Reproductive strategies and sexual conflict in the bed bug *Cimex lectularius*

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To Mum, Dad and Angela

Acknowledgements

Here is my final piece of writing to go at the start of this thesis and I have been warned that it is the most difficult thing to write. I am beginning to realise... My fading memory of the last three years with so many people met along the way means I will have overlooked some, if so I am sorry.

If I am to start anywhere it must begin with the lab. My development in Sheffield is perhaps as much down to the members of sclab than any other factor. In the early days Stu Plaistow both scared and inspired me into reading as many papers as I could manage, and with David Blake always lightened the mood when things looked grim. Jill Thompson showed me how to research sperm competition properly, and settled me into the lab with forays to Club Capri. Simon Forrest made many an arid comment to keep a smile on my face. Alex Cordoba-Aguilar dazzled me and many others with his fantastic genitalia. Jonny Ryder stands as a testament to the thoughtful, rigorous approach to any problem. Helen Crudgington, my colleague in conflict and after a free transfer, Andy Barnes (the Statler to my Waldorf) both guided me through many a semantic argument. All have given me both encouragement and a shove in the right direction over the years. John Thompson and Sophie Armitage were late arrivals making me feel old with memories of me as a demonstrator. Nick, José, Paco, Piedad and Jenny have all passed through the lab over the years and brought new diverse ideas with them. A better place to work I cannot imagine.

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Summary

Reproductive strategies and sexual conflict in the bed bug *Cimex lectularius*

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In this thesis I examine the reproductive strategies of the bed bug *Cimex lectularius*, a traumatically inseminating insect. In Chapter 2 I examine the mating behaviour of *C. lectularius*, including the mating rates of males and females. Remating rates were very high, with females mating with 5 different males during a single reproductive bout. Males copulated for longer with virgin females than non-virgins. Sperm competition was predicted to be an important determinant of male reproductive success, because the ejaculates of an average of 5 males will be concurrent in the female's reproductive tract during a reproductive bout. In Chapter 3 the different gamete allocation strategies used by males were examined. Males allocated more sperm to virgin females than to non-virgins. Sperm migration and storage by females was examined in order to provide a basis from which mechanisms of sperm competition could be predicted. In Chapter 4 the patterns of sperm precedence were examined and a hypothetical mechanism of sperm competition was tested experimentally. Sperm precedence appears to favour the last male to mate due to a positional effect in the spermalege of the last ejaculate inseminated. In Chapter 5 the effect of high mating rates on females was assessed experimentally. Females mating at a high rate were found to die earlier than females mating at an artificially low rate. There was no difference in the rate of egg production of females between these two groups, so females mating at a low rate had a higher lifetime reproductive success. Appendix I investigates the potential benefits females may gain from polyandry. Both direct benefits of mating and a suite of possible fitness traits were assessed. However, no detectable differences in number or quality of offspring were uncovered. Chapter 6 reviews the evidence for a conflict of interest between the sexes over the remating rate and the possible causes of this conflict.

Contents

1 General Introduction

1.1. Sexual selection: an introduction

When Darwin published the Origin of Species (1859) he realised that his proposed mechanism of evolution through natural selection could not explain many of the traits he had observed in the natural world. The peacock's train, for instance, is clearly a hindrance to the survival of the males that bear it. To explain such extravagant features (usually found in males) Darwin (1871) proposed the theory of sexual selection which he defined as: 'the advantages that certain individuals have over others of the same sex and species in exclusive relation to reproduction'. Darwin (1871) suggested that sexual selection could operate through two different pathways. Firstly, competition between members of one sex for access to members of the other. Secondly, one sex could choose members of the other in terms of attractiveness. Since sexual selection usually acts more strongly on males than females (Bateman, 1948), males usually display weapons used for competing with other males, or the epigamic traits that females use to choose between males (Andersson 1994; Clutton-Brock & Parker, 1992; Trivers, 1972).

1.1.1. Post-copulatory sexual selection: sperm competition and cryptic female choice

Darwin (1871) assumed that sexual selection operated up to the point of copulation, and that females were generally sexually monogamous. Consequently the more females a male could copulate with, the more offspring he would father. Parker (1970a; 1970b) was the first to suggest, and demonstrate, that sexual selection could continue after copulation if the sperm of more than one male competed in a female's reproductive tract to fertilise her ova. This is a post-copulatory extension of male-male competition and is known as sperm

competition. More controversially, it has recently been suggested that post-copulatory female choice could operate if females can influence the outcome of sperm competition. The myriad of proposed mechanisms of post-copulatory female effects have been called cryptic female choice (Thornhill 1983; Eberhard 1996). The fact that female sexual monogamy is now considered the exception, rather than the rule, in animal mating systems suggests that post-copulatory sexual selection may play an important role in the fitness of many animals (see Birkhead & Møller, 1998 for review).

The relative strength of selection caused by sperm competition and cryptic female choice is widely debated (Parker, 1984; Parker 1998; Eberhard, 1998). The asymmetry in the strength of sexual selection between males and females is caused by the different reproductive rate of males and females. In his classic study of *Drosophila melanogaster*, Bateman (1948) demonstrated that male reproductive success is limited by the number of females inseminated, whereas females reproductive success is limited by the number of eggs she can produce.

1.1.2. Sexual conflict

Not only does sexual selection operate after copulation but it is also becoming clear that, during courtship and mating, conflicts of interest often occur between the sexes. The traditional view of mating behaviour in animals was that it was essentially a co-operative process where courtship aided the formation of the pair bond. However, over the past twenty years there has been a shift away from this view of benign sexual behaviour towards the concept that males and females are often in conflict (Trivers 1972, 1974; Parker, 1979, 1984: Hammerstein & Parker 1987; Alexander *et al.* 1997). Sexual conflict can be defined as: the evolutionary conflict that occurs between the sexes due to sexual reproduction. Conflict occurs because of the potentially different fitness optima for each sex. Various conflicts can occur, such as conflicts over isogamous and anisogamous reproduction

(Parker, 1972), copulation duration and mating frequency (Parker 1979; Arnqvist 1989, 1997), and relative parental investment (Trivers 1972, 1974). These conflicts can be costly and can result in injury or death to one partner (Birkhead & Møller 1992; Elgar, 1992; Jackson & Pollard, 1997).

Sexual conflicts often arise from adaptations in the male associated with sperm competition (Stockley, 1997). A well studied example of this phenomenon is sperm displacement in *Drosophila melanogaster*. Female *D. melanogaster* are polyandrous and their mates transfer proteins within their ejaculates which act to disable and kill the sperm of rival males. Male success in sperm competition is in part determined by the effectiveness of these proteins (Clark *et al*, 1995). However, a side-effect is that the proteins are toxic to females and so reduce female longevity and, consequently, fitness (Fowler & Partridge, 1989; Chapman *et al.*, 1995; but see Chapman & Partridge, 1996). Rice (1996) demonstrated that this conflict has produced a co-evolutionary arms race between the sexes with females adapting against traits that increase male reproductive success but are detrimental to female fitness. One dramatic and frequently cited example of an assumed sexual conflict is traumatic or extragenital insemination (Carayon, 1966; Thornhill & Alcock, 1983; Eberhard 1986, 1996; Hadrys & Siva-Jothy, 1994).

1.2. Extragenital Insemination

Extragenital insemination (insemination without using female genitalia) is rare but taxonomically widespread in the invertebrates. It has been reported in leeches (Mann, 1962), polychaete worms (Schaller, 1971; Schroeder & Hermans, 1975) at least two groups of molluscs (Purchon, 1977), giant squid (Norman & Lu, 1997), Sacoglossa (gastropoda) (Gascoigne, 1956; Purchon, 1977; Reid, 1964), rotifers (Thane, 1974), onychophorans (Manton, 1938; Schaller, 1971), cestodes (Hyman, 1951), gnathostomulid worms (Sterrer, 1974) and some turbellarian flatworms (Henley, 1974). Extragenital copulation has three main forms: 1. Dermal

copulation, where a spermatophore is attached to the external surface of the female (e.g. onychophorans; Manton, 1938; Schaller, 1971; Curach & Sunnocks, 1999). The spermatophore contains proteases which dissolve through the skin, the sperm are then released from the spermatophore and migrate to the site of fertilisation (Mann, 1984). 2. Hypodermic impregnation, where the previously deposited spermatozoa are injected through the body wall by a second organ that is not associated with the organs that produce the gametes (e.g. platyhelminthes; Lang 1884; Ax 1969; Ax & Apelt 1969; Ax and Borkott, 1969), annelida (Harmer, 1889) and the hirudinidae (Whitman, 1891). 3. Traumatic insemination where the intromittent organ pierces the body wall of the female and inseminates into the body cavity (e.g. cimicid bugs, Carayon, 1966).

1.3. Extragenital copulation in the insects

Extragenital copulation has been described in 2 orders of insects: the closely related heteropteran families of the Cimicidae, Nabidae, Anthocoridae and Plokiophelidae and several families within the order strepsiptera. The Cimicidae are by far the best studied family and the cimicid system is widely cited as an example of sexual conflict (e.g. Eberhard 1985; 1996; Thornhill and Alcock 1983).

Perhaps the most remarkable feature of the cimicids is that females have a complex system of organs which accept and store sperm following insemination. This system is termed the paragenital system and it has a separate embryological origin to the genital tract. Carayon (1966) defined it as; "all the morphological, anatomical, and histological differentiation's that are associated with traumatic insemination". There is great morphological diversity in the paragenital system of the cimicidae.

1.3.1. The Cimicidae

The cimicid bugs are heteropteran ectoparasites of vertebrates. The best known are the human bed bugs *Cimex lectularius* and *C. hemipterus*, however many other species exist and use birds and other mammals (mostly bats) as hosts. In this section I avoid all use of terminology that would infer ancestry in these species since the phylogeny of the Cimicidae is poorly understood and is primarily based on the details of the reproductive systems that are under discussion (Schuh & Stys, 1991).

1.3.2. *Primicimex*

The least differentiated of the cimicid genera is *Primicimex*. Males pierce the female's abdomen mid way up the left hand ventral tergites. The exact site of copulation is very variable and females often have many scars of copulation in different regions of the abdomen (Figure 1.1.a). The sperm storage organs are large sacs and, as with the other cimicids, are of mesodermal origin. The sperm migrate through the haemocoel to the seminal concepticles where the sperm are stored. Sperm migration in the haemocoel is prolonged and diffused compared to *Cimex* (Carayon, 1966).

1.3.3. *Bucimex*

In *Bucimex* the males only copulate into a single region of the females abdomen (this structure was named the ectospermalege in *Cimex lectularius* and is likely to be homologous in all the Cimicidae so that terminology will be used throughout this review (Carayon, 1966)(Figure 1.1.b). The ectospermalege is a cuticular invagination of the tergites which allows the male's intromittent organ access to the tissues below (Figure 1.2). Copulation only takes place at this site though it is unclear whether males are able to copulate elsewhere on the abdomen or whether they gain higher reproductive success by using the ectospermalege. The sperm are injected into a loose collection of cells known as the mesospermalege in

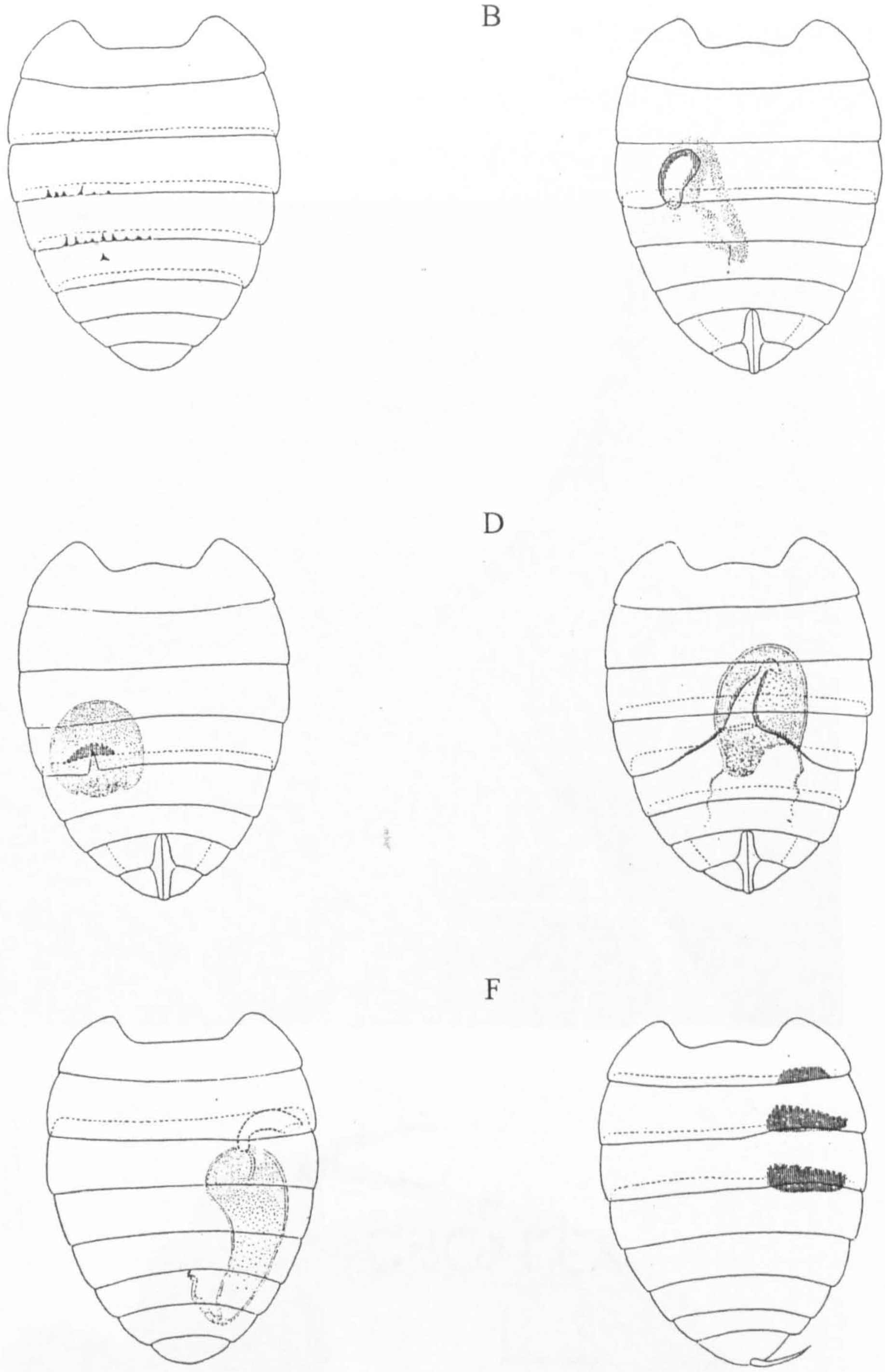
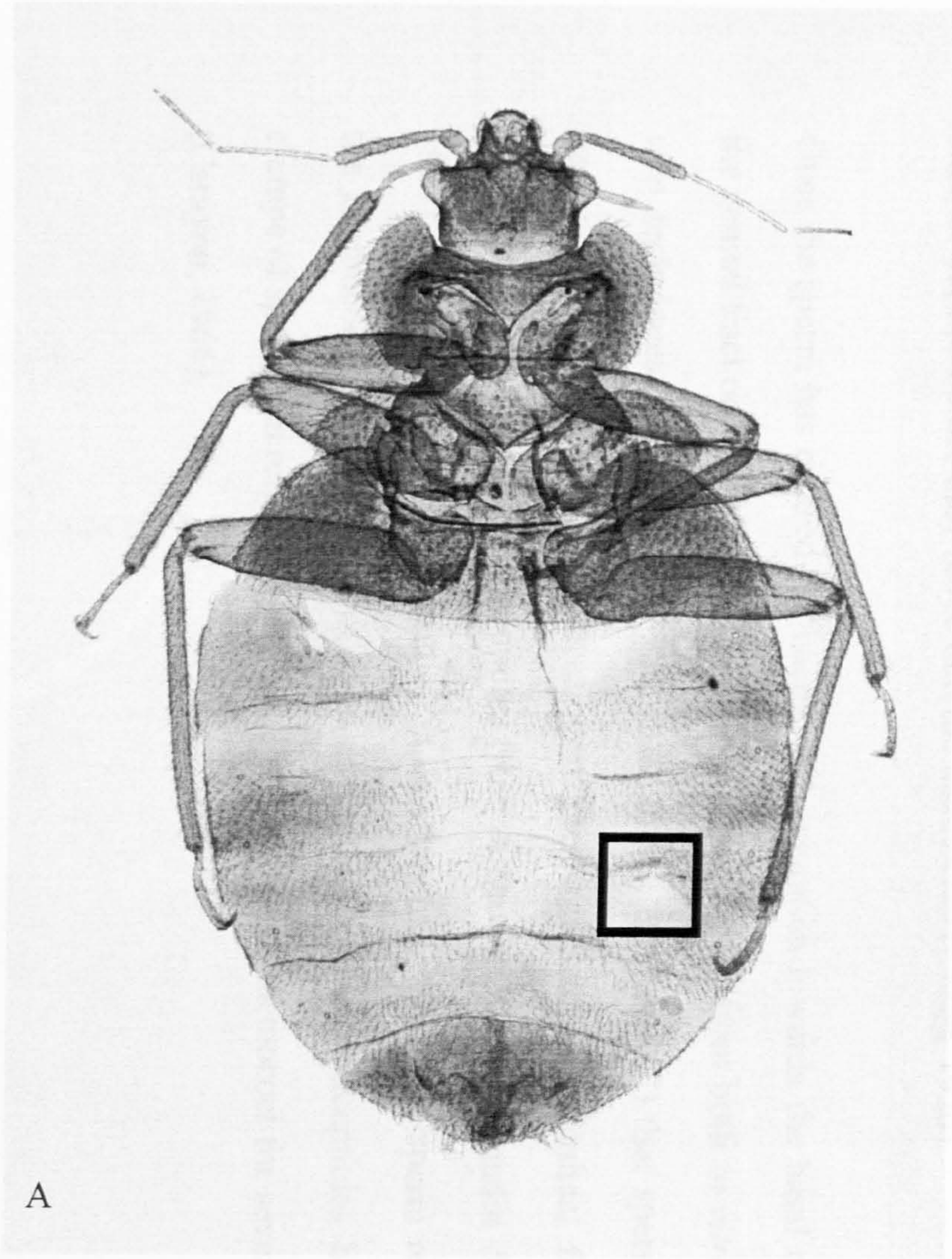


Figure 1.1. The position of the ecto- and meso-spermalege in several genera of the cimididae. A. *Primicimex* with many scars of copulation across the body surface. B. *Bucimex*. C. *Cimex*. D. *Paracimex*. E. *Stricticimex*. F. *Afrocimex* (male). (From Carayon, 1966).



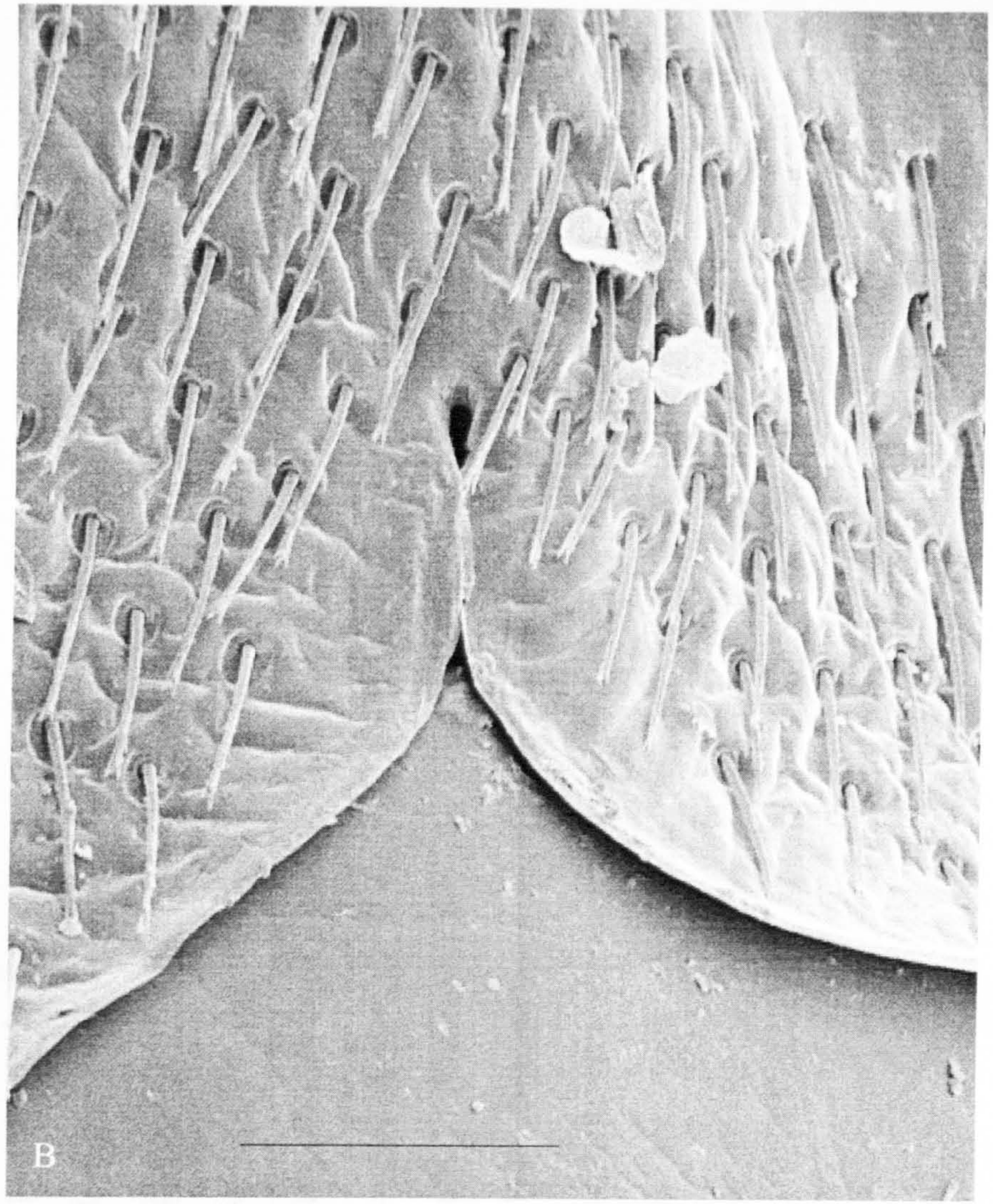


Figure 1.2. The position noted by the black box (A) and morphology (B) of the ecto-spermalege in *Cimex lectularius*

Bucimex, which has no membrane separating it from the haemocoel, and the sperm migrate into the haemocoel. Sperm migration in the haemocoel is again diffuse and prolonged when compared with migration in *Cimex* and the sperm enter the seminal concepticles through specialised lacunae.

1.3.4. *Cimex*

The best examples of extragenital copulation come from studies of the genus *Cimex*. Here an ectospermalege is present and is the only site used for insemination (Figure 1.1.c). During copulation in *C. lectularius* the male punctures the ectospermalege with his intermittent organ (a modified paramere (Figure 1.3) and injects sperm in a single mass into the mesospermalege (Figure 1.4). Copulation takes between 1 and 5 minutes. The sperm become mobile within 30 minutes of injection, and do not distribute themselves randomly within the spermalege (Abraham, 1934). The majority move towards the posterior region of the organ to the 'conductor lobe' from where the sperm move into the haemocoel. Movement into the haemocoel normally occurs 3 to 4 hours after copulation (Cragg, 1920). The dense masses of sperm that are initially injected break up into smaller clumps before they move into the haemocoel (Cragg, 1920).

Once the sperm has entered the haemocoel, migration towards the basal region of the genital tract occurs rapidly. There is good evidence (from both *in vitro* and *in vivo* techniques (Rao & Davis, 1969; Ruknudin & Silver, 1987) that sperm in the haemocoel migrate down an increasing oxygen concentration gradient from the spermalege to the seminal concepticles (Rao & Davis, 1969; Ruknudin & Silver, 1987). There is considerable variation in the amount of time that sperm remain in the haemocoel. The first sperm soon enter the seminal concepticles but large clumps of sperm can remain in the basal region of the haemocoel for several days (Carayon, 1966).

