consequently, by kicking their mates, females appear to shorten, and ultimately bring about the termination of copulation. This result provides experimental support for Rup's (1986) assertion that by kicking their mating partners, females initiate the termination of copulation. Since mate-kicking is frequently accompanied by females walking and dragging their partners behind them (see Chapter 3, section 3.4.1), these traits may act in combination to facilitate genital separation. The results also demonstrated that the genital tracts of females prevented from mate-kicking displayed a greater area of damage (Crudgington and Siva-Jothy 2000) and more genital punctures than females permitted to kick. Mate-kicking may operate to undermine any anchoring processes determined by the male, thereby allowing females to exert greater control of the timing and/or method of genital detachment, and the degree of injury sustained. The associations between mate-kicking and both copulation duration and genital damage suggest that costs arising from one or both of these aspects of copulation underlay the evolution/maintenance of mate-kicking behaviour. However, costs due to other factors may be implicated in the selection of this behavioural trait. For instance, if copulating females suffer increased predation or reduced foraging efficiency relative to single females, selection would favour traits that reduce the frequency and/or duration of copulation. Given that mate-kicking reduces copulation duration, this behaviour could be employed to terminate copulation early, thereby lowering the risk of predation and/or

creating opportunities to forage. Costs of mating due to both of these factors are expressed in females of some species of water-strider (Arnqvist 1989; Fairburn 1993; Rowe 1994). It is difficult to evaluate the potential role of predation in the evolution of mate-kicking behaviour given that information regarding predation rates in the natural inhabit of *C. maculatus* (i.e. human grain stores) is not available. The idea that females terminate copulation by mate-kicking in order to increase foraging efficiency has little relevance for *C. maculatus* because feeding rarely occurs in the adult phase (Larson and Fisher 1938).

Of course other, unknown factors may have influenced the evolution and maintenance of mate-kicking behaviour. Nonetheless, the results of the present study provide support for the hypothesis that this trait currently functions in females to shorten copulation duration and reduce the extent of genital damage sustained. It is possible that mate-kicking evolved in the context of either copulation duration or genital damage, only to confer advantages in the other. Such a dual benefit would intensify the selective advantage enjoyed by females that engage in mate-kicking behaviour.

4.5.1. Potential benefits to females of reducing copulation duration

Several fitness benefits may accrue to females that use mate-kicking behaviour to shorten copula.

Reduced time investment

Adult lifespan in *C. maculatus* is relatively short. This is because adult feeding opportunities are scarce and so resources assimilated as larvae are usually the sole determinants of adult longevity (Larson and Fisher 1938). A relatively brief adult phase could indicate that time allocation strategies are extremely important for fitness. By reducing the period occupied by copulation, females would increase the time available for investment in other fitness-enhancing activities. For example, searching for plant hosts and assessing their suitability as oviposition sites. Time is known to be an important constraint of this behaviour and females that succeed in locating hosts bearing relatively few eggs enjoy increased offspring production (Wilson 1989). Overall, any time-saving benefits of reducing copulation duration are likely to depend on the frequency at which females copulate. Thus, at high mating frequencies females may be under greater pressure to minimise the time invested in each copulation.

Reduced opportunity for the transfer of toxic seminal products

The ejaculates of some male insects contain physiologically active compounds that influence female reproductive decisions in ways that are advantageous to males (Chen 1984; Chen *et al.* 1988, Leopold 1976). An example is that of male *D. melanogaster* which transfer toxic ejaculates that accelerate female death rate (Chapman *et al.* 1995). If male *C. maculatus* transfer seminal compounds with similar detrimental effects on females, selection will act to minimise the negative consequences for female fitness. Means of achieving this include blocking their absorption, rendering them less harmful and reducing the quantity received. If the rate of transfer of male seminal compounds

covaries with copulation duration, one mechanism by which females could lower their intake is to decrease the time spent copulating. In this context, mate-kicking may be used to reduce copulation duration and therefore the time available for males to transfer potentially costly seminal compounds. However, this benefit of reducing copulation duration remains theoretical, as it is not known whether male *C. maculatus* transfer harmful substances to their mating partners.

Reduced copulation duration as a pleiotropic effect of controlling genital separation

Females may not gain from shorter copulations *per se*, but may benefit from influencing the way in which copulation is terminated. During copulation, the aedeagal spines puncture the female genital tract and if the process of genital separation plays a role in determining the degree of injury, females may benefit from controlling this process. To this end, mate-kicking may influence the mode of departure of the aedeagus from the genital tract, and thereby the amount of damage incurred. In the absence of mate-kicking by females the termination of copulation appears to be controlled by similar kicking tactics in males (see section 4.4.1). This apparent transfer of power over genital separation from females to males may underlay the increased genital damage seen in females that cannot kick their mates.

4.5.2 Potential benefits to females of reducing genital damage

The potential fitness costs to females of genital damage have been explored in Chapter 3 (see section 3.5.1). If the scale of such costs is proportional to the severity of the damage, females will benefit from reducing their injuries. Given that mate-kicking

lessens the extent of damage and the number of punctures sustained, this behavioural trait may enable females to reduce the costs paid due to their injuries. In general, the benefits of avoiding cuticular injury include less risk of mortality due to factors such as haemorrhage, dehydration and infection (Chapman 1998) as well as lower physiological costs associated with immune and repair responses (Nappi and Vass 1993; Nayar and Knight 1995; Gillespie *et al.* 1997). If, as discussed earlier (see section 3.5.1), the genital punctures provide routes for the passage of toxic seminal compounds to the female haemolymph, reducing the number of punctures may reduce the quantity that reach the female's body cavity, and therefore any negative consequences for female fitness. Theoretically then, several selective advantages may be gained by females that incur fewer genital injuries through their use of mate-kicking behaviour.

4.5.3 Female counter-adaptations to antagonistic male traits

If a trait in one sex imposes fitness costs on the opposite sex, a conflict of reproductive interests will arise between the sexes (Parker 1979; Parker 1984; Alexander *et al.* 1997). Counter-adaptations are then predicted to evolve in the losing sex to reduce the detrimental effects of the antagonistic trait (e.g. Stockley 1997). Anecdotal support for such antagonistic co-evolution can be found in a study of the blue shark, *P. glauca*, where the skin of females is more than twice as thick as that of males (Pratt 1979). This characteristic is thought to have evolved as a protective response against so-called "courtship wounds" which are inflicted by males during copulation (Pratt 1979). Experimental evidence for a female morphological adaptation to sexual conflict can be found in a study of the water-strider, *Gerris incognitus* (Arnqvist and Rowe 1995). In

this species, conflict over mating decisions is expressed as intense pre-copulatory struggles and males use abdominal claspers and spines on their forelegs to grasp females and overcome their resistance to mating (Arnqvist and Rowe 1995). Females possess modified, elongated abdominal spines and in order to investigate their function, Arnqvist and Rowe (1995) manipulated their length. They found that the abdominal spines undermined the efficacy of the male grasping apparatus, thereby allowing females to repel mating attempts and avoid superfluous matings.

Arnqvist and Rowe (1995) provided the first experimental demonstration of an evolutionary response by females to sexual conflict. The results of the present investigation into the function of mate-kicking in female *C. maculatus* are consistent with the proposal that this behavioural trait has evolved to counter male strategies for controlling the duration of copulation and/or the amount of genital damage inflicted. If so, this study provides important experimental evidence for another female counter-adaptation to an antagonistic male trait(s). However, a necessary caveat to this hypothesis is that a reduction in female fitness due to copulation duration or genital damage must be demonstrated in order to provide definitive evidence that mate-kicking has arisen in the context of sexual conflict to exert control over these components of copulation.

Brown *et al.* (1997) argue that females may be more constrained than males in their capacity to generate counter-adaptations to conflict because females experience greater pressure to trade-off investment in conflict traits with traits operating in other life-

history contexts. In light of this proposal, it is interesting to consider the suggestion by Linley and Hinds (1975) that copulatory kicking, such as that exhibited by female *Culicoides melleus* and *C. maculatus*, may arise by modification of the grooming response to produce so-called 'resistive grooming'. Perhaps the alteration and transfer of an existing behavioural trait from one context to another, in this case from grooming to copulation, is a relatively simple and inexpensive route by which females can counter male manipulatory strategies and regain control of reproductive events.

4.6 Summary

Towards the end of copulation in *C. maculatus*, females direct bouts of stereotyped and repetitive kicking behaviour towards males, which I have called 'mate-kicking'. In this chapter I have demonstrated an association between mate-kicking and both copulation duration and the extent of genital injury females sustain. Females unable to kick their mates had longer copulations and more genital injuries than females able to kick. In the absence of mate-kicking behaviour, males appeared to terminate copulation. These results suggest that copulation duration and/or the genital damage generates costs for females, and that mate-kicking may have arisen as a counter-adaptation to increase female control of these aspects of copulation.

Chapter 5: Mate-kicking, female post-copulatory behaviour and consequences for male fitness

5.1 Introduction

I present results in Chapter 4 that suggest that mate-kicking may have evolved in female *Callosobruchus maculatus* as a counter-adaptation to one or both of the following traits: copulation duration and the extent of genital damage. If these traits form the basis of a sexual conflict, one or both of them may have important consequences for male, as well as female fitness. This chapter is concerned with the potential for males to derive benefits in the absence of mate-kicking by females, in particular whether female post-copulatory behaviour is altered in ways that favour the last male to mate. In theory, males can derive substantial benefits from increased copulation duration and genital damage through the potential of these traits to influence female oviposition and remating behaviour. Because of the high last male sperm precedence in this insect (P_2 = 0.83 - Eady 1991a; Eady and Tubman 1996), any male able to induce increases in female oviposition rates or refractory periods would increase his fitness. If mate-kicking functions in females to regain control of copulation duration and genital injury, it may obscure the aspects of female behaviour that males are attempting to influence (i.e. oviposition rate and remating interval). Experimental prevention of kicking should therefore allow the effect of extended copulation and increased genital damage on

female post-copulatory behaviour to be examined. There are several ways in which imposing longer copulations and greater damage could enable males to induce favourable changes in the oviposition and remating behaviour of their mates.

5.1.1 Potential benefits to males of longer copulations

The effects that male seminal signals have on female post-copulatory behaviour are well documented (e.g. Chen 1984; Ringo 1996; Klowden 1999): these include stimulating oviposition and inhibiting remating. If the ejaculate of male *C. maculatus* contains compounds that function to manipulate female behaviour and physiology, and if the rate of transfer of these compounds to females covaries with copulation duration, extending copulation would result in the transfer of greater quantities. Providing that seminal compounds have a dose-dependant impact on female behaviour, extended copulations/damage may reward males with increased fertilisation success. Gains of this kind may be derived by male houseflies, *Musca domestica*. Males of this species transfer manipulatory seminal compounds during copulation (Leopold *et al.* 1971) and copulation duration is reported to be positively correlated with the duration of the resulting sexual refractory period in females (Reimann and Moen 1967). Moreover, prolonging copulation may enhance the efficacy of seminal signals in terms of their potential to induce sexual non-receptivity in females. This is because longer copulations could allow time for the inhibitory compounds to take effect before genital separation occurs and females become free to mate with rival males.

5.1.2 Potential benefits to males of imposing greater genital damage

Imposing genital damage may enable males to manipulate female post-copulatory behaviour in three non-exclusive ways. First, if females perceive genital injury as a threat to their survival, they may elevate investment in current reproduction as prospects for future reproduction decline (Williams 1966; Stearns 1992). This may be expressed as a rise in oviposition rate following injury, thereby making more eggs available for fertilisation by the last male to mate. Second, if genital punctures provide direct routes for pathogens to enter the female haemocoel, the resultant infection may generate similar alterations in female egg-laying patterns following copulation. The potential for pathogenesis to induce such changes has been demonstrated in female house-crickets, *Acheta domesticus* (Adamo 1999). Adamo (1999) inoculated females with the bacterium, *Serratia marcescens*, and found an immediate increase in egg output. Further investigation revealed that the adaptive increase in the oviposition rate of infected females was mediated by activation of the immune system in response to the invading bacteria, although the precise mechanism was not identified (Adamo 1999). If genital damage results in pathogenesis which stimulates the immune system of female *C. maculatus*, males could benefit from a similar mechanism operating to increase the number of eggs available following copulation. Finally, genital damage may function in males as a remote mate-guarding strategy if it discourages females from remating (Simmons and Siva-Jothy 1998). Theoretical models indicate that males can inhibit female remating by inflicting costly injury during copulation such as genital damage (see Johnstone and Keller 2000). Assuming successive doses of genital damage have a cumulative effect on fitness, females may delay remating in order to avoid receiving

additional injuries. Thus, female remating intervals will be extended and males will increase their share of paternity.

The potential advantages to males of extending copulation and imposing damage in order to influence female behaviour are not mutually exclusive and may even interact to increase male fitness. If long copulations allow males to transfer more seminal compounds and genital damage provides direct routes for their passage to the female haemocoel, the manipulatory effect on females could be accelerated. If mate-kicking by females is a response to such strategies in males, experimental removal of this adaptation may reveal the impact of extended copulations and increased genital damage on the female post-copulatory decisions discussed here.

5.2 Aims

The aim of this chapter is to examine the effect of experimentally increasing genital damage and copulation duration on the magnitude of two fitness traits expressed soon after copulation in female *C. maculatus*. Specifically I aim to quantify the effects of preventing mate-kicking on:

(1) the immediate oviposition rate, and

(2) the remating interval of females following an initial copulation.

5.3 Materials and methods

5.3.1 Male and female body size

Male and female body size was measured using the procedure described in section 2.3.3.

5.3.2 Mate-kicking and copulation duration

Females (n = 102) (aged 24-40 hrs) were allocated to one of three experimental groups: *Restrained* (treatment), *Post-copulatory restrained* (procedural control) and *Free* females (control). Females in the *Restrained* group were immobilised on ice for approximately 5 mins before being secured via their hind legs in a vice. The vice consisted of a pair of forceps fitted with a controlled opening and closing device. The forcep tips were bound with plastic tape to create a padded surface that would minimise compression injury to the female's legs during restraint. Whilst restrained, each female was exposed to two males (aged 24-40 hrs) and allowed to copulate. Once copulation was achieved with one of the males, the surplus male was removed. Females (n = 28) that failed to mate within 30 mins of being placed in the vice were excluded from the experiment (thus, despite being constrained females appeared able to prevent intromission, possibly by refusing to expose their genitalia).

Similarly, females in the *Post-copulatory restrained* and *Free* groups were each exposed to two males and allowed to copulate once. Again, the surplus male was removed. Following copulation, females in the *Post-copulatory restrained* (procedural control) group were immobilised on ice and restrained in the vice for approximately 5 mins.

Females in the *Free* group were not immobilised on ice or restrained. The duration of each copulation was recorded with the exception of three in which recording was prevented due to equipment failure.

The methodology used in this experiment to prevent mate-kicking behaviour involved restraining, rather than ablating, the hind limbs of females (see section 4.3.1). This is because the injuries resulting from the ablation procedure may confound any effect of genital damage on the traits examined here.

5.3.3 Mate-kicking and immediate oviposition rate

Following copulation females were isolated in plastic petri-dishes (90 mm diameter) containing 25 black-eyed beans as sites for oviposition. At 6 hrs intervals over the next 24 hrs females were provided with 25 fresh beans, and the eggs laid during each sampling period were allowed to develop and hatch as normal. This procedure enabled me to quantify the number of eggs laid by each female every 6 hrs for the first 24 hrs after mating.

5.3.4 Mate-kicking and remating interval

28 hrs following the initial copulation, each female was confined with a male in order to provide her with an opportunity to remate. Females that failed to remate within 30 mins were again isolated with 25 fresh beans. 44 hrs following their first mating these females were given a second opportunity to remate. All males were 24-36 hrs old.

5.3.5 Statistical analysis

Data on copulation duration were log-transformed to correct for non-normality and analysed using a parametric test. Data on oviposition rates and body size were also analysed using parametric tests. Finally, Chi-squared tests were used to analyse data on remating intervals.

5.4 Results

5.4.1 Male and female body size

There were no significant differences in the body size of females from the three treatment groups (ANOVA: $F_{2,70} = 0.371$, $p > 0.05$), or of males mated to females from the three treatment groups (ANOVA: $F_{2,64} = 0.195$, $p > 0.05$).

5.4.2 Mate-kicking and copulation duration

The ability of females to kick their mates during copulation had a significant effect on the duration of copulation (ANOVA: $F_{2,68} = 9.967$, $p = 0.0002$). Females that were unable to kick their mates had significantly longer copulations than those able to kick (mean copulation duration: *Restrained* females = 15.8±1.4 mins, $n = 24$; *Post-copulatory restrained* females = 10.6±0.5 mins, $n = 24$; and *Free* females = 10.5±0.8 mins, $n = 23$) (see Figure 5.4.1). Fisher's PLSD multiple comparison test revealed no significant difference in the copulation durations of females from the two

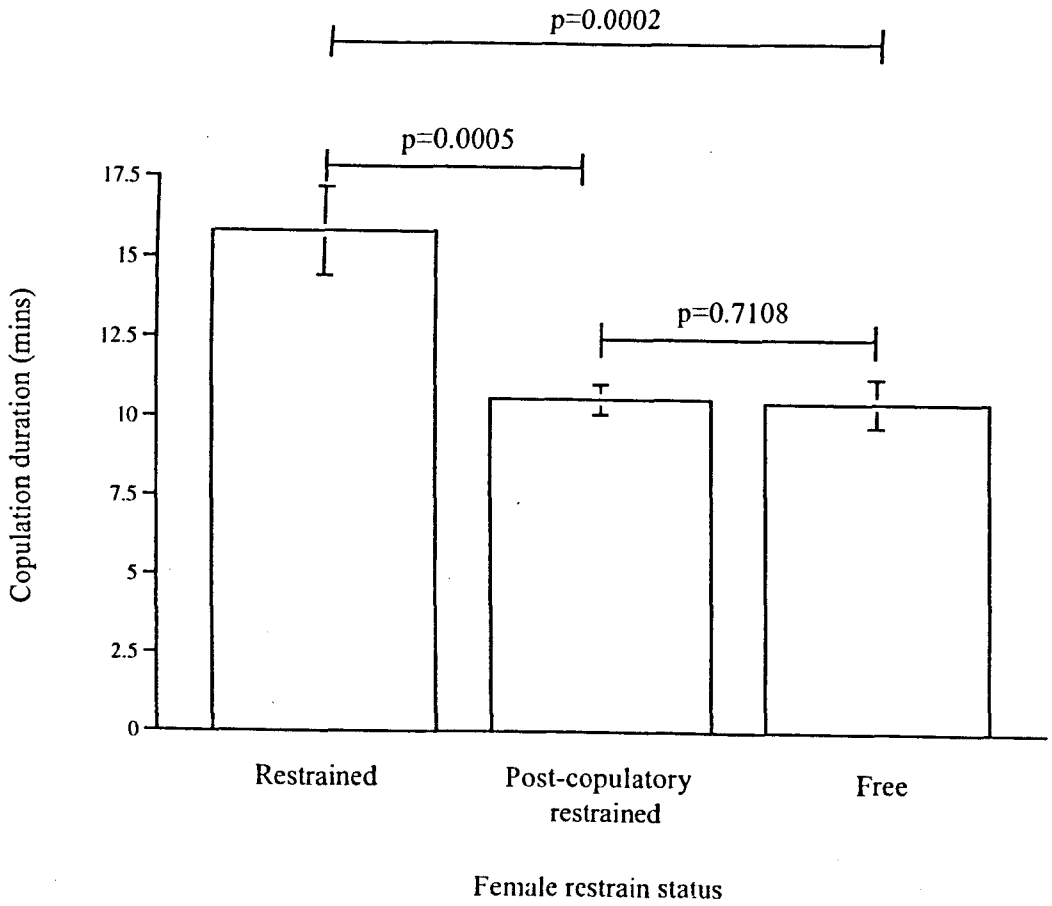


Figure 5.4.1 The effect of mate-kicking ability of the duration of copulation. Copulations involving females that were unable to kick their mates were longer than those involving females that were able to kick their mates.

control groups ($p = 0.71$), but the copulation durations of both control groups differed significantly from that of the treatment group ($p < 0.001$).

5.4.3 Mate-kicking and immediate oviposition rate

Mate-kicking ability had no significant effect on female oviposition rate during the four consecutive 6 hr sampling periods following an initial copulation (Repeated measure ANOVA: $F_{2,71} = 0.362$, $p > 0.05$) (see Figure 5.4.2, also see Figure 5.4.3 for cumulative number of eggs laid). There was also no significant effect of either mate-kicking ability (ANCOVA: $F_{2,69} = 0.349$, $p > 0.05$) or female body size ($F_{1,69} = 1.156$, $p > 0.05$) on total female fecundity for the 24 hrs following mating; mean total number of eggs: *Restrained* females = 9.52±2.22, $n = 25$; *Post-copulatory restrained* females = 7.84±1.72, $n = 25$; and *Free* females = 7.08±1.97, $n = 24$) (see Figure 5.4.4).

5.4.4 Mate-kicking and remating interval

A total of 55 females remated 28 hours following their initial copulation but there was no significant effect of treatment on the number of females that remated (Chi-sq test: $\chi^2 = 0.952$, $p > 0.05$) (see Table 5.4.1). A total of 4 females remated 44 hrs after their initial copulation but the data were of insufficient size to analyse statistically (number of females: 3 *Restrained* females, 1 *Post-copulatory restrained* female, and 0 *Free* females). A total of 15 females failed to remate by the end of the experiment (i.e 44 hours after the initial copulations) but there was no significant effect of treatment on the number of females that failed to remate (Chi-sq test: $\chi^2 = 0.615$, $p > 0.05$) (see Table. 5.4.2).

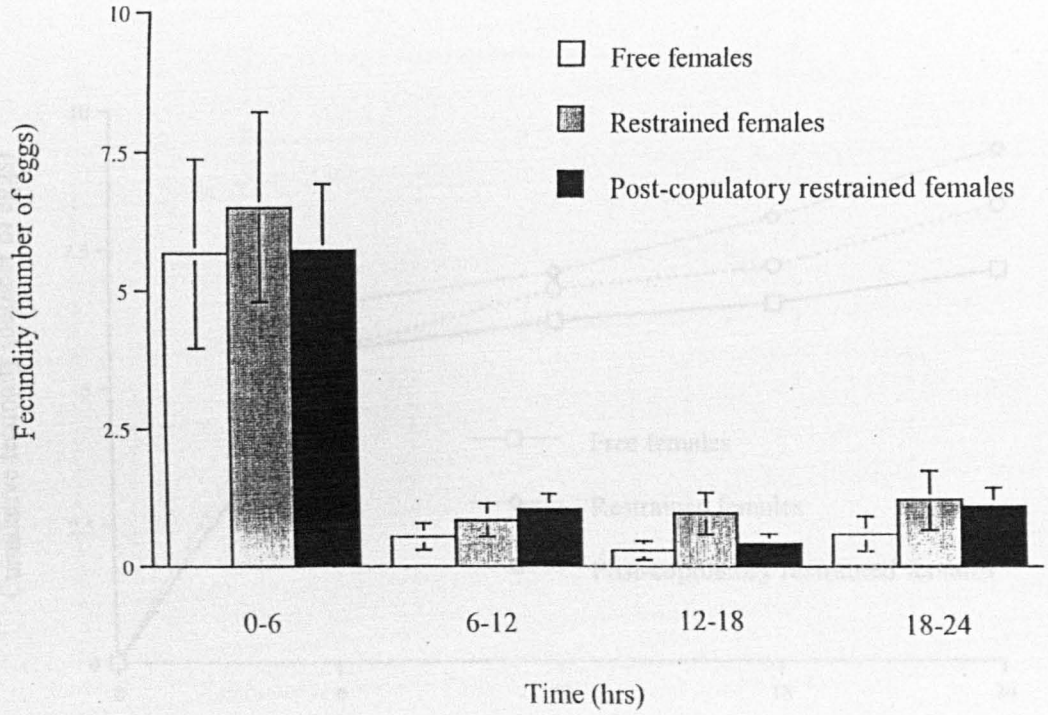


Figure 5.4.2 The effect of mate-kicking ability on female oviposition rate over four 6 hr sampling periods following an initial copulation. There was no significant differences between the treatment groups.

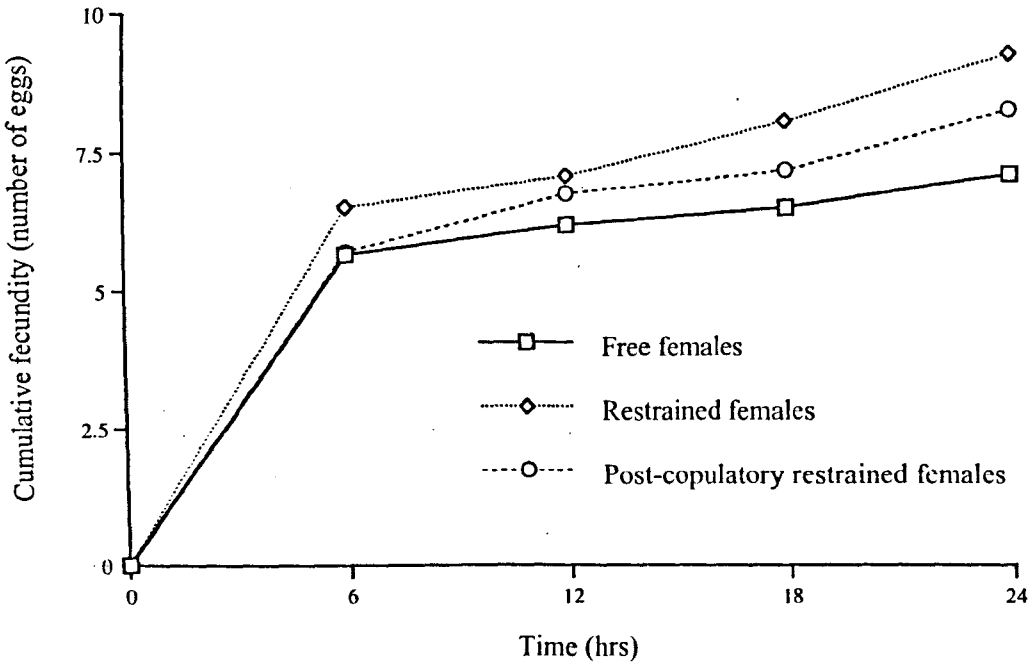


Figure 5.4.3 The effect of mate-kicking ability on cumulative fecundity over the 24 hrs following an initial copulation..

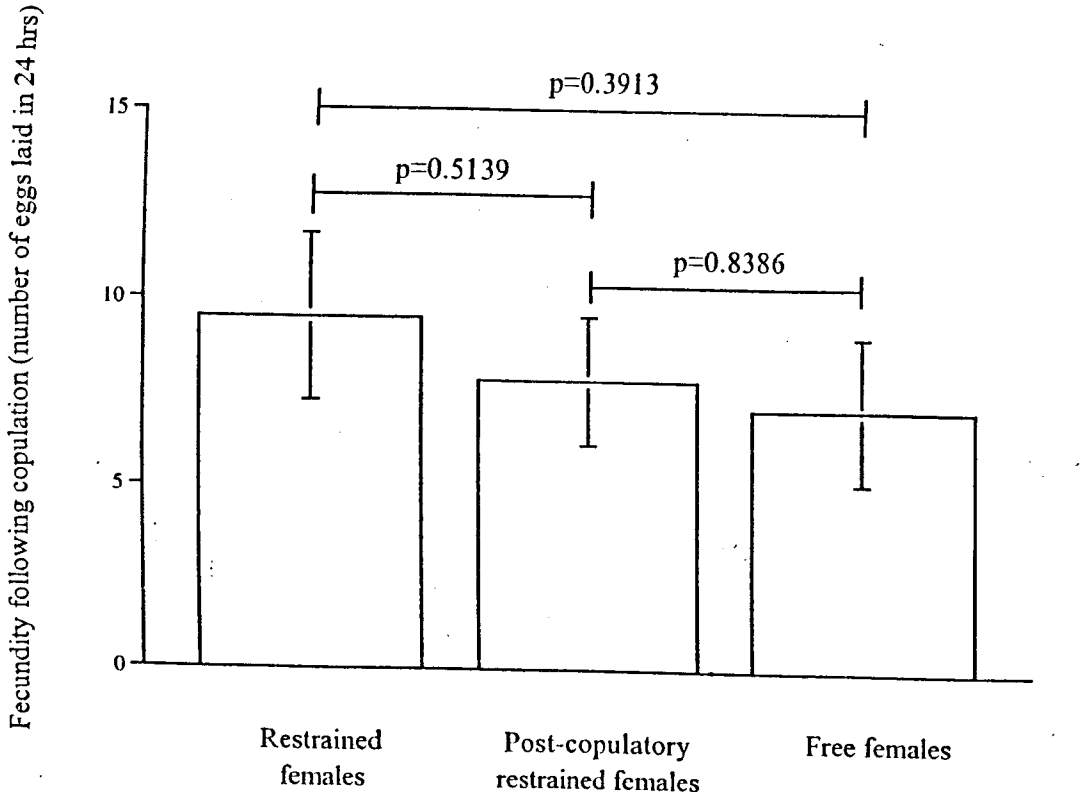


Figure 5.4.4 The effect of mate-kicking ability on total fecundity in the 24 hours following an initial copulation. There was no significant differences between the treatment groups.

Table 5.4.1 Analysis of female remating frequencies 28 hrs after the initial copulations. There was no significant departure from the null model (expected frequencies) according to female mate-kicking ability.

Mate-kicking status	**Remated females**	**Non-remated females**	χ^2	**p**
Restrained	17	8	0.952	NS
Post-copulatory restrained	20	5		
Free	18	6		

Table 5.4.2 Analysis of remating frequencies at the end of the experiment (i.e. 44 hrs after the initial copulations). There was no significant departure from the null model (expected frequencies) according to female mate-kicking ability.

Mate-kicking status	**Remated females**	**Non-remated females**	χ^2	**p**
Restrained	20	5	0.615	NS
Post-copulatory restrained	21	4		
Free	18	6		

5.5 Discussion

The results of this chapter demonstrate that when female *C. maculatus* were experimentally prevented from engaging in mate-kicking behaviour, copulations were extended relative to those involving females permitted to kick. This finding concurs with results reported in Chapter 4 (see section 4.4.1) which also showed that the prevention of mate-kicking was associated with significantly greater genital damage to females (see section 4.4.2). There are several theoretical reasons to predict an effect of extended copulation duration and increased genital damage on the female post-mating behaviours examined here (see e.g. von Helversen and von Helversen 1991; Johnstone and Keller 2000). Increases in female oviposition rate and remating interval would favour the last male to mate by providing more eggs for fertilisation and allowing sperm competition to be avoided prior the female's next copulation (Parker 1970a; Simmons and Siva-Jothy 1998). However, no effects of mate-kicking, or its consequences, on the immediate oviposition rate or the remating interval of females were found. There are several possible explanations for these results.

Firstly, although no relationship between mate-kicking ability and the oviposition rate or remating interval of females was found, the treatment (absence of mate-kicking) may have resulted in unmeasured changes in female reproductive traits. For instance, females may respond to a threat to survival due to increased genital damage by laying larger eggs.

Secondly, the procedure used to prevent mate-kicking involved chilling (see section 5.3.2) and a comparative study of the use of carbon dioxide, nitrogen and low temperature as anaesthetising agents in another bruchid, *Callosobruchus subinnotatus*, indicated an inhibitory effect of all three on reproductive behaviour Mbata *et al.* (1998). If such effects were operating here, females subjected to chilling (i.e. *Restrained* and *Post-copulatory restrained* females) would be predicted to exhibit lower oviposition rates and/or shorter remating intervals than non-chilled females (*Free* females). However, since no variation in female reproductive behaviour was detected across treatments, chilling is unlikely to provide an explanation for the findings presented in this chapter.

It seems reasonable to conclude therefore that under the conditions of the experiments presented here, longer copulations and/or greater genital damage do not induce measurable changes in female oviposition rates or remating intervals that would favour the last male to mate.

5.5.1 No adaptive change in female post-copulatory behaviour

Given the lack of response in females to the treatment imposed in this experiment, questions arise as to why females do not appear to make adaptive alterations in reproductive decisions following increased genital injury. There are at least two possible explanations. Firstly, the ability to respond to threats to survival may be constrained by the mechanism mediating the response. As reported earlier, Adamo (1999) demonstrated an adaptive increase in the oviposition rate of female house-crickets, *Acheta domestica*,

following inoculation with the bacterial pathogen, *Serratia marcescens*. Interestingly however, no such effect was detected when these animals were subjected to invasion by larvae of the fly, *Ormia ochracea*, even though this parasitoid invariably kills its host (Adamo 1999). Different components of the immune system are triggered by these two organisms: an anti-microbial response by the former, and an encapsulation response by the latter (Adamo 1999). Thus, it seems that in the event of a threat to survival, increased investment in current reproduction occurs in some instances but not others. It is therefore possible that genital damage may indeed represent a threat to female survival, and yet is not a mechanism that can produce adaptive adjustments in female reproductive decisions. However, such adjustments may be made if the risk of pathogenesis increases with the extent of genital damage sustained, although given Adamo's (1999) findings, this will probably depend on the nature of the invasion. Under the laboratory conditions of this experiment, rates of pathogenesis and parasitism are likely to be considerably lower than in natural populations. In this case, the potential for increased genital damage to induce adaptive changes in female post-mating decisions through an increased risk of pathogenesis may not be evident.

Another explanation for the apparent inability of females to adjust reproductive behaviour following genital injury is that the levels of damage imposed in this manipulation were not sufficient to mediate such changes. In other words, genital damage did not pose a sufficient threat to survival, and therefore to future reproduction, to warrant a major diversion of resources to current reproduction. This statement would appear to run contrary to the finding that females kick their mates during copulation to

reduce the extent of damage incurred (see Chapter 4, section 4.4.2). However, although injuries from a single copulation may not be severe enough to threaten future reproduction, cumulative injuries over several copulations may be. Thus, females may kick during each copulation in order to reduce the *total* injuries sustained during an entire lifetime, and therefore to lessen any long-term threat to fitness. Biologically realistic levels of damage, that is, those incurred over successive copulations, may be more likely to induce changes in female reproductive strategies. The life-history consequences for females of multiple copulations are investigated in the next chapter.

5.6 Summary

In this chapter I have demonstrated that two female post-copulatory behaviours with potentially important consequences for male fitness, immediate oviposition rate and remating interval, were not influenced by female mate-kicking ability. Given that experimental prevention of mate-kicking increased both copulation duration and the extent of genital damage sustained by females, these findings suggest that males may not induce favourable female post-copulatory responses by extending copulation and/or imposing greater amounts of genital damage on their mates.

Chapter 6: Female mating frequencies and fitness traits

6.1 Introduction

Females of the majority of insect species mate with more than one male during their lifetime (Ridley 1988; Arnqvist and Nilsson 2000), and assuming males cannot force copulations, female mating rates are predicted to reflect strategies aimed a maximising fitness. Theoretical and empirical studies propose that a range of fitness advantages may accrue to multiply mating females (Thornhill and Alcock 1983; Andersson 1994). Direct, material benefits include replenishing sperm supplies, avoiding infertile males, and acquiring nutrients, protection and access to male-defended resources (Boggs and Gilbert 1979; Waage 1979b; Gwynne 1984; Fincke 1986; Ridley 1988; Markow *et al.* 1990; Tsubaki *et al.* 1994; Olsson and Shine 1997; Vahed 1998). Several indirect, genetic benefits have also been invoked to explain polyandry, including increased viability, attractiveness, and genetic diversity of offspring (Loman *et al.* 1988; Madsen *et al.* 1992; Andersson 1994; Zeh and Zeh. 1996; 1997; Evans and Magurran 2000; for a non-adaptive explanation of multiple mating by females see Halliday and Arnold 1987).

Aspects of mating can also be disadvantageous for females. Examples of costs associated with mating include depleted energy reserves, decreased foraging efficiency

and increased of risk predation, pathogenesis and physical injury (Krupa and Sih 1993; Magurran and Seghers 1994; Rowe 1994; Arnqvist and Rowe 1995; Hurst *et al.* 1995; Arnqvist 1997 and see Chapter 3, section 3.1.1). Although often expressed physiologically, costs of mating are sometimes expressed in life-history terms through reduced survival, and most dramatically, through reduced reproductive output (Fowler and Partridge 1989; Chapman and Partridge 1996; Stutt and Siva-Jothy in press; Arnqvist and Nilsson 2000).

In addition to trading-off the costs and benefits of mating, a suite of internal and external factors should be considered when females assess the value of accepting a copulation (Emlen and Oring 1977; Arnqvist and Nilsson 2000). Important internal state variables could include nutritional status, parasite load, and previous mating history, in addition to the quantity of stored sperm and eggs available for fertilisation (Chapman and Partridge 1996; Ringo 1996; Mangan 1997). External variables with the potential to influence female mating decisions include the operational sex ratio, population density, micro-habitat structure and the availability of food and oviposition substrates (Newport and Gromko 1984; Harshman *et al.* 1988; Arnqvist and Nilsson 2000). Since this diverse range of factors are likely to vary, both within and among populations, female mating rates are unlikely to be fixed optima but will be contingent on prevailing local and individual circumstances (Arnqvist and Nilsson 2000).

6.1.1 Mating rates in female *C. maculatus*

This chapter is mainly concerned with the impact of mating frequency and exposure to males on fitness traits in female *C. maculatus*. Data on the natural mating rates of females of this species are not available and accounts of laboratory rates vary from daily remating (Rup 1986; Fox 1993), to once every two or three days (Shu *et al.* 1996). Some of the variation in laboratory mating rates may stem from the use of different animal strains. However, because reproductive decisions in female *C. maculatus* are strongly influenced by environmental parameters (Bellows 1982; Credland *et al.* 1986; Wilson and Hill 1989; Fox and Hickman 1994 and see below), variation may also arise from differences in culturing and experimental conditions.

Several investigations of the relationship between mating rate and female fitness traits have been conducted in this species (e.g. Credland and Wright 1989; Fox 1993; Savalli and Fox 1999; Wilson *et al.* 1999). For instance, Savalli and Fox (1999) found a reduction in female lifespan with multiple mating. This effect was assumed to be due to a corresponding increase in lifetime fecundity, although this hypothesis was not tested. In an earlier study using a different strain, Fox (1993) detected no effect of polyandry on longevity when females were fed, but lifespan was extended when females were deprived of food. Further to this, Fox (1993) found no difference in the lifetime fecundities of females mated once and those confined permanently with males. However, lifetime fecundity was elevated when females were mated on alternate days. A study by Wilson *et al.* (1999) also demonstrated a fecundity benefit in mulitply-mated females.

In general, when investigating the relationship between female mating frequencies and fitness traits, it is vital to distinguish between the potentially confounding effects of copulation and reproduction on female longevity, in other words between the costs of copulation and those of reproduction (Partridge and Harvey 1985; Fowler and Partridge 1989; Stearns 1992). This can be achieved by controlling egg production whilst varying mating opportunities. Furthermore, given the potential for environmental parameters to influence female mating decisions, the results generated will achieve greater realism if the experimental conditions mimic, as far as possible, conditions encountered naturally (Newport and Gromko 1984; Harshman *et al.* 1988).

6.1.2 The ecology of *C. maculatus*

The ecology of *C. maculatus* is well known and conditions prevailing in its natural environment are relatively easy to replicate in the laboratory. This species typically inhabits human grain stores where suitable food sources for larvae are abundant but those for adults are rarely available (Larson and Fisher 1938), and where females may encounter temporary shortages of oviposition sites (Wilson and Hill 1989). These are factors which are likely to influence the relationship between female mating rates and fitness traits. For instance, a scarcity of suitable oviposition sites constrains egg-laying behaviour (Credland *et al.* 1986).

The natural environment of female *C. maculatus* also includes a gregarious lifestyle. Although this ensures the availability of mates, it may also generate costs due to competition with other females for access to suitable oviposition sites (Bellows 1982).

Other potential costs of gregarious living may arise from non-mating exposure to males such as sexual harassment (see Odentaal *et al.* 1989; Magurran and Seghers 1994; Stone 1995; Clutton-Brock and Langley 1997; McLain and Pratt 1999 for examples in butterflies, guppies, solitary bees, tsetse flies, and bugs respectively). Indeed, female *C. maculatus* frequently resist persistent male courtship (pers. obs.), which at the very least may place energetic demands on females (e.g. Arnqvist 1997).