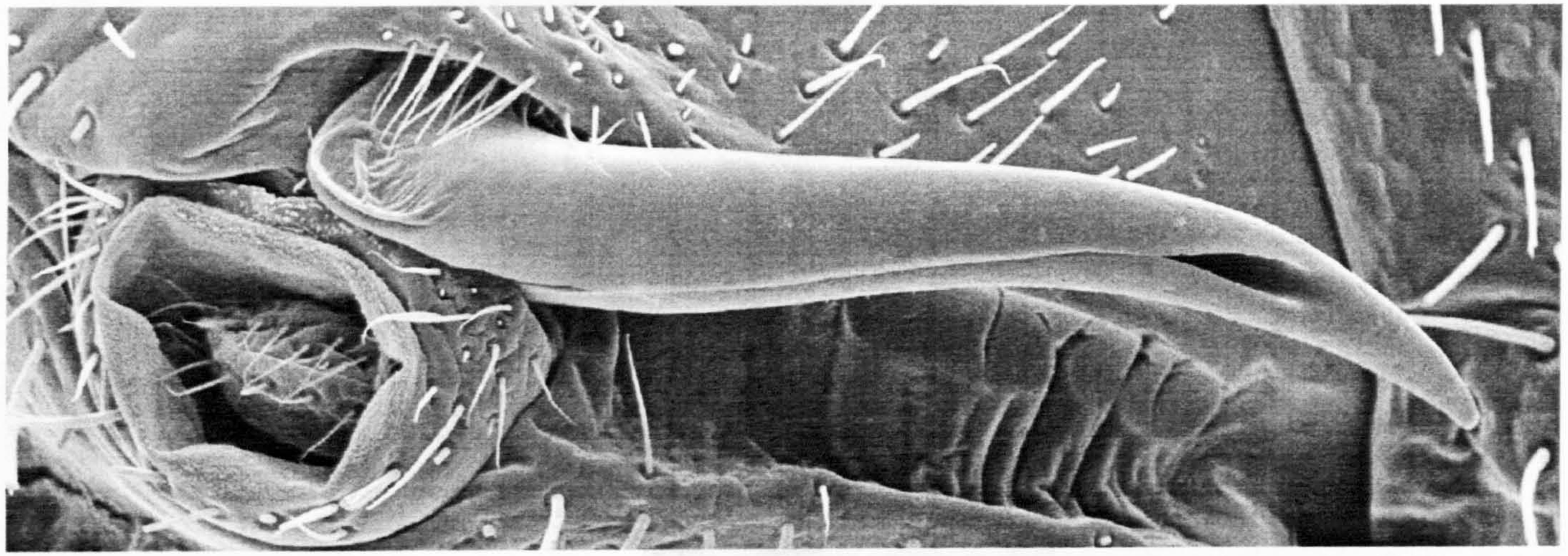


Figure 1.3. The paramere of a male *C. lectularius*

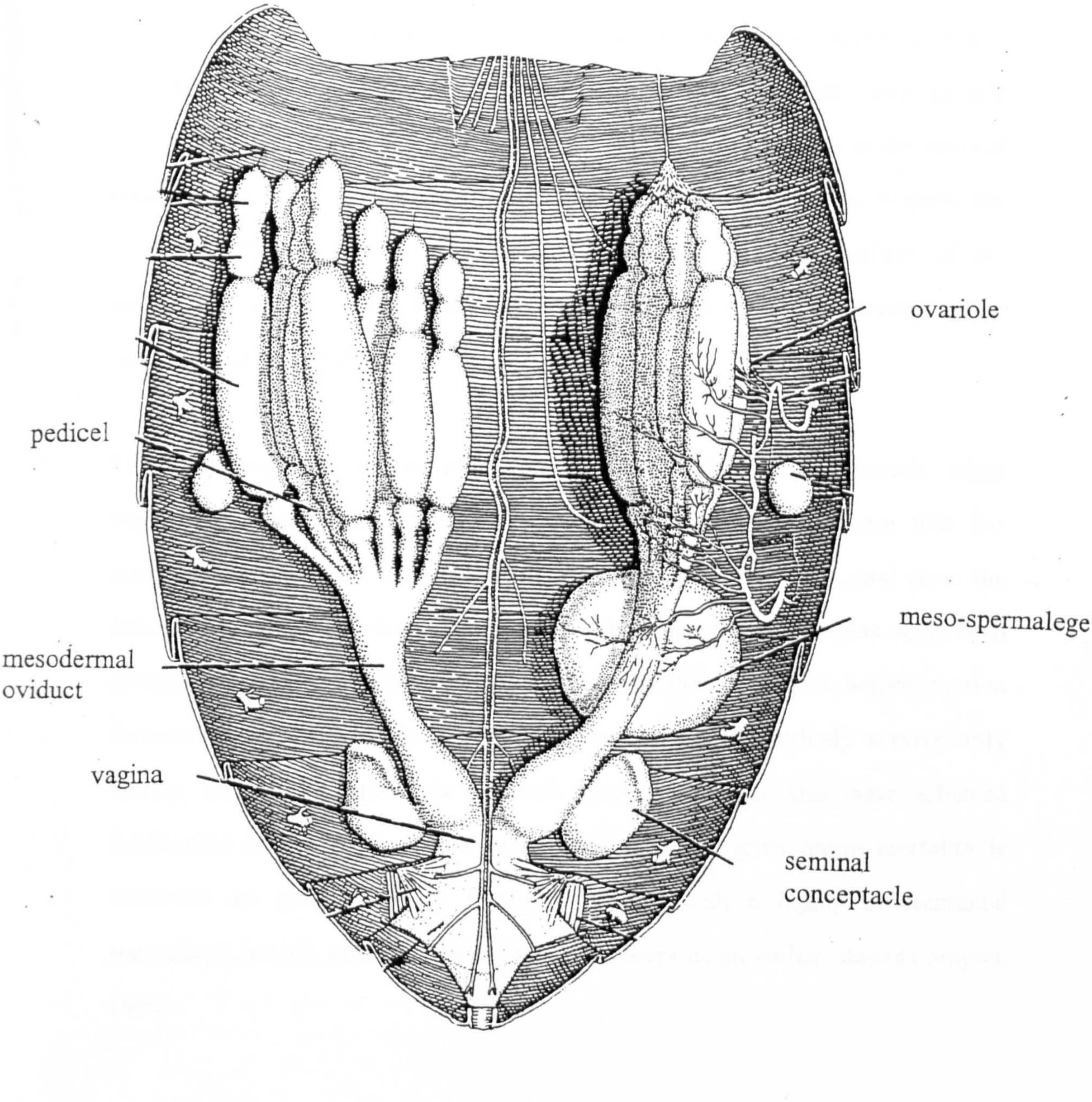


Figure 1.4. The paragenital system of a female bed bug *C. lectularius* (From Davis, 1956)

The movement of sperm in the intragenital phase (from the seminal concepticles to the ovarioles) is the least well studied period of sperm migration. Abraham's (1934) description is the only published account of sperm migration in the intragenital region of the female. Sperm are stored in the seminal concepticles in large numbers: Some resorption of the sperm occurs here (in the same way as was described in the spermalege), although this resorption effect was greatly exaggerated by Abraham (1934) (Carayon, 1966). Soon after arrival in the seminal concepticles the sperm disperse into the haemochrism zone that surrounds the oviduct (Figure 1.5). Sperm converge towards the posterior orifices of the spermodes, and enter the base close to the ovariole before dispersing in the syncitial bodies (Figure 1.5).

The final stage of sperm migration to the ovaries only proceeds when vitellogenesis occurs in the posterior oocyte of the ovary. Sperm that has congregated at the base of the ovariole, cross the follicular body, and enter the follicle to gain access to the syncitial body. Although fertilisation has never been directly observed it is assumed that it occurs at this point just before chorion formation. The great excess of sperm that enter the syncitial body survive only briefly, and all the sperm is resorbed apart from those that have achieved fertilisation of an oocyte. The syncitial bodies, where great sperm mortality is observed, are greatly reduced in those cimicids with a highly differentiated spermalege (which greatly reduces sperm numbers at an earlier stage)(Carayon, 1966).

1.3.5. *Paracimex*

The ectospermalege of *Paracimex* is considerably more complex than any of the above genera and has been described as a copulatory tube similar to those in the Anthocoridae (Carayon 1966)(Figure 1.1.d). The tube is blind in virgin females and is not damaged by the action of the male's intromittent organ, but instead is

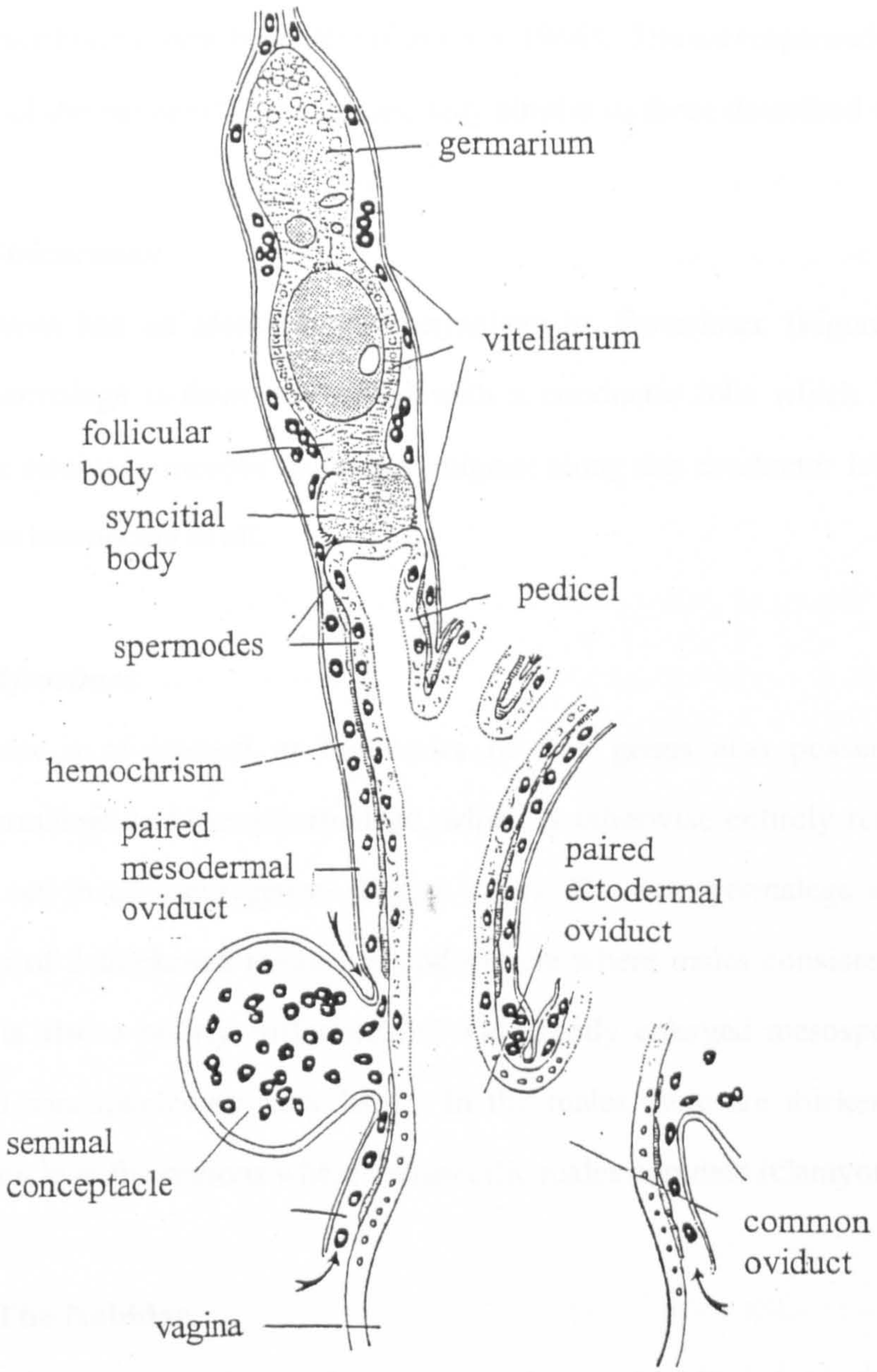


Figure 1.5. The intra-genital phase of sperm migration in *C. lectularius* (From Carayon, 1966). Amoebocytes are represented in black with white nuclei.

ruptured by the pressure caused by ejaculation. This mode of insemination has been described as semi-traumatic (Carayon, 1966). The mesospermalege and other aspects of the paragenital system are very similar to those described in *Cimex*.

1.3.6. *Stricticimex*

Stricticimex has an identical ectospermalege to *Paracimex* (Figure 1.1.e). The mesospermalege is three segmented with a conductor lobe which is continuous with the seminal concepticles. Sperm migrate along this conductor lobe and do not enter the haemocoel at all.

1.3.7. *Afrocimex*

Afrocimex is of interest as the males of this genus also possess a type of ectospermalege and mesospermalege, which is otherwise entirely restricted to the female sex in all other genera (Figure 1.1.f). The ectospermalege in the females consists of 2 thickened regions of endocuticle where males consistently copulate. There is also a poorly differentiated and greatly enlarged mesospermalege. The seminal concepticles are very large. In the males there are thickened regions of endocuticle in the regions where conspecific males copulate (Carayon 1959).

1.3.8. The Nabidae

In this family of predatory heteropteran bugs (the damsel bugs) the mode of insemination is rather varied. In the genus *Prostemma* sperm are injected through the roof of the vagina, into the lumen of the oviducts or even into a mesospermalege type pouch associated with the genitalia (Carayon 1952a;b). Uniquely in the genus *Alloerhynicus* sperm injection occurs as in *Prostemma* sp. or through the integument. The cues associated with the switch in mode of copulation is not known (Carayon 1952a).

1.3.9. The Plokiophelidae

In this under-studied heteropteran family only very little can be gleaned from the literature (Carayon's unpublished observations). Sperm are injected through the integument but no ecto- or mesospermalege is present. The sperm remain in the haemocoel for long periods of time before migrating into the genital tract and no sperm storage organs are present. Other data from this potentially important family are lacking.

1.3.10. The Anthocoridae

In this diverse heteropteran family only the genus *Xylocoris* has received attention. These have a copulatory tube ectospermalege which is almost certainly homologous to that of *Stricticimex*. Indeed the Cimicidae and Anthocoridae are frequently grouped together taxonomically (Schuh & Stys, 1991). The mesospermalege (probably not analogous) is a pocket shaped diverticulum of the ectodermal genital ducts.

1.3.11. The Strepsiptera

All the families and genera discussed so far have been closely related and similarities could be the result of a single evolutionary event (Schuh & Stys, 1991). Evidence for the independent evolution of extragenital copulation in the insects is found in the Order Strepsiptera. These minute insects are parasites of other insects. The early larval stages and adult females generally live within the host but adult males are winged and free living. Many families of Strepsiptera have been described but data on copulation is rare. When the female is sexually mature she pushes her cephelothorax (a fusion of the head and thoracic segments) out of the host and emits a pheromone. This pheromone attracts the winged males which copulate with the cephelothorax. In some species belonging to the family elenchus the male's barbed aedeagus pierces the brood canal (a tube which leads to the haemocoel used by the viviparous young to escape from their mother)

and injects sperm into the coelum. The aedeagus of the male then breaks off and the male dies (Kathirithamby & Hamilton, 1992). In some species belonging to the family acroschismus copulation is apparently not traumatic and sperm simply pass along the brood canal into the haemocoel. All the species so far described are viviparous, and the eggs develop in the haemocoel (Retnakaran & Percy, 1985).

1.4. Proposed hypotheses for the evolution of traumatic insemination in the Cimicidae

Remarkably little work has been carried out on the evolution of this system despite its biological novelty. Only two explanations have been proposed and neither are supported by experimental evidence. Hinton (1964) suggested that the "large" ejaculates and frequent copulations observed between adult males, and females, juveniles, and even other males, could be explained as a strategy of food sharing during periods of starvation. Although this is a group selectionist argument it was claimed that the almost non-existent levels of gene flow between populations of bed bugs would mean that populations would consist largely of kin. However, it is difficult to see how mating with other males which are reproductive competitors could evolve via this mechanism, also starved males will not copulate with conspecifics.

More recently Eberhard (1985) has suggested that haemoceolic insemination evolved to bypass cryptic female choice mechanisms which he suggests, may occur within the female's normal reproductive tract. Eberhard (1996) has subsequently argued that the paragenital system has evolved in females to allow cryptic female choice despite the occurrence of haemoceolic insemination, and that the primary role of the spermalege is to kill sperm. Eberhard (1996) does not consider other possibilities (i.e. that females may be reducing the costs of mating by reducing mortality or gaining nutritional benefits).

The costs of haemoceolic insemination to females may result from mortality (or reduced immune system function) caused by the introduction of micro-organisms into the haemocoel during traumatic insemination. By having a spermalege females could reduce this possibility. Second, once the spermalege has evolved, females may be able to reduce the costs of producing and maintaining it by digesting the sperm, and thereby gaining nutritional or "rare compound" benefits. It is important to point out that a digestive function for the spermalege does not preclude a "sperm selection" function for this organ, especially if females digest the sperm which fail to pass any selection criteria determined by the spermalege.

1.5. The study species

Bed bugs have an impressive folklore due to their association with humans. The true bed bugs (*Cimex lectularius* in temperate regions and *Cimex hemipterus* in the tropics) are cosmopolitan pests of human dwellings however they are only two representatives of the Cimicidae.

All members of the Cimicidae display traumatic insemination although it varies in mode and in the complexity of the paragenital system (see section 1.3.1). *C. lectularius*, the temperate human bed bug, is found in human dwellings and often infests poultry houses (Figure 1.6). The bugs are nocturnal and all the life history stages are haemophagous though they do not live on the host but live in harbourages near beds. The hosts are located by body heat and the bugs will only spend 5-10 minutes feeding on the host. The activity of bugs is retarded at low temperatures but at 20°C bugs will attempt to feed from a host at approximately weekly intervals (Mellanby, 1939). However, bugs are also capable of surviving for several months without feeding although all development and reproductive activity will cease after approximately 10 days without a blood meal.

Figure 1.6. Copulating bed bugs. The male is on top of the female and is about to insert his paramere into the female's spermalege which lies on the ventral surface of her abdomen.

C. lectularius makes a good model organism for the study of traumatic insemination for several reasons. *C. lectularius* is the best studied of all the Cimicidae and it has a paragenital system that is intermediate in complexity compared with the diversity seen within the Cimicidae. *C. lectularius* is relatively easy to culture in the laboratory and has a short generation time of 6 weeks from egg to adult. They go through 5 juvenile instars before eclosion and are sexually mature as soon as they have taken their first blood meal. A clutch of eggs is laid 3-4 days after mating.

1.6. Aims and thesis outline

This thesis asks functional questions about traumatic insemination and its consequences in the common bed bug *Cimex lectularius*. The main aims of this thesis are: 1. to examine the reproductive strategies of males and females, and 2. to identify potential conflicts of interest that arise from these strategies.

Chapter 2: examines the function of behavioural reproductive strategies of males and females. The mating system is classified and reproductive behaviours are quantified to produce predictions concerning the important mechanisms of sexual selection which are likely to be operating.

Chapter 3: examines functional aspects of sperm transfer and migration. Variation in sperm allocation to females is considered, and sperm migration and storage is measured to gain understanding of possible mechanisms of sperm competition.

Chapter 4: investigates variation in male reproductive success through sperm competition. Sperm competition mechanisms are investigated experimentally using predictions formulated from observations of sperm migration in Chapter 3.

Chapter 5 (and Appendix I): investigate the fitness payoffs associated with multiple mating for females. Longevity, fecundity and offspring quality are measured in females under different levels of polyandry.

Chapter 6: discusses the evidence that traumatic insemination is an example of a male driven reproductive strategy and that a sexual conflict has resulted over the mating rate.

2 Reproductive behaviour and mating system

2.1. Introduction

One widespread pattern of reproductive behaviour in the animal kingdom is polyandry: when females mate with more than one male (Birkhead & Møller, 1998). There are many reasons why females may mate with more than one male (Walker, 1980; Thornhill & Alcock, 1983; Ridley, 1988; Ridley, 1989; Ridley, 1990; Choe & Crespi, 1997). Possible benefits have been suggested via direct routes such as increasing female fecundity, or through indirect mechanisms via genetic benefits producing high quality or genetically compatible offspring. Although there is some evidence for the operation of indirect benefits (e.g. Gilburn & Day, 1994; Tregenza & Wedell, 1998) they are likely to provide a weaker selection pressure than the direct benefits of polyandry (Parker, 1984; Kirkpatrick & Ryan, 1991). These two possible benefits are not mutually exclusive. In the arctiid moth *Utetheisa ornatrix* females mate preferentially with larger males and body mass is heritable (Iyengar & Eisner, 1999). By choosing larger males females also gain larger quantities of nutrients and defensive alkaloids from the male's spermatophore (Iyengar & Eisner, 1999). This study demonstrates that females can gain both types of benefits through mate choice.

In a recent meta-analysis of female benefits arising from polyandry in insects Arnqvist and Nilsson (In press) found that females generally gain directly in terms of lifetime offspring production as a result of polyandry. However at very high mating rates females tended to have reduced longevity and therefore reduced lifetime offspring production. The optimal female mating rate is therefore intermediate between monandry and high levels of polyandry. One prediction that arises from this general pattern is that when females have little or no control

over the mating rate, then sub-optimal mating rates for females should be observed. Such, sub-optimal mating rates should be observed when females are forced or coerced into copulation.

2.1.1. Sexual coercion as sexual selection

Several authors have advocated that sexual coercion should be considered as a third sub-division of sexual selection alongside intra-sexual competition and inter-sexual mate choice (Smuts & Smuts, 1993; Clutton-Brock & Parker, 1995). Examples of sexual coercion are widespread in animal taxa and have been sub-divided into three classes: Firstly forced copulation where females have lost control over copulation. Secondly, sexual harassment where females can resist copulation by males but males continue to harass females until they allow copulation to take place. Thirdly, when males intimidate females into copulating with them (Clutton-Brock & Parker, 1995). Examples of forced copulation are widespread in insect taxa (for review see: Thornhill & Alcock, 1983). In members of the grasshopper tribe Melanoplini (Acrididae) males pounce on females and attempt to copulate immediately. Females have no pre-copulatory mechanisms of mate choice available to them, and males have genital claspers that function to extend their copulation duration once they have begun insemination (Cantrall & Cohn, 1972; Alexander et al., 1997).

Several predictions can be made as to how males and females should behave when forced copulation is used as a strategy by one sex (usually males). Smuts & Smuts (1993) have suggested that forced copulation should only occur where it is difficult for males to monopolise females. Consequently guarding behaviour should be rarely observed in mating systems with forced copulation. It has been suggested that because males have a higher potential rate of reproduction they receive a higher fitness gain if they win conflicts of interest between the sexes (Clutton-Brock & Parker, 1995). When this occurs male mating rates should be

close to optimum and females will have sub-optimal fitness as a result of high mating rates (Chapman & Partridge, 1996). Moreover a lack of courtship (other than that necessary for species recognition) would be predicted as female choice mechanisms will be redundant (Brown et al., 1997). Conversely, if females have control over mating, then male courtship which either manipulates a female's pre-existing sensory bias (coercive mating: e.g. Eberhard & Cordero, 1995) or provides reliable information to the female about male "quality" (persuasive mating: e.g. Wilkinson et al., 1998) should occur. In some cases male harassment for matings may be energetically costly for females (Stone, 1995). Where costly harrassment by males occurs females may mate to make the 'best of a bad job' (Thornhill & Alcock 1983). Such 'convenience polyandry' has been suggested to occur in several species of water strider (for review see: Arnqvist, 1997). The final prediction that comes from forced copulatory sexual conflicts is that females are predicted to show counter-adaptations which reduce the cost, or likelihood, of the occurrence of forced copulation (Dawkins & Krebs, 1979; Parker, 1979). Convincing evidence that this has occurred has been found in the water strider *Gerris incognitus*. Females in this species have genital spines that allow females to avoid superfluous forced copulations by males (Arnqvist & Rowe, 1995). Female control over mating rate is not the only mechanism by which females can control the relative payoffs of forced copulations to males and females. A growing body of research is now testing whether females may have an influence on fertilisation outcomes after copulation with several males (Eberhard, 1996).

2.1.2. Forced copulation and female control of fertilisation

The fact that sexual selection continues after copulation in the form of sperm competition is now widely accepted as a strong selective force in animal mating systems (e.g. Birkhead & Møller, 1998). The concept that females may be able to control the outcome of sperm competition is more controversial (Eberhard, 1998). However some evidence that females may be able to control the

fertilisation of their eggs is now being produced (e.g. Clark et al., 1999). How females should bias the paternity of their offspring is far from clear. If forcing behaviour in males has a heritable basis, then females should choose males which are particularly good at forcing copulation via cryptic female choice mechanisms. However, this will be traded off again any cost associated with loosing control over mating frequency. The cost of avoiding copulation will also have an effect on this trade off and could easily produce 'convenience polyandry' in females (see Arnqvist, 1989).

2.1.3. Reproductive behaviour of *C. lectularius*

Other than a description of the mode of copulation (Patton & Cragg, 1913; Cragg, 1915) the reproductive behaviour of *C. lectularius* is unstudied. Traumatic insemination results in potentially novel routes to fitness for males. Preliminary observations in the anthocorid *Xylocoris maculipennis*, suggested males often copulate with other males. Carayon (1974) suggested that such homosexual copulation may be a method of 'copulation by proxy'. He envisaged that males traumatically inseminated other males and that the sperm migrated to the testes of the copulated male. This was then ejaculated by the copulated male and so the copulating males could gain fertilisation success by proxy. Although fertilisation by proxy has been demonstrated in a beetle (Haubrudge, et al. 1999) the mechanism is quite different to that proposed by Carayon (1974). Fertilisation success through ejaculate 'parasitism' has not been tested in *C. lectularius*.

Males of *C. lectularius* have been observed to copulate with pre-adults as well as with other males (Rivney, 1933). A second novel route to fitness is that males may gain fertilisation success by inseminating pre-adult females. In most insects males gain no fertilisation success by inseminating juvenile females since the genital tract is ectodermal. This lining is shed at the moult to adult eclosion and so any sperm within the female's genital tract would be lost. However, in *C.*

lectularius and other cimicid bugs, sperm is transferred into, and stored in, mesodermal structures. The mesoderm remains intact during eclosion in hemimetabelous insects and so insemination before sexual maturation may result in fertilisation success for males. Males may even mate with pre-adult females in order to avoid the costs associated with inseminating through the spermalege or to gain sperm precedence. The spermalege has been reported to kill sperm (Carayon 1966) and one hypothetical function has been to allow females to exercise cryptic choice over the sperm from different males (Eberhard 1996).

2.2. Aims

The aim of this chapter is to quantify aspects of the reproductive behaviour of *C. lectularius*. The mating system of *C. lectularius* has not previously been studied. The importance of examining it here is to provide a basis from which to produce biologically accurate predictions of what reproductive strategies are likely to be important to males and females.

I assess the mating rates of males and females and examine copulation between adult males and pre-adult females as well as with other adult males. Finally I examine an alternative hypothesis: males attempt to copulate with conspecifics of both sexes because of restricted mate recognition abilities.