Clearly, components of gregarious living can interact or conflict with female reproductive strategies. Consequently, incorporating gregarious living conditions will introduce important elements of realism into studies of female mating rates and fitness traits. However, precisely because of the potential to influence female reproductive decisions and fitness, gregarious conditions, as well as other experimental conditions, must be standardised across different mating regimes (e.g. Partridge and Harvey 1985; Fowler and Partridge 1989; Chapman and Partridge 1996). By ensuring gregarious conditions are kept constant, the effect of varying mating frequencies may be identified.

6.2 Aims

The aims of this chapter are to examine the relationship between fitness traits in female *C. maculatus* and:

1) varying opportunities to mate, and

2) sexual harassment by males.

For the experiments reported in this chapter I use the term "harassment" to mean "non-mating exposure to males". Three experiments are reported, the first of which aims to quantify the effect of mating rate on female longevity. In order to distinguish the effects of copulation from those of egg production, oviposition rates are held constant across different treatment groups. The aim of the second experiment is to quantify the effect of varying exposure to males on female reproductive output. The inclusion of gregarious living conditions in the design of this experiment increases realism. Finally, the aim of the third experiment is to test the assumption that gregarious living conditions generate costs of harassment for females. To this end, the effect of sexual harassment on female fitness traits is quantified.

6.3 Materials and methods

6.3.1 Female body size

Female body size was measured using the procedure described in section 2.3.3.

6.3.2 Female mating rate and longevity

Female mating rates

Females (n = 123) (aged 16-24 hrs) were assigned to one of three treatment groups. Females in the *Single-mated* group were mated once, females in the *Double-mated* group

were mated twice (with an interval of 2 days) and females in the *Triple-mated* group were mated three times (with intervals of 2 days). Males were aged 16-40 hrs.

Oviposition conditions

Females were housed individually in plastic petri-dishes (90mm diameter) containing 25 black-eyed beans as sites for oviposition. Oviposition rates were held constant across treatment groups by limiting the availability of oviposition sites since a scarcity of this resource constrains oviposition behaviour in female *C. maculatus* (Credland *et al.* 1986). Thus, the initial supply of oviposition sites was not replenished. Attempts to constrain egg-laying through denying access to oviposition sites were unsuccessful (see Appendix 1).

Female longevity and lifetime fecundity

Females were examined at intervals of 24 hrs and any found dead were recorded as having died in the previous 24 hr sampling period. Female longevities were defined as the period between adult emergence and the observed time of death, and used to derive a rate of death and for each treatment group. Following death, the lifetime fecundities of females were determined by counting the number of eggs laid on the beans provided. Two females that failed to lay any eggs were eliminated from the analysis (both were *Single-mated* females).

6.3.3 Exposure to males and female fitness traits

Female mating opportunities

Females (n = 172) (aged 24-30 hrs) were assigned to one of three treatment groups. Females in the *Single-mated* group were mated once, those in the *Double-mated* groups were mated twice (with an interval of 3 days), and those in the *Continuously-exposed* group were confined permanently with intact males at a sex ratio of 1:1. Males were aged 24-30 hrs. Five females allocated to the *Double-mated* group failed to remate within 30 mins of being placed with males. Consequently, the replicates (see below) to which they belonged were excluded from the experiment.

Gregarious living conditions

Females were housed in mixed-sex replicates consisting of four females and four males. In order to control for the sexual harassment experienced by the *Continuously-exposed* females, emasculated males (see below) were included in the *Single-* and *Double-mated* replicates at a sex ratio of 1:1 Emasculated males sexually harass but cannot copulate with females (virgin females placed with emasculated males do not produce offspring - pers. obs.), and the rate at which they harass did not differ significantly from that of intact males (see Appendix 2). Thus, gregarious conditions were standardised but females received varying opportunities to mate according to the treatment group to which they belonged.

Oviposition conditions

Every day each replicate (n = 38) was provided with a fresh supply of 25 black-eyed beans. Replenishing the supply of oviposition sites ensured that female oviposition behaviour was not constrained by a shortage of this resource. Eggs laid daily by each replicate were allowed to hatch, develop and pupate as normal.

Female longevity and offspring production

Female longevities were derived as in section 6.3.2. Daily offspring production was defined as the number of adults emerging from the eggs laid daily by each replicate. Values for daily offspring production were summed to give the lifetime offspring production of each replicate. Due to the communal housing conditions, each datum is the mean value for a replicate of four females.

Emasculated males

Emasculated males were obtained in the following way: after immobilisation on ice, males were restrained in a small polystyrene vice. The posterior region of each male was left protruding from the block and, by applying gentle pressure on either side of the block, the aedeagus could be everted. The aedeagus was then severed as close to the point of attachment to the male's body as possible. Once normal body temperature was restored, the emasculated males resumed locomotory behaviour. Because emasculated males died sooner than intact ones (pers. obs), all males, including intact ones, were replaced every two days with younger males (aged 24-36 hrs).

6.3.4 Sexual harassment and female fitness traits

Harassment regimes

Females (n = 39) (aged 16-24 hrs) were allocated to the *Single-mated and harassed* group, mated once and confined permanently with emasculated males (see section 6.3.3 and Appendix 2) at a ratio of 2 males: 1 female. This experimental design ensured that females were exposed to sexual harassment from males without receiving further mating opportunities. Thus, the effects of harassment were uncoupled from those of copulation. I elected to use a biased sex ratio in order to increase the probability of detecting an effect of sexual harassment on the traits measured. Emasculated males were replaced every two days with younger males (aged 24-36 hrs) because of their reduced survival probability.

Female death rate, longevity and lifetime fecundity

Females were provided with identical containers and oviposition conditions to those described in section 6.3.2. The death rates, longevities and lifetime fecundities of females were derived as in section 6.3.2 and compared to those of the *Single-mated* females from the experiment described in section 6.3.2. In this case, *Single-mated* females constituted a control group.

6.3.5 Statistical analysis

Data on female death rates were used to generate survival curves and analysed with Mantel Cox log rank tests. Data on female body size and fitness traits were analysed using parametric tests.

6.4 Results

6.4.1 Female body size

There were no significant differences in female body size according to treatment group in any of the three experiments: section 3.3.2 experiment (ANOVA: $F_{2,120} = 0.043$, $p > 0.05$); section 3.3.3 experiment (ANOVA: $F_{2,35} = 1.767$, $p > 0.05$, and section 3.3.4 experiment (t-test: $t_{88} = 0.007$, $p > 0.05$).

6.4.2 Female mating rate and longevity

Mating rate had a significant effect on both the death rate (Mantel Cox log rank test: $\chi^2_2 = 6.398$, $p = 0.0408$) (see Figure 6.4.1), and longevity of females (ANCOVA: $F_{2,119} = 3.783$, $p = 0.0255$) but there was no significant effect of female body size ($F_{1,119} = 1.796$, $p > 0.05$). There was a significant reduction in longevity after the second copulation and a marginally non-significant increase after the third (mean longevity: *Single-mated* females = 9.92±0.22 days, $n = 51$; *Double-mated* females = 9.03±0.26 days $n = 40$; and *Triple-mated* females = 9.78±0.30 days, $n = 32$) (see Figure 6.4.2).

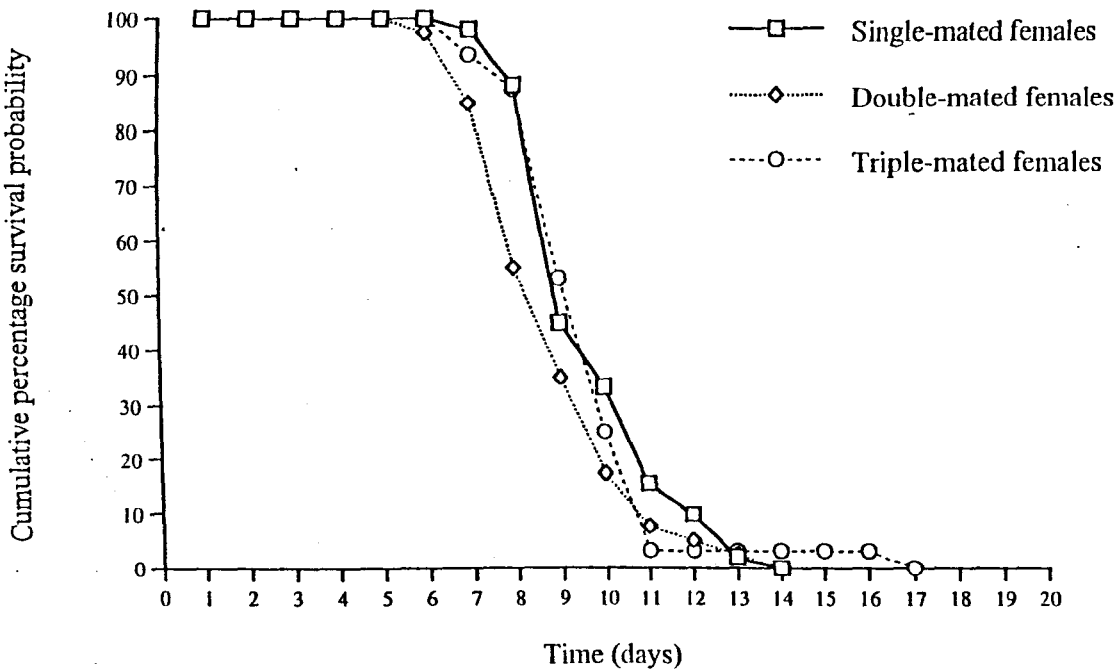


Figure 6.4.1 Survival curves for females mated once, twice and three times. The cumulative survival probability is the percentage of females left alive at the end of a given sampling period (24 hr). Lifetime fecundities were standardised across different mating frequencies by constraining female oviposition behaviour.

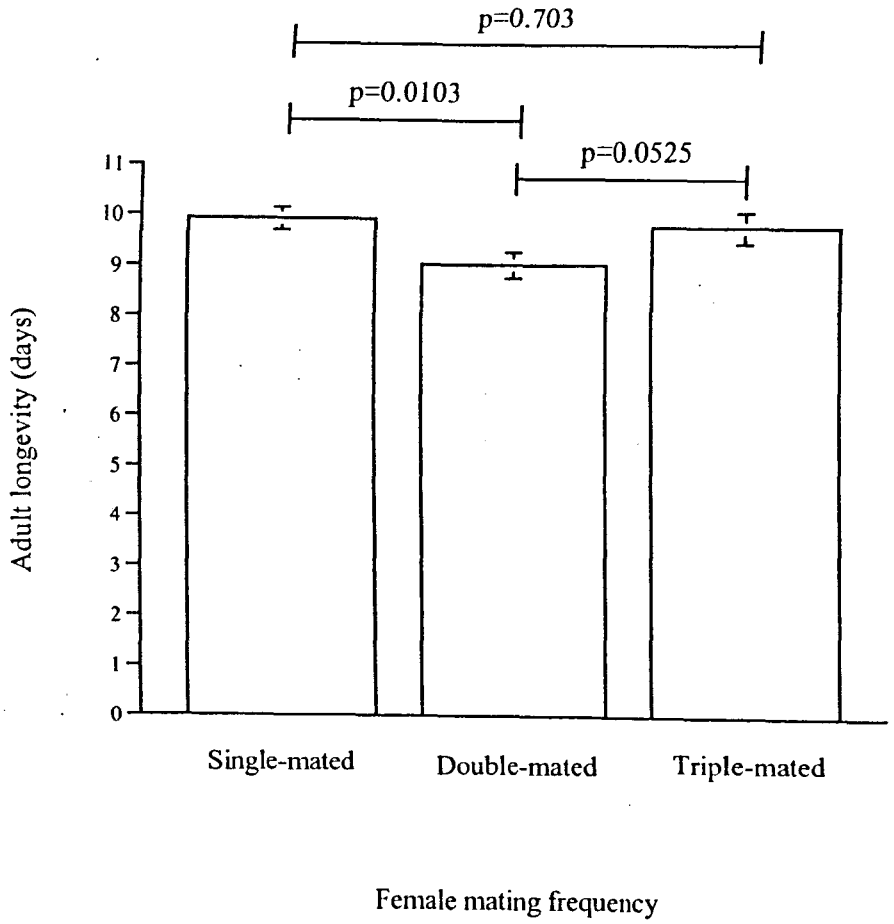


Figure 6.4.2 Mean longevities of females mated once, twice or three times. Lifetime fecundities were partially standardised across different mating frequencies by constraining female oviposition behaviour.

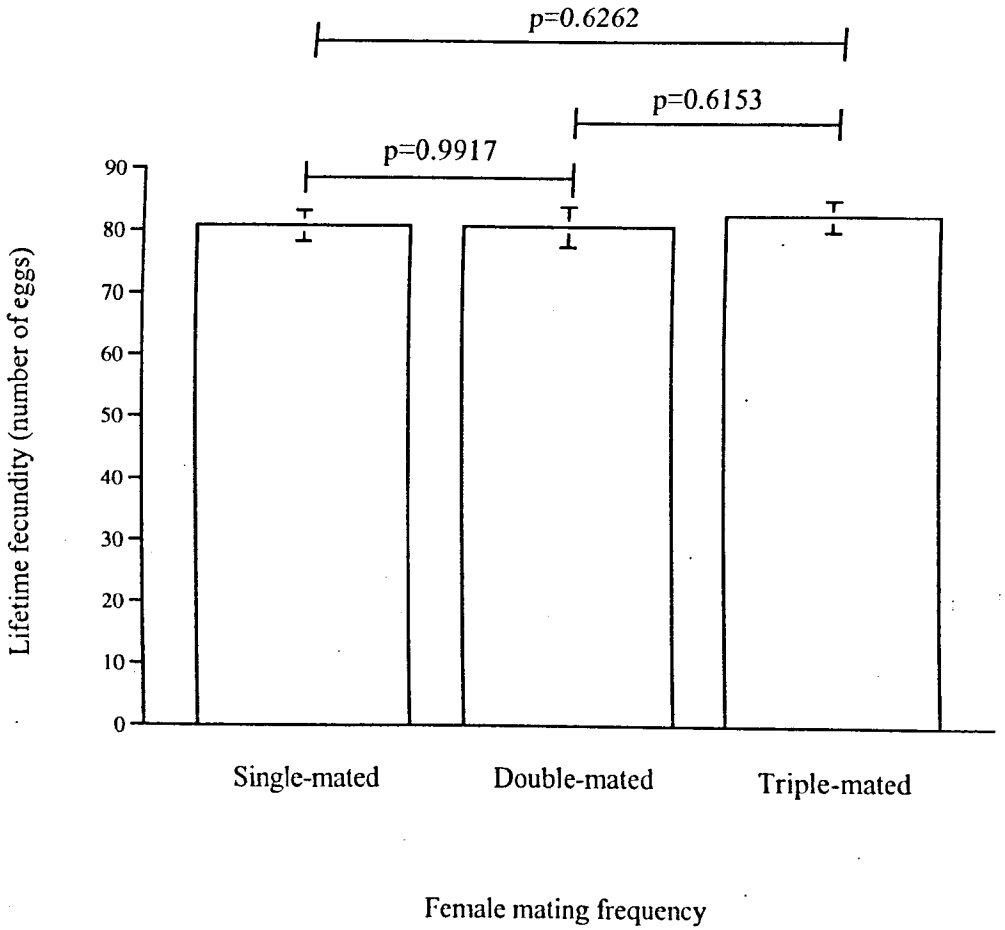
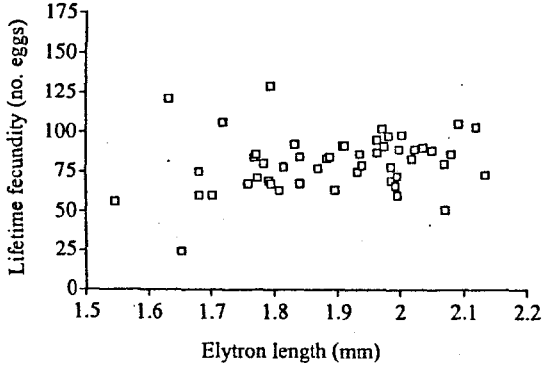
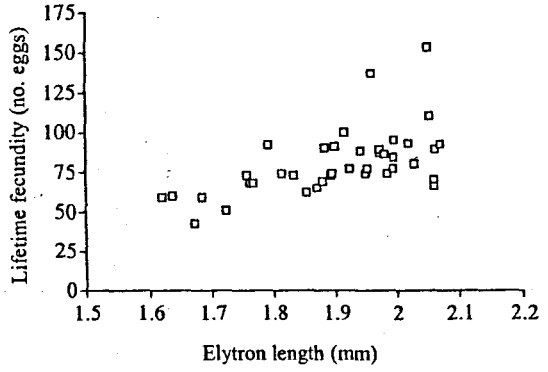


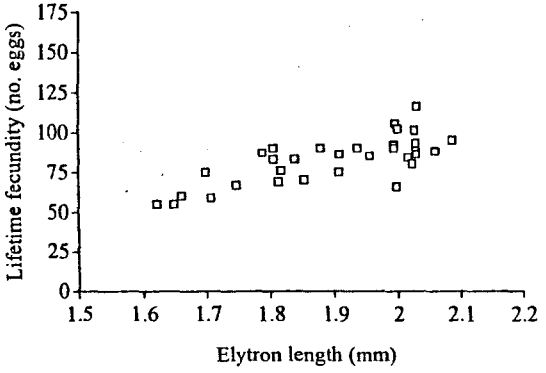
Figure 6.4.3 Mean lifetime fecundities of females mated once, twice or three times. Lifetime fecundities were partially standardised across different mating frequencies by constraining female oviposition behaviour.



Single-mated females



Double-mated females



Triple-mated females

Figure 6.4.4 Relationship between the body size and lifetime fecundity of females mated once, twice or three times.

There was a significant effect of both treatment (ANCOVA: $F_{2,117} = 3.781$, $p = 0.0256$), and female body size ($F_{1,117} = 38.557$, $p < 0.0001$) on female lifetime fecundity and an interaction between treatment and female body size ($F_{2,117} = 3.814$, $p = 0.0248$) (see Figure 6.4.4); mean number of eggs: *Single-mated* females = 80.78±2.49, $n = 51$; *Double-mated* females = 80.75±3.22, $n = 40$; and *Triple-mated* females = 82.56±2.59, $n = 32$) (see Figure 6.4.3).

6.4.3 Exposure to males and female fitness traits

The opportunity to mate had a significant effect on female longevity (ANCOVA: $F_{2,34} = 4.914$, $p = 0.0133$) but there was no significant effect of female body size ($F_{1,34} = 0.002$, $p > 0.05$). Females with more mating opportunities had shorter longevities than those with fewer mating opportunities (mean longevity: *Single-mated* females = 7.32±0.22 days, $n = 14$; *Double-mated* females = 6.95±0.25 days, $n = 9$; and *Continuously-exposed* females = 6.48±0.11 days, $n = 15$) (see Figure 6.4.5).

The opportunity to mate had a significant effect on female lifetime reproductive output (ANOVA: $F_{2,34} = 9.374$, $p = 0.0006$) but there was no significant effect of female body size ($F_{1,34} = 1.025$, $p > 0.05$). Females with increased opportunities to mate produced more offspring (mean number of offspring: *Single-mated* females = 37.429±3.385, $n = 14$; *Double-mated* females = 46.194±3.814, $n = 9$; and *Continuously-exposed* females = 55.733±2.799, $n = 15$) (see Figure 6.4.8) (for mean daily offspring production and cumulative mean daily offspring production see Figures 6.4.6 and 6.4.7 respectively).

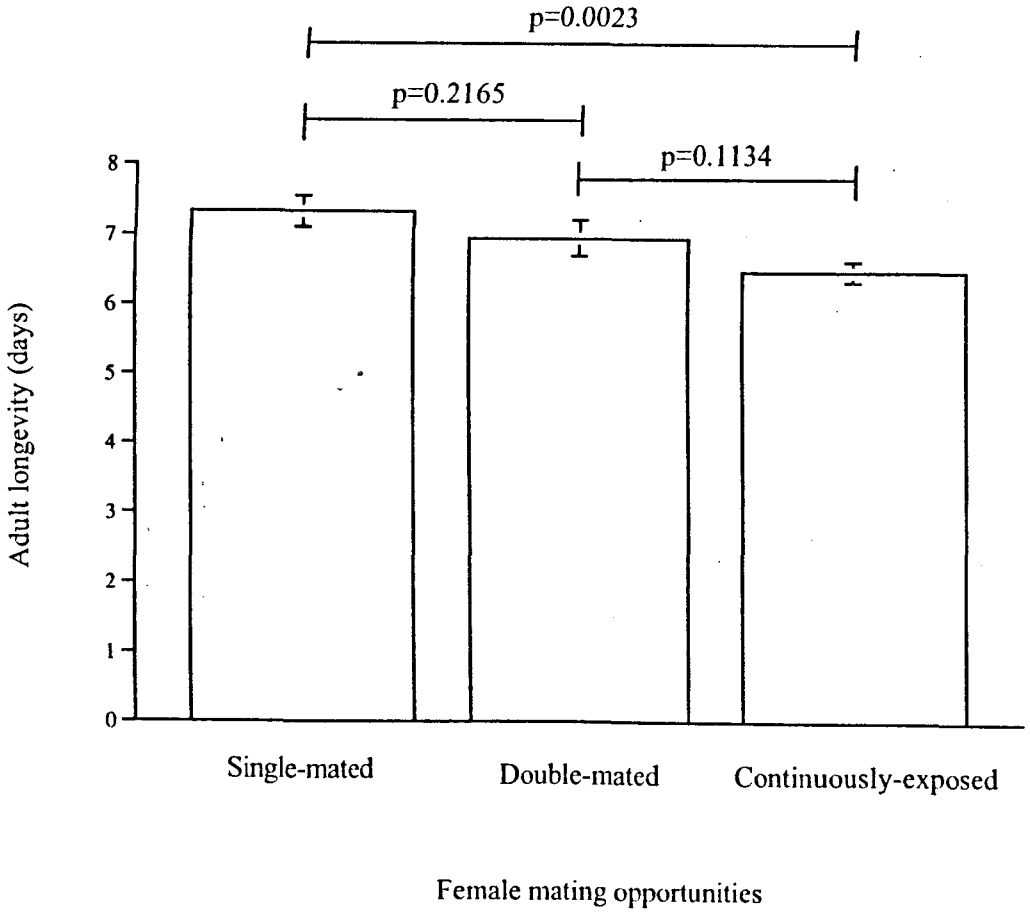


Figure 6.4.5 Mean longevities of females mated once, twice or exposed continuously to intact males. Gregarious conditions were standardised across treatment groups and each datum is the mean of four females belonging to a single replicate.

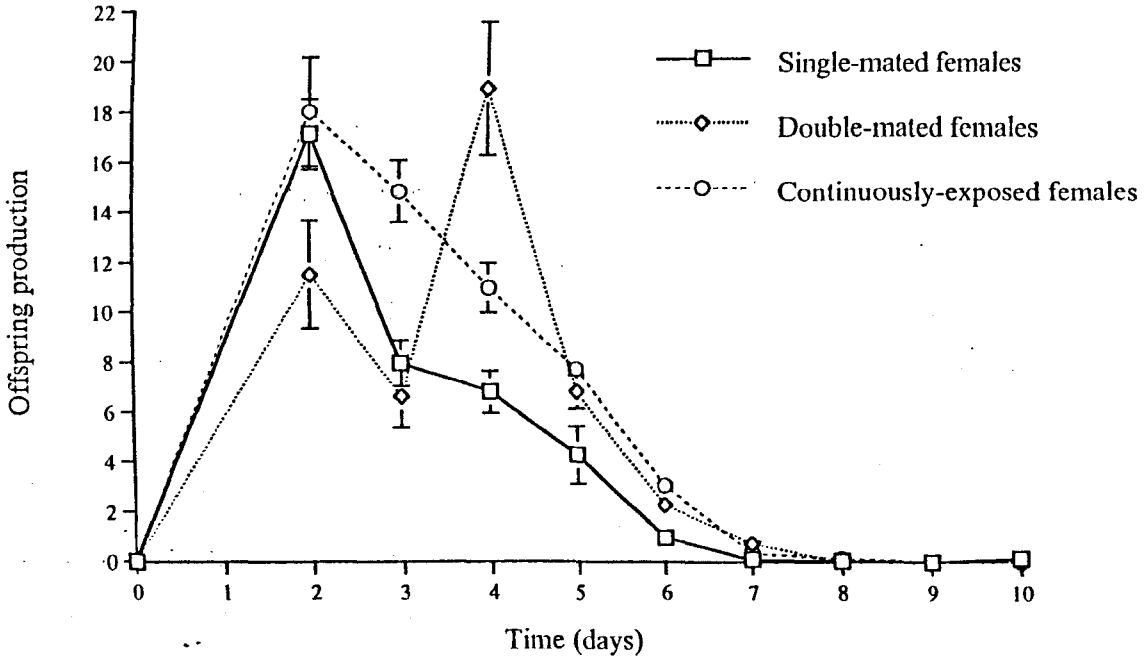
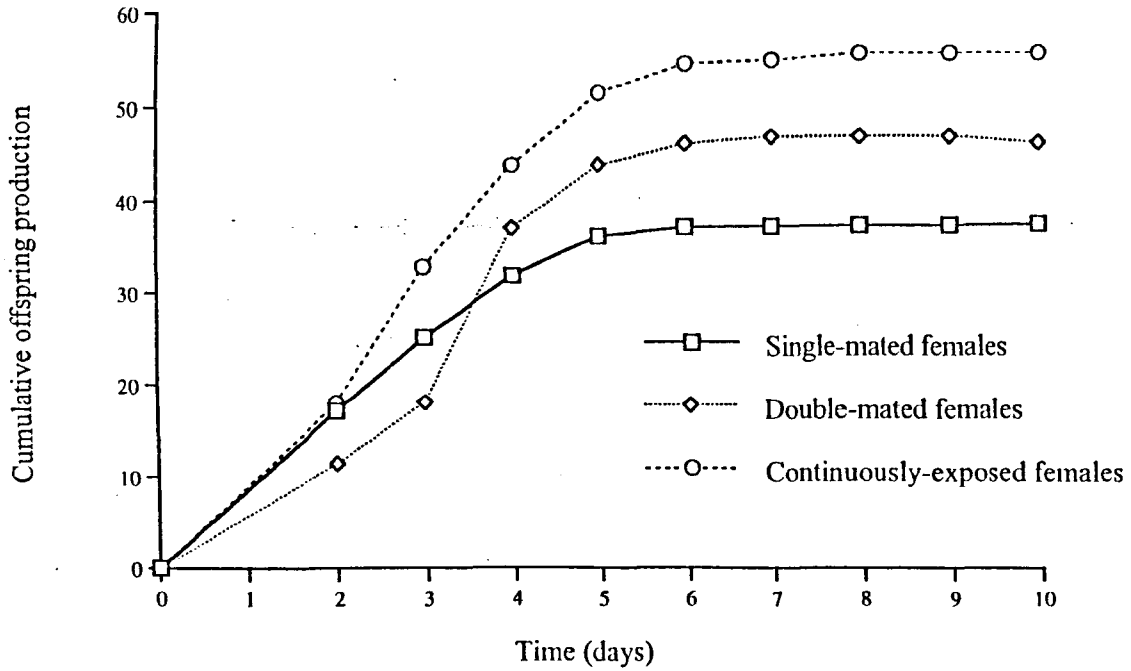


Figure 6.4.6 Mean daily offspring production by females mated once, twice or exposed continuously to intact males. Gregarious conditions were standardised across treatment groups and each datum is the mean of four females belonging to a single replicate.



6.4.7 Cumulative mean daily offspring production by females mated once, twice or exposed continuously to intact males. Gregarious conditions were standardised across treatment groups and each datum is the mean of four females belonging to a single replicate.

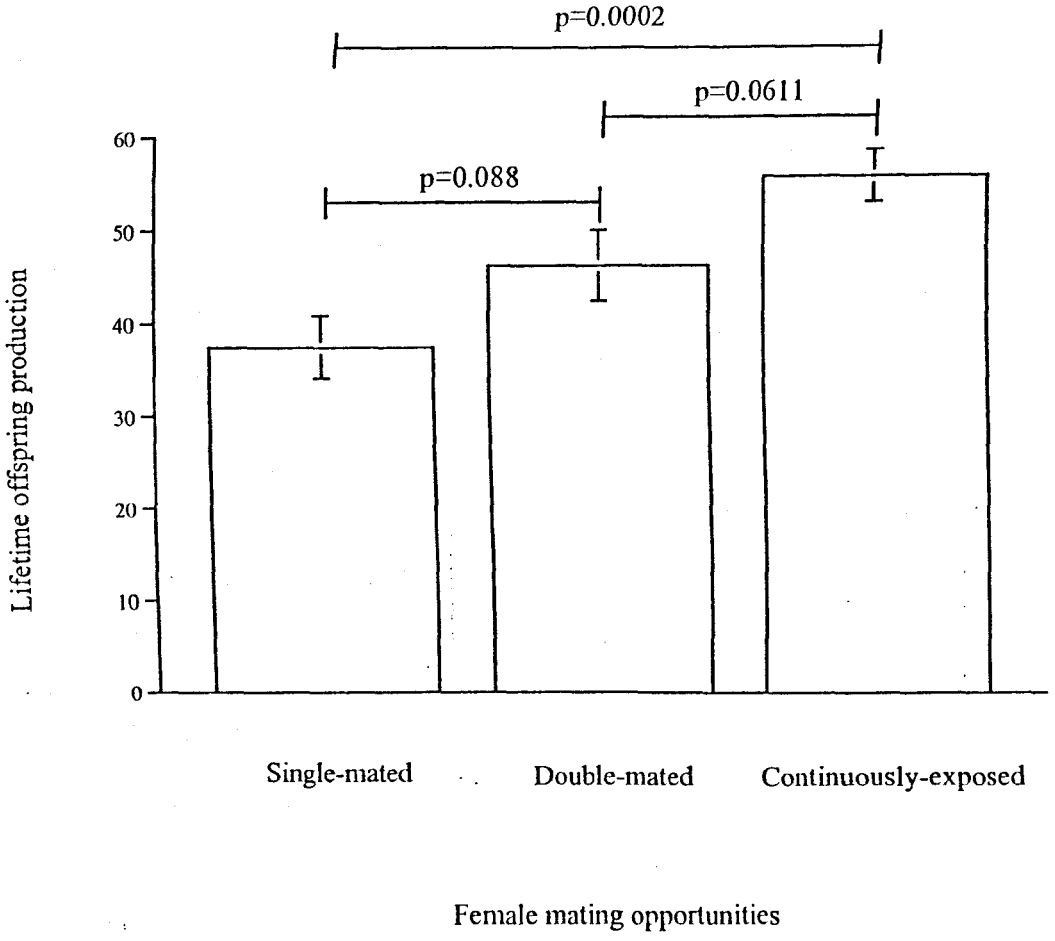


Figure 6.4.8 Lifetime offspring production of females mated once, twice or exposed continuously to intact males. Gregarious conditions were standardised across treatment groups and each datum is the mean of four females belonging to a single replicate.

6.4.4 Sexual harassment and female fitness traits

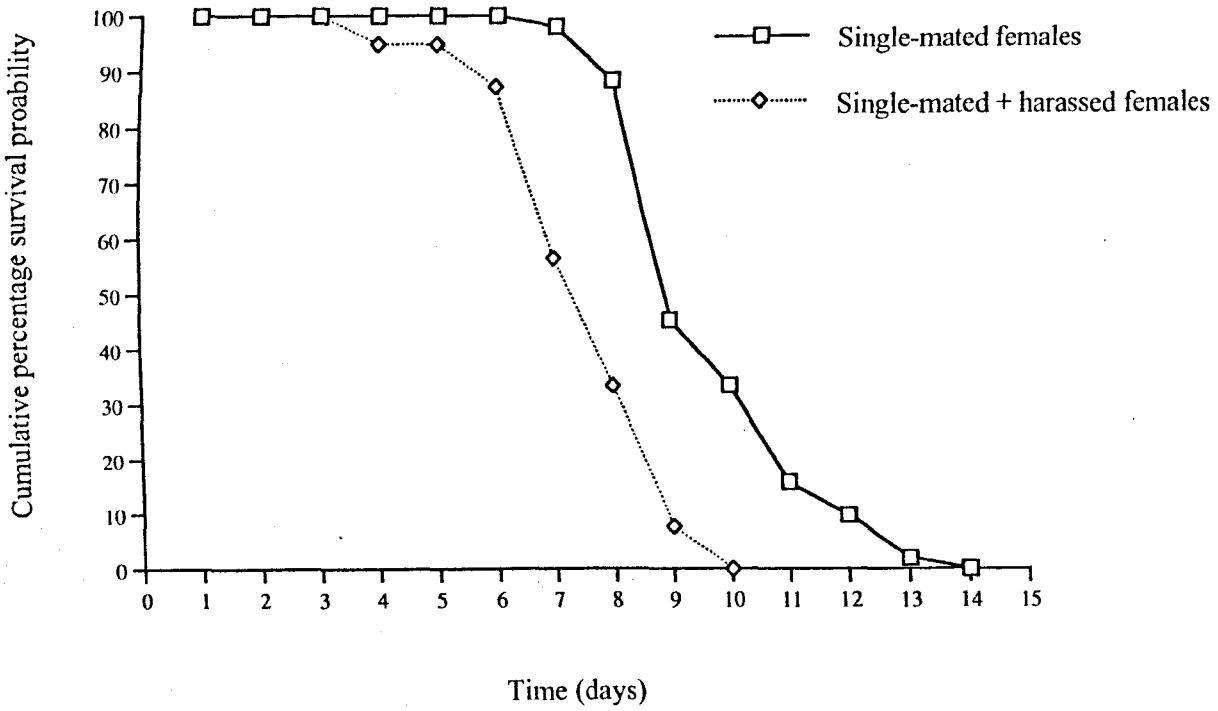
Confinement with emasculated males had a significant effect on both the death rate (Mantel Cox log rank test: $\chi^2_1 = 35.752, p < 0.0001$) (see Figure 6.4.9) and the longevity of females (ANCOVA: $F_{1,87} = 37.549, p < 0.0001$) but there was no significant effect of female body size ($F_{1,87} = 2.207, p > 0.05$). Females confined with emasculated males had shorter longevities than females housed alone; mean longevity: *Single-mated* females = 9.92±0.22 days, $n = 51$; and *Single-mated and harassed* females = 8.03±0.21 days, $n = 39$) (see Figure 6.4.10).

Despite attempts to standardise oviposition rates across treatment groups, there was a significant effect of both the presence of emasculated males (ANCOVA: $F_{1,87} = 11.749$, $p = 0.0009$) and female body size ($F_{1,87} = 18.037, p < 0.0001$) on female lifetime fecundity; number of eggs: *Single-mated* females = 80.78±2.49, $n = 51$; and *Single-mated and harassed* females = 68.59±3.02, $n = 39$) (see Figure 6.4.11).

6.5 Discussion

6.5.1 Remating reduces female lifespan

The first investigation of this chapter demonstrated a significant effect of mating frequency on female death rate (see Figure 6.4.1). Under experimental conditions that were designed to standardise oviposition rates across different mating frequencies



6.4.9 Survival curves for once-mated females housed individually or with two emasculated males. The cumulative survival probability is the percentage of females left alive at the end of a given sampling period (24 hr). Oviposition behaviour was constrained in an attempt to standardise egg production costs across treatment groups.

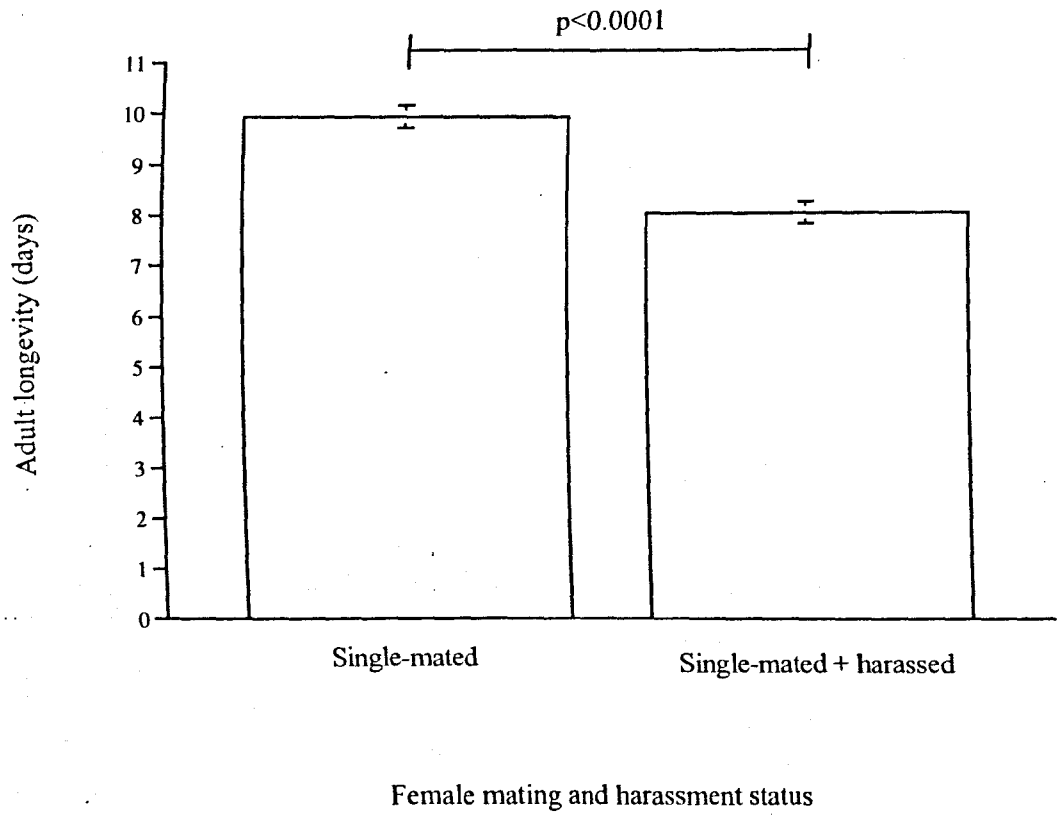


Figure 6.4.10 Mean longevities of once-mated females housed individually or with two emasculated males. Oviposition behaviour was constrained in an attempt to standardise egg production costs across treatment groups.

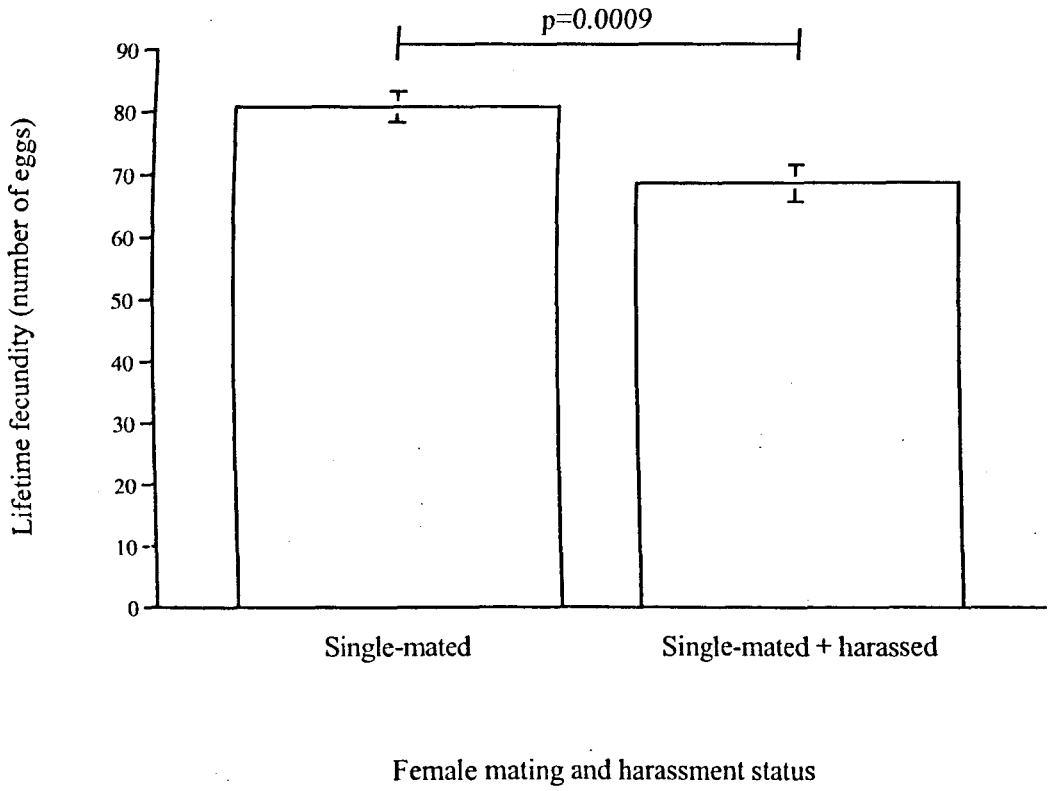


Figure 6.4.11 Mean lifetime fecundities of once-mated females housed individually or with two emasculated males. Oviposition behaviour was constrained in an attempt to standardise costs of egg production across treatment groups.