2.3. Methods

2.3.1. General stock maintenance

C. lectularius was cultured in the laboratory using a laboratory stock sourced from the Medical Entomology Centre, Cambridge Road, Fulbourn, Cambridge, UK. These bugs have been in laboratory culture for many years. The bugs where maintained in an incubator at 26±1°C and ca. 70% relative humidity (maintained by a saturated sodium chloride solution). Bugs were fed weekly on rabbit blood using the protocol of Davis (1956). Bugs in these conditions went through a

juvenile instar every ca. 6 days and eggs hatched within 10 days of laying. Final instar bugs were separated from the stock cultures every week and isolated in individual containers after feeding to produce constant aged virgins for all the experiments. All virgin bugs were sexed under the dissecting microscope by examining the ventral surface of the abdomen for the presence of the ectospermalege (a female specific structure). All experimental animals were measured using an image analysis system (Software: Optimus 6, Washington 98011, USA) and the total length and maximum thoracic width was recorded. Size measurements were always taken on bugs starved for 5 days to control for the expanded size of the abdomen after feeding.

2.3.2. Mating system observations

Observations of copulations were carried out under dim red light conditions (60W bulb) in a constant temperature room (26±2˚C). Satiated bugs were individually marked using enamel paint and then placed into a 5cm diameter petri dish lined with clean filter paper. The dish was then placed under a video camera (Sony Handicam) and was recorded for up to 3 days. The frequency of copulation, duration of copulation, remating interval and individuals involved were recorded for each animal. Two different replicate mating trials were used. Firstly, 5 males were isolated with 5 females. Secondly, 5 males were isolated with 5 females and 5 penultimate (5^{th}) instar females. Both trials were replicated 4 times with different individuals.

2.3.3. Natural sex ratios of *C. lectularius* populations

Sex ratios are often manipulated in studies of insect mating and the responses of male and female reproductive strategies are measured (e.g. Gage & Baker, 1991). Samples of bugs from 7 different natural populations were collected and sexed to determine if sex ratio biases occur in the field.

2.3.4. Dissections following intrasexual copulation

Following intrasexual copulation the recipient males were chilled on ice and dissected within 30 minutes of insemination. Dissections were carried out under a stereo microscope (Leitz M8) in 200µl of 0.15M Hepes buffer adjusted to pH 7.2 using 0.1M KOH (Ruknudin & Raghavan, 1988). This saline maintains sperm activity for several hours. Any dissections that caused the rupture of the testes or genital tract were discarded to prevent the scoring of self sperm. The presence or absence of sperm, or a sperm mass was scored under the dissection microscope before examination of the buffer under a compound microscope (Leitz Diaplan) to look for traces of sperm.

2.3.5. Assessment of fertilisation success by males copulating with pre-adults

100 virgin pre-adult females were fed a blood meal and each placed in a $7cm^3$ plastic tube with a virgin male. Each male was then allowed to copulate freely with the pre-adult for two days. Freshly fed males were replaced on day 2 and 4 of the experiment. Males were removed on the 7th day of the experiment and the pre-adults were allowed to eclose in isolation. A control group of 100 pre-adult females were treated in the same way though no males were allowed to contact the pre-adults at any point during the experiment. This was carried out to determine if *C. lectularius* could reproduce parthenogenetically. Following eclosion both the control and experimental animals were fed a blood meal and the number of fertile eggs counted.

2.3.6. Female fertility

Several direct benefits of remating have been suggested for females (e.g. Ridley, 1989). One potential benefit of remating is to maintain sperm stores, thereby allowing maximum fertility of eggs. To investigate this 20 females recieved a single copulation and the number of fertile eggs produced were counted over 8 clutches (1 clutch per week).

2.3.7. Statistical analysis

All analysis was performed using Statview 5.0 (SAS Institute Inc., SAS Campus Drive, Cary, NC 27513, U.S.A.) statistical software for the Macintosh operating system. Data were checked for normality and homogeneity of variances. Where these assumptions were violated the appropriate non-parametric test was used. Means are displayed ± the standard error and medians ± the upper and lower quartiles unless otherwise stated.

2.4. Results

2.4.1. Sex ratios of natural *C. lectularius* populations

None of the populations sampled showed any deviation from a 1:1 sex ratio (Table 2.1). Sex ratios at unity were therefore used throughout the experimental designs in this thesis.

2.4.2. Copulation in *C. lectularius*

Copulation in *C. lectularius* is not preceded by any overt courtship or other intersexual association. When a male encounters a receptive female he moves rapidly onto the female's dorsal surface and, whilst grasping the female's thorax, probes his paramere around the right hand-side of the female's abdomen before inserting the paramere into the ectospermalege. The time between encounter and genital contact is less than five seconds. The male remains stationary in this position until copulation is terminated. Copulation lasts 88.63±5.36 seconds (n=98) but is affected by a number of variables which are discussed in section 2.4.4. As soon as copulation has finished the male moves off the female, and shows no post-copulatory mate association.

Population	No. of males	No. of females	Expected no. of either sex	Chi squared	P. value
U. A. E. Population 1	8	8	8	0	>0.05
U. A. E. Population 2	9	5	7	4.0	>0.05
U. A. E. Population 3	13	10	11.5	0.38	>0.05
U. A. E. Population 4	4	7	5.5	0.38	>0.05
U. A. E. Population 5	26	21	23.5	0.53	>0.05
U. A. E. Population 6	9	8	8.5	0.06	>0.05
U. A. E. Population 7	8	11	9.5	0.38	>0.05
Total	**88**	**76**	**82**	**0.87**	**>0.05**

Table 2.1. The sex ratios of natural populations and expected values of a sex ratio at unity, collected from chicken farms around Dubai, United Arab Emirates.

2.4.3. Mating system

Starved male and female *C. lectularius* do not copulate (Mellanby, 1935). Copulations always follow a blood meal (Figure 2.1): immediately after feeding there is a period of intense reproductive activity. During the first 24 hours after feeding females have a median remating interval of 17 minutes (Q1= 5.0, Q3=134.1, n=41). Medians are provided as an estimate of the average remating intervals due to the skew in the data. Although there is variation in mating frequency, the data demonstrate that sperm competition is likely to be important, since single females copulate with an average of 5.0 (±3.16, n= 20) males during the period of reproductive activity.

2.4.4. Copulation duration

The mean copulation duration for bugs in the mating system experiments was 88.63 ±5.36 (n=89) seconds. Several factors affected this copulation duration. A male copulating with a virgin female copulated for significantly longer (106.10 seconds ±8.49 n=40) than a male copulating with a non-virgin (71.17 seconds ±5.38 n=40)(Mann-Whitney U test: U=404, P<0.001)(Figure 2.2). This effect occurred whether the male copulated with (see data above) or without other males present (with virgins and other males present: 80.5 seconds ±17.5, n=10)(with non-virgins and others males present: 45.76 seconds ± 4.74, n=39)(Mann-Whitney U test: U=82.5, P=0.005)(Figure 2.2).

2.4.5. Copulation with conspecific males

Copulation attempts occur with other males (see Table 2.2.a) but rarely lasted longer than five seconds. The most parsimonious explanation is that males failed to distinguish between the sexes. This was tested by producing a null model of expected numbers of copulations if mating attempts occurred at random (Table 2.2a). The frequency of copulations did not differ significantly from the null

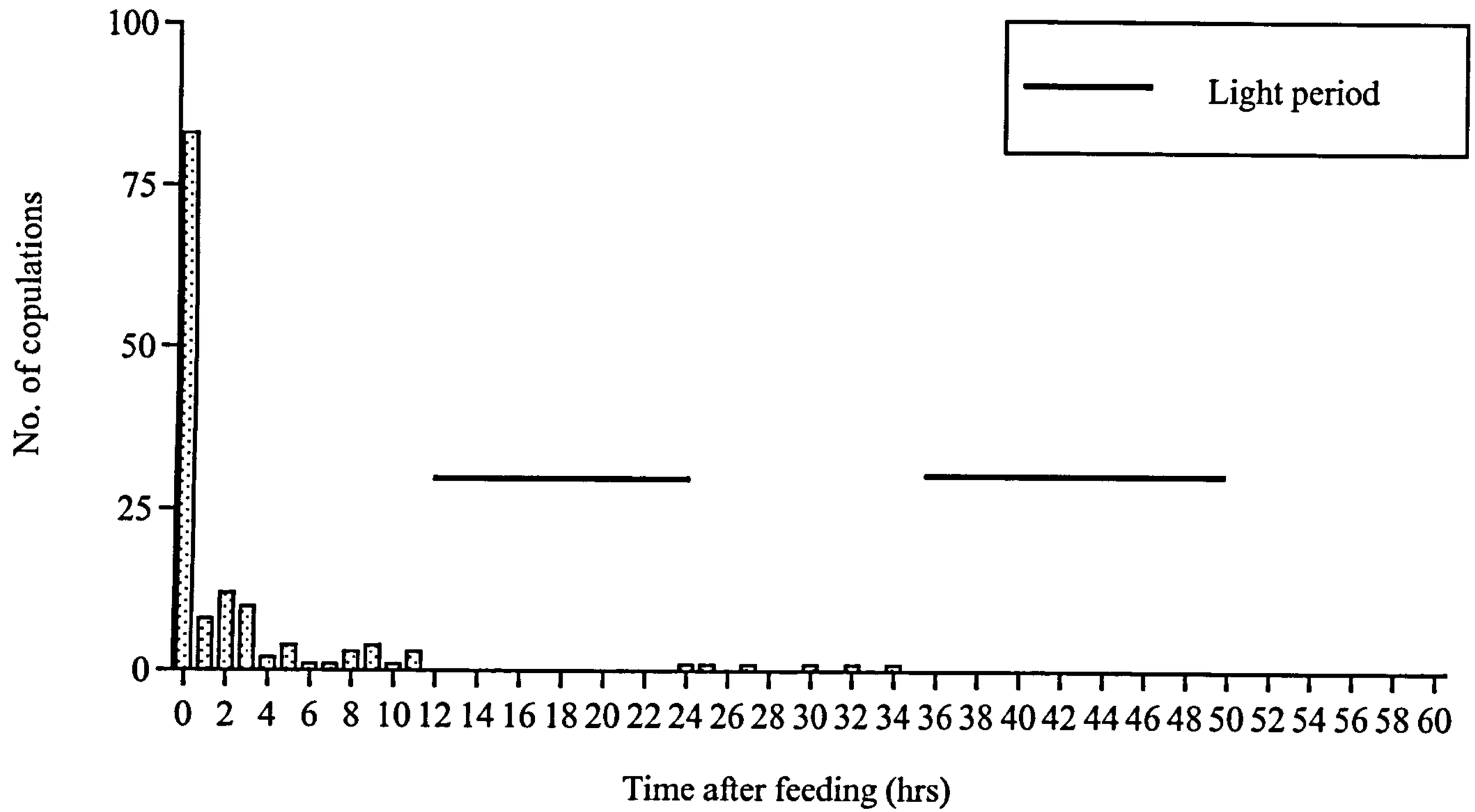


Figure 2.1. The frequency of copulations over time following a blood meal. Pooled data from 4 experiments with 5 males and 5 females.

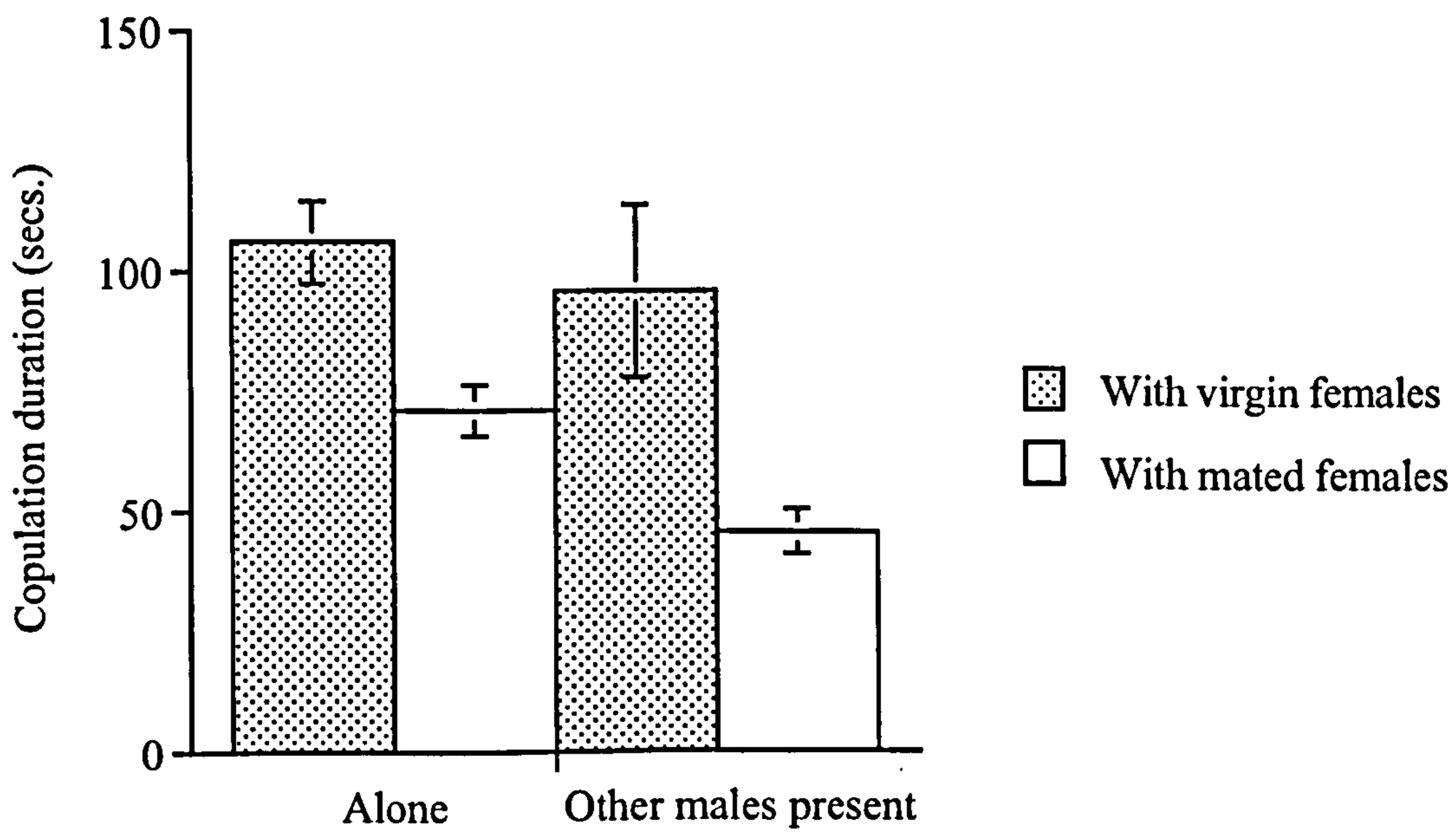


Figure 2.2 The copulation durations of males copulating with virgin and once mated females. The effect of the presence of conspecifics is also displayed. Bars show the standard error.

A

Interaction	Expected value (%)	Observed value	Chi squared	P value
Male: male	44.44	36.22	3.28	>0.05
Male: female	55.56	63.78		

B

Interaction	Expected value (%)	Observed value	Chi squared	P value
Male: male	28.57	18.22	6.04	<0.05
Male: female	35.71	44.66		
Male: PAF	35.71	37.12		

Table 2.2. A. Frequency of copulation attempts of 20 males and 20 females in a 5:5 sex ratio. There was no significant departure from the null model of random mate choice (expected value). The Chi squared value was adjusted by Yates correction because the degrees of freedom = 1. (Sokal & Rohlf 1995) **B.** The frequency of copulation attempts 20 males, 20 females and 20 Pre-adult females (PAFs) in a 5:5:5 ratio of individuals. The frequency of copulations departed significantly from the null model when pre-adults were present with males copulating more frequently than predicted with females and less frequently than predicted with other males.

model when only females were present (χ^2 = 3.28, df =1 P>0.05). However when pre-adult females were present males appeared to be able to reduce the frequency of copulation attempts with other males (χ^2 = 6.04, df =2 P>0.05) and copulate more frequently with mature females. None of the males that were observed to have been mounted by another male (n=20) had sperm in the haemocoel when dissected.

2.4.6. Copulation with pre-adult females

As with the copulations with males copulations with pre-adult females occur frequently in the experimental set up and accounted for 37% of total (n= 20) copulation attempts. The frequency of copulations with pre-adult females were also compared with a null model of random mate choice by males (Table 2.2b). Males copulated more frequently than expected with mature females than with males (χ^2= 6.04, df =2 P>0.05) but the frequency of copulation with pre-adult females was not significantly different from the null model (Table 2.2b). These data suggest that males cannot distinguish between pre-adults and adult females before entering copulation. However, this result could also be explained if pre-adult females had some reproductive value to males. When males were allowed to copulate with pre-adults they gained no reproductive success (n=100). No pre-adult females exposed to males contained sperm in their seminal concepticles when dissected (n=100).

2.4.7. Remating rate and the maintenance of maximum fertility

Females were able to maintain maximum fertility for a minimum of 4 clutches after a single copulation. After this clutch the number of infertile eggs slowly increased (Figure 2.3).

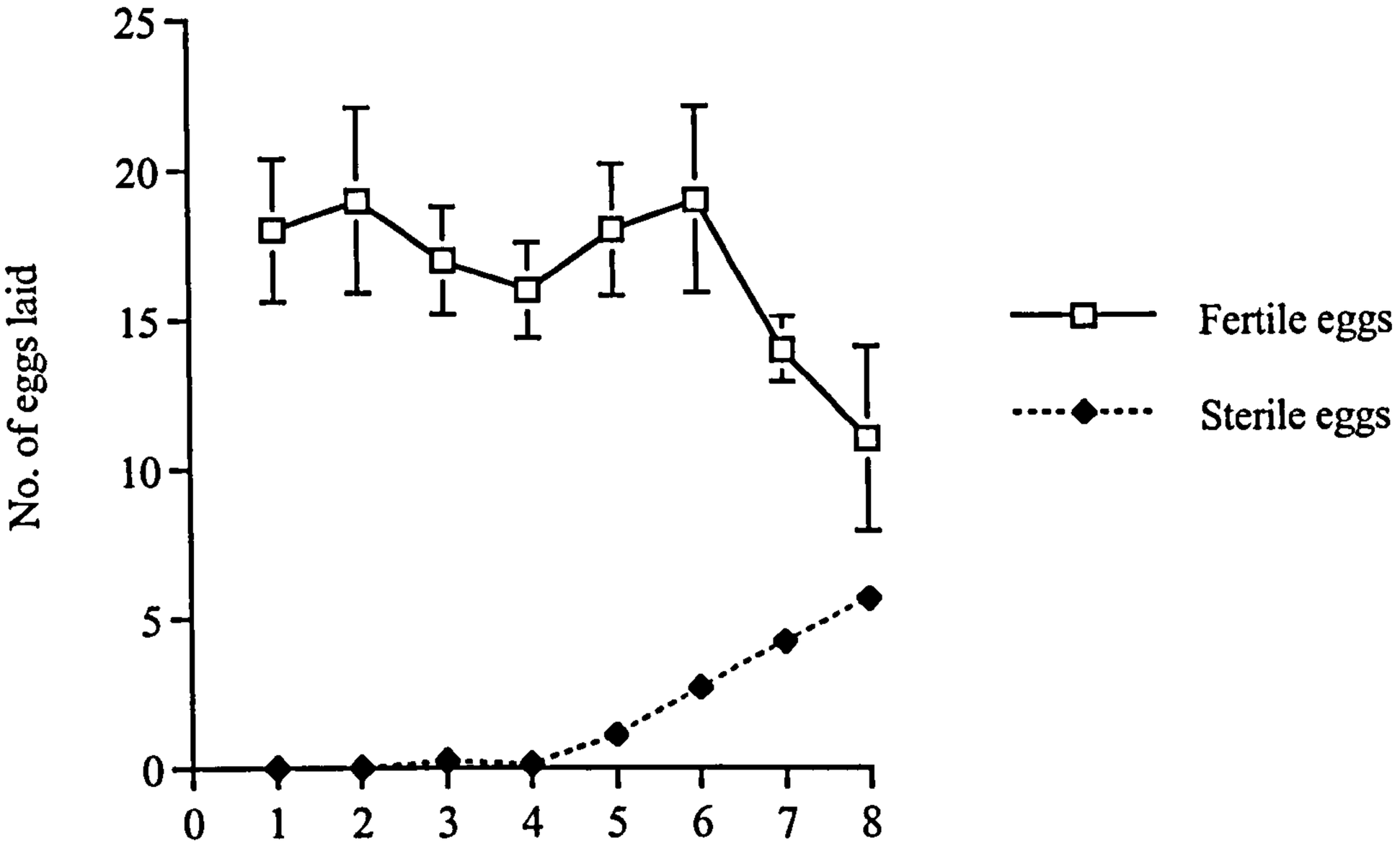


Figure 2.3. Mean number of fertile and sterile eggs laid by a once mated female over time (n=20).

2.5. Discussion

The mating system of the bed bug can be defined as promiscuous (Thornhill & Alcock, 1983). Males copulate repeatedly with females during the intense period of reproductive activity following a blood meal, and attempt to mate with as many females as possible: this can be considered a type of scramble competition. Females showed no overt resistance to multiple copulations and remate every 17 minutes in the few hours following a blood meal. These observations had several important implications. Firstly sperm competition is likely to be intense in this system since the ejaculates of 5 males overlap within the female's paragenital system during a single reproductive episode. Sperm competition theory would predict that in this situation males should allocate their ejaculate expenditure to optimise their reproductive success (Parker, 1998). This prediction is tested in more detail in Chapters 3 and 4. However, some evidence that males may be allocating gametes differentially to females can be found by examining the copulation durations of males. The copulation duration of males was found to be affected by two main factors. Firstly, males copulated for much longer when they copulated with a virgin female than a female who was previously mated. Variation in copulation duration caused by female mating status has been demonstrated in butterflies (Cook & Gage, 1995; Wedell & Cook, 1998), and is often correlated with the amount of sperm transferred (Svärd & Wiklund, 1986). This is especially likely to be true in insects where physical sperm removal does not occur and copulation functions only to transfer gametes. The relatively simple genitalia of the male bed bug suggests that physical sperm removal does not occur in this species (See Figure 1.3.).

Secondly, males copulate longer when no other males are present compared with when copulating in the presence of other males. Males have been shown to increase their ejaculate expenditure in response to the presence of other males, as

male presence could signal an increased risk of sperm competition (e.g. Gage, 1991; Gage & Baker, 1991; Simmons et al., 1993). The observation of increased copulation duration in *C. lectularius* goes against the prediction of how males should respond to cues of sperm competition risk (Parker, 1998), however the predictions of male strategies in response to sperm competition are complex and discussed fully in Chapter 3.

2.5.1. Possible sexual conflicts arising from mating rate

During their life-time female bed bugs copulate approximately 20 times more frequently than they require to maintain maximum fertility. There are several potential evolutionary consequences of this high mating rate. If, as it appears, females cannot resist male mating attempts then the rate of mating observed may be approaching the optimum for males. If remating carries a cost in *C. lectularius* then females should attempt to mate as few times as possible to maintain fertility. Females may be gaining indirect benefits for their offspring by mating with several males and this is discussed further in Appendix I. The remating rates of females may be an artefact of the method of observation. Males and females were fed simultaneously at weekly intervals in the laboratory, and this is very similar to the patterns of bug feeding observed in natural populations (Mellanby, 1939). One way that females could reduce their remating rate is by dispersing away from areas where they may encounter other males, and this cannot occur when the animals are contained in a 5cm diameter petri dish. However two observations suggest that this may not be an unrealistic mating arena. Bed bugs often occur in huge densities (King et al., 1989) and in the populations sampled there was no bias in the sex ratios observed (as would be expected if females dispersed away from males after feeding). Secondly, bed bugs secrete an aggregation pheromone and following the first few hours of activity males and females form aggregations (Usinger, 1966).

Collecting quantitative data on forced copulation is difficult. Observation can only provide data on whether overt female choice is occurring and the concept of forced mating or 'rape' is unhelpfully anthropomorphic (Thornhill, 1980; Hilton, 1982; Kirkpatrick & Turner, 1991; Mesnick & Leboeuf, 1991). In some systems males change their reproductive strategies depending on factors like the operational sex ratio, and in these cases different types of copulation can be objectively classified (Arnqvist, 1997). However, there were no alternative mating strategies observed in *C. lectularius*. One prediction from mating system theory does provide a framework to examine forced copulations. Brown et al., (1997) predicted that where forced mating occurs the mating rate will be closer to the male's than the female's optimum mating rate (a full discussion of the evidence for the pay-offs of different mating rates to males and females is provided in Chapter 6). However, I would predict that due to the mode of insemination then mating will carry a fitness cost to females when mating at the high rates observed in the mating observations (see Chapter 5).

2.5.2. Male-male & Pre-adult female copulations

The male-male copulations that have been reported in cimicids (Carayon, 1974) may have been different to those observed in *C. lectularius*. In this species male-male copulations appear to be a maladaptive product of the scramble competition mating system. Males do not appear to transfer sperm during these short copulations and the copulation attempts may simply be the result of absent or inoperative female recognition systems. The same may be true for copulations with pre-adult females. Although these copulation attempts tended to be much longer than those with males, no sperm were transferred. If the absence of mate recognition hypothesis is correct then why did males attempt to copulate with pre-adult males? The answer may be that 5th instar males are much smaller than 5th instar females especially after feeding. Fifth instar females take the largest

blood meal of any *C. lectularius* life history stage and, when engorged, are similar in size to engorged adult females (Titschack, 1930). Males may simply be attempting to copulate with any large engorged conspecifics that they encounter as a strategy for mate location. However, when pre-adult females are present males appear to be able to avoid copulating with other males and instead copulate more frequently with mature females. How males judge mating partners is unclear. However if males copulate with any engorged conspecific over a certain size this could explain why males appear to avoid copulation with other males when pre-adult females are present. Engorged pre-adult females are very similar in size to adult females whereas males are considerably smaller. Where the probability of copulating with a mature female by random is 50% males may use a strategy of random mate choice. However, when pre-adults are present the probability of copulating with a mature female is ca.33%. In this situation males may switch their mate locating behaviour by increasing the magnitude of the size threshold of individuals with which they copulate. If this occurs then males would be predicted to copulate with mature females and pre-adult females preferentially therefore increasing the probability of copulating with a mature female.