(i.e limited oviposition site availability), remated females had shorter lifespans than once-mated females (see Figure 6.4.2). However, there was a significant effect of mating frequency on reproductive output with single-mated females exhibiting higher fecundities than both double- and triple-mated females when body size was taken into account. Despite this, the experimental conditions were successful in constraining reproductive output to a considerable degree, thereby partially standardising reproductive output across mating frequencies. Thus, the effect of mating frequency on longevity is unlikely to be entirely attributable to any energetic or physiological costs associated with reproductive output (see section 6.3.2 and Figure 6.4.3). The reduction in lifespan is therefore likely to be at least partially attributable to the act of copulation itself. Copulation *per se* has similar consequences for females of some other invertebrate taxa (Fowler and Partridge 1989; Gems and Riddle 1996; Chapman *et al.* 1998; Stutt and Siva-Jothy in press). In *Drosophila melanogaster* (Chapman *et al.* 1995) lower survival of remated females is due to the transfer of toxic male seminal compounds during copulation. The underlying mechanism in *C. maculatus* is unknown but candidates include costs related to energy depletion, sperm storage, the contraction of sexually transmitted diseases, as well as male seminal compounds. Clearly, another possible mechanism is the genital damage caused by the male genitalia (see Chapter 3). In this case, the proximate costs of genital injury, such as dehydration and/or immune and repair system activation, may generate a phenotypic trade-off with lifespan (see Stearns 1992). These potential mechanisms are not mutually exclusive and two or more of them may act in concert to undermine female survival. If the cost underlying the reduction in lifespan of remated females is of sufficient magnitude to threaten reproductive output, it

could form the basis of a conflict between the sexes over mating rates. Such a conflict probably exists in the bed-bug, *Cimex lectularius*, where females mating at natural frequencies die earlier, and as a consequence produce fewer offspring, than females mating at experimentally reduced frequencies (Stutt and Siva-Jothy in press).

Interestingly, copulation frequency did not have a dose-dependant linear effect on female longevity: triple-mated females lived approximately as long as single-mated females (see Figure 6.4.2). Two explanations could account for the non-monotonic effect of copulation frequency on female lifespan. First, the costly component(s) of copulation is not dose-dependant in its effect, and second, that it is dose-dependant but the resultant costs are offset by linked beneficial components of copulation (e.g. the receipt of male seminal nutrients). The latter could arise because, in general, relationships between mating costs and copulation frequency are predicted to be linear (because costs are additive), whereas those between mating benefits and copulation frequency are predicted to be asymptotic (because benefits deliver diminishing returns) (Arnqvist and Nilsson 2000). Thus, interactions of the costs and benefits of copulation could produce complex patterns in life-history traits over different mating frequencies, even though the costs increase linearly.

The effect of mating frequency on female longevity demonstrated here does not concur with the finding by Fox (1993) that lifespan was extended by multiple mating. The use of different mating regimes may explain these divergent results (Fox mated females every two days until death, I imposed a maximum of three copulations). Of greater

likely importance is the use of different strains of *C. maculatus* (Californian by Fox, see Savalli and Fox 1999; and Brazilian in this study). The importance of geographical origin is further illustrated by reference to a third strain from South India in which polyandry has been shown to have no impact on either longevity or reproductive output (T. Taylor; pers. comm.).

6.5.2 Continuous exposure to males increases female reproductive output

The second investigation in this chapter demonstrated that females provided with greater mating opportunities showed elevated lifetime reproductive output (see Figure 6.4.8). Thus, despite the negative consequences for the lifespan of remated females, remating and continuous exposure to males increased female fitness. Indeed, on average, females appear to exhaust their reproductive capacity prior to the point at which reduced longevity due to multiple mating takes effect (see Figure 6.4.7 - the reproductive output of *Continuously-exposed* females plateaus before mean longevity is reached).

These findings concur with those of Wilson *et al.* (1999) where females mated three times exhibited higher fecundities than females mated once or twice. However, they do not confirm those of Fox (1993) who failed to detect a fecundity benefit to females confined permanently with males. Again, the explanation for these contradictory findings could lay in the use of different strains of animals or experimental design. In particular, Fox (1993) neglected to control for the effects of permanent exposure to males. Such an omission would impose sexual harassment on females confined

permanently with males, the costs of which may be sufficiently large to offset any beneficial effect of multiple mating on reproductive output.

Within the methodological framework adopted here, the direct benefits of polyandry to female *C. maculatus* are clear. The mechanism underlying this benefit is unknown but may be due to the transfer of nutrients (Fox 1993) or oviposition stimulants (Wilson *et al.* 1999) in the male ejaculate. In another bruchid, *Acanthoscelides objectus*, male seminal compounds have been shown to play a stimulatory role in egg production (Das *et al.* 1980). Nutritive and stimulatory mechanisms could act in a non-exclusive manner to increase reproductive output in female *C. maculatus*. Whatever the cause, the peak in reproductive output following a second mating (see *Double-mated* females - Figure 6.4.6) suggests that resources acquired during copulation may be invested in current reproduction with immediate beneficial effects on the rate of offspring production.

6.5.3 Potential costs of gregarious living

The final investigation of this chapter demonstrated the potential for non-mating exposure to males, including sexual harassment, to erode female fitness traits. With controlled copulation rates, females exposed to non-copulating males died faster and had shorter lifespans than females housed alone (see Figures 6.4.9 and 6.4.10). Furthermore, despite attempts to standardise reproductive output across treatment groups, the lifetime fecundities of harassed females were on average 15% lower than those of non-harassed females (see Figure 6.4.11). However, these costs of harassment may be unrealistically high. This is because although emasculated males harass females at similar rates to

intact males (see Appendix 2), they are incapable of mating and consequently females cannot reduce harassment costs by accepting copulations. In addition, this experiment used a biased sex ratio (2 males: 1 female). The importance of harassment in natural populations will depend on female abilities to avoid such costly interactions (Clutton-Brock and Parker 1995b). Under laboratory conditions of continuous confinement with males, the success of female decamping and avoidance responses will be limited (see Newport and Gromko 1984; Harshman *et al.* 1988). Consequently, the expression of costs may be further exaggerated. Nonetheless, these findings illustrate the potentially detrimental effects on females of sexual harassment and suggest that females may gain from precise evaluations of the costs and benefits associated with both resisting and accepting copulations. In general, if the costs of resisting persistent courtship outweigh the costs of compliance, females may be induced to mate at frequencies beyond those necessary to maximise fitness (Thornhill and Alcock 1983; Clutton-Brock and Parker 1995b; Arnqvist 1997).

6.5.4 Expression and detection of mating costs

In the laboratory population of *C. maculatus* used in the present study, a clear reduction in female longevity as a consequence of copulation frequency has not been demonstrated but the reduction in longevity of re-mated females suggests copulation carries some costs (see Figure 6.4.2). Any costs to females of copulation may have been offset by associated benefits since fitness outcomes were positive. In addition to being offset by the benefits derived from mating, be they nutrients or stimulants, the costs of copulation may be reduced by female-based mechanisms. In particular, cumulative costs may be

regulated through the control of mating frequency and/or ameliorated by counter-adaptations (Parker 1979; Stockley 1997). For instance, if genital damage underlies the reduction in lifespan associated with remating, mate-kicking by females may reduce the probability that cumulative costs due to such injuries are expressed in terms of net fitness outcomes.

The experiments reported here demonstrate the importance to studies of female mating rates and fitness traits of considering a range of potential life-history consequences of exposure to males. This is because, for instance, egg production costs may confound those of copulation, thereby making it difficult to determine if a reduction in lifespan is due to costs of egg production, or those of copulation (e.g. see Partridge and Harvey 1985; Fowler and Partridge 1989). Thus, by controlling oviposition rates across different mating frequencies, the effects of copulation on female lifespan may be determined. Similarly, by standardising gregarious living conditions, the effects of varying mating opportunities on female reproductive output can be identified. Furthermore, by introducing elements of realism through the inclusion of gregarious conditions, the findings can be applied to natural populations with some confidence.

6.6 Summary

In this chapter I have demonstrated a longevity cost to twice-, but not thrice-mated female *C. maculatus*. Although the mechanism of longevity reduction in twice-mated females is not known, the effect is unlikely to be due to expenditure in reproductive

output. Under standardised gregarious living conditions, increased exposure to males elevated female reproductive output. In addition, the potential for non-mating exposure to males to reduce both the lifespan and reproductive output of females was shown. These investigations demonstrate the value of devising experimental approaches that allow the various effects of exposure to males on female fitness traits to be distinguished.

Chapter 7: General discussion - evaluating the evidence for sexual conflict in *C. maculatus*

7.1 Introduction

This thesis has used a model insect system to examine the functions, causal relationships and life-history consequences of particular aspects of reproductive morphology and behaviour within the framework of sexual conflict theory. This chapter summarises the results of the thesis and assesses the evidence for a conflict of reproductive interests between the sexes in *C. maculatus*. Finally, promising avenues for further investigation of the problems raised in the study are explored.

7.2 Thesis summary

Chapter 1 provided a historical and theoretical background to the evolutionary concepts underpinning the work in this thesis, namely sexual selection and inter-sexual conflict. The study organism, *Callosobruchus maculatus*, was introduced, and its life-cycle and benefits as a model system in sexual selection research were outlined. I also presented *a priori* reasons for predicting the presence of sexual conflict in this model.

Chapter 2 described the gross genital anatomy of male and female *C. maculatus*. The female genital tract consists of an endocuticular tube whilst the distal tip of the male intromittent organ, or aedeagus, is covered in sharp cuticular spines. Dissections of the genitalia of *in situ* mating pairs revealed the relative positions attained by the genitalia of each sex during copulation: The aedeagal spines unfurl within the central region of the female genital tract and a distal sac is inflated within the female bursa copulatrix. Possible functions for the major genitalic traits in each sex were discussed and the potential for the aedeagal spines to injure females during copulation was highlighted.

Chapter 3 was largely concerned with examining the incidence of genital injury in female *C. maculatus*. A causal relationship between the action of the male intromittent organ during copulation and damage to the genital tract of mated females was established. The aedeagal spines puncture the endocuticular lining of the female genital tract during copulation, causing injury. Following injury, female repair systems are activated and the punctures are manifest as dark patches, probably due to melanised haemocytes having plugged the punctures. This outcome is readily visible sixteen hours after copulation. An essay for quantifying the extent of injury was developed and used to show that genital damage varied among once-mated females, and increased with each additional copulation (one, two and three copulations imposed). A suite of behavioural and morphological traits were quantified but none were found to correlate with the degree of damage sustained. Finally, the potential costs to females of incurring genital damage were discussed.

Chapter 4 investigated the function of mate-kicking behaviour, whereby female *C. maculatus* direct vigorous kicks at their mating partners towards the end of copulation. Females experimentally prevented from engaging in kicking behaviour had longer copulations and greater genital damage than females permitted to kick. Kicking therefore appears to function to initiate the termination of copulation and reduce both copulation duration and the severity of the injuries sustained. These findings suggest that one or both of these components of copulation (copulation duration and genital damage) are costly for females and that mate-kicking may have evolved as a counter-adaptation to ameliorate the associated costs.

Chapter 5 examined the relationship between mate-kicking and two female post-copulatory traits that are linked to fitness, namely oviposition and remating behaviour. These traits have important potential consequences for the reproductive success of male *C. maculatus* because of the advantage in sperm competition enjoyed by the last male to mate. An absence of mate-kicking was associated with increased copulation duration and genital damage and there are several theoretical reasons to predict an effect of these traits on both oviposition and remating by females. Mate-kicking was experimentally prevented so that its impact on female post-copulatory behaviour could be examined. No measurable effects were found on either the immediate oviposition rate or the remating interval of females following an initial copulation. Males do not, therefore, appear to benefit from longer copulations / increasing female genital damage by inducing higher oviposition rates and longer remating intervals in their mates.

Chapter 6 examined the relationship between the level of exposure to males and fitness traits in female *C. maculatus*. When oviposition rates were partially standardised across different treatment groups, there was a significant effect of mating frequency on female survival. Twice-mated females had reduced longevities relative to once-mated females, a finding that is unlikely to be due to costs associated with reproductive output. However, the relationship between copulation frequency and female lifespan was not linear: thrice-mated females lived approximately as long as once-mated ones. In the context of standardised gregarious living conditions where females were subject to sexual harassment, increased opportunities to mate caused a substantial increase in female reproductive output. Thus, despite the reduced lifespan of remated females, multiple-mating and increased exposure to males elevated female fitness. Possible mechanisms underlying the effects of polyandry were discussed. Finally, the potential for non-mating exposure to males to undermine female fitness traits was demonstrated.

Appendix 1 investigated the relationship between exposure to males and female longevity. To this end, methods to control costs related to both sexual harassment and egg production were tested. Addressing these problems allows the effect of copulation on female lifespan to be distinguished from other costs. However, the methods used to standardise the costs of harassment and egg production were largely unsuccessful. Despite the methodological inadequacies of this investigation, the results provide the necessary groundwork for experiments reported in Chapter 6 because the potential for increased exposure to males to reduce female lifespan was demonstrated.

Appendix 2 investigated the rates at which emasculated and intact males sexually harass once-mated females. The harassment rates of manipulated and non-manipulated males were not significantly different. This result underlies the use of emasculated males in two experiments reported in Chapter 6 to impose effects of non-mating exposure to males on females.

7.3 Is genital damage costly for female *C. maculatus*?

This thesis represents the first study to have (a) provided direct qualitative evidence that male genitalic traits can be causally related to the damaged genital tracts of mated females, (b) developed and used an assay to quantify the extent of genital injuries in individual females, and (c) provided evidence suggsting a female counter-adaptation to genital injury. This study has not provided direct evidence that injury to the genital tracts of female *C. maculatus* is costly. Moreover, given that a phenomenon such as genital damage, which appears to be due to 'deliberate' male-based strategies to cause harm, is particularly susceptible to anthropomorphic interpretations relative to other aspects of animal behaviour (see Kennedy 1992), it is tempting to assume the costs to females are considerable. With this in mind, I feel it is reasonable to assume that the *potential* detrimental effects of genital damage are non-trivial (see Chapter 3): this thesis provides several indirect lines of evidence that genital damage may have negative consequences for females.

7.3.1 Repair system activation

Female *C. maculatus* respond to genital injury by initiating systems involved in the repair of cuticular trauma (see Chapter 3). The process of repair is probably triggered fairly rapidly following injury (indeed, the process is underway ten hours after mating (pers. obs.)) and appears to be complete within approximately sixteen hours of copulation. Cuticular wounding in general poses a fundamental threat to survival and is therefore a powerful selection pressure acting on insects (Chapman 1998). The ability to raise effective repair responses in the event of cuticular trauma is vital and such adaptations were undoubtedly present when speciation events gave rise to *C. maculatus*. Although in insects generally, damage to the endocuticle is likely to be less common than that to the exocuticle, adaptations to exocuticular trauma are probably effective in responding to endocuticular trauma. Thus, once aedeagal spines and damaging tactics arose in male *C. maculatus*, females may have been relatively well adapted to deal with the resultant injuries. Nonetheless, regardless of the pre-existence of repair and immune responses in females, mobilising them against genital damage is not likely to be free of costs. The costs are likely to depend on the severity of the injuries sustained during copulation, and during successive copulations over the lifetime of a female. Thus, although the magnitude of female repair responses and associated costs were not quantified in this study, it is reasonable to assume that repair mechanisms and immune responses to pathogens introduced as a result of genital damage, have some cost: consequently genital wounding may form the basis of a reproductive conflict.

7.3.2 Remating intervals

Following copulation in *C. maculatus*, female pheromone production and sexual receptivity decline rapidly (Shu *et al.* 1996) and further matings are typically resisted for one or two days (pers. obs; P. Eady, pers. comm.). Despite persistent copulation attempts, females avoid remating via a combination of resistive behavioural traits (specific investigation of these traits were not undertaken but were observed during the course of the study) (also see Fox and Hickman 1994). Initially, females reject courting males by decamping and kicking with their hind legs (see Chapter 3). If unwanted courtship progresses to the stage of mounting, females can still prevent intromission by refusing to expose their genitalia (see Chapter 4, section 4.3.2 and Fox and Hickman 1994). The energetic expenditure necessary for decamping and kicking responses indicates that some aspect(s) of copulation is probably costly for females. Anecdotal support for this proposal is provided by the observation that resistive behaviours seemed to be deployed against males in a non-discriminatory manner, suggesting they enable females to control mating rates rather than to exercise pre-copulatory mate choice (see Andersson 1994). If so, a conflict of interests between males and females over mating rates is possible.

Genital damage may underlie the refractory period seen in recently mated females if non-receptivity arises from the need to repair the damage before further risks of injury are encountered. This proposal is not necessarily contradicted by the finding that indirect manipulation of the damage did not alter female remating intervals (see Chapter 5). This is because the duration of the repair process may not covary with the extent of damage.

In other words, the length of time required for repair, and therefore the length of the refractory period, may not be dependent on the severity of the injuries. However, it is also possible that non-receptivity is induced by inhibitory seminal compounds transferred by males during copulation (see Chen 1984). Interestingly, whether under male or female control, a delay in remating may benefit any female that sustains injuries to her genital tract during copulation if a refractory period allows further injury to be avoided whilst the original injuries are repaired.

7.3.3 Costs and benefits of remating

Notwithstanding the arguments in favour of genital damage generating physiological costs, the fundamental question is whether these costs are sufficient to threaten female fitness traits. This study has shown that remating caused a small but significant reduction in female lifespan that is unlikely to be entirely attributable to any trade-off between somatic maintenance and the physiological costs of reproductive output (see Chapter 6). Genital damage may be the mechanism responsible since the extent of injury increased following a second copulation (see Chapter 3). However, such a relationship has not been demonstrated and other mechanisms such as toxic male seminal signals may be implicated (see Chapman 1995). Furthermore, the finding that female lifespan did not decline linearly with copulation frequency (see Chapter 6) could indicate that genital damage is not the cause given that the injuries increased linearly with copulation frequency (see Chapter 3). However, a non-monotonic effect of mating frequency on lifespan could be generated by an interaction between the costs and benefits associated with copulation.

Whatever underlies the reduction in lifespan of remated females, the cost is not sufficient to undermine female fitness. This is evidenced by the finding in Chapter 6 that females with greater mating opportunities enjoyed higher reproductive output than females with fewer opportunities. Indeed, continuous exposure to males elevated offspring production by a massive 50% relative to a single mating. Thus any costs imposed by males during copulation were compensated by the direct benefits derived by females, be they nutrient donations (Fox 1993), oviposition stimulants (Wilson *et al.* 1999), or both. However, it is interesting to note that expression of copulation costs in another species has been shown to be context-specific. When female *D. melanogaster* are maintained under standard laboratory conditions the delivery of toxic male seminal compounds reduces their survival but not their reproductive output (Chapman and Partridge 1996). But when food is made super-abundant, females mate more frequently and mating frequencies shift towards the male optimum. Under these circumstances, the resultant increase in seminal compound transfer is sufficient to reduce female survival to the extent that female reproductive output is eroded (Chapman and Partridge 1996). These findings indicate that the cost of mating is only made apparent when certain environmental conditions are altered and suggest that mechanisms for determining mating frequencies are not optimal under all conditions (Chapman and Partridge 1996). Mating rates in female *D. melanogaster* appear to have evolved to ensure that, under a familiar feeding regime, the dose-dependant costs of male seminal proteins do not threaten fitness (Chapman and Partridge 1996).

It is possible that expression of the longevity cost seen in remated female *C. maculatus* is similarly dependent on prevailing conditions. For instance, if females were induced to mate at frequencies beyond those necessary to maximise reproductive output, the cost may reveal itself in terms of a net fitness reduction. Expression of mating costs could also depend on male mating history. Eady (1991b) showed that males become sperm depleted after four consecutive matings. If males transfer seminal nutrients, it is possible that the size of the donation shows a similar decline over successive matings. Given that seminal nutrients may be used to offset the costs of copulation, if females receive smaller donations when copulating with recently mated males, the benefits derived may not be sufficient. The use of virgin males in many laboratory experiments, including those in Chapter 6, may not accurately reflect male mating status in natural conditions (Savalli and Fox 1999). If, under natural conditions females receive fewer benefits per mating, their ability to offset copulation costs may be reduced, thereby increasing the probability that such costs will be expressed more strongly.

7.3.4 Mate-kicking

Undoubtedly the most persuasive evidence that genital damage is costly for female *C. maculatus* comes from the investigation of mate-kicking behaviour. By kicking their mates, females reduce the extent of genital damage sustained (see Chapter 4). Indeed, when females were experimentally prevented from kicking, the increase in damage was immense (on average, 85% - Chapter 4). Mate-kicking therefore appears to have evolved as a counter-adaptation to male damaging tactics and, in combination with the functionally diametric aedeagal spines, probably constitutes an example of sexually

antagonistic coevolution (see e.g. Rice 1996). If the detrimental effects of genital damage are dose-dependent, mate-kicking will significantly reduce the costs incurred. Although mate-kicking does not prevent the occurrence of genital damage altogether, this adaptation may prevent the expression of costs in terms of a net reduction in female fitness.

In light of the relationship between mate-kicking and the potentially costly genital damage, it is interesting to note Alexander *et al.'s* (1997) suggestion that the extent of sexual conflict can be estimated from the degree of effort used by females to free themselves from males. Although such reasoning probably relies too heavily on anthropomorphic interpretations of animal behaviour (see Kennedy 1992), aggressive traits or physical struggles between the sexes may provide valuable cues to investigators to the existence of reproductive conflicts. Of course, females in general may respond to conflict with adaptations that are far less conspicuous than overt aggression.

To conclude, females reduce the extent of genital damage by mate-kicking, they repair the damage following copulation, and, as a result of their post-mating refractory periods, avoid additional injuries whilst the repair process is underway. These traits suggest that females deal with the imposition of injuries by males relatively effectively. However, their very existence indicates the importance of genital damage and suggests it has the potential to generate fitness costs in their absence.

7. 4 What is the function of the aedeagal spines?

Johnstone and Keller (2000) proposed that the evolution of male genital spines and aggression may be driven by the potential for these antagonistic traits to induce favourable post-copulatory responses in females that are subjected to them. In particular, females may lengthen their remating intervals in response to harm. In this sense, imposing harm is analogous to remote male mate-guarding strategies such as the deposition of mating plugs following insemination (Parker 1970a; Simmons and Siva-Jothy 1998). This idea was explored in Chapter 5 where the effect of genital damage on female post-copulatory decisions was tested indirectly via the manipulation of mate-kicking behaviour. No effects were detected, indicating that males may not induce favourable post-mating responses in females through the injuries caused by their aedeagal spines.

If the function of the aedeagal spines in male *C. maculatus* is not to impose harm in order to influence female post-mating behaviour, what selective forces underlie the maintenance of such complex genital anatomy? Inflicting harm on females may not be the principal function of the aedeagal spines. Rather, genital damage may arise as a pleiotropic effect of male genital morphology. Several potential benefits are apparent, including that of direct sperm removal although this particular function has been discussed and discounted in Chapter 2 (section 2.6.2). Other possible functions include the maintenance of genital contact and a role in indirect sperm displacement.

7.4.1 Maintaining genital contact

By penetrating the endocuticular lining of the female genital tract, the aedeagal spines are likely to improve the anchoring ability of mating males. Such a function could be selectively advantageous in both intra- or inter-sexual competition (see Chapter 2, section 2.6.2). Copulating pairs receive persistent harassment by non-mating males seeking access to females (pers. obs). Males able to achieve a stable anchorage during copulation via the spines would therefore be favoured due to their ability to prevent take-overs by rival males and avoid sperm competition (Parker 1970a). These benefits could be supplemented if improved anchorage prevents premature termination of copulation by females. Although highly plausible, the hypothesis that the aedeagal spines function to maintain genital contact during copulation remains untested.

In the absence of mate-kicking this thesis has shown that genital coupling continues beyond the point where copulations are terminated naturally - in other words, copulation duration is extended (see Chapter's 4 and 5). This suggests that males and females have varying optima for copulation duration. If genital contact is maintained via the aedeagal spines males may be able to coerce copulation duration to a point nearer their own optimum. Several theoretical advantages of prolonging copulation exist. For example, males may gain sperm competitive benefits from longer copulations if the rate of sperm transfer correlates with copulation duration and sperm precedence is influenced by the number of sperm transferred. However, these traits do not determine sperm competitive success in *C. maculatus* (Eady 1994). Nonetheless, prolonged copulation may increase the probability that sperm are stored successfully. In *C. maculatus*, sperm begin to

migrate from the female bursa to the spermatheca approximately five minutes after copulation is terminated (Eady 1994b). Since in the absence of mate-kicking, copulation duration was extended by an average of six minutes, any male able to prolong copulation, possibly via his aedeagal spines, may ensure that the process of sperm storage has commenced prior to genital separation when his mate is free to copulate with rivals.

7.4.2 Indirect sperm displacement

Another potential function of the aedeagal spines is in determining patterns of sperm precedence. The precise mechanism underlying the last male sperm competitive advantage in *C. maculatus* is unclear, but Eady (1991b) proposed that success is achieved via indirect displacement of sperm from the female spermatheca. The outward-movement of sperm from this storage organ is believed to be controlled by contraction of the spermathecal muscle (Eady 1991b). The action of the aedeagal spines during copulation may play a role in stimulating contraction of this muscle, thereby causing the contents to be expelled. This hypothetical function cannot be discounted until the mechanism for sperm precedence in this insect is determined. Eady (1991b) also showed that the degree of sperm precedence varied considerably among males but was unable to identify the reason for this. The extent of genital damage among mated females shows considerable variation (see Chapter 3) and the possibility that these two phenomena are causally related is intriguing.

7. 5 Conclusions

Demonstrating sexual conflict is not straightforward (Parker 1979). This is because most conflict is hidden by cycles of coevolution between males and females as effective counter-adaptations to antagonistic traits arise in the losing sex and conceal the effects of the conflict (e.g. Rice 1996). Consequently, demonstrating the presence of conflict in co-evolved populations is problematic. In order to expose the underlying dynamics of any conflict it may be necessary to alter the balance of power between males and females since this can exaggerate expression of traits associated with conflict (e.g. Rice 1996). This type of experimental approach was taken in Chapters 4 and 5 when female mate-kicking behaviour was manipulated to order for its potential effect on copulation duration, the extent of genital damage, and female post-copulatory behaviour to be investigated. Manipulations of this kind are also required to identify the fitness consequences to each sex, both beneficial and detrimental, of the putative conflict traits.

However, the main obstacle to this approach to studying sexual conflict in *C. maculatus* is the difficulty of manipulating the aedeagal spines or the injuries they cause directly. This problem stems from the relatively small size of the animal, and of its genitalia. Altering both the length of the spines and extent of the injuries would permit identification and quantification of the benefits derived by males and the costs incurred by females as a consequence of these phenomena.

7.6 Future Work

Several experimental approaches are likely to prove particularly valuable in overcoming the practical obstacles encountered in this model system. In so doing, some of the remaining questions regarding sexual conflict in this insect may be addressed:

7.6.1 Selection experiments

Does mate-kicking reduce female mating costs?

The establishment of selection lines in which mate-kicking is either permitted or prevented may allow the potential role of mate-kicking in reducing female mating costs to be elucidated. Mating crosses within and among selection and reference lines could be used to examine whether females belonging to kicking lines had less genital damage and fewer life-history costs than females from non-kicking lines. If so, investigating the relationship between genital injury and the propensity for remating in females from kicking and non-kicking lines could reveal whether male harming tactics generate conflicts between the sexes over mating frequencies.

Does post-copulatory sexual selection maintain damaging tactics in males?

Selection lines can also be used to determine whether male harming tactics are maintained by post-copulatory sexual selection. In this instance, lines would be based on either monandrous or polyandrous mating regimes (see Holland and Rice 1999). Using random mate assignment, females from monandrous lines would receive one copulation,

whilst from polyandrous lines would receive two but with no opportunity for pre-copulatory mate choice or male-male competition. If post-copulatory sexual selection maintains the putative male conflict trait in this species, monandrous females should exhibit less genital damage than polyandrous females.

7.6.2 Inter-strain crosses

Is genital damage the mechanism underlying female mating costs?

Indian-strain females do not incur mating costs in the form of reduced longevity (T. Taylor; pers. comm.). Although the reason for this difference is not clear, it can be used to examine the underlying mechanisms and life-history consequences of sexual conflict in *C. maculatus*. The finding that crosses between Brazilian females and Indian males generate less genital damage and lower longevity costs in females than crosses between Brazilian males and females would provide some support for the proposal that genital damage underlies the longevity cost shown in remated Brazilian females. Preliminary investigation of the gross genital morphology of Indian males revealed no obvious differences when compared to Brazilian males (pers. obs.). However, the incidence and extent of genital damage in Indian females is not yet known.

7.6.3 Sperm competition studies

Does genital damage benefit males in sperm competition?

C. maculatus shows a considerable last male advantage in sperm competition. Although indirect sperm displacement is believed to produce this pattern, the mechanism responsible remains unclear (Eady 1991b). Identification of males that consistently impose either greater or fewer injuries on females may elucidate the potential role of genital damage in determining sperm precedence patterns in this insect. If genital damage benefits males in sperm competition, and all else is equal, high-damaging males should show elevated sperm precedence values relative to low-damaging males.

Do females influence sperm competition through their susceptibility to genital damage?

Using full-sisters and non-sisters, Wilson *et al.* (1997) conducted reciprocal sequential matings with males from different strains to show that female genotype had a considerable influence on the outcome of sperm competition in *C. maculatus*. A similar experimental design could be used to investigate whether female genotype influences male fertilisation success via the degree of genital damage sustained. If this is the case, the amount of genital damage (and patterns of sperm precedence) should show higher repeatabilities among males mated to full-sisters than among males mated to non-related females.

7.6.4 Physiological studies

Do males use chemical signals to mediate changes in female post-copulatory behaviour?

By injecting female Australian sheep blowflies, *Lucilia cuprina*, with extracts of male accessory glands, Smith *et al.* (1989) demonstrated the potential for components of the male ejaculate to stimulate female oviposition behaviour. The same methodology could be employed to determine whether the ejaculate of male *C. maculatus* contains substances that manipulate the post-copulatory behaviour of their mates to their advantage. Whilst controlling for copulation frequency, injections of extracts of male gonads are predicted to induce higher oviposition rates and/or longer remating intervals in females.

Does genital damage provide a route for chemical signals to the female body cavity?

If male *C maculatus* transfer seminal signals that induce favourable post-mating responses in their mates, males may benefit from direct deposition within the female body cavity. Radio-labelling techniques could be used to track the progress of the male ejaculate through the female reproductive tract and haemocoel. If genital damage provides routes for sperm and seminal compounds directly to the female body cavity, the labelled compounds are predicted to reach their target by passing through the genital punctures created during copulation.

7.7 Final conclusions

This thesis presents novel evidence for the existence of a sexual conflict in *Callosobruchus maculatus*. During copulation the male genitalia imposes genital damage on females to which they appear to have responded with a behavioural adaptation, mate-kicking, to reduce the extent of injuries. Remating appears to impose a longevity cost on females. However, this is not sufficient to threaten reproductive output and females increase their fitness by mating multiply. The precise costs to females of genital damage remain unidentified, as does the function of the aedeagal spines. Despite the difficulty of manipulating the conflict traits directly, the findings presented here demonstrate the value of this model system for sexual conflict research. Further investigation may provide a unique opportunity to understand precisely how sexual conflict is manifested in this insect and to identify the mechanism(s) maintaining the putative conflict traits in each sex.

Appendix 1: The effect of varying exposure to males on female lifespan

A1.1 Objective

The objective of this appendix is to determine the effect of varying exposure to males on the lifespan of female *C. maculatus*. To this end, females were allocated to one of the three following treatment groups:

1) mated once and isolated for the remainder of their lives,

2) confined permanently with two males, or

3) mated once and confined permanently with two manipulated males that could sexually harass but not copulate with females.

A1.2 Experimental design

Sex ratio

I elected to use a biased sex ratio (2 males: 1 female) in order to increase the probability of detecting an effect of confinement with males on longevity.

Controlling harassment costs

Inclusion of the third treatment group arose from the need to distinguish between the effects on female longevity of (a) sexual harassment from males and (b) copulation. The procedure used to render males incapable of copulation involved placing a droplet of glue (UHU® Super Glue Gel) on the surface of the cuticle located external to their genitalia. However, this procedure proved unsuccessful due to failure of the glue to remain in place beyond a day or so of application. Consequently, some males were able to achieve copulation and for this reason the third treatment group was eliminated from the experiment.

Controlling oviposition costs

In order to distinguish between the effects on female longevity of (a) egg production and (b) copulation, the experiment was designed to prevent oviposition by all females, thereby eliminating any consequences for longevity of egg production. Attempts to fulfil this objective involved denying females access to oviposition sites since such conditions constrain egg-laying behaviour in female *C. maculatus* (Credland *et al.* 1986). However, this element of experimental design proved inadequate due to variation in the reluctance of females to oviposit on the surface of the containers in which they were housed.

General methods

This experiment was conducted at 22±2°C. All animals were 18-24 hrs old. Males that died before the female with which they were confined were replaced with younger males (again, aged 18-24 hrs). Females were housed in plastic petri-dishes (35 mm diameter).

Finally, female longevities and female death rates were determined using the methods described in Chapter 6 (see section 6.3.2).

A1.3 Results

There was no significant difference in the body size of females from the two treatment groups (t-test: $t_{77} = -0.042$, $p > 0.05$). Females confined permanently with two males died significantly faster (Mantel Cox log rank test: $\chi^2_1 = 62.077$, $p < 0.0001$) (see Figure A2.1), and had significantly shorter longevities than females mated once (ANCOVA: $F_{1,76} = 45.742$, $p < 0.0001$), but there was no significant effect of body size ($F_{1,76} = 0.006$, $p > 0.05$); mean longevity: once-mated females = 19.1±0.9 days, $n = 40$; and females confined with two males = 12.3±0.5 days, $n = 39$) (see Figure A2.2).

A1.4 Conclusions

This investigation demonstrated that females confined permanently with males died faster and had shorter lifespans than females mated once. This effect may be due to costs related to sexual harassment from males (e.g. Odentaal *et al.* 1989; Magurran and Seghers 1994; Stone 1995; Clutton-Brock and Langley 1997; McLain and Pratt 1999), as well as to copulation itself (Fowler and Partridge 1989; Chapman and Partridge 1996). However, given that the method used to constrain female oviposition behaviour proved inadequate, the reduction in longevity may also be partly due to costs associated with egg production (see Partridge and Harvey 1985; Stearns 1992).

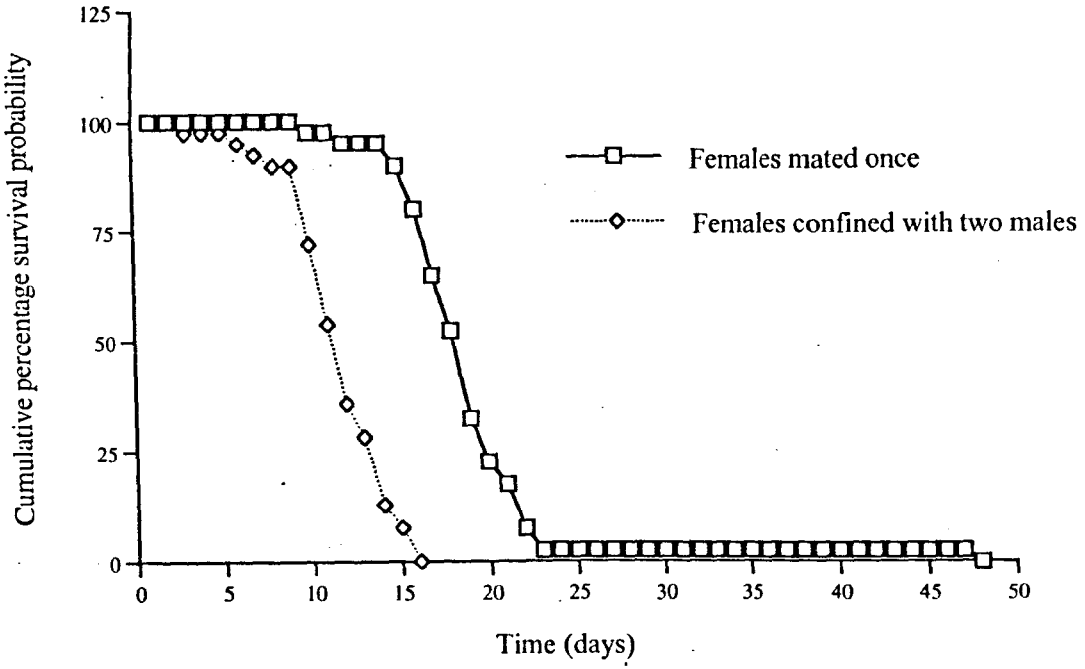


Figure A1.1 Females confined permanently with males died significantly faster than females mated once. Access to oviposition sites was denied.

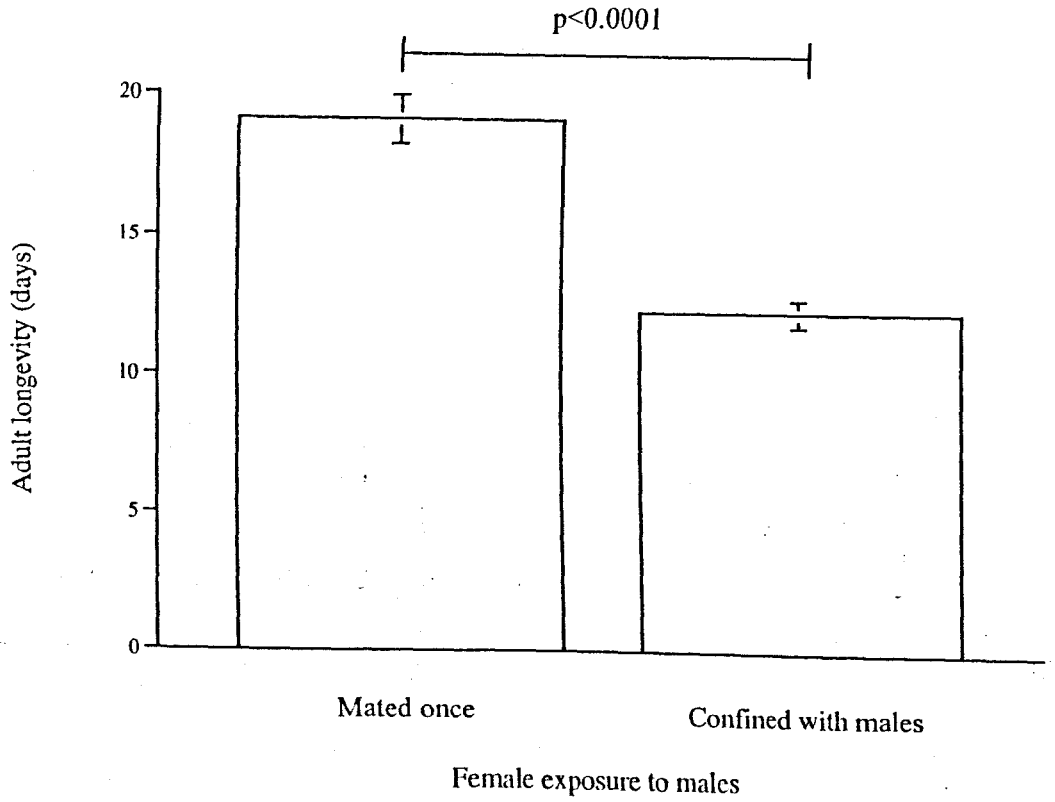


Figure A1.2 Females confined permanently with males had shorter lifespans than females mated once. Access to oviposition sites was denied.

This experiment indicates the potential for, as yet, unidentified aspects of exposure to males to undermine the longevity of female *C. maculatus*. However, it also highlights the necessity of devising reliable methods of controlling the costs associated with both male harassment and female oviposition across different mating frequencies. By standardising these costs, the effects of various aspects of exposure to males on female fitness traits can be distinguished, particularly those of copulation. The problem of controlling both male harassment and female oviposition rates are addressed in Appendix 2 and Chapter 6 respectively.