2.6. Summary

In this chapter I have described the promiscuous mating system of *C. lectularius* and the mating rates of both males and females. I have identified a potential conflict of interest between the sexes over the mating rate. The relative pay-offs of the mating rate to males and females will be examined in Chapters 4 and 5 and Appendix I. I have also presented some evidence to suggest that males have control over mating frequency. Males also copulate for different durations with females of different mating status and the relationship of these copulations to gamete allocation will be investigated in Chapter 3.

ejaculates (Parker, 1984; Parker et al., 1993). Interspecific studies have demonstrated that male investment in ejaculates often increases as polyandry (and therefore sperm competition) increases (e.g. Gage, 1994). Ejaculate size can be an important determinant of success in sperm competition under two main conditions. Firstly, where sperm compete on a numerical basis then the more sperm added by a given male the greater the chance his sperm has of fertilising the female's eggs (Simmons, 1987a; Wedell, 1991). This is the raffle model of sperm competition (Parker, 1990). The second way in which ejaculate size can effect success in sperm competition is when the ejaculate is used to displace sperm volumetrically within a female's sperm storage organs (Otronen, 1990; Otronen and Siva-Jothy, 1991; Clark et al., 1995; Simmons et al., 1999).

Although there is good evidence that males can increase their success in a single bout of sperm competition by increasing their ejaculate size it may not pay males to inseminate females with as much sperm as possible. Males need to trade off the allocation of sperm from the current reproductive event against those of the future as there may be non-trivial costs associated with the production of ejaculates (see Dewsbury, 1982 for review). Parker (1990), and others (Parker et al., 1990; see also Ball and Parker, 1996; Parker et al., 1996; Ball and Parker, 1997; Parker et al., 1997) suggested selection would favour males that tailored their ejaculate size to the number of conspecific ejaculates that were likely to be encountered in competition.

Using an 'evolutionarily stable strategy' (ESS) theoretical approach, Parker (1990; Parker et al., 1990; Parker et al., 1997) first considered the effect of 'sperm competition risk[1]' on a male's gametic strategy. Risk is the situation where there is likely to be a low probability of a male's ejaculate competing with

[1] Sperm competition risk is defined by Simmons & Siva-Jothy (1998) as: "The probability (between zero and one) that females will engage in promiscuous mating activity that will result in the temporal and spatial overlap of the ejaculates from two or more males" (p. 434).

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that of another (i.e. the number of competitors is likely to be between 0 and 1) in a single copulation. Under these conditions the male's allocation in ejaculate is predicted to match the perceived risk of sperm competition. When the perceived risk of sperm competition is zero then a male is predicted to inseminate only enough sperm to maintain maximum fertility of the female and use the remaining sperm reserve for future reproductive investment. However when a male perceives that the risk of sperm competition is high (i.e. there is chance that his ejaculate will be competing with that of another male) then he should increase the size of his ejaculate in order to gain maximal fertilisation success under sperm competition.

The same theoretical approach has been extended to examine how males should react to an increase in the number of potential competitors in sperm competition. The term 'sperm competition intensity'[2] has been proposed to deal with cases where the number of ejaculates that compete within a female (in internally fertilising species) is 2 or more (Parker et al., 1996; Ball & Parker, 1997). The prediction of these models is that when competing with a single ejaculate a male should ejaculate the maximum number of sperm (this is the same as in 'risk' models (Parker, 1990; Parker et al., 1990). However, as the number of competitors increases beyond one then the amount of sperm that should be allocated is predicted to decrease.

Caution is required before raffle models are applied to many taxa as knowledge of sperm competition mechanisms are still poorly understood (Simmons and Siva-Jothy, 1998). The key assumption of the 'risk' and 'intensity' models is that the mechanism of sperm competition acts as a raffle (Parker, 1990; Parker et al., 1990). In a fair raffle there is a fixed price for a ticket and a fixed value for the

[2]'Intensity' has been defined by Simmons & Siva-Jothy (1998) as: "The extent of overlap between the ejaculates of different males once competition occurs. Intensity is determined by the relative numbers of sperm from different males and the absolute number of males engaged in competition for the ova of a single female" (p. 434).

prize. If the probability is that you are the only competitor in the raffle then it would pay you to buy the minimum number of tickets that would gain the prize. However, if it is likely that you will have one competitor then it would pay you to buy as many tickets as possible to maximise your chance of winning (this is analogous to the risk models). As the number of potential competitors increase then the value of each ticket is reduced and it would pay you to reduce your investment in the raffle (this is analogous to the intensity model).

3.2 Aims of the chapter

The aims of this chapter are to investigate sperm migration patterns and sperm storage in female bed bugs to gain an understanding of the possible mechanisms of sperm competition. The relationship between copulation duration and ejaculate transfer and the data demonstrating that males copulating with virgin females copulated for significantly longer than those copulating second (section 2.4.4.) led me to investigate if males who copulate second inseminate less sperm than those who copulate first. The effect of female mating status and socio-sexual situation on ejaculate expenditure was examined. The suitability of the 'raffle' models of sperm competition to studies of *C. lectularius* are critically assessed.

3.3 Methods

3.3.1 General

Virgin age controlled, males and females where cultured as in Chapter 2 and used for all experimental procedures. Copulations took place under standardised conditions of light and temperature (see section 2.3.2).

3.3.2 Measurement of ejaculate sizes

Ejaculates were collected by placing females in liquid nitrogen immediately following copulation. These females were then stored at –20°C until they where dissected. All dissections were done under 0.15M Hepes buffer which was

adjusted to pH 7.2 using 0.1M K OH (Ruknudin & Raghavan, 1988). This buffer prevented tissues from changing in size as it is isotonic with respect to the tissues. Dissections where carried out under a stereo microscope (Leitz M8). The spermalege was removed from the female and placed on a microscope slide under a drop of Hepes buffer. A coverslip bridge preparation was then made of the tissue. A 0.1 mm coverslip was placed on either side of the sample and a thicker (0.2mm) coverslip was placed over the top. Excess saline was removed using a piece of filter paper until a constant thickness preparation was produced (Siva-Jothy and Hooper, 1996).

The slide was then viewed under a compound microscope (Leitz Diaplan) which was connected to an image analysis system (Optimus 6.0.) via a digital camera (Pulnix). The area of the sperm mass was calculated and then converted into its volume using the equation $V = TA$. Where T is the thickness of the preparation and A is the measured area of the sperm mass.

The ejaculate volume in each seminal concepticle was measured in the same way with the exception that females were not placed in liquid nitrogen until 6 hours after copulation. The space left in the seminal concepticles was assessed by the remaining available volume of the structure that was not filled with sperm. When observed in the coverslip bridge preparation the sperm remain in a tight bundle and do not distribute themselves evenly throughout the seminal concepticle.

3.3.3 Assessment of arrival of sperm in the ovaries

The assessment of the timing of the arrival of sperm in ovaries of females was more problematic as sperm migrate through the tissues of the female's oviducts and ovarioles in small numbers. However females were prepared in the same way as for the ejaculate size observations but instead of placing the ovaries into a coverslip bridge preparation they where dissected out and stained for ten minutes

in 20mg/ml Hoechst #33258 solution. The stained preparation was then viewed under indirect u.v. illumination in a fluorescence microscope (Leitz Diaplan). When placed under epifluorescent illumination the cell nuclei appear blue (Figure 3.1.). As sperm nuclei are compacted and elongate they can be easily distinguished from those of the female's somatic tissues. The presence of sperm after different periods following mating were scored.

3.4 Results

3.4.1 Sperm transfer

There was a significant relationship between copulation duration and the amount of ejaculate transferred. Under natural conditions the longer the copulation duration the more sperm were transferred ($r^2 = 0.549$, $P< 0.001$, N=30)(Figure 3.2.). This positive relationship also occurred when copulations were interrupted ($r^2 = 0.749$, $n = 20$, $P<0.001$)(Figure 3.3.). More of the variation in ejaculate size in the experimental group was explained by the copulation duration, suggesting that under natural conditions some males do not immediately terminate copulation after insemination has finished.

3.4.2 Ejaculate size

Males inseminated significantly larger ejaculates into virgin than non-virgin females (Mann Whitney U test: $U = 6$, $N_1 = N_2 = 20$, $P>0.001$).

3.4.3 Seminal concepticle filling

The amount of ejaculate stored in the seminal conceptacles was measured and expressed as a percentage of the total space available. The mean percentage filling of the seminal conceptacles after a copulation that lasted an average of 97.25±9.47 seconds (n=20) was 80.69 ± 3.88% (n=20). Sperm storage was also asymmetrical with the left seminal concepticle filling completely 100±0% (n=20)

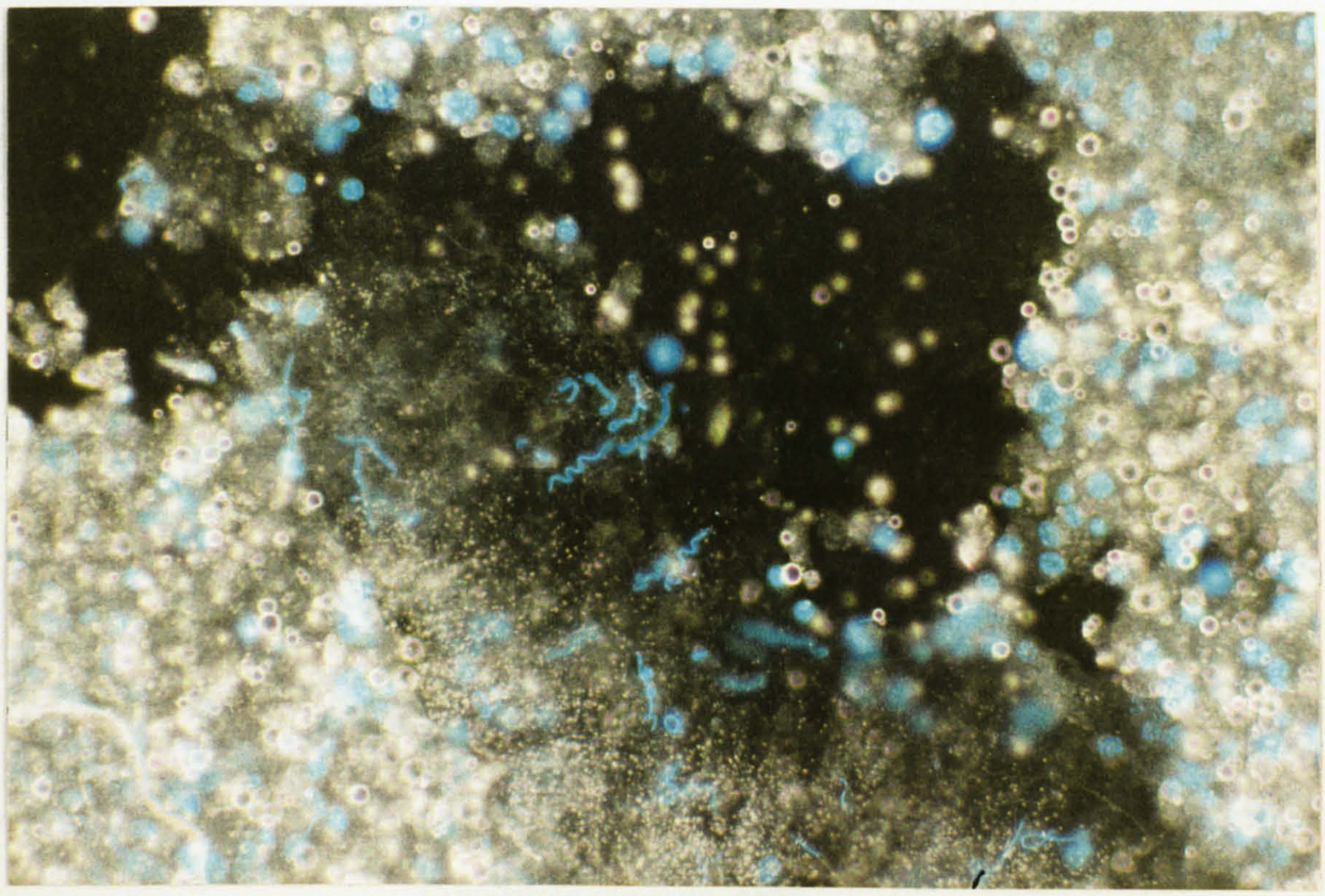


Figure 3.1. Sperm in the female meso-spermalege stained with Hoechst #33258 solution. The sperm nuclei appear blue and elongate and are easily distinguished from the nuclei of the female somatic tissue. (Magnification: x40).

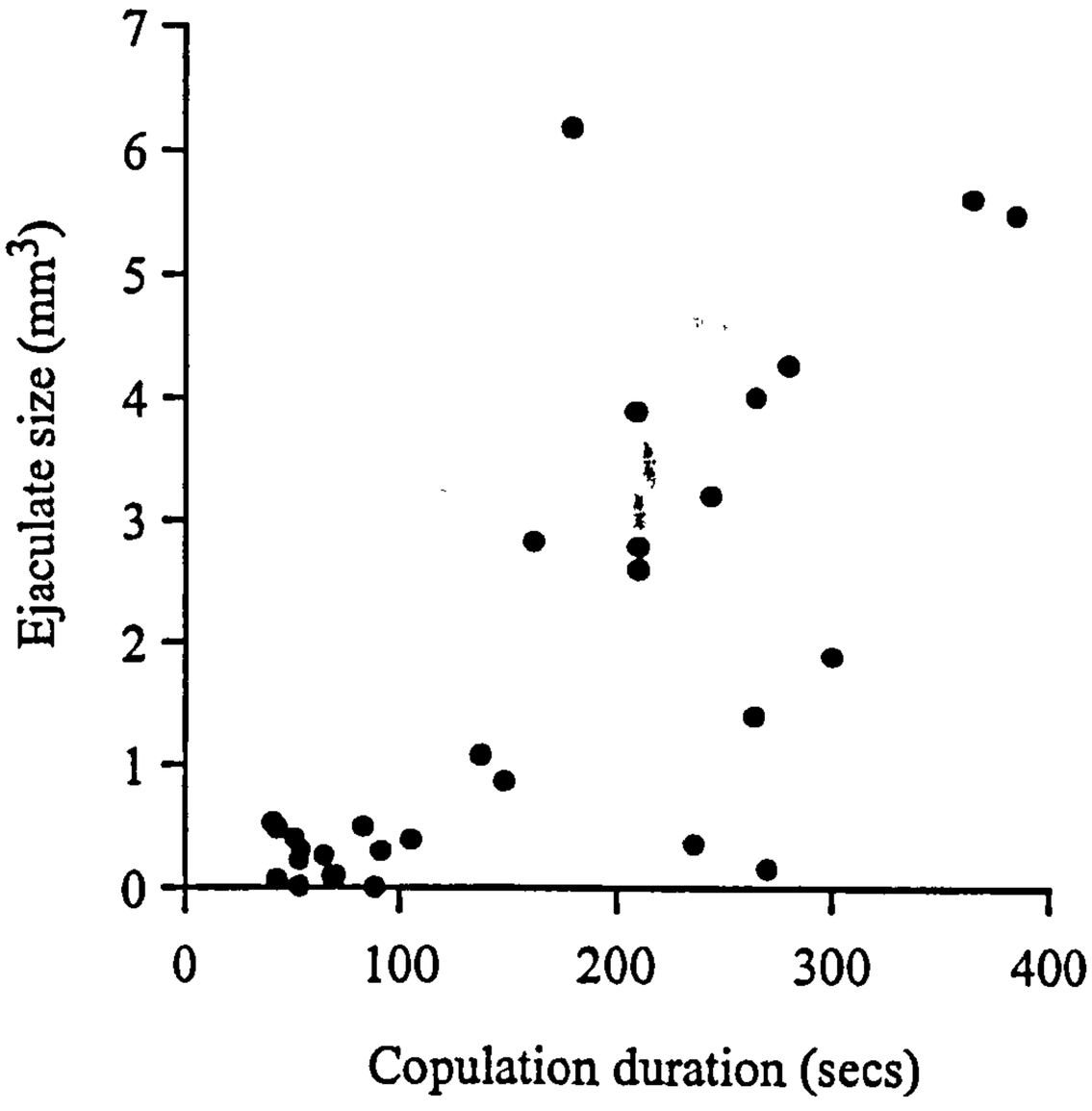


Figure 3.2. Ejaculate volume against natural copulation duration in *Cimex lectularius*. $r^2 = 0.549$, $P < 0.01$, $N = 30$

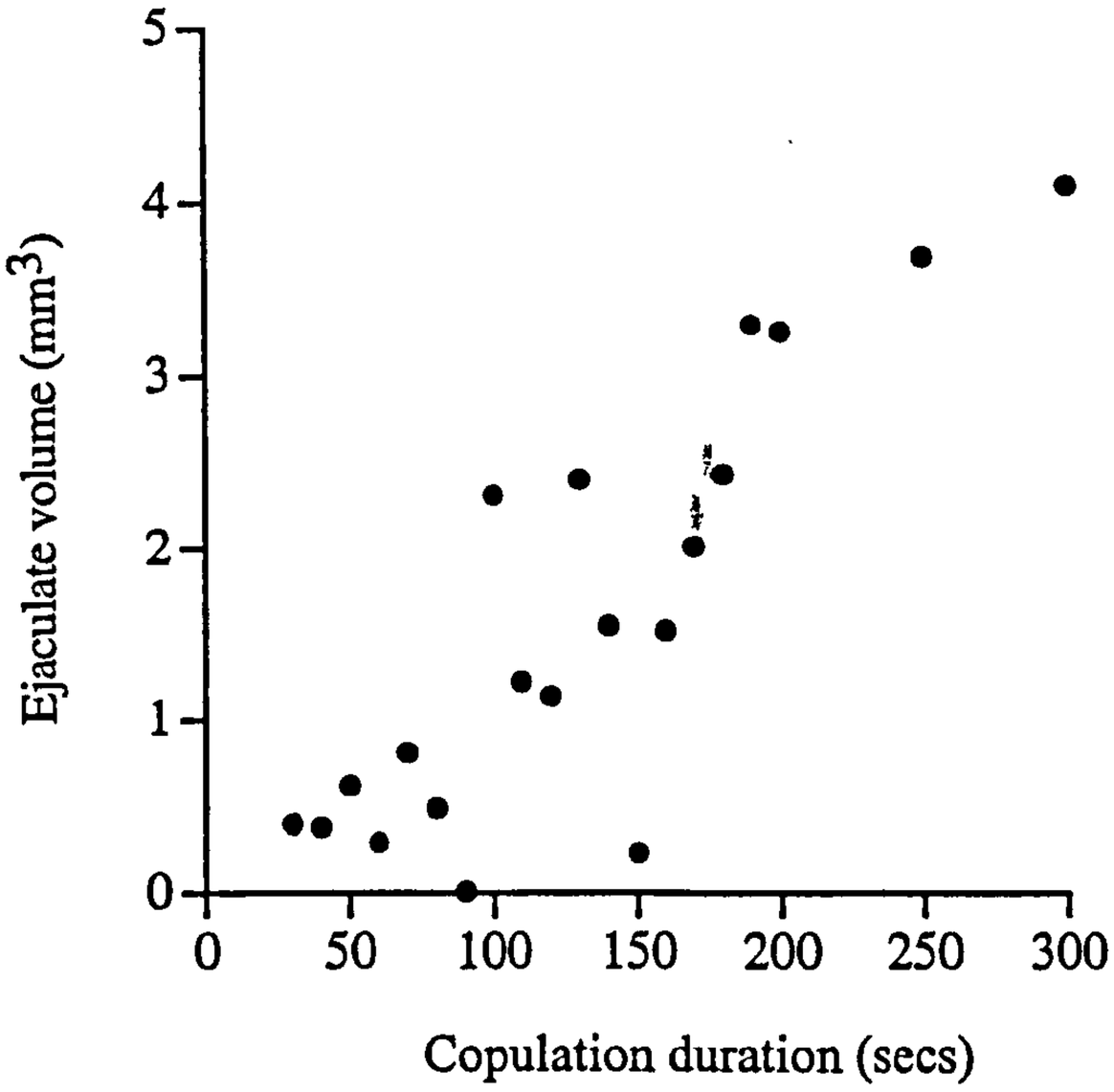


Figure 3.3. The effect of manipulating copulation termination on ejaculate size.

after a single copulation while the right concepticle fills to 3.45±0.71% (n=20) its volume.

3.4.4. Sperm in ovaries

Following staining, the presence and absence of sperm was scored in twenty females after 5 different time periods. Sperm began to migrate into the ovary in 40% of females after 12 hours. All females had sperm present in the ovary after 20 hours (Fig 3.4). Further confirmation that fertilisation is occurring in the ovary comes from the observation that females mated 4 days previously had developing zygotes present in the eggs contained in the ovarioles. This phenomenon is known as ovo-viviparity (Retnakaran & Percy, 1985).

3.5. Discussion

One of the most striking patterns in sperm transfer in *C. lectularius* is the difference in ejaculate sizes when males inseminate females of different mating status. Males inseminated nearly twice as much sperm into virgin females than into mated females. Does this pattern of sperm allocation fit the predictions of sperm allocation models and are those models appropriate for use in *C. lectularius*? The key assumption of all the 'raffle' based models of sperm competition is that sperm compete numerically (Parker, 1990) . This means that species with sperm removal are unsuitable for the application of raffle models. Sperm removal is widespread in insect taxa, and is associated with specialised male genital anatomy used to remove sperm from the female's sperm storage organs (e.g. Waage, 1979). The simple paramere of the bed bug seems unlikely to be able to remove sperm from the female and appears to function in the same way as a hypodermic needle (see Figure, 1.3.) No sperm has been observed adhering to the copulating males paramere after copulation with a non-virgin female (pers. obs.). The sperm storage organs (seminal conceptacles) are also remote to the site of copulation and are not accessible to the male's genitalia

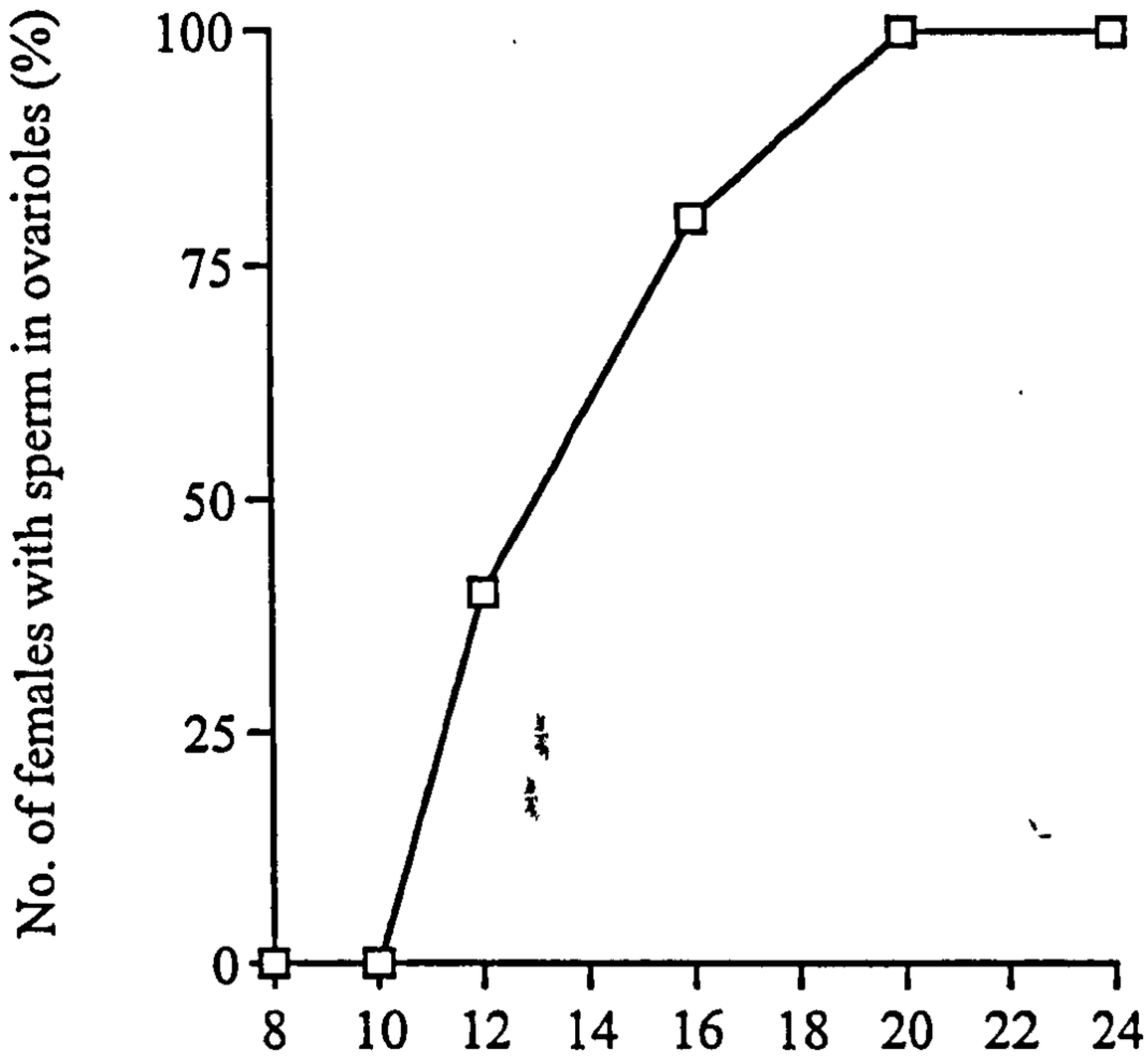


Figure 3.4. The number of females with sperm present in the ovaries at different periods following copulation (n = 20).

during copulation. Furthermore, the linearity of sperm transfer with small copulation durations yielding small ejaculates and longer copulations inseminating larger ejaculates (even when manipulated) suggests copulation functions only to inseminate sperm. In most species that carry out sperm removal the early phase of copulation is usually concerned with sperm removal: sperm transfer occurs after this initial stage (see Waage, 1984). Ejaculates have been observed to overlap in females (pers. obs.) and the sperm storage organs are relatively small and ovoid. This suggests that sperm should mix over time in the sperm storage organs. However whether sperm compete numerically is difficult to interpret from the data in this chapter and more attention is paid to this question in Chapter 4. The second assumption of the raffle models is that there is a trade-off between current investment in sperm production and future mating opportunities (Parker, 1990). Sperm depletion is a commonly reported phenomenon in a wide range of taxa (e.g. Birkhead & Fletcher, 1995; Pitnick, 1993), and the high mating rate of males after feeding is likely to cause a similar effect in *C. lectularius*.

If we accept that the first two assumptions of the raffle models are likely to be at least partially valid then which of the three proposed models are most suitable for application to *C. lectularius*? Parker et al's (1990) sneaks and extra-pair copulations games can be excluded initially as both mate guarding and paternal investment appear to be of little importance in *C. lectularius* mating systems (see Chapter 2, Appendix I). Parker's (1990) models of sperm competition can also be discounted as they assume very low levels of sperm competition where a male's ejaculate has a given probability of competing with an other ejaculate. Given a female bed bug copulates an average of 5 times during a single reproductive bout (see Chapter 2) a model that is based on higher levels of sperm competition is required. This is provided by Parker et al's (1997) 'intensity' model of sperm competition. This model has the same assumptions as the 'risk' model of sperm competition however it also assumes that the raffle is loaded. A

loaded raffle is where there is inequality in the chance of a sperm from one ejaculate gaining fertilisations (i.e. when there is a mating order effect). This assumption is discussed more fully in Chapter 4 but can be accepted for the current discussion. What predictions do Parker et al.'s (1997) model have for a situation like that found in the *C. lectularius* mating system? There are three main predictions that come from the model. Firstly if males have no information on individual females then expenditure should increase linearly from zero with the increasing probability (q) of sperm competition in the population. Where males have full information on females then males should have minimal ejaculation with 'no risk' females and should expend equally with 'past' and 'future' risk females. Finally where males have partial information (e.g. virgin or mated) expenditure increases with q, so males are predicted to allocate more sperm on females representing higher risk (e.g. mated females).

To consider which prediction should be used in the current context we need to consider the question; how much information does a male have on a female's mating status? The fact that males inseminate less sperm into non-virgin females when no other cues are present provides some evidence that males may be able to detect female mating status. So, as the assumptions of this model appear to be valid, how does the male's ejaculate expenditure match the predictions of the model? Where males have some information on female mating status males should allocate larger ejaculates to mated females than virgins (Parker et al., 1997). This is opposite of what appears to be occurring in *C. lectularius*. The possible reasons why males appear to use this paradoxical ejaculate strategy is discussed in Chapter 4 along with its effect on P_2.

Sperm storage by females is asymmetrical and males do not inseminate enough sperm to fill both sperm storage organs. This observation when combined with the confirmation of ovarian fertilisation in this system allows mechanisms of

sperm competition to be predicted. These data are discussed in the context of sperm precedence in Chapter 4.

Summary

In this chapter I have demonstrated that males allocate sperm in response to a female's mating status. Copulation duration was found to be a good predictor of ejaculate size. This relationship allows the role of ejaculate size on sperm precedence to be examined. Sperm storage was asymmetrical in the female and fertilisation occurs in the ovary.

4 Sperm competition

4.1. Introduction

Parker (1970) first suggested, and subsequently demonstrated, that sexual selection could continue after copulation when the sperm of different males competed for the fertilisation of a given female's ova. He termed this sperm competition, and it has proven to be an important determinant of male reproductive success in a wide diversity of taxa (for review, see Birkhead & Møller, 1998)

Sperm competition in insects has been studied using one of two main methods. The sterile male technique where some males are given sub-lethal doses of x or γ irradiation. The sperm of irradiated males function normally but eggs fertilised by these sperm carry dominant lethal mutations that cause early embryo death. Eggs fertilised by irradiated males can therefore be easily distinguished from those sired by normal (i.e. non-irradiated) males (Boorman & Parker, 1976). The second method of determining paternity utilises the genetic marker technique. This uses either colour morphs of known inheritance pattern, enzyme polymorphisms, or more recently DNA markers to determine paternity (Dickinson, 1988; Eady, 1991; Cooper et al., 1996). In both methods the proportion of eggs sired by the second male to copulate with a doubly mated female is termed the P_2 value and is the convention used to describe paternity in all insect sperm competition studies (see Boorman & Parker, 1976).

4.1.1. Mechanisms of sperm competition

Examining the paternity of offspring from multiply mated females is only part of the study of insect sperm competition. In order to understand how sexual

selection acts on males and females the underlying mechanisms need to be examined. Sperm competition in insects has been well studied and the mechanism by which some males gain high levels of sperm precedence over other males is well understood in some insect taxa (e.g. Waage, 1979). Knowledge of the mechanism of sperm competition allows prediction of the more subtle strategies that males may be using to maximise their reproductive success (e.g. ejaculate adjustment (Parker, 1990; Parker et al., 1990). Unlike other taxa, such as birds, where a single mechanism of sperm precedence has been proposed (Colegrave et al., 1995) the behavioural, morphological and physiological diversity of insects means that large numbers of different mechanisms are likely to occur, and mechanisms of sperm competition across insect taxa are never likely to be well understood (Simmons & Siva-Jothy, 1998; Ridley, 1999). This caveat means that caution needs to be used when interpreting the pattern of sperm precedence if the mechanism by which it is derived is not understood. Parker (1990; also Parker et al., 1990) advocated the use of mathematical ESS models to predict mechanisms of sperm competition in insects. Two main mechanisms have been modelled: random sperm mixing where a male's sperm acts like a lottery ticket (the more sperm inseminated the higher the chance of gaining paternity), and sperm displacement where a male uses his ejaculate to displace the previous male's sperm. These models are useful where knowledge of sperm migration, storage and transfer has been elucidated. The other method of examining mechanisms relies on verbal arguments of mechanisms following studies that determine patterns of sperm migration, storage and usage in the female's reproductive tract (Simmons & Siva-Jothy, 1998).

4.1.2. Sperm competition avoidance mechanisms

With sperm competition proving to be an important determinant of male reproductive success, males are predicted to produce adaptations that would prevent their sperm competing with those of other males; this has been termed

sperm competition avoidance (Parker, 1970a; Simmons & Siva-Jothy, 1998). These adaptations are not mutually exclusive from sperm displacement and precedence but they include mate guarding (proximate and remote)(Alcock, 1994) and multiple copulations with the same female (Hunter et al., 1993). Sperm competition avoidance mechanisms that occur during copulation usually function to avoid sperm competition with previous males' sperm. Avoidance mechanisms that occur after copulation often prevent inseminated sperm from competing with the sperm of future males.

i. Avoidance of sperm competition with previously stored sperm

One mechanism to avoid sperm competition with previously stored sperm is to remove or reposition the previous males' ejaculates. These sperm displacement mechanisms were defined by Simmons and Siva-Jothy (1998) as: 'The spatial displacement of sperm derived from a female's previous mate(s) by the copulating male with the consequence that self sperm is more likely to fertilise ova, while displaced sperm is less likely to do so (p. 433)'. Three commonly cited mechanisms of sperm displacement are physical removal of sperm (Waage, 1979); sperm flushing (Simmons et al., 1999) and sperm repositioning (Waage, 1984). Studies of sperm displacement are the only good examples in insect taxa where sperm competition mechanisms have been studied in detail (Clark et al., 1995; Clark et al., 1999).

ii. Avoidance of sperm competition with future males

As shown in Chapter 2 neither proximate mate guarding nor repeated copulations with a single female occur in *C. lectularius* so I will concentrate on examples of remote mate guarding. Remote mate guarding is any physical or chemical mechanism which prevents females from effective remating (Simmons & Siva-Jothy, 1998). One common example found in insects is the insertion of a post-copulatory mating plug by the male (Boorman & Parker, 1976; Parker, 1984).

These mating plugs act to physically prevent other males from copulating with a female. Compounds within the ejaculate have also been shown to manipulate female behaviour. In several species of Diptera males transfer compounds in the seminal fluid which increases the remating interval (e.g. Thornhill, 1976; Kalb et al., 1993). Specialist sperm morphs have also been implicated in delaying female remating in some Lepidoptera (Cook & Wedell, 1999). Spermathaecal filling has been suggested as a mechanism to avoid sperm competition (Retnakaran, 1974). Although originally proposed to explain the bimodal distribution of P_2 values observed in the Lepidoptera it has been used as an explanation for patterns of P_2 in several other orders (Wilkes, 1965; Gwynne & Snedden, 1995; Suzuki et al., 1996). One novel method of preventing remating has been suggested in a bush cricket where males use a modified subgenital plate to remove the sperm of previous males the removal process also damages the reproductive tract and prevents the female from remating. It remains unclear whether the damage is an intentional strategy by the male to prevent the female from remating or a incidental effect of the sperm removal mechanism (Helversen & Helversen, 1991).

4.1.3. Strategic ejaculation

Parker (1990; Parker et al., 1996; 1997) suggested that where sperm compete numerically, males should adjust the number of sperm they inseminate into a female according to the likelihood of the sperm having to compete with those of other males. Males should also invest in their ejaculates depending on their future reproductive potential as ejaculates can be costly to produce (Dewsbury, 1982; Van Voorhies, 1992). So where sperm compete numerically the amount of sperm allocated to each ejaculate should match the perceived risk of competition. Therefore the best place to examine strategic ejaculation is in species where there is no sperm displacement.

In species which carry out physical sperm removal of the previous male's ejaculate, males would be predicted not to vary the size of their ejaculates according to the risk of sperm competition as sperm numbers would have little bearing on the fertilisation success (Siva-Jothy & Tsubaki, 1989a; Siva-Jothy & Tsubaki, 1989b). However, where a male's ejaculate is used to flush out the sperm of previous males then ejaculate size (rather than sperm numbers) is of importance. There is good comparative evidence that this occurs in the Lepidoptera where males in monandrous species inseminate relatively small spermatophores and males in species that are polyandrous inseminate relatively large spermatophores (Svärd & Wicklund, 1989; Gage, 1994). However, this finding is also consistent with the hypothesis of Retnakaran (1974) that male Lepidoptera avoid sperm competition by filling the sperm storage organs of females. This leads to a bimodal distribution of P_2 where the modes occur at 0 and 1.

4.1.4. Female roles in sperm precedence

Lewis and Austad (1990) were the first to point out the ubiquity and importance of intraspecific variation in P_2. The variance was partitioned out and revealed a potential role for females to control sperm storage and usage. Since this study many examples have demonstrated that females cannot be assumed to be passive arenas in which sperm compete (for review see Eberhard & Cordero, 1995; Eberhard, 1998). Female causation of variance in P_2 has been demonstrated in the bruchid beetle *Callosobruchus maculatus* although the mechanism by which females cause this variation is still unknown (Wilson et al., 1997). Careful studies in two species of Diptera have also demonstrated a female role in sperm displacement (Clark et al., 1999; Simmons et al., 1999). Although the problems of examining female roles in sperm competition are manifold, species where intraspecific variation in P_2 is high should be highlighted as candidates for female effects on paternity (Siva-Jothy & Hadrys, 1998).

4.1.5. Possible mechanisms of sperm competition in *C. lectularius*

As levels of sperm competition in traumatically inseminating insects are not known predicting mechanisms is problematic. However, other taxa can be used to suggest possible mechanisms and patterns of sperm precedence. In *C. lectularius* physical removal can be ruled out for reasons outlined in Chapter 3. The use of the male's ejaculate to displace sperm from the sperm storage organ is also unlikely as insemination takes place in the spermalege away from the site of sperm storage (see Chapter 3). However repositioning of sperm towards the anterior of the spermalege may occur if the remating interval of the female is less than three hours (the time taken for the first male's ejaculate to leave the spermalege). As sperm can only migrate out of the posterior of the spermalege then this could provide the second male with a positional advantage in the spermalege before the sperm migrate towards the seminal conceptacles. Since in this scenario the second male's ejaculate will reach and fill the seminal conceptacles first, this could prevent the access of the second male's sperm (see Chapter 3 and Wilkes, 1965; Retnakaran, 1974; Gwynne & Snedden, 1995; Suzuki et al., 1996). Evidence suggests that spermathaecal filling may be important in *C. lectularius* comes from the observation that sperm remain in the female's haemoceol around the seminal conceptacles for several days following multiple inseminations (see Chapter 3; Carayon, 1966).

If sperm repositioning and seminal concepticle filling are part of the mechanism of sperm competition in *C. lectularius* then I would predict that sperm precedence would be second male biased when there was a short remating interval. However, when the remating interval is longer the time taken for the sperm of the first male to migrate out of the spermalege, first male sperm precedence would be expected. I used this as a working hypothesis to investigate sperm competition and its possible mechanism.

4.2. Aims

The aims of this chapter are; to examine the pattern of short- and long-term sperm precedence (P_2) in *C. lectularius*. To assess the role of different male ejaculate allocation strategies on paternity. Finally, to examine the potential mechanisms of sperm precedence, using data on sperm migration from Chapter 3, and using an experimental approach to alter patterns of sperm precedence by altering the remating interval of males.

4.3. Methods

Paternity was assessed using the irradiated male technique of Boorman & Parker (1976). This is a standard technique for assessing paternity in laboratory studies (Eady, 1991). Males were irradiated using 30 Krads of γ-irradiation from a closed Cs^{137} source at Liverpool University (Atomic Energy of Canada Ltd, Gamma cell 1000). The average dose rate was 384 rads/min All the animals used were controlled for age, nutritional and mating status (i.e. were virgins at the beginning of the experiment). All matings occurred in a constant temperature room (26 ±1°C) under diffuse red lighting conditions. The copulation durations of all the matings were recorded. Following double matings females were isolated in individual $7cm^3$ tubes and kept in standard conditions (see section 2.3.1.) to oviposit. Once the last eggs of the first clutch were laid females were given a further blood meal and allowed to oviposit a second clutch. The number of fertile and sterile eggs were counted to assess paternity. The number of eggs fertilised by the second male in the first clutch was termed short term P_2. The number of eggs fertilised by the second male in the second clutch laid was termed long term P_2 (Siva-Jothy et al., 1996). To correct for variation in the natural fertility of the males in the population as well as variation in the effectiveness of the irradiation protocol sterile-sterile and fertile-fertile control matings were carried out. The data from this experimental design were entered into a mathematical correction to

control for the variation in natural and experimentally induced sterility (Boorman & Parker 1976). The equation is given below.

$$P_R = \left[1-\frac{x}{p}\right] + \left[\frac{z}{p}\times\frac{1-\left[\frac{x}{p}\right]}{1-\left[\frac{z}{p}\right]}\right]$$

Where P_R is the number of offspring sired by the irradiated male, x is the mean natural fertility, z is the mean level of sterility in the irradiated males and p is the number of offspring sired by the second male to mate in a doubly mated female.

The reciprocal sterile: fertile and fertile: sterile matings were used to assess the relative competitive ability of normal and irradiated sperm. The experiment was split into two distinct parts. The first experiment used the treatments outlined above but the remating interval between first and second males was 17 minutes. (the median remating interval observed in laboratory observations (see section 2.4.3.). The second experiment used a remating interval of four hours. This remating interval was used because after four hours the first male's sperm will have migrated out of the spermalege and will have begun to enter the seminal conceptacles (see Chapter 3). The second experiment therefore examines if sperm 'interactions' in the spermalege and filling of the seminal conceptacles contribute to the mechanism by which second males are gaining sperm precedence.

4.4. Results

4.4.1. Short remating interval

4.4.1.1. Experimental male sizes

There was no significant difference in male size in any of the experimental groups when considering mating order or sterilisation protocol) (ANOVA: $F_{3,116}=2.54$, NS).

4.4.1.2. Copulation duration of 1st & 2nd males

The copulation duration of males copulating first was significantly longer (129.3±13.6secs., N=30) than males copulating second (66.8±5.6secs., N=30)(ANOVA: $F_{3,116}$=33.65, P<0.001) however there was no significant effect of the sterilisation protocol on the copulation durations of males copulating first (105.6±5.7secs., N=30) or second (67.2±7.2secs., N=30)(ANOVA: $F_{3,116}$=1.78, P=0.183)(Figure 4.1.). There was no significant interaction between these factors (ANOVA: $F_{3,116}$=1.92, P=0.168).

4.4.1.3. Short term P_2

Short term sperm precedence (i.e. the number of eggs sired by the second male in the first clutch) was second male biased (0.68±0.03, N=20). There was considerable variation around this mean value (Figure 4.2a).

4.4.1.4. Long Term P_2

After 14 days the P_2 value had declined significantly to an intermediate level of sperm precedence (0.48±0.02)(Wilcoxon signed rank test: $T = 37$, $N = 40$, P<0.001). Again there was considerable variation around this mean value (Figure 4.2b).

4.4.1.5. Effect of ejaculate size on P_2

The effect of ejaculate size on the level of P_2 was estimated by the relationship between the level of P_2 a male achieved against the ejaculate size of the second male. There was no effect on short-term P_2 of ejaculate size of either the first (Spearmans rank correlation r^2 =0.19, P=0.22), second (Spearmans rank correlation r^2 =0.05, P=0.97) or the 1st males ejaculate size divided by the 2nd males (Spearmans rank correlation r^2 =-0.23, P=0.14)(Figure 4.3a,b,c).

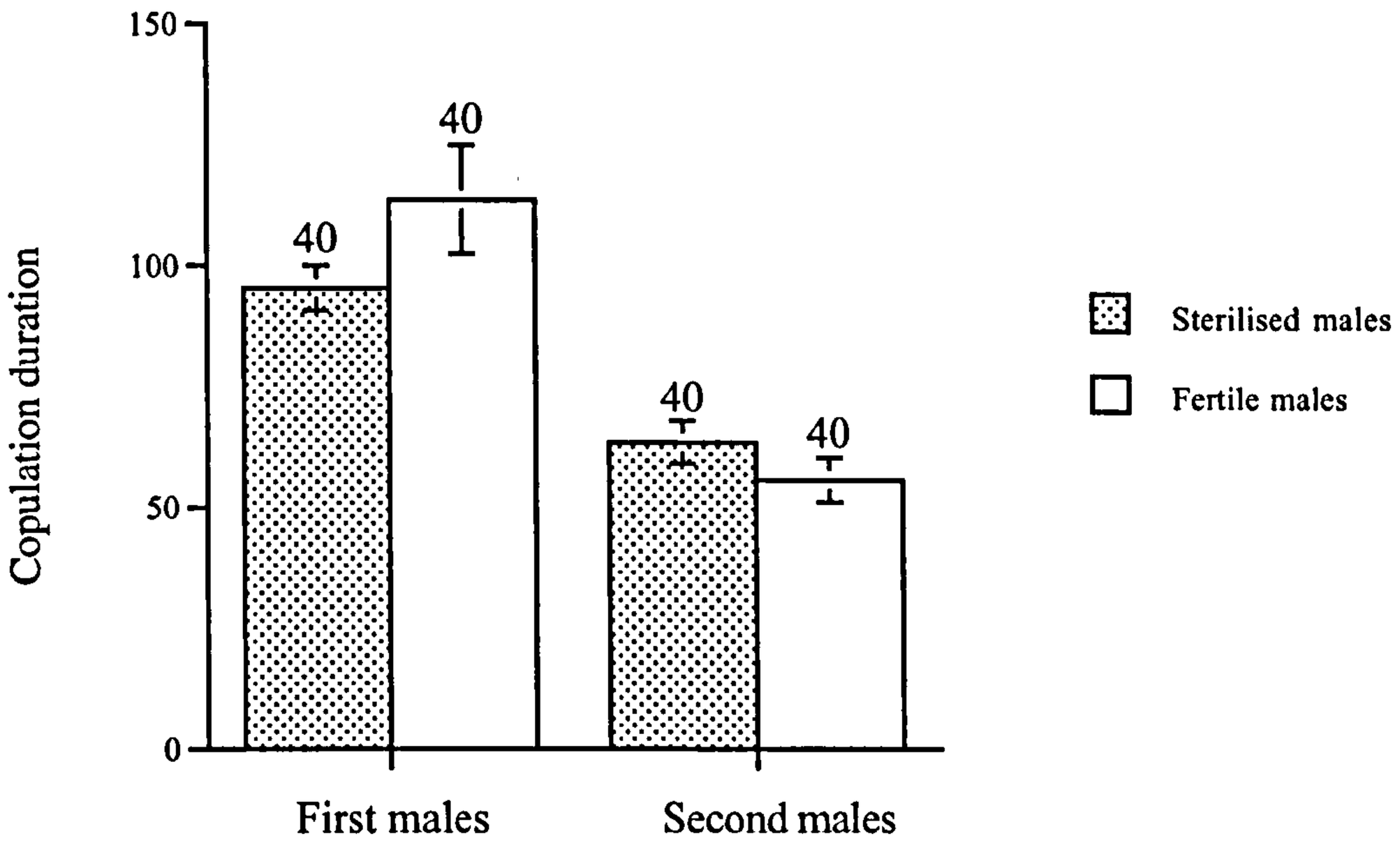


Figure 4.1. Copulation durations of sterile and normal males copulating with virgin and non-virgin females with a remating interval of 17 minutes.

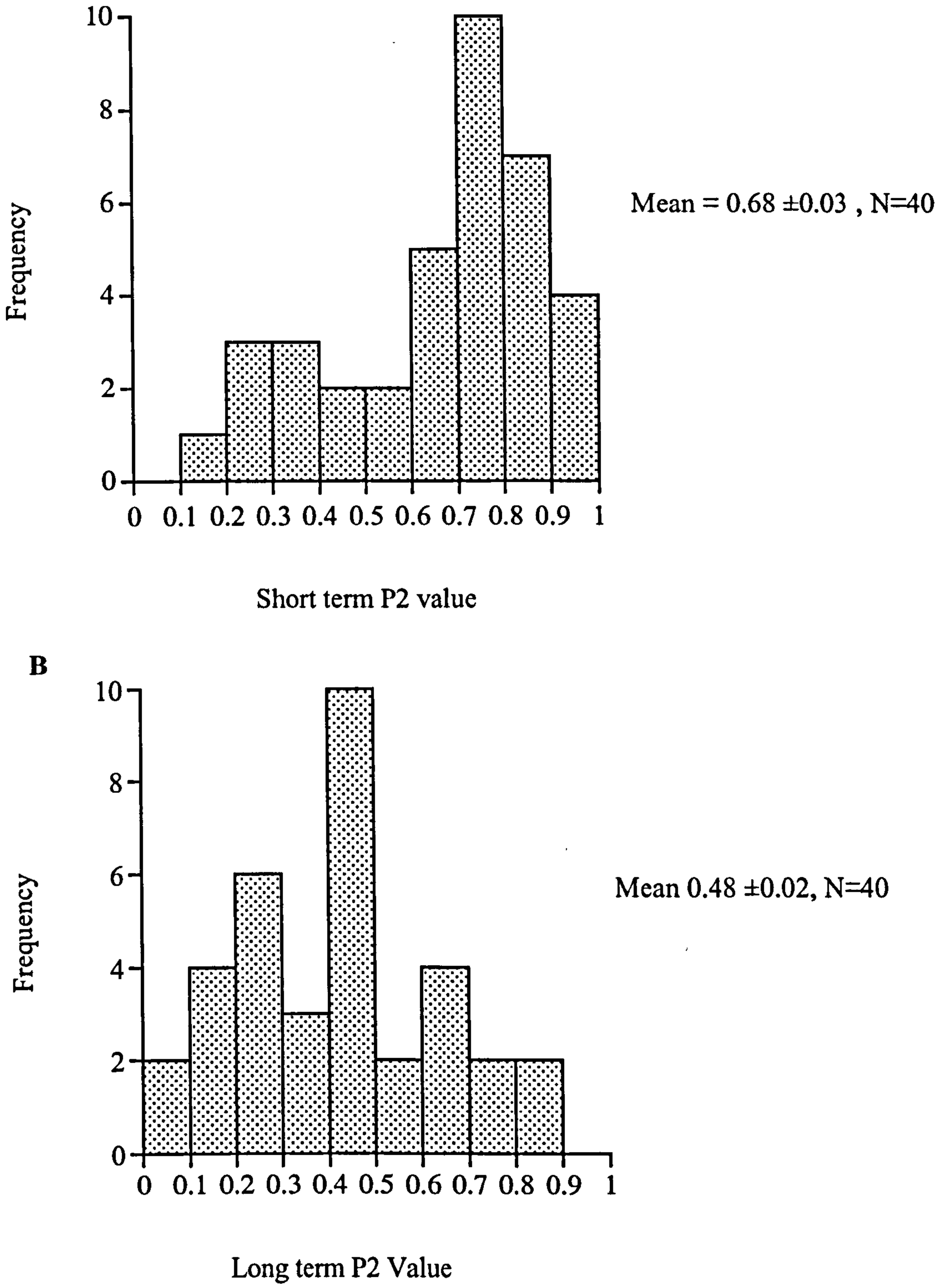


Figure 4.2 Frequency distributions for short- (A) and long- (B) term P2 values with a remating interval of 17 minutes.