Appendix 2: Rates of harassment by intact and emasculated males

A2.1 Objective

The objective of this appendix is to determine the rates at which emasculated and intact males sexually harass once-mated females. The results of this investigation are relevant to experiments in Chapter 6 (see sections 6.3.3 and 6.3.4) which utilised non-virgin females. Therefore, non-virgin females were used in this investigation.

A2.2 Experimental design

Males were assigned to one of two treatment groups: *Emasculated-* or *Intact*-males. Males allocated to the former group were emasculated using the methods described in Chapter 6 (see section 6.3.3), whilst those in the latter group remained unmanipulated. Males were confined in mixed sex replicates consisting of three males and three once-mated females, and each replicate was housed in plastic a petri-dish (35mm diameter). All replicates were filmed for 1 hr after the onset of photophase using a camcorder (Sony Handicam video Hi8). I elected to make recordings at this time of day since sexual activity in *C. maculatus* is reported to peak during early photophase (Shu *et al.* 1996). Harassment rates were determined by counting the number of failed copulation

attempts made by intact and emasculated males. A copulation attempt was defined as any occasion a male antennated and mounted a female from the rear. A mean harassment rate for each replicate was derived by dividing the total number of failed copulation attempts by three. Thus, each datum is the mean harassment rate of three males. All animals were 18-24 hrs old.

A2.3 Result

There was no significant difference in the rates at which *Emasculated*- and *Intact*-males harassed once-mated females (Mann-Whitney test: $U = 27.00$, $p = 0.2332$; median number of failed copulation attempts per hr: *Intact*-males = 33.333, $n = 9$; and *Emasculated*-males = 16.333, $n = 9$) (see Figure A2.1).

A2.4 Conclusions

The results of this investigation indicated that the rates at which intact and experimentally emasculated males harassed once-mated females did not differ significantly (see Figure A2.1). This finding is relevant to two experiments reported in Chapter 6. The first of these used emasculated males to standardise non-mating exposure to males whilst varying female mating opportunities (see section 6.3.3). The second experiment tested an assumption implicit in the experimental design of the first experiment, that non-mating exposure to males imposes life-history costs on females (see section 6.3.4). The results of this appendix indicate that the use of emasculated males in the two experiments reported in Chapter 6 is justified.

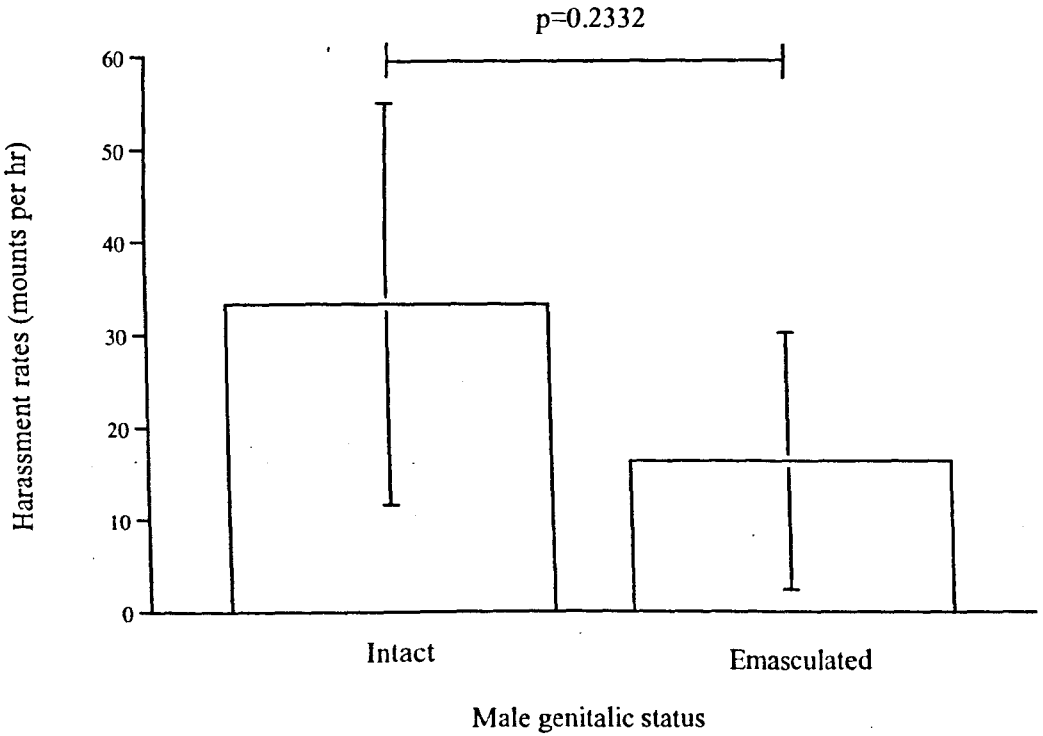


Figure A2.1 Rates of sexual harassment by intact and emasculated males. Median values are shown with inter-quartile ranges and each datum is the mean harassment rate for three males.

References

Adamo, S. 1999. Evidence for adaptive changes in egg laying in crickets exposed to bacteria and parasites. *Animal Behaviour,* **57,** 117-124.

Alcock, J. and Forsyth, A. 1988. Post-copulatory aggression toward their mates by males of the rove beetle *Leistotrophus versicolor* (Coleoptera: Staphylinidae). *Behavioral Ecology and Sociobiology,* **22,** 303-308.

Alexander, R. D. and Borgia, G. B. 1979. Origin and basis of the male-female phenomenon. In: *Sexual Selection and Reproductive Competition in Insects.* (Ed. by M. S. Blum and N. A. Blum), New York: Academic Press.

Alexander, R. D., Marshall, D. C. and Cooley, J. R. 1997. Evolutionary perspectives on insect mating. In: *The Evolution of Mating Systems in Insects and Arachnids.* (Ed. by J. C. Choe and B. J. Crespi), Cambridge: Cambridge University Press.

Andersson, M. 1994. Sexual Selection. Princeton: Princeton Press.

Arnqvist, G. 1989a. Multiple mating in a water strider - mutual benefits or intersexual conflict. *Animal Behaviour,* **38,** 749-756.

Arnqvist, G. 1989b. Sexual selection in a water strider - the function, mechanism of selection and heritability of a male grasping apparatus. *Oikos,* **56,** 344-350.

Arnqvist, G. 1997. The evolution of water strider mating systems: causes and consequences of sexual conflicts. In: *The Evolution of Mating Systems in Insects and Arachnids* (Ed. by J. Choe and B. Crespi), Cambridge: Cambridge University Press

.

Arnqvist, G. 1998. Comparative evidence for the evolution of genitalia by sexual selection. *Nature,* **393,** 784-786.

Arnqvist, G. and Rowe, L. 1995. Sexual conflict and arms races between the sexes - a morphological adaptation for control of mating in a female insect. *Proceedings of the Royal Society of London Series B,* **261,** 123-127.

Arnqvist, G. and Nilsson, T. 2000. The evolution of polyandry: multiple mating and female fitness in insects. *Animal Behaviour,* **60,** 145-164.

Arnqvist, G., Edvarsson, M., Friberg, U. and Nilsson, T. 2000. Sexual conflict promotes speciation in insects. *Proceedings of the National Academy of Sciences USA,* **97,** 10460-10464.

Bateman, A. J. 1948. Intra-sexual selection in *Drosophila. Heredity,* **2,** 349-368.

Baumann, H. 1974. Biologial effects of paragonial substances PS-1 and PS-2, in females of *Drosophila funebris*. *Journal of Insect Physiology*, **20**, 2347-2362.

Bellows, T. S. 1982. Analytical models for laboratory populations of *Callosobruchus chinensis* and *C. maculatus* (Coleoptera, Bruchidae). *Journal of Animal Ecology*, **51**, 263-287.

Birkhead, T. R. 1998. Cryptic Female Choice: Critera for establishing female sperm choice. *Evolution*, **52**, 1212-1218.

Birkhead, T. R. 2000a. Defining and demonstrating postcopulatory female choice - again. *Evolution*, **54**, 1057-1060.

Birkhead, T. R. 2000b. Promiscuity: an evolutionary history of sperm competition and sexual conflict. London: Faber and Faber Ltd.

Birkhead, T. R. and Møller, A. P. 1998. Sperm competition and sexual selection. London: Academic Press.

Boggs, C. L. and Gilbert, L. E. 1979. Male contribution to egg production in butterflies: evidence for transfer of nutrients at mating. *Science*, **206**, 83-84.

Brown, W. D., Crespi, B. J. and Choe, J. C. 1997. Sexual conflict and the evolution of mating systems. In: *The Evolution of Mating Systems in Insects and Arachnids*. (Ed. by J. C. Choeand B. J. Crespi), pp. 352-378. Cambridge: Cambridge University Press.

Chapman, R. F. 1998. The insects: structure and function. Third edition. London: Hodder and Stoughton.

Chapman, T., Liddle, L. F., Kalb, J. M., Wolfner, M. F. and Partridge, L. 1995. Cost of mating in *Drosophila melanogaster* females is mediated by male accessory-gland products. *Nature*, **373**, 241-244.

Chapman, T., Neubaum, D. M., Wolfner, M. F. and Partridge, L. 2000. The role of male accessory gland protein Acp36DE in sperm competition in *Drosophila melanogaster*. *Proceedings of the Royal Society of London Series B*, **267**, 1097-1105.

Chapman, T. and Partridge, L. 1996. Female fitness in *Drosophila melanogaster*: an interaction between the effect of nutrition and of encounter rate with males. *Proceedings of the Royal Society of London Series B*, **263**, 755-759.

Chen, P. S. 1984. The functional morphology and biochemistry of insect male accessory glands and their secretions. *Annual Review of Entomology*, **29**, 233-55.

Chen, P. S., Stumm-Zollinger, E., Aigaki, T., Balmer, J., Bienz, M. and Bohlen, P. 1988. A male accessory gland peptide that regulates reproductive behaviour of female *Drosophila melanogaster*. *Cell*, **54**, 291-298.

Choe, J. C. and Crespi, B. J. 1997. The Evolution of Mating Systems in Insects and Arachnids. Cambridge University Press.

Clark, A. G., Aguadé, M., Prout, T., Harshman, L. G. and Langley, C. H. 1995. Variation in sperm displacement and its association with accessory gland protein loci in *Drosophila melanogaster*. *Genetics*, **139**, 189-201.

Clutton-Brock, T. and Langley, P. 1997. Persistent courtship reduces male and female longevity in captive tsetse flies *Glossina morsitans morsitans* Westwood (Diptera: Glossinidae). *Behavioural Ecology*, **8**, 392-395.

Clutton-Brock, T. H. and Parker, G. A. 1995a. Punishment in animal societies. *Nature*, **373**, 209-216.

Clutton-Brock, T. H. and Parker, G. A. 1995b. Sexual coercion in animal societies. *Animal Behaviour*, **49**, 1345-1365.

Clutton-Brock, T. H., Price, O. F. and MacColl, A. 1992. Mate-retention, harassment and the evolution of ungulate leks. *Behavioural Ecology*, **3**, 234-242.

Colegrave, N. 1995. The evolution of larval competitive strategies in *C. maculatus* and *C. analis*. PhD Thesis, University of Sheffield.

Credland, P. F., Dick, K. M. and Wright, A. W. 1986. Relationship between larval density, adult size and egg production in the cowpea seed beetle, *Callosobruchus maculatus*. *Ecological Entomology*, **11**, 41-50.

Credland, P. F. and Wright, A. W. 1989. Factors affecting female fecundity in the cowpea seed beetle, *Callosobruchus maculatus* (Coleoptera: Bruchidae). *Journal of Stored Products Research*, **25**, 125-136.

Crudgington, H. S. and Siva-Jothy, M. T. 2000. Genital damage, kicking and early death. *Nature*, **407**, 855-856.

Daly, M. 1978. The cost of mating. *American Naturalist*, 771-774.

Darwin, C. 1859. On the Origin of Species by Means of Natural Selection. London: Murray.

Darwin, C. 1871. The Descent of Man, and Selection in Relation to Sex. Princeton, N.J.: Princeton University Press.

Das, A. K., Huignard, J., Barbier, M. and Quesneau-Thierry, A. 1980. Isolation of the two paragonial substances deposited into the spermatophores of *Acanthoscelides objectus* (Coleoptera, Bruchidae). *Experientia,* **36,** 918-920.

Dworkin, A. 1974. Woman Hating. New York: Dutton.

Eady, P. E. 1991a. Sperm competition in *Callosobruchus maculatus* (Coleoptera: Bruchidae): a comparison of two methods used to assess paternity. *Ecological Entomology,* **16,** 45-53.

Eady, P. E. 1991b. Sperm competition in *Callososbruchus maculatus.* PhD thesis, University of Sheffield.

Eady, P. E. 1994a. Intraspecific variation in sperm precedence in the bruchid beetle *Callosobruchus maculatus. Ecological Entomology,* **19,** 11-16.

Eady, P. E. 1994b. Sperm transfer and storage in relation to sperm competition in *Callosobruchus maculatus. Behavioural Ecology and Sociobiology,* **35,** 123-129.

Eady, P. E. 1995. Why do male *Callosobruchus maculatus* beetles inseminate so many sperm? *Behavioral Ecology and Sociobiology,* **36,** 25-32.

Eady, P. E. and Tubman, S. 1996. Last-male sperm precedence does not break down when females mate with three males. *Ecological Entomology,* **21,** 303-304.

Eberhard, W. G. 1994. Evidence for widespread courtship during copulation in 131 species of insects and spiders, and implications for cryptic female choice. *Evolution,* **48,** 711-733.

Eberhard, W. G. 1985. Sexual Selection and Animal Genitalia. Cambridge, Mass.: Harvard University Press.

Eberhard, W. G. 1991. Copulatory courtship and cryptic female choice in insects. *Biological Reviews of the Cambridge Philosophical Society,* **66,** 1-31.

Eberhard, W. G. 1993. Copulatory courtship and genital mechanics of 3 species of *Macrodactylus* (Coleoptera Scarabaeidae Melolonthinae). *Ethology, Ecology and Evolution,* **5,** 19-63.

Eberhard, W. G. 1994. Evidence for widespread courtship during copulation in 131 species of insects and spiders, and implications for cryptic female choice. *Evolution,* **48,** 711-733.

Eberhard, W. G. 1996. Female Control: Sexual Selection by Cryptic Female Choice. Princeton, New Jersey: Princeton University Press.

Eberhard, W. G. and Cordero, C. 1995. Sexual selection and cryptic female choice on male seminal products - a new bridge between sexual selection and reproductive physiology. *Trends in Ecology and Evolution,* **10,** 493-496.

Edvardsson, M. and Arnqvist, G. 2000. Copulatory courtship and cryptic female choice in red flour beetles *Tribolium castaneum. Poceedings of the Royal Society of London Series B,* **267,** 559-563.

Emlen, S. and Oring, L. 1977. Ecology, sexual selection, and the evolution of mating systems. *Science,* **197,** 215-223.

Endler, J. A. 1986. Defense against predators. In: *Predator-prey relationships: Perspectives and Approaches form the Study of Lower Invertebrates.* (Ed. by M. E. Federand G. V. Lauder). Chicago: University of Chicago Press.

Evans, J. P. and Magurran, A. E. 2000. Multiple benefits of multiple mating in guppies. *Proceedings of the National Academy of Sciences USA,* **97,** 10074-10076.

Ewer, R. F. 1973. The Carnivores. Ithica, New York: Cornell University Press.

Fairburn, D. J. 1993. The costs of loading associated with mate-carrying in the water-strider *Aquarius remigis. Behavioural Ecology,* **4,** 224-231.

Fincke, O. M. 1986. Underwater oviposition in the damselfly (Odonata: Coenagrionidae) favours male vigilance, and multiple mating by females. *Behavioural Ecology and Sociobiology,* **18,** 405-412.

Fowler, K. and Partridge, L. 1989. A cost of mating in female fruit-flies. *Nature,* **338,** 760-761.

Fox, C. W. 1993. Multiple mating, lifetime fecundity and female mortality of the bruchid beetle, *Callosobruchus maculatus* (Coleoptera: Bruchidae). *Functional Ecology,* **7,** 203-208.

Fox, C. W. and Hickman, D. L. 1994. Influence of oviposition substrate on female receptivity to multiple mating in *Callosobruchus maculatus* (Coleoptera: Bruchidae). *Behavior,* **87,** 395-398.

Gavrilets, S. 2000. Rapid evolution of reproductive barriers driven by sexual conflict. *Nature,* **403,** 886-889.

Gems, D. and Riddle, D. L. 1996. Longevity in *Caenorhabditis elegans* reduced by mating but not gamete production. *Nature,* **379,** 723-725.

Gerber, G. H., Church, N. S. and Rempel, J. G. 1971. The anatomy, histology, and physiology of the reproductive systems of *Lytta nuttalli* Say (Coleoptera: Meloidae). I. The internal genitalia. *Canadian Journal of Zoology*, **49**, 253-533.

Giga, D. P. and Smith, R. H. 1983. Comparative life history studies of four *Callosobruchus* species infesting cowpeas with special reference to *Callosobrucus rhodesianus* (Pic.) (Coleoptera: Bruchidae). *Journal of stored Product Research*, **19**, 189-198.

Gillespie, J., Kanost, M. and Trenczek, T. 1997. Biological mediators of insect immunity. *Annual Review of Entomology*, **42**, 611-643.

Goodall, J. 1986. The Chimpanzees of the Gombe. Massachusetts: Belknap Press.

Greer, G. 1970. The Female Eunuch. London: MacGibbon and Kee.

Gupta, A. 1966. External genitalia of the Meloidae (Coleoptera). I. The genitalia and their musculature in *Lytta vulnerata*. *Annals of the Entomological Society of America.*, **59**, 736-746.

Gwynne, D. T. 1984. Courtship feeding increases female reproductive success in bushcrickets. *Nature*, **307**, 361-363.

Halliday, T. and Arnold, S. J. 1987. Multiple mating by females: a perspective from quantitative genetics. *Animal Behaviour*, **35**, 939 - 941.

Hamilton, W. D. 1964. The genetical evolution of social behaviour. *Journal of Theoretical Biology*, **7**, 1-52.

Hamilton, W. D. 1970. Selfish and spiteful behaviour in an evolutionary model. *Nature*, **228**, 1218-1220.

Hammerstein, P. and Parker, G. A. 1982. The asymmetric war of attrition. *Journal of Theoretical Biology*, **96**, 647-682.

Harshman, L. G., Hoffmann, A. A. and Prout, T. 1988. Environmental effects on remating in *Drosophila melanogaster*. *Evolution*, **42**, 312-321.

Harshman, L. G. and Prout, T. 1994. Sperm displacement without sperm transfer in *Drosphila melanogaster*. *Evolution*, **48**, 758-766

Heifetz, Y., Frongillo, E. A. and Wolfner, M. F. 2000. The *Drosophila* seminal fluid protein Acp26Aa stimualtes release of oocytes by the ovary. *Current Biology*, **10**, 99-102.

Higashi, M. and Yamamura, N. 1994. Resolution of evolutionary conflict: a general theory and its applications. *Research Population Ecology*, **36**, 15-22.

Hinton, H. E. 1964. Sperm transfer in insects and the evolution of haemocoelic insemination. *Symposium of the Royal Entomological Society of London*, 2 (Insect Reproduction).

Holland, B. and Rice, W. R. 1998. Perspective: Chase-away sexual selection: antagonistic seduction versus resistance. *Evolution*, **52**, 1-7.

Holland, B. and Rice, W. R. 1999. Experimental removal of sexual selection reverses intersexual antagonistic coevolution and removes a reproductive load. *Proceedings of the National Academy of Sciences USA*, **96**, 5083-5088.

Hurst, G. D. D., Sharpe, R., Broomfield, A. H., Walker, L. E., Majerus, T. M. O., Zakharov, I. and Majerus, M. E. N. 1995. Sexually-transmitted disease in a promiscuous insect, *Adalia bipunctata*. *Ecological Entomology*, **20**, 230-236.

Jackson, R. R. and Pollard, S. D. 1997. Jumping spider mating strategies: sex among cannibals in and out of webs. In: *The Evolution of Mating Systems in Insects and Arachnids*. (Ed. by J. C. Choe and B. J. Crespi). Cambridge: Cambridge University Press.

Johnstone, R. A. and Keller, L. 2000. How males can gain by harming their mates: sexual conflict, seminal toxins, and the cost of mating. *American Naturalist*, **156**, 368-377.

Kadow, P. E. 1954. An analysis of sexual behavior and reproductive physiology in the guppy, *Lebistes reticulatus* (Peters). New York University.