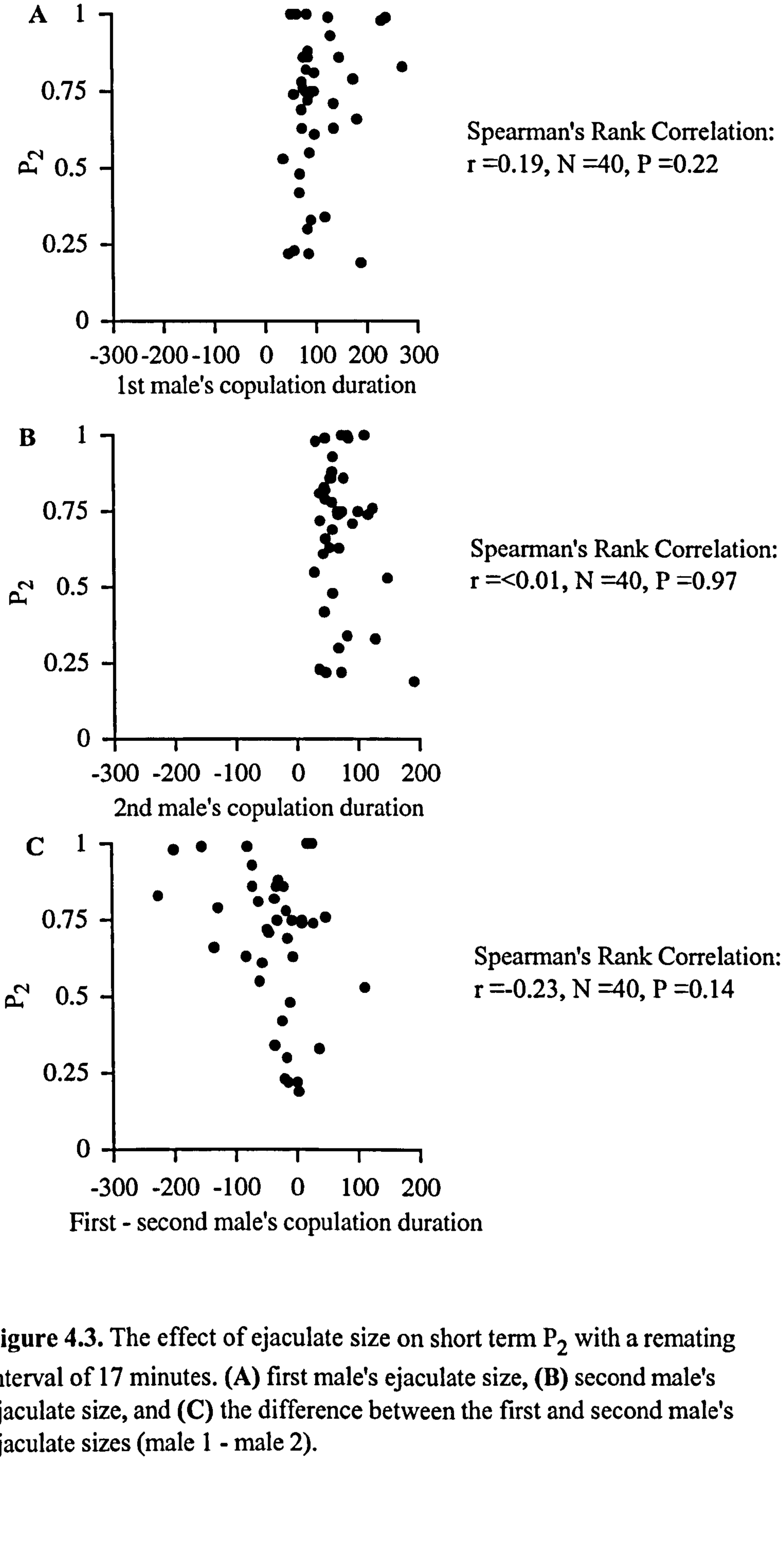


Figure 4.3. The effect of ejaculate size on short term P_2 with a remating interval of 17 minutes. **(A)** first male's ejaculate size, **(B)** second male's ejaculate size, and **(C)** the difference between the first and second male's ejaculate sizes (male 1 - male 2).

4.4.2. Long remating interval

4.4.2.1. Experimental male sizes

Males were split into their experimental groups according to their mating order and their sterilisation treatment. There was no difference in the size of males in any of the treatment groups (ANOVA, $F_{3,116} = 3.11$, NS).

4.4.2.2. Copulation duration of 1st & 2nd males

As with the short remating interval study the copulation duration of males copulating first was significantly longer (129.33±13.66secs., N=30) than that of males copulating second (66.80±5.62secs., N=30)(ANOVA: $F_{3,116}$=33.82, P<0.001).. There was also no significant effect of the sterilisation protocol on the copulation durations of males copulating first (105.63±5.70secs., N=30) or second (67.23±7.20secs., N=30)(ANOVA: $F_{3,116}$=1.65, P=0.201). There was no significant interaction between these factors (ANOVA: $F_{3,116}$=2.34, P=0.313).

4.4.2.3. Short term P_2

The mean number of eggs sired by the second male to mate following double matings with a 4 hour remating interval was low (0.29± 0.03 n=20). As before there was considerable variation around this P_2 value (Figure 4.4a)

4.4.2.4. Long Term P_2

After 14 days the P_2 value had increased significantly to an intermediate level of sperm precedence (0.43±0.06 n=20)(Wilcoxon signed rank test: $T = 7$, $N = 40$ P=0.004). Again there was considerable variation around this mean value (Figure 4.4b).

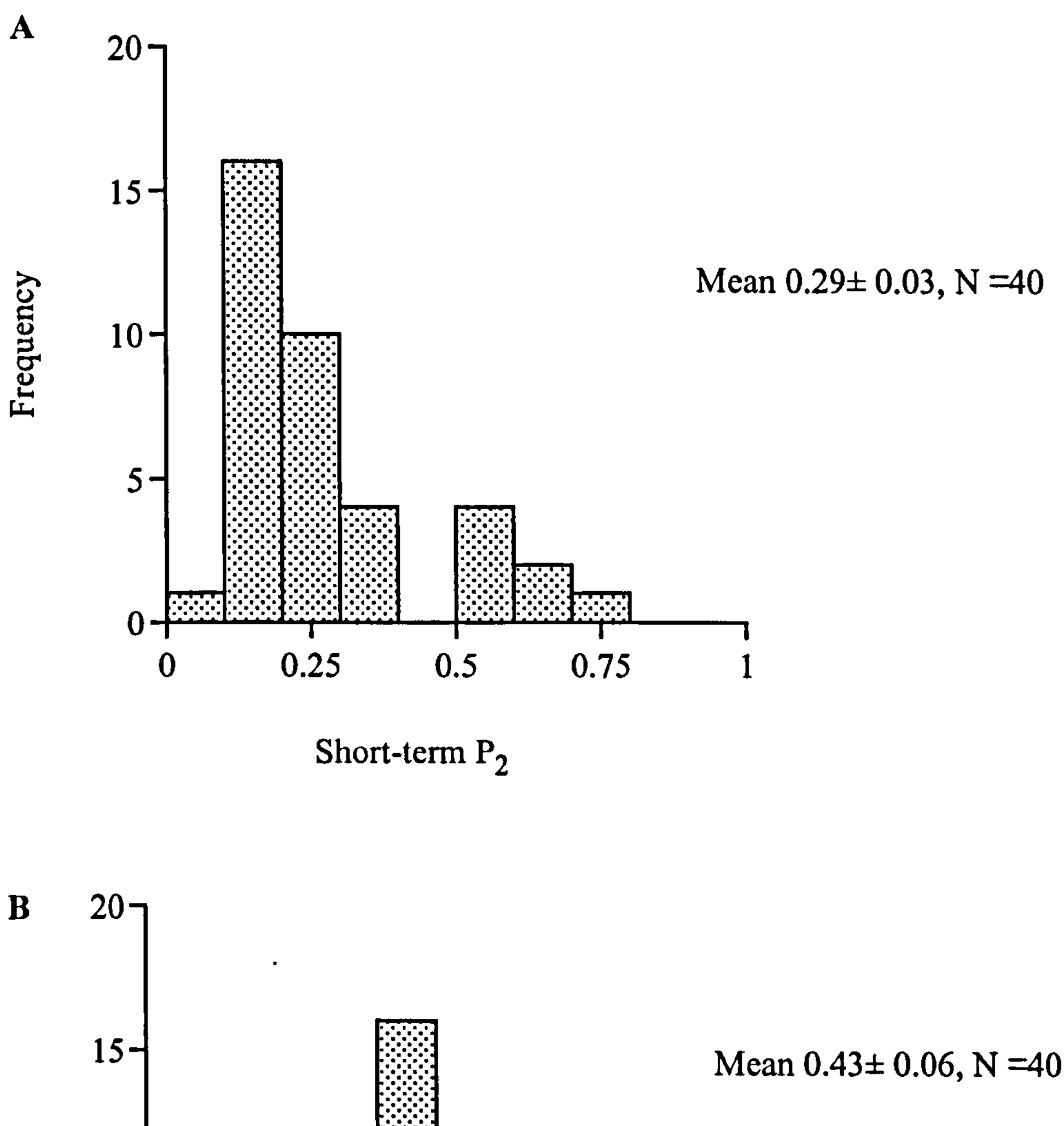


Figure 4.4. Frequency distributions for short- (A) and long-term P_2 values (B) with a remating interval of 4 hours.

4.4.2.5. Effect of ejaculate size on P_2

The effect of ejaculate size on the level of P_2 was again estimated by the relationship between the level of P_2 a male achieved against the ejaculate size of the second male. There was no effect on P_2 of ejaculate size of either the first (Spearmans rank correlation r^2 =0.13, P=0.40), second (Spearmans rank correlation r^2 =-0.12, P=0.45) or the 1st males ejaculate size divided by the 2nd males (Spearmans rank correlation r^2 =0.19, P=0.31).

4.4.2.6. Differences between P_2 with short and long remating intervals

The median short-term P_2 value when the remating interval is 17 minutes is significantly higher than when the remating interval is 4 hours (Mann-Whitney U test: U=57, N= 40, P<0.001). The pattern of P_2 is reversed with c. 68% of fertilisations going to the second male when remating interval is short and c. 70% of fertilisations going to the first male when remating intervals are long. Long-term P_2 values are not significantly different when the remating interval is altered (Mann-Whitney U test: U=754, N= 40, P= 0.705).

4.5. Discussion

Sperm competition has not previously been studied in a traumatically inseminating insect. In this chapter I have demonstrated that sperm competition occurs in *C. lectularius* and resulted in non-random paternity amongst males. The short term P_2 value was only moderately high in comparison to other published studies of sperm competition in insects (see Simmons & Siva-Jothy, 1998) and dropped to an intermediate level (equal precedence for each male) within 14 days of copulation. By increasing the remating interval of the female the P_2 pattern was reversed with sperm precedence being achieved by the first male. This reversal of the P_2 pattern suggests the outcome of sperm competition is complex in *C. lectularius*. This is not surprising as the sperm storage organs of

the female cannot be directly manipulated by males. The mechanisms of sperm competition avoidance may well occur because of this.

4.5.1. Role of sperm numbers

Males who copulated with virgin females inseminate almost twice the quantity of sperm of those who copulate with a once mated female. Large ejaculates are expected to have an effect on P_2 in two different situations. Firstly, where sperm mix randomly in the sperm storage organs and where numerical sperm superiority results in higher fertilisation success for that male (Parker, 1990; Parker et al., 1990). Secondly, where the ejaculate of the copulating male is used to displace the sperm of the previous male or prevent sperm from entering the sperm storage organs by filling the sperm storage organs (Retnakaran, 1974; Simmons et al., 1999). The fact that larger ejaculates have no effect on P_2 suggests that random sperm mixing is not the mechanism males use to gain high levels of paternity, however large first male ejaculates may be an attempt to fill the sperm storage organs of the female to prevent the sperm of future males entering the seminal conceptacles (see Chapter 3).

4.5.2. Mechanisms of sperm competition

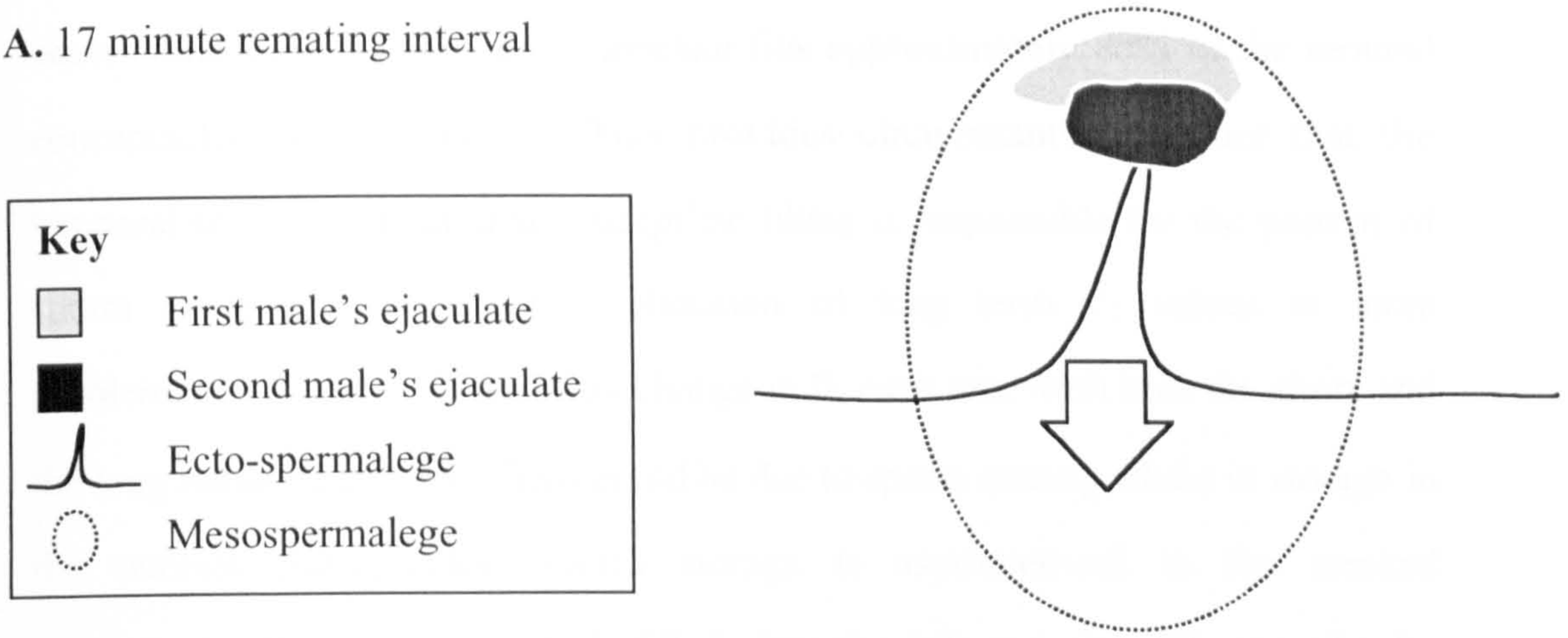
Fertilisation in *C. lectularius* occurs in the ovarioles (see Chapter 3). However sperm must first migrate into the seminal conceptacles before they enter the wall of the oviduct and move into the ovarioles. Sperm that enter the seminal conceptacles first thus have a positional advantage (in that they are closer to the ovary). However the first sperm to enter the seminal conceptacles is also likely to be the first sperm out as the point of entry to the seminal conceptacles is furthest away from the point of exit. Sperm release in other insects is from the spermathecae and is usually under female control, with sperm being released as the egg moves down the oviduct (Chapman 1998). This is not the case in *C. lectularius* and the unusual migration pattern (i.e. a conduit system rather than a

single entrance/exit system) should be born in mind during the rest of this discussion. The change in short-term P_2 caused by varying the remating interval of females supports the idea that different mechanisms of sperm precedence are occurring. With the 17 minute remating interval the ejaculate of the first male to copulate is still in a position that could allow the second male's ejaculate to pre-empt it. This is not the case when the remating interval is long enough to allow the first male's ejaculate to migrate to the female's seminal concepticles which are inaccessible to the second male.

The role of ejaculate size in sperm displacement in the spermalege is unclear. It could be predicted that the second male to copulate with a female should allocate a large ejaculate in order to displace the sperm of the previous male completely. This is pattern of allocation is also predicted by the intensity model of sperm competition (Parker, et al., 1997). However, a male who copulates with a non-virgin female is unlikely to have information on the mating history of that female other than the fact that she is not a virgin and this may explain why a smaller ejaculate is allocated. No effect of ejaculate size on P_2 was found in either of the sperm precedence experiments. One possible explanation of the pattern of sperm allocation observed is that the benefits that a male gains from allocating a large ejaculate are only expressed over longer temporal scales than investigated in these experiments. Female *C. lectularius* live and produce eggs for several months (see Chapter 5), therefore males allocating large ejaculates may be gaining fertilisation success over these much greater time scales. This hypothesis requires further investigation.

It seems likely that short-term second male sperm precedence, after a short remating interval, is caused by the displacement of the first ejaculate in the spermalege followed by the filling of the seminal conceptacles by the second male's sperm (Figure 4.5.a). At long remating intervals the first male's sperm can

A. 17 minute remating interval



B. 4 hour remating interval

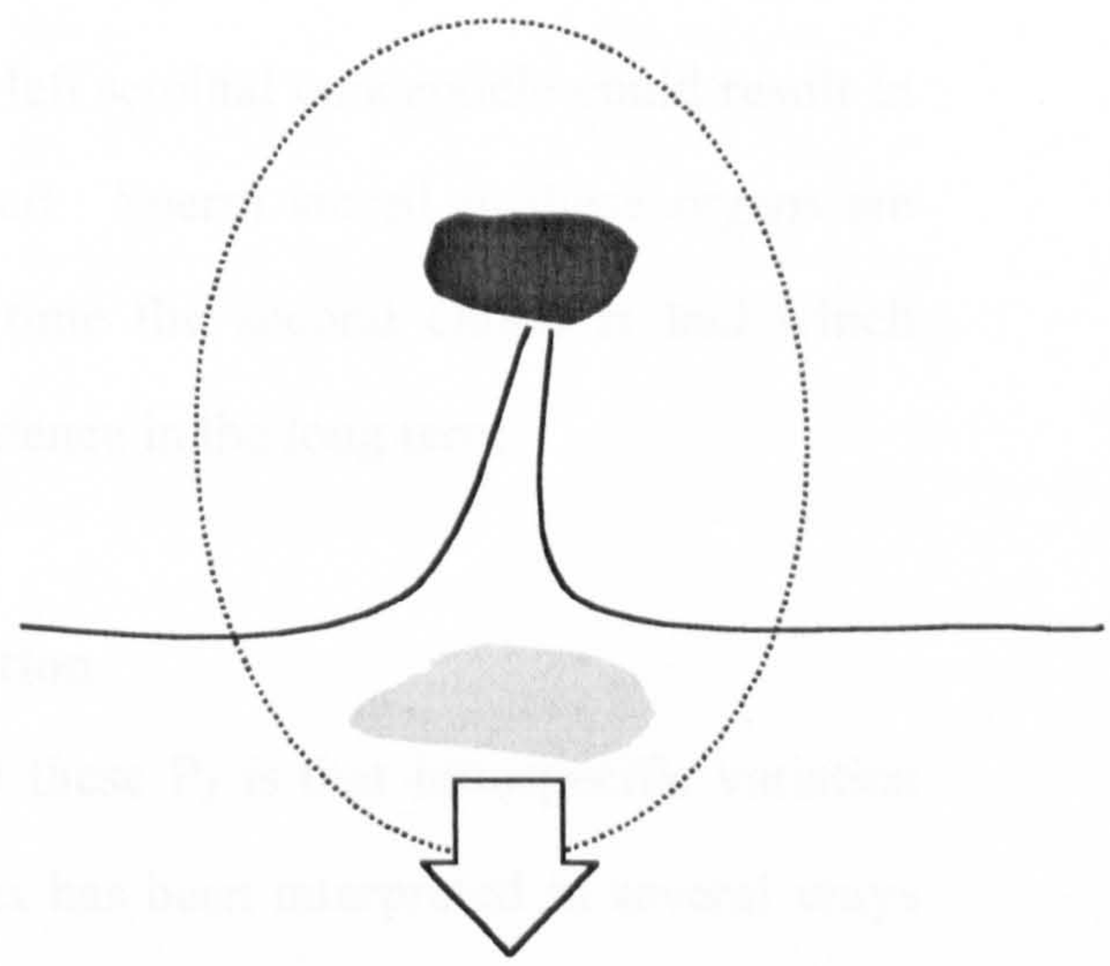


Figure 4.5. Possible sperm interactions in the female's spermalege. **A.** With a remating interval of 17 minutes the second males sperm pre-empts the first males sperm and so is the first to leave the meso-spermalege. **B.** When the remating interval is 4 hours the first males sperm has left the spermalege before the second males sperm can pre-empt it and so is the first sperm to fill the seminal concepticles. See Figure 1.4. for anatomical detail.

fill the seminal conceptacles before the second male's sperm have a chance to pre-empt the first male's sperm in the spermalege (Figure 4.5.b). The mean short-term P_2 value of 0.3 is consistent with the predicted value based on the observation that the first male's ejaculate fills approximately 80% of the seminal conceptacles (see Chapter 3). This provides circumstantial evidence that the temporal sequence of seminal concepticle filling is responsible for the pattern of sperm precedence observed. Explanation of long term P_2 values is more problematic. There is a significant change in P_2 over time with both the short and the long remating intervals. This could be due to sperm mixing whilst in storage in the seminal conceptacles. Sperm storage is asymmetrical in the seminal conceptacles the right concepticle fills before the left and also fills completely. The left concepticle only partially fills following a single copulation (See Chapter 3). The space remaining in the left concepticle is presumably filled by the second male's sperm. The mixing of sperm in the left seminal concepticle could result in the incomplete sperm precedence observed. Sperm stored in these organs are likely to have mixed completely by the time the second clutch is laid which would explain the decrease in sperm precedence in the long term.

4.5.3. Female effects on sperm competition

One obvious pattern occurring throughout these P_2 is that intraspecific variation in P_2 was high. This variance in P_2 values has been interpreted in several ways (Lewis & Austad, 1990; Simmons & Siva-Jothy, 1998). High variation in P_2 is suggestive of female effects influencing the outcome of sperm competition. Female effects have been a much neglected aspect of sperm competition and the evidence is mounting that females can influence paternity (Eberhard, 1996; Eberhard, 1998; Simmons & Siva-Jothy, 1998). The potential mechanisms for female control of paternity in *C. lectularius* are unusual when compared to those of other insects. As sperm migrate through the spermalege and then through the haemocoel the female has the opportunity to manipulate sperm using her immune

system (See Chapter 3). There is evidence that females of *C. lectularius* are capable of resorbing sperm in the haemoceol (Carayon, 1966). How much of the variation in P_2 can be attributed to these potentially female controlled processes is currently unclear.

4.5.4. Conclusion

Sperm competition is an important determinant of male reproductive success in *C. lectularius*. The mechanisms that underlie non-random paternity remain unclear but it seems likely that more than one mechanism is operating. The high levels of remating in bed bugs is probably a consequence of the ability of males to force copulation and last male sperm precedence (see Chapter 2). This may have important life history consequences for females and the potential costs of being repeatedly copulated by males are investigated as a possible sexual conflict in Chapter 5. The establishment of an estimate of sperm precedence in *C. lectularius* has provided important data to investigate other potential fitness traits and strategies used by males and females in traumatically inseminating insects.

5 Costs of mating

5.1. Introduction

Sexual conflicts occur when an adaptation that increases the reproductive success of one sex causes a reduction in fitness in the other sex (Parker, 1979). Many conflicts of interest between the sexes are caused by adaptations associated with sperm competition (Stockley, 1997). The conflict can be expressed in many different ways, the most dramatic of these conflicts is where copulation itself causes a direct fitness cost to females. In the waterstrider *Gerris incognitus* males frequently attempt to force females to copulate using specialised spines and claspers. Males attempt to copulate as frequently as possible with females as this enhances their reproductive success, however multiple insemination in females carries a fitness cost as superfluous matings greatly increase the risk of predation and female's energy expenditure (Arnqvist and Rowe, 1995; Arnqvist, 1997). Rowe *et al.*'s (1994) study of *G. incognitus* provides an example how sexual conflicts can occur over the mating rate. For males, an increase in the number of matings usually brings a corresponding increase in reproductive success (Bateman 1948), however in females this is rarely the case.

Mating can frequently carry a cost to females (see above). In *Drosophila melanogaster* costs of reproduction have been well studied from the female perspective. It had long been known that high reproductive rates caused high mortality in females and this was assumed to be the result of an energetic trade off of somatic maintenance against egg production (Partridge & Andrews, 1985). However, Fowler & Partridge (1989) demonstrated that mating *per se* had a fitness cost to females. Females with a high rate of mating suffered reduced longevity and lifetime reproductive success compared with females in the low

mating group (Fowler & Partridge 1989). The expression of these costs varies with remating frequency, length of exposure to males and nutrition. When *D. melanogaster* is reared on poor quality food the remating rate and egg production of females drops and the cost of mating disappears. However as the quality of food medium increases so does the rate of remating and egg production and the cost associated with mating becomes apparent and increases in magnitude (Chapman & Partridge, 1996). Similar costs of mating were also found in the Mediterranean fruit fly *Ceratitis capitata* (Chapman et al., 1998). Chapman et al. (1998) also review the evidence for mating costs in all the Diptera and although the results were equivocal several careful studies have revealed mating costs in 4 phylogenitically independent taxa (Maynard Smith, 1958; Fowler & Partridge, 1989; Clutton-Brock & Langley, 1997; Mangan, 1997). Whether the underlying mechanism of the cost is same in all the Diptera is still unknown.