Kempenaers, B., Foerster, K., Questiau, S., Rpbertson, B. C. and Vermeirssen, E. L. M. 2000. Distinguishing between female sperm choice versus male sperm competition: a comment on Birkhead. *Evolution*, **54**, 1050-1052.

Kennedy, J. S. 1992. The New Anthropomorphism. Cambridge: Cambridge University Press.

Klowden, M. J. 1999. The check is in the male: male mosquitoes affect female physiology and behavior. *Jounal of the American Mosquito Control Association.*, **15**, 213-220.

Konig, C. and Schmid-Hempel, P. 1995. Foraging activity and immunocompetence in workers of the bumble bee, (*Bombus terrestris* L.). *Proceedings of the Royal Society of London Series B*, **260**, 225-227.

Krupa, J. J. and Sih, A. 1993. Experimental studies on water strider mating dynamics: spatial variation in density and sex ratio. *Behavioural Ecology and Sociobiology.*, **33,** 107-120.

Lackie, A. M., Takle, G. and Tetley, L. (1985). Haemocytic encapsulation in the locust *Schistocerca gregaria* (Orthoptera) and in the cockroach *Periplaneta americana. Cell Tissue Res.* 240, 343-351.

Larson, A. O. and Fisher, C. K. 1938. The bean weevil and the southern cowpea weevil in California. *Technical Bulletin of the U.S. Department of Agriculture,* **593,** 1-70.

Le Boeuf, B. J. and Mesnick, S. 1990. Sexual behaviour of male northern elephant seals: I. Lethal injuries to adult males. *Behaviour,* **116,** 143-162.

Leopold, R., Terranova, A., Thorson, B. and Degrugillier, M. 1971. The biosynthesis of the male housefly accessory secretion and its fate in the mated female. *Journal of Insect Physiology,* **17,** 987-1003.

Leopold, R. A. 1976. The role of male accessory glands in insect reproduction. *Annual Review of Entomology,* **21,** 199-221.

Lessells, C. M. and Boag, P. T. 1987. Unrepeatable repeatabilities: a common mistake. *Auk,* **104,** 116-121.

Lessells, C. A. 1999. Sexual conflict in animals. In: *Levels of Selection in Evolution* (Ed. by L. Keller). Princeton: Princeton Universtiy Press.

Lewis, C. T. and Pollock, J. N. 1975. Engagement of the phallosome in blowflies. *Journal of Entomology (A),* **49,** 137-147.

Linley, J. R. and Adams, G. M. 1972. A study of the mating behaviour of *Culicoides melleus* (Coquillett) (Diptera: Ceratoponidae). *Transactions of the Royal Entomological Society of London,* **124,** 81-121.

Linley, J. R. and Adams, G. M. 1975. Sexual receptivity in *Culicoides melleus* (Diptera: Ceratopogonidae). *Transactions of the Royal Entomological Society of London,* **126,** 279-303.

Linley, J. R. and Hinds, M. J. 1975. Quantity of the male ejaculate influenced by female unreceptivity in the fly *Culicoides melleus. Journal of Insect Physiology,* **21,** 281-285.

Linley, J. R. and Mook, M. S. 1975. Behavioural interaction between sexually experienced *Culicoides melleus* (Coquillett) (Diptera: Ceratoponidae). *Behaviour,*

Lockhart, A. B., Thrall, P. H. and Antonovics, J. 1996. Sexually transmitted diseases in animals: ecological and evolutionary implications. *Biological Reviews of the Cambridge Philosophical Society*, **71**, 415-471.

Loman, J., Madsen, T. and Hakansson, T. 1988. Increased fitness from multiple matings, and genetic heterogeneity: a model of a possible mechanism. *Oikos*, **52**, 69-72.

Magnhagen, C. 1990. Predation risk as a cost of reproduction. *Trends in Ecology and Evolution*, **26**, 183-186.

Magurran, A. E. and Seghers, B. H. 1994. The costs of sexual harassment in the guppy, *Poecilia reticulata*. *Proceedings of the Royal Society of London. Series B*, **258**, 89-92.

Mangan, R. L. 1997. Effects of strain and access to males on female longevity, lifetime oviposition rate and egg fertility of the Mexican fruit fly (Diptera: Tephritidae). *Ecology and Behavior*, **90**, 944-954.

Markow, T. A., Gallagher, P. D. and Krebs, R. A. 1990. Ejaculate derived nutritional contribution and female reproductive success in *Drosophila mojavensis* (Patterson and Crow). *Functional Ecology*, **4**, 67-73.

Mbata, G. N., Ramaswamy, S. B. and Reichmuth, C. 1998. Comparative effect of short term exposures of *Callosobruchus subbinotatus* to carbon dioxide, nitrogen , or low temperature on behavior and fecundity. *Entomologica Experientalis et Applicata.*, **89**, 243-248.

Mbata, G. N., Shu, S. and Ramaswamy, S. B. 1997. Rhythmicity of mating and oviposition in *Callosobruchus subinnotatus* (Pic) (Coleoptera: Brucidae). *Journal of Insect Behaviour*, **10**, 409-423.

McComb, K. and Clutton-Brock, T. 1994. Is mate choice copying or aggregation responsible for skewed distributions of females on leks? *Proceedings of the Royal Society of London. Series B*, **255**, 13-19.

McKinney, F., Derrickson, S. R. and Mineau, P. 1983. Forced copulation in waterfowl. *Behaviour*, **86**, 250-294.

Madsen, T., Shine, R., Loman, J. and Hakansson, T. 1992. Why do female adders copulate so frequently? *Nature*, **355**, 440-441.

McLain, D. K. and Pratt, A. E. 1999. The cost of sexual coercion and heterospecific sexual harassment on the fecundity of a host-specific, seed-eating insect (*Neacoryphus bicrucis*). *Behavioral Ecology and Sociobiology*, **46**, 164-170.

Merritt, D. J. 1989. The morphology of the phallosome and accessory gland material transfer during copulation in the blowfly, *Lucilia cuprina* (Insecta, Diptera). *Zoomorpholgy*, **108**,

Mesnick, S. and Le Boeuf, B. J. 1991. Sexual behaviour of male northern elephant seals: II. Female response to potentially injurious encounters. *Behaviour*, **117**, 262-280.

Michiels, N. K. and Newman, L. J. 1998. Sex and violence in hermaphrodites. *Nature*, **391**, 647.

Millett, K. 1971. Sexual Politics. London: Hart-Davis.

Nappi, A. J. and Vass, E. 1993. Melanogenesis and the generation of cytotoxic molecules during insect cellular immune reactions. *Pigment Cell Research*, **6**, 117-126.

Nayar, J. K. and Knight, J. W. 1995. Wounding increases intracellular encapsulation (melanization) of developing *Brugia malayi* (Nematoda: Filarioidea) larvae in thoracic muscles of *Anopheles quadrimaculatus*. *Comparative Biochemistry and Physiology*, **112**, 553-557.

Newport, M. and Gromko, M. A. 1984. The effect of experimental design on female receptivity to remating and its impact on reproductive success in *Drosophila melanogaster*. *Evolution*, **38**, 1261-1272.

Odentaal, F. J., Turchin, P. and Stermitz, F. R. 1989. Influence of host-plant density and male harassment on the distribution of female *Euphydryas anicia* (Nymphalisae). *Oecologia*, **78**, 283-288.

Olsson, M. and Shine, R. 1997. Advantages of multiple matings to females: a test of the infertility hypothesis using lizards. *Evolution*, **51**, 1684-1688.

Otronen, M. and Siva-Jothy, M. T. 1991. The effect of postcopulatory male behaviour on ejaculate distribution within the female sperm storage organs of the fly, *Dryomyza anilis* (Diptera: Dryomyzudae). *Behaviorial Ecology and Sociobiology*, **29**, 33-37.

Parker, G. A. 1970a. Sperm Competition and its evolutionary consequences in the insects. *Biological Reviews*, **45**, 525-567.

Parker, G. A. 1970b. The reproductive behaviour and the nature of sexual selection in *Scatophaga stercoraria* L. (Diptera: Scatophagidae). II. The fertilization rate and the spatial and temporal relationships of each sex around the site of mating and oviposition. *Journal of Animal Ecology*, **39**, 205-228.

Parker, G. A. 1979. Sexual Selection and sexual conflict. In: *Sexual Selection and Reproductive Competition in Insects* (Ed. by M. S. Blum and N. A. Blum). London: Academic Press.

Parker, G. A. 1984. Sperm competition and the evolution of animal mating strategies. In: *Sperm competition and the evolution of animal mating systems* (Ed. by R. L. Smith), London: Academic Press.

Parker, G. A. 1998. Sperm competition and the evolution of ejaculates: towards a theory base. In: *Sperm Competition and Sexual Selection* (Ed. by T. R. Birkhead and A. Møller). London: Academic Press.

Parker, G. A., Baker, R. R. and Smith, V. G. F. 1972. The origin and evolution of gamete dimorphism and the male-female phenomenon. *Journal of Theoretical Biology*, **36,** 529-553.

Parker, G. A. and Partridge, L. 1998. Sexual conflict and speciation. *Philosophical Transactions of the Royal Society of London. Series B,* **353,** 261-274.

Partridge, L. and Harvey, P. H. 1985. Costs of reproduction. *Nature,* **316,** 20.

Pitnick, S. and Brown, W. D. 2000. Criteria for demonstrating female sperm choice. *Evolution,* **54,** 1052-1056.

Pizzari, T. and Birkhead, T. R. 2000. Female feral fowl eject sperm of subordinate males. *Nature,* **405,** 787-789.

Poulin, R. 1998. Evolutionary Ecology of Parasites: From Individuals to Communities. London: Chapman and Hall.

Pratt, H. 1979. Reproduction in the blue shark, *Prionace glauca. Fishery Bulletin,* **77,** 445-470.

Price, C. S. C., Dyer, K. A. and Coyne, J. A. 1999. Sperm competition between *Drospohila* males involves both displacement and incapacitation. *Nature,* **400,** 449-452.

Prout, T. and Clark, A. G. 2000. Seminal fluid causes temporarily reduced egg hatch in previously mated females. *Proceedings of the Royal Society of London Series B,* **267,** 201-203.

Qi, Y. and Burkholder, W. E. 1982. Sex pheromone biology and behaviour of the cowpea weevil *Callosobruchus maculatus* (Coleoptera: Bruchidae). *Journal of Chemical Ecology,* **8,** 527-534.

Reale, D., Bousses, P. and Chapuis, J. 1996. Female-biased mortality induced by male sexual harassment in a feral sheep population. *Canadian Jouranl of Zoology,* **74,** 1812-1818.

Reimann, J. G. and Moen, D. J. 1967. Female monogamy and its control in houseflies. *Journal of Insect Physiology,* **13,** 407-418.

Rice, W. R. 1996. Sexually antagonistic male adaptation triggered by experimental arrest of female evolution. *Nature,* **381,** 232-234.

Ridley, M. 1988. Mating frequency and fecundity in insects. *Biological Reviews of the Cambridge Philosophical Society,* **63,** 509-549.

Ridshill Smith, T. J. 1970. The behaviour of *Hemithynnus hyalinatus* (Hymenoptera: Tiphiidae), with notes on some other Thynninae. *Journal of the Australian Entomological Society,* **9,** 196-208.

Ringo, J. 1996. Sexual receptivity in insects. *Annual Review of Entomology,* **41,** 473-494.

Rodriguez, R. 1998. Possible female choice during copulation in *Ozophora baranowskii* (Heteroptera: Lygaeidae): female behaviour, multiple copulations, and sperm transfer. *Journal of Insect Behaviour,* **11,** 725-739.

Rohlf, F. J. and Sokal, R. R. 1995. Statistical Tables. Third edition. W. H. Freeman, New York.

Rowe, L. 1994. The costs of mating and mate choice in water striders. *Animal Behaviour,* **48,** 1049-1056.

Rowe, L., Arnqvist, G., Sih, A. and Krupa, J. 1994. Sexual conflict and the evolutionary ecology of mating patterns - water striders as a model system. *Trends in Ecology and Evolution,* **9,** 289-293.

Rup, P. J. 1986. Mating and its attendant behaviour in *Callosobruchus maculatus* (F.) (Coleoptera: Bruchidae). *Journal of Stored Products Research,* **22,** 77-79.

Ryan, M. J. 1990. Sexual selection, sensory systems and sensory exploitation. *Oxford Surveys in Evolutionary Biology,* **7,** 157-195.

Sakaluk, S. K., Bangert, P. J., Eggert, A. K., Gack, C. and Swanson, L. V. 1995. The gin trap as a device facilitating coercive mating in sagebrush crickets. *Proceedings of the Royal Society of London Series B,* **261,** 65-71.

Savalli, U. M. and Fox, C. W. 1999. The effect of male mating history on paternal investment, fecundity and female remating in the seed beetle, *Callosobruchus maculatus. Functional Ecology,* **13,** 169-177.

Shu, S., Koepnick, W. L., Mbata, G. N., Cork, A. and Ramaswamy, S. N. 1996. Sex pheromone production in *Callosobruchus maculatus* (Coleoptera: Bruchidae):

electoantennographic and behavioral responses. *Journal of Stored Product Research,* **32,** 21-30.

Simmons, L. W. and Siva-Jothy, M. T. 1998. Sperm competition in insects: mechanisms and the potential for selection. In: *Sperm Competition and Sexual Selection* (Ed. by T. R. Birkheadand A. P. Møller), pp. London: Academic Press.

Siva-Jothy, M. T., Tsubaki, Y. and Hooper, R. E. 1998. Decreased immune response as a proximate cost of copulation and oviposition in a damselfly. *Physiological Entomology,* **23,** 274-277.

Smith, P. H., Barton Browne, L. and van Gerwen, A. C. M. 1989. Causes and correlates of loss and recovery of sexual receptivity in *Lucilia cuprina* females after their first mating. *Journal of Insect Behavior,* **2,** 325-337.

Smith, R. H. and Lessells, C. M. 1985. Oviposition, ovicide, and larval competition in granivorous insects. In: *Behavioural Ecology: Ecological Consequences of Adaptive Behaviour.* (Ed. by R. M. Sibly and R. H. Smith). Oxford.: Blackwells.

Smith, R. L. 1984. Sperm competition and the evolution of animal mating systems. Orlando: Academic Press Inc.

Smuts, B. B. and Smuts, R. W. 1993. Male aggression and sexual coercion of females in nonhuman primates and other mammals: evidence and theoretical implications. *Advances in the Study of Behaviour,* **22,** 1-63.

Sokal, R. R. and Rohlf, F. J. 1997. Biometry. Third Edition. New York: Freeman.

Southgate, B. J. 1978. The importance of the Bruchidae as pests of grain legumes, their distribution and control. In: *Pests of grain legumes: Ecology and Control.* (Ed. by S. R. Singh and H. F. van Emden). London: Academic Press.

Southgate, B. J. 1979. Biology of the Bruchidae. *Annual Review of Entomology,* **24,** 449-473.

Southgate, B. J., Howe, R. W. and Brett, G. A. 1957. The specific status of *Callosobruchus maculatus* (F.) and *Callosobruchus analis* (F.). *Bulletin of Entomological Research,* **48,** 79-89.

Stearns, S. C. 1992. The Evolution of Life Histories. Oxford: Oxford University Press.

Stockley, P. 1997. Sexual conflict resulting from adaptations to sperm competition. *Trends in Ecology and Evolution,* **12,** 154-159.

Stone, G. N. 1995. Female foraging responses to harassment in the solitary bee *Anthophora plumipes. Animal Behaviour,* **50,** 405-412.

Stutt, A. D. and Siva-Jothy, M. T. 2001. Traumatic insemination and sexual conflict in the bed-bug *Cimex lectularius*. *Proceedings of the National Academy of Sciences USA*, **in press.**

Tadler, A. 1999. Selection of a conspicuous male genitalic trait in the seedbug *Lygaeus simulans*. *Proceedings of the Royal Society of London Series B*, **266,** 1773-1777.

Thornhill, R. 1980. Rape in *Panorpa* scorpionflies and a general rape hypothesis. *Animal Behaviour*, **28,** 52-59.

Thornhill, R. 1983. Cryptic female choice and its implications in the scorpionfly *Harpobittacus nigriceps*. *American Naturalist*, **122,** 765-788.

Thornhill, R. and Alcock, J. 1983. The Evolution of Insect Mating Systems. Cambridge, Massachusetts: Harvard University Press.

Thornhill, R. and Sauer, K. P. 1991. The notal organ of the scorpionfly (*Panorpa vulgaris*): an adaptation to coerce mating duration. *Behavioral Ecology*, **2,** 156-164.

Thrall, P. H., Antonovics, J. and Dobson, A. P. 2000. Sexually transmitted diseases in polygynous mating systems: prevalence and impact on reproductive success. *Proceedings of the Royal Society of London Series B*, **267,** 1555-1563.

Tregenza, T., Butlin, R. K. and Wedell, N. 2000. Sexual conflict and speciation. *Nature*, **407,** 149-150.

Trivers, R. L. 1972. Parental investment and sexual selection. In: *Sexual Selection and the Descent of Man* (Ed. by B. Campbell). Chicago: Chicago.

Trivers, R. H. 1974. Parent-offspring conflict. *American Zoologist*, **14,** 249-264.

Trivers, R. L. and Hare, H. 1976. Haplo-diploidy and the evolution of the social insects. *Science*, **191,** 249-263.

Tsubaki, Y., Siva-Jothy, M. T. and T, O. 1994. Re-copulation and post-copulatory mate guarding increases immediate female reproductive output in the dragonfly *Nannophya pygmaea*. *Behavioural Ecology and Sociobiology*, **35,** 219-225.

Tufton, T. 1993. The cost of reproduction in *Callosobruchus maculatus*. PhD Thesis, University of Sheffield.

Vahed, K. 1998. The function of nuptial feeding in insects: a review of empirical studies. *Biological Reviews of the Cambridge Philosophical Society*, **73,** 43-78.

van der Meer, J. M. 1979. The specification of metameric order in the insect *Callsosbruchs maculatus* Fabr. (Coleoptera). *Journal of Embryology and Experimental Morphology*, **51**, 1-26.

von Helversen, D. and von Helversen, O. 1991. Pre-mating sperm removal in the bushcricket *Metaplastes ornatus* Ramme 1931 (Orthoptera, Tettigonoidea, Phaneropteridae). *Behavioral Ecology and Sociobiology*, **28**, 391-396.

Waage, J. K. 1979a. Dual function of the damselfly penis: sperm removal and transfer. *Science*, **203**, 916-918.

Waage, J. K. 1979b. Adaptive significance of postcopulatory guarding of mates and nonmates by male *Calopteryx maculata* (Odonata). *Behavioural Ecology and Sociobiology*, **6**, 147-154.

Walker, W. F. 1980. Sperm utilization strategies in non-social insects. *American Naturalist*, **115**, 780-799.

Watson, P. J., Arnqvist, G. and Stallmann, R. R. 1998. Sexual conflict and the energetic costs of mating and mate choice in water striders. *American Naturalist*, **151**, 46-58.

Westneat, D. F. and Sargent, R. C. 1996. Sex and parenting: the effects of sexual conflict on parental strategies. *Trends in Ecology and Evolution*, **11**, 87-91.

Williams, G. C. 1966. Adaptation and Natural Selection. Princeton: Princeton University Press.

Wilson, K. 1989. Evolution of oviposition behaviour in *Callosobruchus maculatus*. PhD thesis, University of Sheffield.

Wilson, K. and Hill, L. 1989. Factors affecting egg maturation in the bean weevil *Callosobruchus maculatus*. *Physiological Entomology*, **14**, 115-126.

Wilson, N., Tufton, T. J. and Eady, P. E. 1999. The effect of single, double and triple matings on the lifetime fecundity of *Callosobruchus analis* and *Callosobruchus maculatus* (Coleoptera: Bruchidae). *Journal of Insect Behavior*, **12**, 295-307.

Wolfner, M. F. 1997. Tokens of love: functions and regulation of *Drosophila* male accessory gland products. *Insect Biochemistry and Molecular Biology*, **27**, 179-192.

Zeh, J. A. & Zeh, D. W. 1996. The evolution of polyandry I: Intragenomic conflict and genetic incompatibility. *Proceedings of the Royal Society of London. Series B*, **263**, 1711-1717.

Zeh, J. A. & Zeh, D. W. 1997. The evolution of polyandry II: Post-copulatory defences against genetic incompatability. *Proceedings of the Royal Society of London. Series B,* **264,** 69-75..