Theory predicts that females should attempt to counteract any of the costs associated with mating and so a coevolutionary arms race is expected to occur between the sexes (Rice & Holland, 1997; Holland & Rice, 1998; Parker & Partridge, 1998; Partridge & Hurst, 1998). Empirical evidence that such a conflict occurs was found in *D. melanogaster*. Rice (1996) took lines of male and female flies from a base population. He then allowed the males to coevolve with a second female line taken from the stock population. The first female line was prevented from coevolving with males for 41 generations using recombination techniques. These females were then allowed to mate with the coevolved males. The females mating with these coevolved males showed longer refractive periods and higher rates of mortality (and lifetime reproductive success) than when mated with males from the base population. The males also had higher reproductive success in sperm competition when competing against males from the base population. It is substances involved with sperm competition that cause the cost of mating to females (see section 5.1.1.). Some of the consequences of this co-

evolutionary arms race were investigated by Holland & Rice (1999). Lines of *D. melanogaster* were placed under monogamous or polyandrous (control) mating regimes. After 47 generations males in the monogamous lines had become more benign to females. When males of the monandrous line competed against control males they performed less well in sperm competition, and females from the monandrous lines when mated with control males suffered higher mortality than control females. However, the monandrous populations had higher reproductive outputs than control populations, this indicates that antagonistic sexual conflict produces a suppressive reproductive load on the population, and when the sexes are forced into co-operation this load is removed.

5.1.1. Proximate mechanisms producing costs of mating

A series of elegant experiments have demonstrated that the cost of mating in *D. melanogaster* is mediated by the main cell products contained in the male's ejaculate (Chapman et al. 1995). These products are advantageous to the male by both increasing the females refractory period (the period after copulation that the female will refuse to copulate with other males) and killing the sperm of other males. Both of these phenomena are important as they increase a male's success in sperm competition an important determinant of reproductive success in *D. melanogaster* (Clark et al., 1995). The cost to the female is caused by the toxic side effect of these chemicals.

Another possible mechanism for costs associated with mating was examined by Siva-Jothy *et al.* (1998) in the damselfly *Matrona basilaris japonica.* Although Siva-Jothy *et al.* (1998) did not examine if a cost of mating was present they found that males had decreased immune function immediately after copulation a phenomenon not found after the energetically costly activity of fighting. This observation suggests that the decrease in immune system function is caused by a physiological cost other than an energy based trade-off. As immune system

function is assumed to be a physiological determinant of fitness components (Følstad & Karter, 1992) this reduction in immune function could manifest itself as a cost of mating.

The third reported mechanism of a cost of mating is physical damage to the female caused by adaptations for sperm competition. In the tree cricket *Metaplastes ornatus* the males use a specially adapted sub-genital plate in order to physically remove the sperm of previous males before insemination (Helversen and Helversen, 1991). This physical sperm removal damages the females reproductive tract and Helversen and Helversen (1991) have suggested that this may reduce the likelihood of remating in this species. Physical sperm removal is a common mechanism of sperm competition in insects (Simmons & Siva-Jothy 1998) and how costly this sperm removal is to females requires further investigation. In the bean beetle *Callosobruchus maculatus* a cost of mating mediated through damage of the reproductive tract was suggested by Tufton (1993). A more detailed study of these costs has found a cost of mating to females. The proposed mechanism is that spines on the males aedeagus pierce the female's reproductive tract and reduce longevity (H. Crudgington, pers. com.).

5.1.2 Possible sexual conflicts in *C. lectularius*

Traumatic insemination in bed bugs is a paradigm for a sexual conflict arising from mating (Thornhill & Alcock 1983). Despite this, there has been no empirical demonstration that traumatic insemination is costly to females. Indeed one hypothesis as to the evolution of the paragenital system of females is that it functions to reduce the cost of traumatic insemination to females (i.e. it has evolved through natural selection). Although this hypothesis infers a different mechanism to that envisaged by Eberhard (1996) it does not preclude the fact that it may also function as a mechanism by which females can distinguish

between males by cryptic female choice. Also the evidence that the spermalege may absorb or incapacitate sperm following insemination means that females are pre-adapted to use males ejaculates as a nuptial gift (see Appendix I). These nuptial gifts could be used by females to ameliorate the costs of traumatic insemination.

Examining the data on remating rates and fertility in *C. lectularius* (see Chapter 2) reveals that females copulate much more frequently than is necessary to maintain maximum fertility. Females appear to have little control over mating frequency and so mating frequency may be set by the reproductive strategies of males. Within a single reproductive bout (following a blood meal) a male will copulate an average of five times with available females. A female however only requires a single copulation to maintain maximum fertility for about a month. Given an equal sex ratio, females will receive an average of twenty times more copulations than are required to maintain their maximal reproductive rate. The asymmetrical optima for remating rates of each sex may cause a sexual conflict if copulation carries a cost to females. This chapter aims to test this prediction experimentally.

5.2. Aims

The aim of this chapter is to examine the effect of different mating rates on female fitness. The possible mechanisms of any cost of mating and the role that mating costs have on the reproductive strategies of females are discussed.

5.3. Methods

Culturing bugs for the experiments was carried out as outlined in section 2.3.1. Virgin female bugs were collected on the day after adult eclosion and measured using the image analysis method discussed in section 2.3.1. During the experiment all bugs were kept at 26±1°C and 70% relative humidity on a 12hr

light: 12hr dark cycle. These females were then allocated at random to 2 experimental groups. In the first group 45 females were fed to satiation and allowed to copulate once with a virgin male before being isolated in a 7cm^3 plastic vial containing clean filter paper. Each female was then allocated a male whose paramere was glued (Super Attak, Loctite UK Ltd.) into the genital groove and so was unable to mate. This acted as a control for the presence of a male without further copulations and is here after called the low-mating treatment. These females were fed every 7 days and the number of eggs produced were counted each week. After 4 weeks the females were allowed to copulate again with a virgin male. This was done to maintain the female's fecundity at the same level as in the other mating group.

The second group was treated identically to the first group except females in this group were allocated virgin males at the beginning of the experiment and were allowed to copulate at a natural level. Previous data showed that an isolated male and female will copulate an average of 5.00±1.41 (n = 20) times in a 7 day period. This group was termed the control mating group. Although it should be noted that the control mating treatment has a similar remating rate as was noted in the observations in section 2.4.2. The experimental design to test for a cost a mating should be considered as a normal mating group and an artificially low mating group.

5.4. Results

5.4.1. Animal sizes for control and low mating experimental groups

There was no significant difference in the size index measurements for the females allocated to the control (13.72±0.24) or low mating (14.03±0.21) experimental groups (unpaired *t* test t_{88} =0.977, P =0.33)(see section 2.3.1). Males allocated to the control and low mating groups were of similar size (high 12.69±0.24; low 12.73±0.22; unpaired *t* test t_{88} =0.107, P =0.91).

5.4.2. Longevities of females under different mating regimes

Females in the control mating group died at a significantly higher rate than females in the low mating group (Log rank test: Chi-Square 20.48, df = 1, P<.0001)(Figure 5.1.)(Miller, 1981; Statview manual, 1998). Females from the low mating group also survived significantly longer (147.15± 8.37, n=45) than females in the control mating group (110.91±4.98, n=45; unpaired *t* test t_{88} = 3.72, P =0.0004).

5.4.3. Total egg production of females under different mating regimes

There was no significant difference between egg production of females in the control and low remating groups before death at any time during the experiment (Mann-Whitney U Tests, P>0.05 this value was adjusted for repeated sampling using the sequential bonferoni method (Rice, 1989))(Figure 5.2) suggesting that mating rate does not effect egg production. However, females in the low mating group produced significantly more eggs in their lifetime (294.35 ±9.89, n=45) than females in the control mating group (224.71±16.06) (unpaired *t* test t_{88} = 3.69, P = <0.001)(Figure 5.3) due to their higher longevities.

5.4.4. Egg hatchabilities of females under different mating regimes

The infertility of eggs was very low in both mating groups (mean proportion of infertile eggs for pooled data = 0.019±0.003 per clutch). There was no significant difference in infertility between the high and low mating groups (Kruskal Wallis H_{60}= 7.20, P =>0.99).

5.5. Discussion

5.5.1. Evidence for sexual conflicts over mating rate

The evidence that females who experience normal levels of remating suffer reduced lifetime reproductive success provides evidence that the conditions for a

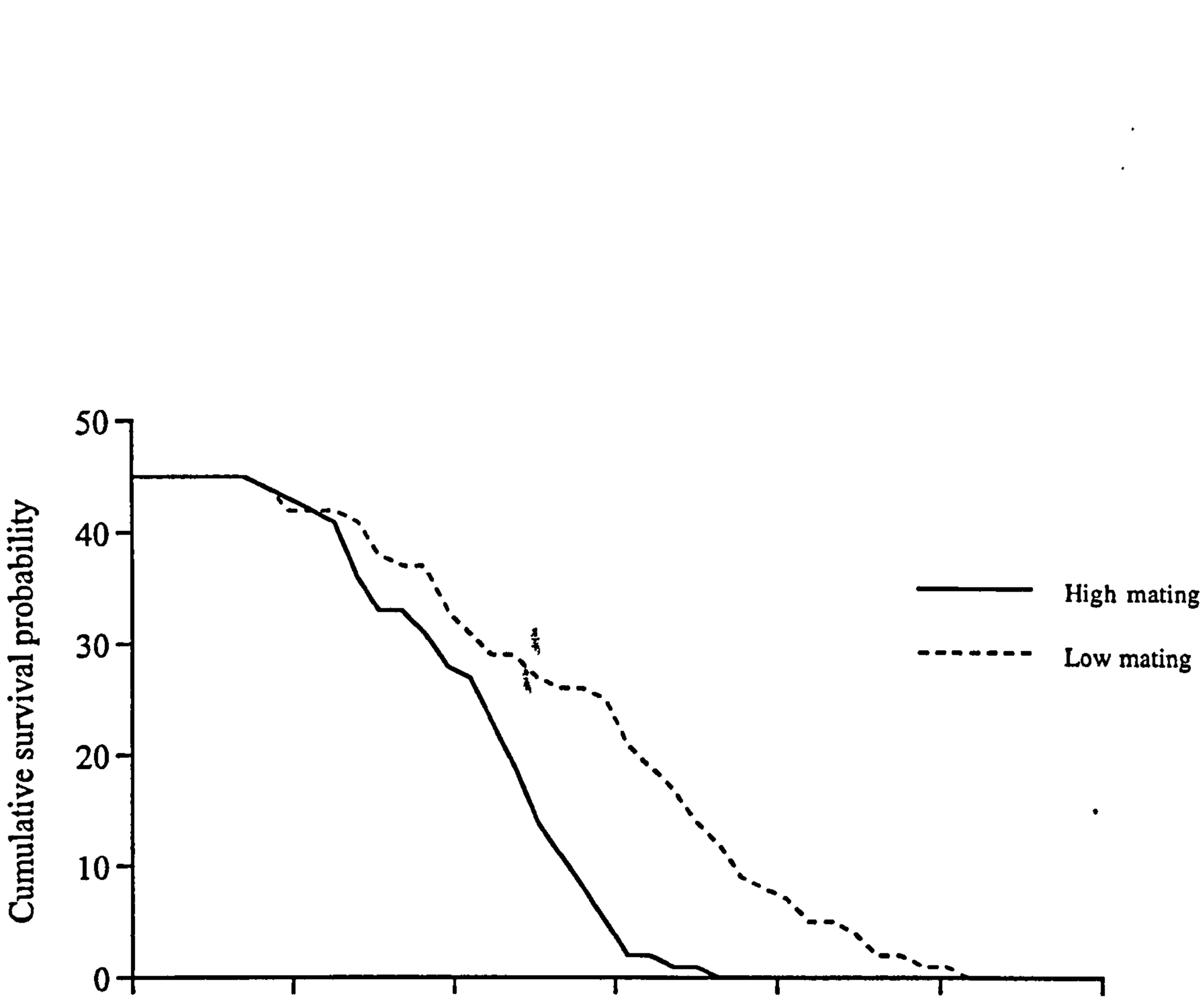


Figure 5.1. Survival curves for females in the high and low mating female experimental groups. The cumulative survival probability is the number of females alive at the end of a given sampling period (7days).

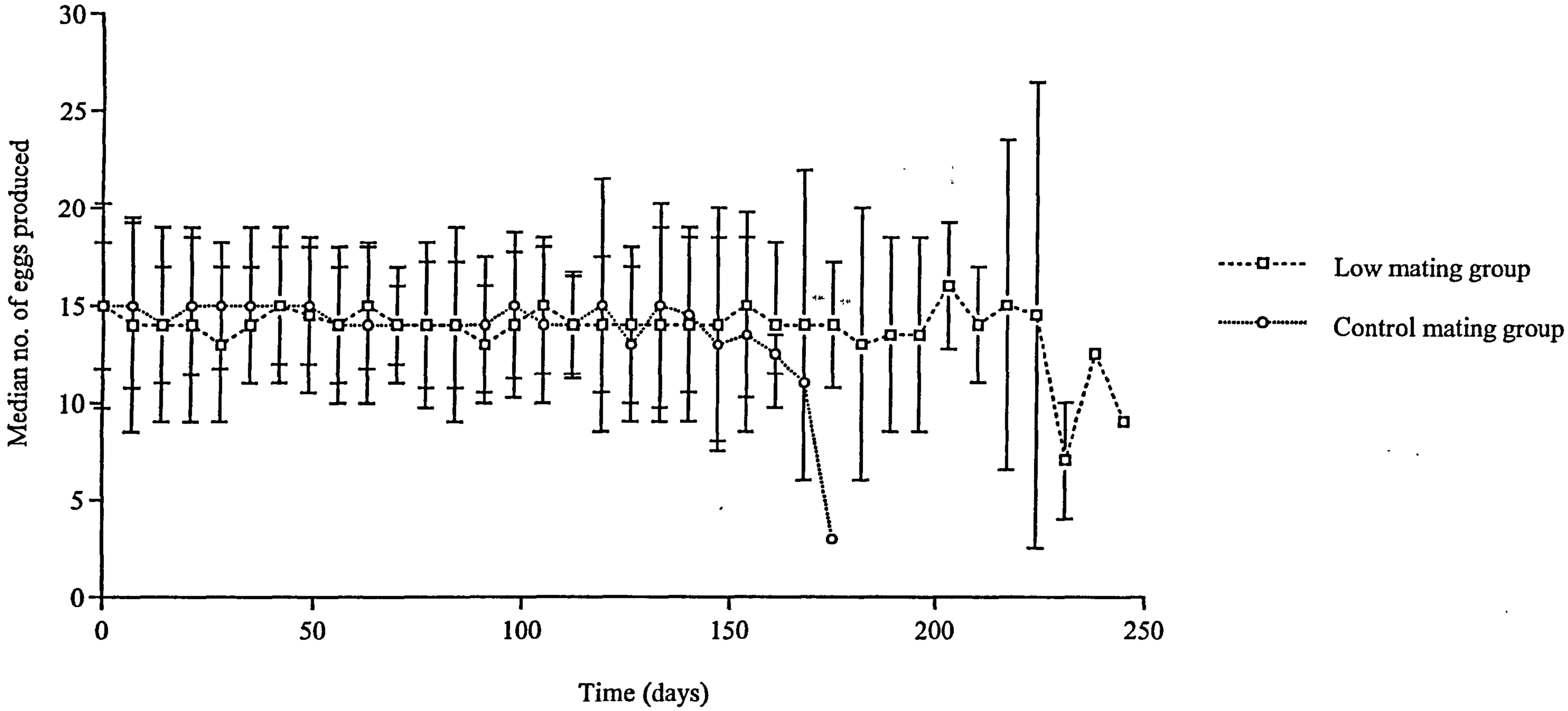


Figure 5.2 Median egg production of females mating at low and control rate. Bars show inter-quartile range.

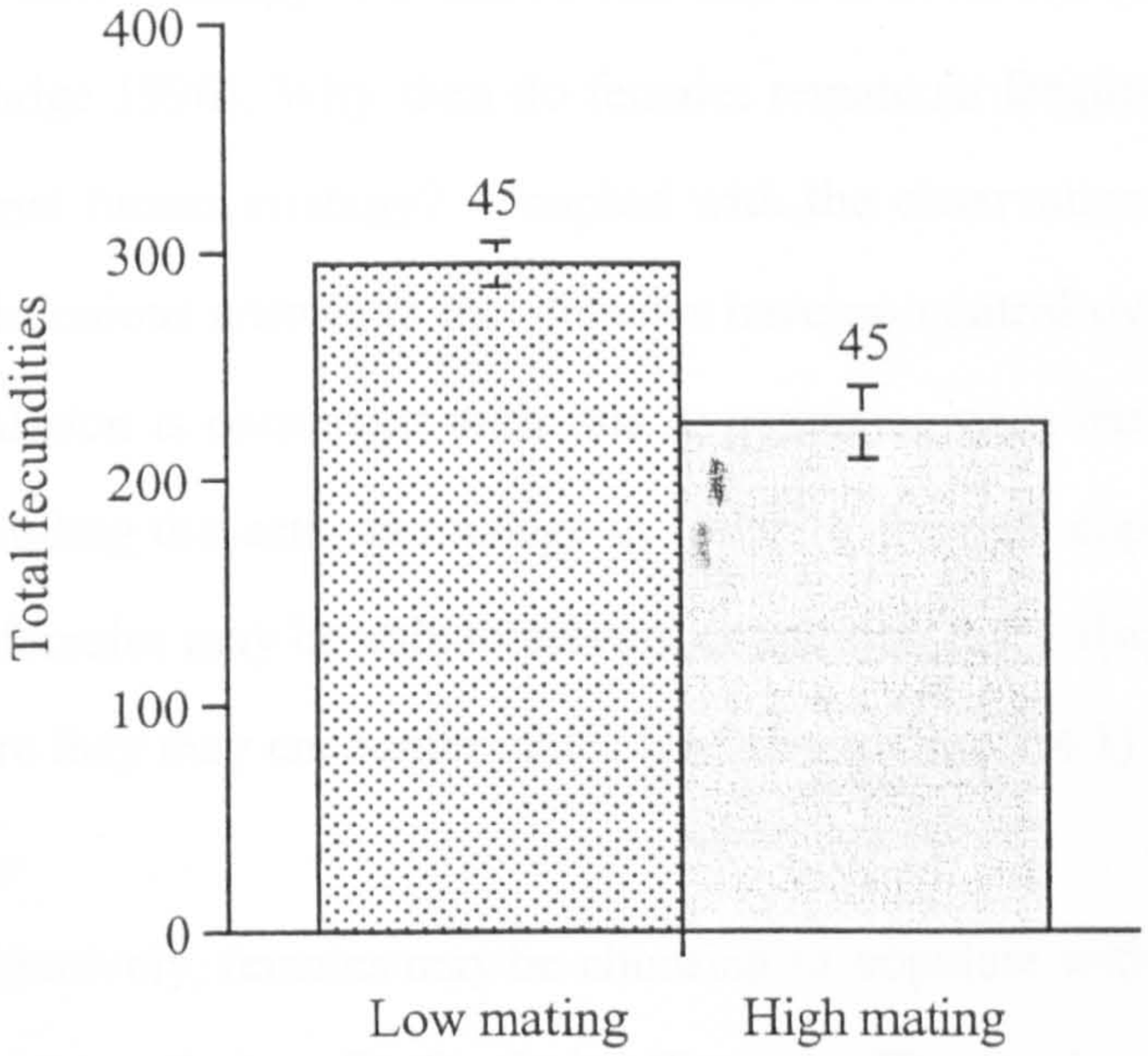


Figure 5.3. Total numbers of eggs produced in a lifetime in the high and low mating experimental groups.

sexual conflict over the remating rate are present in *C. lectularius*. Females should copulate approximately every four reproductive bouts in order to maximise their lifetime reproductive success. Females who copulate at a higher rate suffer a fitness cost as their longevities are reduced. As there is no significant difference in the fecundities of females that mate multiply this reduction in longevity results in a lower lifetime reproductive success (see also Appendix I). This result mirrors the pattern of a mating cost in *D. melanogaster* (Fowler & Partridge 1989). However in *D. melanogaster* the remating rate of females is dependent on their nutritional status so females have some control over their remating rate and so the expression of the mating cost (Chapman & Partridge 1996). Why then do females remate so frequently when this is a sub-optimal fitness strategy? Coupled with the observations in Chapter 2 the most parsimonious answer is that females have no control over mating. The mode of copulation is consistent with this hypothesis, since females have no method of controlling the ecto-spermalege in order to prevent copulation. The only way that females may be able to prevent copulation is by dispersing away from areas where they may encounter males (see also section 2.4.1).

Alternatively, females may be choosing to copulate with multiple males in order to gain genetic benefits for their offspring. The good genes hypothesis of sexual selection would predict that females that have mated with several different males should have more viable offspring as they will be sired by genetically superior males that have competed through sperm competition or cryptic female choice (Kirkpatrick & Ryan, 1991; Eberhard, 1996). Although this is a possible benefit of polyandry no viability differences were found in a suite of putative fitness traits in the offspring of singularly or multiply mated females (Appendix I). A second proposed benefit of multiple mating is that females may be avoiding genetically incompatible sperm that would lead to infertility of eggs and also a possible reduction in offspring fitness (Zeh & Zeh, 1996; Zeh & Zeh, 1997). As

no difference in the number of infertile eggs laid by the high and low mating group was found then this is again unlikely to be a strong fitness benefit for females. Also neither of these hypotheses explain why a female will repeatedly copulate with the same male during a reproductive bout.

5.5.2. Possible proximate costs of mating in *C. lectularius*

The mechanism that causes the reduced longevity of females exposed to high rates of remating is unclear. Any of the potential mechanisms discussed in section 5.1.1. may be occurring and are not mutually exclusive. However, the mode of copulation and insemination in *C. lectularius* produces potentially novel mechanisms for the cost of mating which is absent in insects where insemination is not traumatic. Firstly copulation in *C. lectularius* is likely to cause the production of an immune response. The mounting of an immune response against repeated copulation may cause an energetic trade-off for the female. This trade-off may then be manifested in reduced longevity for those females who have to invest highly in immune function.

A second possible mechanism for the cost of mating in *C. lectularius* is that secondary infection by pathogens may occur during insemination. Exposure to sexually transmitted diseases is a cost of mating that has been well documented in several taxa (Hurst et al., 1995; Lockhart et al., 1996). This cost is perhaps more likely to be an important factor in traumatically inseminating since genital copulation effectively takes place effectively outside of the body which is obviously not the case in traumatic insemination. The fact that very little pathogenesis is seen in the early stages of the mating cost experiment is suggestive that simple exposure to pathogens is not enough to cause the increased mortality of the high mating group. However, it could be that after repeated exposure females are unable to mount an immune response against sexually

transmitted pathogens. This would result in the increased rate of mortality as females became more senile.

The physical damage caused by repeated mating means that the body wall of the female will be repeatedly open to the environment, and so be susceptible to pathogenic invasion, as well as needing to be repaired. Cuticle repair in insects is potentially costly in energetic terms (Chapman, 1998) and so will cause a diversion of resources away from the female's somatic maintenance.

5.5.3. Function of the spermalege

Does the evidence for a cost of mating weaken the hypothesis that the function of the spermalege is to reduce the costs associated with traumatic insemination? Simply by finding a cost associated with high levels of insemination does not allow any inference of the function of the spermalege. This remains a key question in understanding of the evolution of the paragenital system. A female counter-adaptation to the fitness reduction of multiple insemination is predicted by theory (Rice & Holland, 1997; Holland & Rice 1998; Parker & Partridge 1998; Partridge & Hurst 1998) and empirical evidence for a coevolutionary arms race between the sexes has been demonstrated in *D. melanogaster* (Rice, 1996). So if the spermalege functions to reduce the cost of mating in *C. lectularius* then why is the amelioration of the cost incomplete? Further work is required to answer this question.

5.6. Summary

In this chapter I have demonstrated that female *C. lectularius* suffer reduced longevity when exposed to normal remating rates compared with females that have an artificially low remating rate. The reduced longevity is also translated into reduced lifetime reproductive success for females as females in the normal remating group also produce fewer offspring. These data provide evidence for an

inter sexual conflict over remating rates. Males would appear to gain high fertilisation success by inseminating multiple females whereas females should attempt to copulate at as low a rate as possible to maintain sperm stores (approximately every four reproductive bouts).

6 General discussion

The aim of this thesis was to examine functional questions about traumatic insemination in the bed bug *Cimex lectularius*. Although discussed in the literature none of the conjecture is supported by empirical evidence of the function of this mode of copulation (e.g. Hinton, 1964; Thornhill & Alcock, 1983; Eberhard, 1996). In this Chapter I will discuss the evidence for conflicts of interest between the sexes and its possible causes in this species. Several avenues for future work are then examined.

6.1. Résumé

In Chapter 2 the mating system and mating rate of male and female *C. lectularius* was examined. No overt female choice behaviour was observed and the mode of copulation was consistent with the hypothesis that it was forced by males. The difficulty of testing hypotheses of forced copulations are discussed in section 6.2. Females appear to be mating at a higher rate than is necessary to maintain maximum fertility. This observation produced several hypotheses as to the function of polyandry in *C. lectularius*. Two specific hypotheses of the function of inter-sexual copulation and copulations with pre-adults were examined. It has long been suggested that traumatic insemination may allow males novel routes to fitness. Firstly it has been suggested that by inseminating other males, a male may gain fertilisations by proxy, i.e. by parasitising the recipient male's ejaculate (Carayon, 1974). The second novel route to fitness that males could exploit is by copulating with pre-adult females, this could result in fertilisation success because the sperm storage organs are not shed at eclosion. Both types of copulation have been recorded in mating system observations in this thesis (also observed by Rivnay, 1933) but neither type of copulation resulted in sperm

transfer or fertilisation success for males. I suggested that these copulation attempts with conspecifics other than mature females may be a result of the mate location strategies used by males.

In Chapter 3 I examined the ejaculate allocation strategies of males and the migration of sperm within the female's paragenital system. Male copulation duration was found to be a good predictor of the ejaculate size of males. Males were found to allocate more sperm to virgin females than non-virgins and this was suggested to be a result of the mechanism of sperm competition as predicted from several mathematical models. Sperm storage was also estimated and the site of fertilisation was confirmed to be within the ovary. The conduit system of sperm migration found in *C. lectularius* is different from the cul-de-sac method of sperm storage and usage found in other insects (Simmons & Siva-Jothy, 1998). The effect of this conduit system on mechanisms of sperm precedence are discussed in Chapter 4.

Chapter 4 examined the pattern of sperm precedence in *C. lectularius*. When the remating interval for a females was 17 minutes the immediate mean P_2 value was 68% which dropped to 48% after 2 weeks. The observation that sperm precedence was second male biased raised two important questions. Firstly, did the last male sperm precedence cause the high rates of remating observed in Chapter 2? If there is a last male advantage to sperm precedence then it would pay males to attempt to copulate last with a given female. Where this occurs males would be predicted to show proximate or remote mate guarding behaviours. No evidence to support this was found. It could be that males can get higher fitness payoffs by attempting to inseminate as many females as possible rather than attempting to monopolise a small number. The second question which remains is: how does the second male to mate gain sperm precedence? A hypothetical mechanism of sperm precedence was proposed (based on an

anatomical inference) and tested experimentally. If sperm are displaced or pre-empted in the spermalege then I predicted that when the remating interval exceeded the time taken for the first ejaculate to migrate out of the spermalege, the pattern of sperm precedence should be reversed (i.e. become first male biased). This prediction was supported with observed P_2 switching to 30% when the remating interval was 4 hours.

In Chapter 5 the cost to females of the high rates of insemination observed were investigated experimentally. It was found that females which copulated at an artificially low rate did not have a reduced fecundity when compared with females that copulated at the rate observed in Chapter 2. However, females in the low mating group died at a significantly lower rate when compared to females in the normal mating rate group, resulting in a significantly higher longevity and lifetime reproductive success. This cost of mating was in the order of a 30% reduction in lifetime reproductive success for females in the normal mating group. The mechanism by which longevity is reduced remains unclear, however it appears to be a cost of mating *per se* and not caused by a cost of living with males or of reproductive output.

Appendix I attempted to examine any direct or indirect benefits of polyandry to females. The fecundity and offspring of females mating once and 5 times were compared. No effect of polyandry was found on total fecundity, egg fertility, egg development rate, egg size or adult size of offspring. Although no effect of indirect benefits was detected their role cannot be discounted as the traits which determine reproductive success are still unclear in *C. lectularius*. The evidence for a potential conflict of interest between the sexes over the mating rate, and its causes, will now be examined.

6.2. Forced copulation and sexual conflict

Two issues raised in this thesis require evidence from all of the Chapters to be reviewed. Firstly, the concept that traumatic insemination in *C. lectularius* is an example of forced copulation needs to be critically assessed. Extra-genital and traumatic insemination does not mean that females have lost control over copulation rate *per se*. Females could potentially evolve counter adaptations to resist matings in the same way as when copulation is genital (Arnqvist & Rowe, 1995). However, because copulation appears to be forced does not provide evidence that females are not in control of copulation (this is essentially an anthropomorphic argument *sensu* Kennedy (1992)). To assess if copulation is forced requires the testing of predictions arising from a hypothesis that copulation is forced. Brown *et al* (1997) suggested that where copulation is forced the mating rate should be closer to the forcing sex's optima than that of the sex which has little control over mating. Evidence from this study suggests that females pay a cost of mating that results in a sub-optimal fitness outcome for copulation rate. Whether males are mating at the optima for their reproductive success is less clear. Males mate at a relatively high rate and it would appear that this is a response to the pattern of last male sperm precedence. Secondly, Alexander *et al* (1997) suggested that where forced copulations take place no courtship should occur. This prediction is also applicable to *C. lectularius*. Overt courtship is absent and males appear to make regular mistakes when attempting to locate mature females with which to copulate. The frequent copulation attempts with pre-adult females and conspecific males may be a consequence of this lack of overt courtship. The fundamental point when examining hypotheses of forced copulation is that the effect of "force" needs to be expressed in fitness terms.

Secondly, this study has produced evidence of a sexual conflict resulting from traumatic insemination. The sexual conflict appears to arise over the mating rate

with females copulating more frequently than necessary to accrue any measurable benefit of polyandry. Females copulating at the rate observed in Chapter 2 have, as a result, reduced lifetime reproductive success than those which copulate 20 times less frequently. The high level of remating may be beneficial for males in terms of reproductive success through sperm competition.

6.3. Evidence for and against existing hypotheses of the function and evolution of traumatic insemination in *C. lectularius*.

Hinton (1964) proposed that traumatic insemination was a method of food sharing in colonies of cimicid bugs. Although his hypothesis relied on group selection, and so is unlikely to evolve, females could still be using the ejaculates of males as a nuptial gift (Vahed, 1998). This hypothesis was tested directly although no evidence that females were increasing their fecundity when mated at higher rates was found. Females could still be incorporating some nutrients from the ejaculate of males although this seemed to have no effect on female fitness. Radiotracer studies of ejaculate incorporation are required to finally answer this question.

Lloyd (1979) suggested that males evolved traumatic insemination as a method of gaining sperm precedence over males inseminating into the female's reproductive tract. No evidence that this is the pathway that caused the evolution of this mode of insemination was found in this study. Males never attempt to inseminate into the females reproductive tract but this is likely to be a constraint on males rather than a reproductive strategy adopted by them (male genitalia is unlikely to be able to fit into the female's reproductive tract).

Thornhill and Alcock (1983), and later Eberhard (1996), both suggested that the evolution of the paragenital system was likely to have evolved to allow females to regain control over fertilisation. Variation in P_2 has been interpreted as evidence that females are having influence on sperm precedence. The high variation in P_2 observed in my sperm precedence experiments are consistent with

this hypothesis. However, it remains unclear whether the benefits that females gain from any cryptic choice would be strong enough to drive the evolution of the paragenital system itself.

6.4. Future work

This is the first study to examine hypotheses of the function of traumatic insemination from an evolutionary perspective. Clearly much more work is required to gain an understanding of the selective pressures that are likely to produce this unusual male insemination strategy. One problem which is obvious from the observations of behaviour and patterns of sperm precedence is that the fitness basis for the male strategies of mate location and paternity assurance remain unclear in this system. For example, why don't males guard females after they copulate if there is last male sperm precedence? The best way to examine this question is to repeat the mating system observations and genotype the offspring that are produced using molecular markers to assign the paternity of individual males in the population. This would provide a powerful test of hypotheses relating to alternative mating strategies. This molecular approach would also allow an estimate of sperm precedence patterns when females are mated more than twice. As the number of copulations a female undergoes during a reproductive bout is approximately 5, estimates of P_3, P_4 and P_5 are both relevant and potentially interesting (Zeh & Zeh, 1994). The use of molecular markers in this system does have one potential drawback. Bed bugs live in relatively small, isolated populations and as a result inbreeding tends to be very high. Preliminary work revealed very high levels of monomorphism at over 15 allozyme loci (personal observations). The best approach would perhaps be to examine polymorphic molecular markers such as RAPDs or microsatelites and to collect as many individuals from natural populations as possible.

One neglected aspect of this study has been to examine the role of females in the outcome of sperm competition. As no predictors of male quality have been uncovered in this study investigating how females may bias sperm usage to favour certain males becomes problematic (Birkhead, 1998). One method of examining female roles has been carried out by Wilson et al. (1998) working on *Callosobruchus maculatus*. Here a P_2 experiment was designed so that females were mated with different strains of *C. maculatus* and the repeatability of the P_2 pattern was calculated for females mating with the same strain or a different strain. The pattern of P_2 was repeatable in within strain matings but was not repeatable when females mating with males that were from the other strain. This design allowed the role of females in biasing sperm competition outcomes to be revealed. A similar design is possible in bed bugs using either different inbred populations or even examining sperm competition outcomes when *C. lectularius* is mated with its sub-species *C. hemipterus* (Dewberry, 1988; Price, 1997). This would allow an estimate of female roles in the large amount of variation observed in the sperm precedence patterns.

This study has produced data on both sperm precedence and costs of mating for females. The underlying mechanisms of both these phenomena remain poorly understood. Several technical options remain open to examine the mechanism of sperm precedence. Firstly, the labelling of ejaculates with different coloured dyes would allow direct examination of sperm interactions within the spermalege and ultimately the identification of which sperm become stored and used. This would provide more information on the mechanism than the experimental approach used in this study.

Secondly the cause of the cost of mating to females needs to be examined. The potential mechanisms of this cost were discussed in Chapter 5. However, several techniques could allow a better mechanistic understanding of this phenomena.

The role of components of the ejaculate could be assessed by using the artificial insemination techniques of Davis (1965). Seminal fluid, sperm and accessory gland product could all be injected separately and at different concentrations to establish their effect on female life history traits. The second method of understanding costs of traumatic insemination is to measure the response of the immune system to insemination. A variety of techniques are now available to assess the immune response of insects and this could provide evidence of costly female resistance either to the ejaculate itself or to secondary infection due to the piercing of the cuticle (Gillespie et al., 1997).

Ultimately the best framework in which to examine both the function and evolution of traumatic insemination is likely to be comparative (Harvey and Pagel, 1991). The diversity of paragenital morphology among the Cimicidae is spectacular and examining the sperm precedence patterns and costs of mating to females in several key species would provide powerful tests of adaptive hypotheses. Although there are many species within the Cimicoidea superfamily which display interesting behavioural and morphological differentiation several species are of particular importance. Key genera in the understanding of traumatic insemination include: Primicimex (no spermalege); Cimex (intermediate in paragenital morphology); Stricticimex (greatly enlarged spermalege and no haemoceol sperm migration). The phylogenetic relationship of these individuals needs to be assessed using molecular techniques to produce a phylogeny that is independent of the genitalic traits that are under examination (Schuh & Stys, 1991). One species which is also of particular note is *Afrocimex constrictus*. In this species males have a spermalege and intra-sexual copulations are reported to be common (Ferris & Usinger, 1957; Carayon, 1959). An examination of selection pressures on males could provide interesting answers to the function of the spermalege in both males and females.

6.4. General conclusions

This study has demonstrated there is a cost of polyandry and multiple mating to female *C. lectularius.* No potential benefits of multiple mating were uncovered. The level of polyandry is high and probably due to the high mating rates of males attempting to gain last male sperm precedence coupled with the apparent inability of females to resist male copulation attempts. The cost of mating in longevity and lifetime reproductive success in females has revealed the first evidence that traumatic insemination produces a conflict of interest between the sexes.

Appendix I Female benefits of polyandry

Introduction

The traditional view of male investment in insect mating systems was that it was absent, with parental care being viewed as the exception rather than the rule. Obvious cases do exist: the male water bug *Abedus herberti* brood the eggs laid by the female until hatching (Smith, 1979). However most male investment in insects seems to take place during copulation by the transfer of sperm. Males then appear to abandon the female to provide the majority of the parental investment in offspring. The concept that male investment could occur during copulation by the male providing nutrient gifts to the female as a type of male investment is more controversial. Nuptial feeding, as it is known, encompasses any form of nutrient transfer from the male to the female during, or directly after courtship and/or copulation (Vahed, 1998). In insects the investment varies from captured prey items donated to females (Thornhill, 1976), to the secretion of specific chemicals from glands on the male such as uric acid feeding in cockroaches (Mullins, 1980), even parts of the male's body (Eggert & Sakuluk, 1994), or the male's body itself, may act as the gift in sexually cannibalistic species (Lawrence, 1992). The male derived gift may at times be so large that sex role reversal occurs. In some bushcrickets, during times of nutrient limitation, females compete over males that can produce the nutrient rich spermatophores as these have become the limiting resource for a given female's reproduction (e.g. Gwynne & Simmons, 1990).

A common gift donated by males from at least 4 major orders of insects is the spermatophore or substances contained in the ejaculate. These gifts vary from the simple investment of a large protein packet found on the spermatophores of many Orthoptera: the spermaphylax (Gwynne, 1997), that increase female

fecundity, to cantharidin ingested by male pyrochroid beetles (*Neopyrochroa flabellata*) that is incorporated into the ejaculate and is in turn incorporated by the female into her eggs. Eggs with male donated cantharidin have reduced predation rates than those without (Eisner et al., 1996a,b). Both these investments by males are believed to increase male fitness.

Nuptial gifts: Mating effort or paternal investment?

There has been much discussion on whether nuptial gifts should be considered as parental effort or as mating effort by males. Trivers (1972) defined parental investment as: 'any investment by the parent in an offspring that increases the offspring's chance of surviving at the cost of the parent's ability to invest in other offspring'. This specific definition was extended by Simmons & Parker (1989) whose definition included 'any increase in a given male's total surviving progeny by increasing the reproductive output of a given female'. This definition explicitly includes nuptial gifts by males as parental investment. The complication is that by increasing their paternal investment males may also be increasing their chance of gaining fertilisations or mating effort. This means that the evolution and/or maintenance of nuptial gifts may be unrelated to parental investment. These two hypotheses are not mutually exclusive and the operation of one of these mechanisms does not preclude the operation of the other.

To empirically demonstrate that nuptial gifts function as parental effort two phenomena need to be demonstrated: 1. The gift causes an increase in female fecundity or an increase in offspring quality. 2. The male has genetic representation in the offspring that have received his gift (Simmons & Parker, 1989). If both these facts are demonstrated then parental effort can be assumed to be occurring, however this does not preclude the idea that the gift also functions as mating effort. Wickler (1986) suggested that parental investment would be rare due to the patterns of sperm precedence and the incorporation rate

of nutrients. In species where the female frequently remates males will often gain paternity of offspring to which previous males will have donated a nutrient gift. In this case the gift cannot be considered as parental effort.

Vahed (1998) conducted an exhaustive review of studies of nuptial feeding in insects and finally concluded that there was considerable evidence for the mating effort hypothesis. However, due to a number of studies demonstrating that the donating male has no genetic representation in the offspring that had received his nutrients incorporated (e.g. Helversen & Helversen, 1991; Wedell, 1993; Markow, 1988; Oberhauser, 1992) the generality of the parental effort hypothesis had to be questioned.

Indirect benefits of multiple mating for females

Where no fecundity benefit occurs through multiple mating then polyandry becomes paradoxical when examined from the female perspective. Although multiple mating has obvious benefits for males in fitness terms the same cannot be said for females. To address this problem behavioural ecologists have suggested a whole suite of indirect benefits to females of multiple mating. Firstly by mating with multiple males females may, through sperm competition, gain offspring which are more viable or have a higher competitive ability than her previous mates (Andersson 1994; Simmons, 1987a,b).

Females may also be avoiding genetic incompatibility by mating with several genetically different males. Genetic incompatibility can have a range of effects from zygote death through to reduced adult fitness caused by abnormal gametogenesis (Zeh & Zeh, 1996; 1997). Although these hypotheses produce testable predictions they are rarely mutually exclusive and are difficult to examine where the fitness traits for a particular taxon are poorly understood. However, in a study of the cricket *Grylus bimaculatus* Tregenza and Wedell (1998) managed

to differentiate between the indirect benefits of mate choice and concluded that females did multiply mate to prevent infertility of eggs through genetic incompatibility. However, *Grylus bimaculatus* has extremely high levels of natural infertility and so the generality of this finding may be questionable.

Finally it needs to be stressed that both direct and indirect benefits to females can operate simultaneously. In the arctiid moth (*Utetheisa ornatrix*) females gain both directly and indirectly from mating with large males (Iyengar & Eisner, 1999). Male size is a sexually selected trait and females choose larger males preferentially. Male size is heritable and large males also provide larger quantities of a nutrient and protective alkaloid which increases female fecundity and the chance of those offspring surviving to reproduce (Iyengar & Eisner, 1999).

Nuptial gifts in the bed bug

No study has examined the role of the ejaculate of bed bugs as a nuptial gift. Hinton (1964) suggested that traumatic insemination evolved as a strategy for food sharing in the population during times when a host is absent. Hinton's (1964) arguments relied on group selection operating and even in highly inbred populations it seems that this is unlikely to be true. However, there are good *a priori* reasons to suspect that female bed bugs may be gaining nutritional benefits from male ejaculates. Firstly males produce relatively large ejaculates and a female can only store one and a half ejaculates before her sperm storage organs are full (see Chapter 3). In a single reproductive bout females will mate with an average of five males (see Chapter 2) and the excess ejaculate remains in the female's haemocoel. This sperm has disappeared from the haemocoel 5 days after insemination. Also sperm migration occurs within the somatic tissues of the female so she may be pre-adapted to absorb the nutrients that are present in the ejaculates. What nutrients would females be expected to absorb from the

ejaculate? Females will only mate following a blood meal so if any nutrient is limiting the number of eggs a female can produce in a reproductive bout (i.e. following a blood meal) she would be expected to absorb any of these nutrients that were available from the ejaculate. Although blood meals are rich in protein some of the B complex vitamins are completely absent (Lehane, 1991). These vitamins are synthesised by symbiotic bacteria that live within the mycetomes of male and female bugs. Interestingly these mycetomes are always associated with the testes in males (Carayon, 1966). It is possible that these are the only indirectly synthesised nutrients that are required in which case these are good candidates for a nuptial gift.

Aim of this appendix

This appendix aims to examine if multiple insemination by males acts to increase the fecundity of females. A female can fertilise all the eggs in a clutch from a single ejaculate for up to five clutches before fertility drops (see Chapter 2). By allowing virgin females to copulate for one or five times with males we can examine if females are gaining a short term fecundity advantage from the insemination of multiple ejaculates. Possible fitness traits of the offspring of the two experimental groups were measured to examine if multiple inseminations resulted in an increase in measures of offspring quality. This appendix does not attempt to examine female longevity (considered in Chapter 5). This is potentially confounding variable if females are using ejaculates for somatic maintenance or repair.

Methods

Virgin females (controlled for size and age) where allocated at random to one of two experimental treatments. In the first treatment females were allowed to copulate with a single virgin male and in the second treatment females were allowed to copulate with 5 virgin males. Copulation durations for all matings

were recorded. Females were then isolated and fed at weekly intervals and the eggs collected for 5 clutches.

The number of fertile and sterile eggs were counted and all eggs were measured (as in section 2.3.1.) as was the hatching date for each egg. The offspring were fed at weekly intervals and the date of adult eclosion was also taken.

Results

There was no significant difference between the sizes of males (Un-paired t-test: t_{37}=1.07, P= 0.29) or females (Un-paired t-test: t_{37}=0.24, P= 0.81) used in the treatment groups.

No significant difference could be found between the single and multiply mated treatment in any of the variables measured. Egg number (Mann Whitney U tests, P>0.05), egg size (ANOVA $F_{1,20}$=0.981, P=0.32), egg developmental rate (ANOVA $F_{1,20}$=0.380, P=0.53) and adult size (ANOVA $F_{1,20}$=0.02, P=0.88) were all unrelated to the number of times a female copulates (Fig. A1a-d).

Female fertility began to drop in the monandrous group after four weeks but egg production did not, suggesting the sperm stores were becoming depleted without a drop in fecundity.

Discussion

The evidence was not consistent with the idea that females use male seminal products to increase the number, or quality, of their offspring. However these results need to be treated with caution. Firstly the sample sizes, and therefore the statistical power of the tests, were small (and so the probability of a type I error is high). Secondly the traits measured for offspring 'quality' may not have been the ones in which the investment by the male was expressed. Although egg

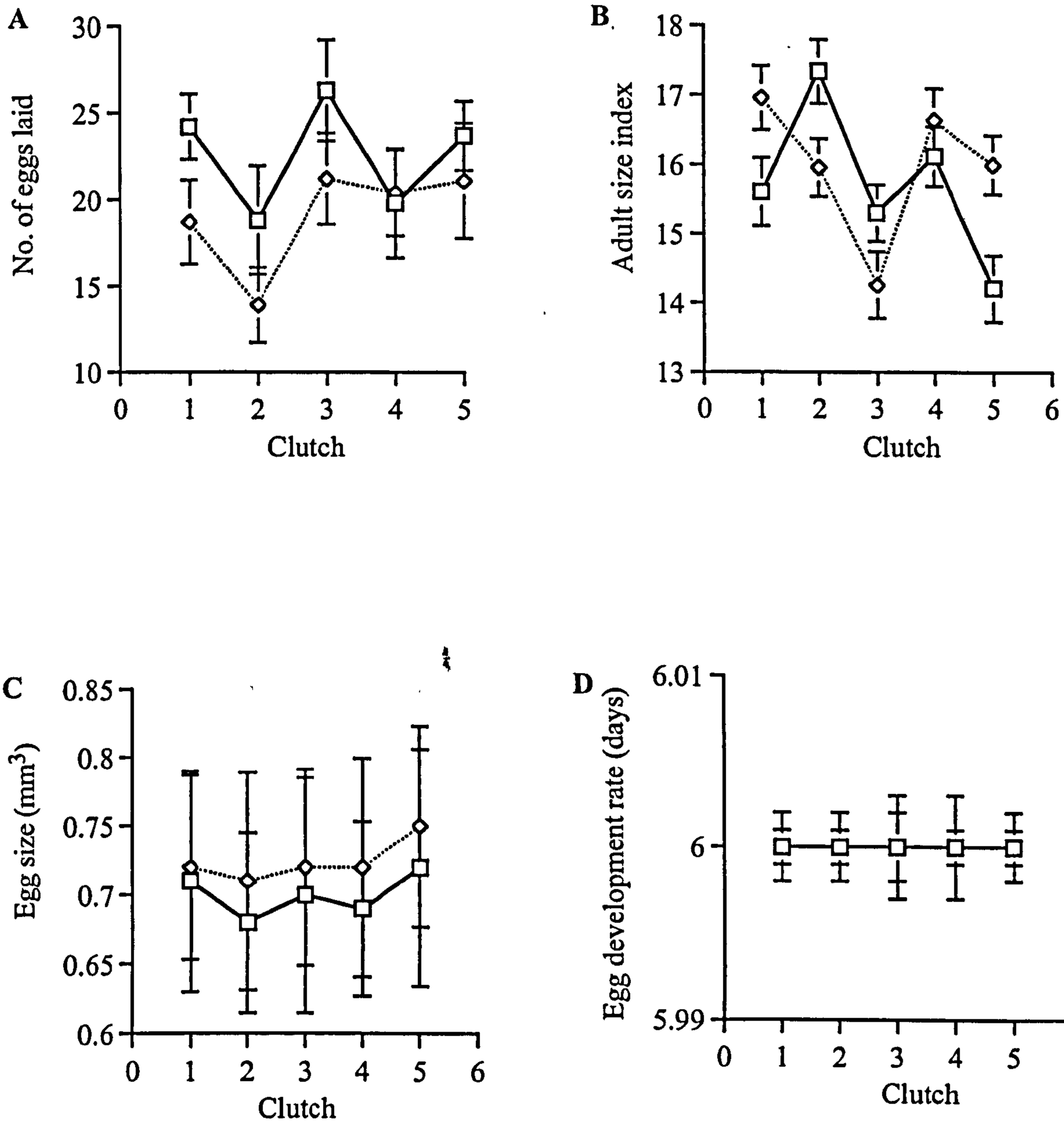


Figure A.1 The effect of multiple mating on several putative fitness components: A. Fecundity, B. Adult size of offspring, C. Egg size, and D. Development rate of eggs. The squares are for high mating females and the diamonds are for low mating females.

size, fecundity and development time have been demonstrated to be effected by nuptial gifts and indirect mechanisms in other insects, many other traits could also be under the influence of male investment (see Iyengar & Eisner, 1999; Stockley & Simmons, 1998).

Does the finding of no fecundity increase, or increase in offspring 'quality' mean that male seminal fluids are not assimilated by females? On one level assimilation of seminal fluids must occur in females as they are inseminated by more sperm than they can store in a single reproductive bout and 3 days after insemination these sperm masses have disappeared from the females haemocoel (See Chapter 3). However the nutritional value of these seminal products may be insignificant compared to that of a blood meal. Females may also be using the seminal products for their own somatic maintenance and the longevity of females would thus be predicted to increase with increased inseminations. The nutrient value of the ejaculate could ameliorate the effect of being multiply pierced by males and the cost of multiple insemination could balance any nutritional benefit that they gain. However, high remating rates caused reduced longevity in females, with no increase in fecundity, and this provides evidence against this hypothesis (Chapter 5). Female use of male investment may also be context specific: an ejaculatory product may protect females from starvation when hosts are scarce and this may benefit males as sperm can survive long periods in the seminal conceptacles. However, as females only mate following a blood meal this is unlikely to be an important effect.

Males inseminate larger ejaculates into virgin females than into females that have already mated. However this increase in male investment does not increase the level of sperm precedence he achieves (See Chapter 3 & 4). This observation is difficult to interpret since males are predicted to increase their investment in a female if it increase their fitness through paternal investment or increases their

genetic representation in the offspring through mating effort. Neither prediction is upheld in this case.

The data collected here are consistent with the idea that there is no significant male investment, although I cannot rule out the possibility that the female incorporates male seminal products either into her own somatic tissues or into investment in her offspring.

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