The evolutionary significance of reproductive traits in *Drosophila melanogaster*

Thesis submitted for PhD

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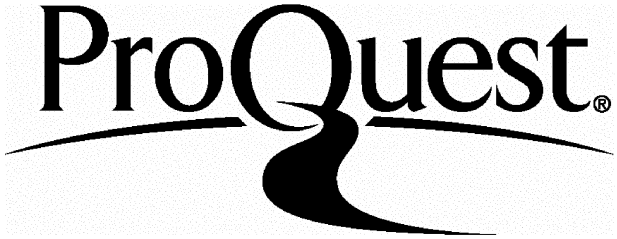


Abstract

As Darwin himself recognised, reproductive success is affected by events that happen both before and after mating. In *Drosophila melanogaster*, proteins produced in the male accessory glands (Acps) are passed to females during mating and strongly influence male post-mating success by affecting sperm transfer, storage, displacement, female oviposition rate and sexual receptivity. The first experiment described in this thesis demonstrates a correlation between male accessory gland size and mating propensity. This suggests that males with large accessory glands have greater Acp resources to achieve high post-mating success for a greater number of successive matings than males with small accessory glands. In addition, a correlation was found between male size and sperm displacement ability, raising the possibility that larger males can pass Acps to females more rapidly than can smaller males. Females too can influence male post-mating success; genetic variation between *D. melanogaster* females is correlated with variation in the sperm precedence achieved by males mated to those females. This thesis describes a correlation between variation in female sperm storage organ morphology and the temporal pattern of offspring production by females, and this temporal pattern could influence the outcome of sperm competition.

Some Acps induce post-mating changes in females that reduce the opportunity for other males to compete for fertilisations. I confirm that the Acp sex-peptide is required for the full reduction of female receptivity and elevation of oviposition that follows a normal mating. At least one Acp is detrimental to female fitness, and is of special interest in the light of recent research suggesting a role for sexual conflict in the evolution of the *D. melanogaster* mating system. However, I find no evidence that a candidate protein, Acp62F, is responsible for this cost. I discuss current and future directions for investigating the influence of sexual conflict on the *D. melanogaster* mating system.

Table of Contents

List of Figures

Chapter 1

Chapter 3

Chapter 4

Chapter 5

Chapter 6

Chapter 7

List of Tables

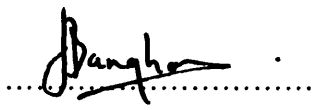
Chapter 3

Chapter 4

Chapter 5

Declaration

I declare that the work presented in this thesis is my own except where duly noted.



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Chapter 1. General Introduction

1.1. Selection in relation to sex

For over a century, sexual selection has helped to explain the selection pressures on traits that affect access to gametes (Darwin 1871). Sexual selection can influence traits that affect mating success (section 1.2) or post-mating fertilisation success (section 1.3). Traits might influence reproductive success because of their effect on competition between males or on sexual interactions between the sexes. In the insects, such traits might include body size, sperm size, sperm number and the morphology of genitalia (section 1.3.1). In species of Diptera, male fertilisation success is affected by a suite of seminal fluid proteins that change female physiology and behaviour. These seminal fluid proteins are particularly well characterised in *Drosophila melanogaster* (section 1.4).

Recently, there has been considerable interest in traits that confer advantages to one sex, but costs to the other. Theory suggests that the persistence of such traits can result in irresolvable evolutionary chases between the sexes (Parker 1979). There is currently widespread interest in the influence of sexual conflict on the evolution of mating systems (Chapman et al. 2003). At the end of this chapter (section 1.5), I describe behavioural and genetic evidence consistent with the influence of sexual conflict on the evolution of the mating system of *D. melanogaster*. This species is an excellent focus for studies of sexual selection and sexual conflict. The genetics of *D. melanogaster* is relatively well understood, providing the means for investigating these traits using powerful genetic tools.

1.2. Pre-copulatory sexual selection

Until about 30 years ago, studies of sexual selection examined traits that affected only mating success. A correlation between a trait, such as body size, and mating success suggests that sexual selection influences the evolution of that trait. Males of some species compete physically or through agonistic displays for access to females. In species of this type, male body size is often positively correlated with male mating success, for example in the fly *Dryomyza anilis* (Otronen 1984a), two species of thrip,

Elaphrothrips tuberculatus (Crespi 1986) and *Hoplothrips karnyi* (Crespi 1988) and in the bee *Amergilla dawsoni* (Alcock 1996). In contrast, smaller males are sometimes favoured in species where agility is required during scramble competition, for example, in the cerambycid beetle *Trachyderes mandibularis* (Goldsmith and Alcock 1993) and in the firefly *Photinus Pyralis* (Vencl and Carlson 1998). In the fruit fly *D. subobscura* (Steele and Partridge 1988), small males are better than larger males at tracking females during courtship. Small males may also have an advantage during flight in male-male competition, for example, in two species of Lepidoptera, *Parapediasia teterrella* and *Agriphila plumbifimriella* (Marshall 1988).

Large males may also have higher mating success than small males when the mating success of males depends on female choice or male-coercion. Large males are, in some species, better able to find, attract, stimulate or coerce potential mates than small males (e.g. in the white spotted sawyer *Monochamous scutellatus*, Hughes and Hughes 1985; in the field cricket *Gryllus bimaculatus*, Simmons 1986, 1988; and in species of seaweed fly, Crean et al. 2000). Some females select mates according to the direct benefits offered by males, such as nutritional gifts (e.g. in the hanging flies *Hylobittacus apicalis* and *Harpobittacus nigriceps*, Thornhill 1976, 1980, 1983; the alfafa butterfly *Colias eurotheme*, Rutowski 1979; and species of bush-cricket, Simmons 1990). Direct benefits may also include parental care (for example in the fish, *Cottus bairdi*, Downhower et al. 1983; the moorhen *Gallinule choragus*, Burley 1981; the red-winged blackbird *Agelaius phoeniceus*, Muldel et al. 1986; and the sedge warbler *Acrocephalus schoenobaenus*, Buchanan and Catchpole 2000; reviewed by Krebs and Davis 1993). For some females, direct benefits constitute access to good territories (e.g. in the fish *Pseudolabrus celidotus*, Jones 1981; the frog *Rana clamitans*, Wells 1977; and in the bee *Anthidium manicatum*, Severinghaus et al. 1981). Females may also discriminate between males based on indirect benefits offered; choosing males that can offer greater genetic benefits for a female's offspring (e.g. in the long-tailed widow bird *Euplectes progne*, Andersson 1982; in the cockroach *Nauphoeta cinerea*, Moore 1994; and in the fruit fly *D. montana*, Hoikkala et al. 1998).

1.3. Post-copulatory sexual selection

Mating at high frequency is not enough to guarantee a male high reproductive success. Male sperm must also successfully negotiate the female reproductive tract for access to eggs, and males may achieve high reproductive success by out-competing rivals during post-mating competition. A female that stores the sperm of two or more males simultaneously (e.g. in the fruit fly *D. melanogaster*, Marks et al. 1988; Harshman and Clark 1998; Imhof et al. 1998; in the damselfly *Ischnura elegans*, Cooper et al. 1996; and in the earwig *Euborellia plebeja*, Kamimura 2003) will precipitate post-copulatory competition between her mates. Since its inception (Parker 1970), the idea of sperm competition has lead to considerable reinterpretation of the selection pressures affecting a number of male traits (such as body size) in the light of their effects on post-copulatory competition. For example, sexual selection may influence body size not just because of the effect of body size on mating success but also because of its effects on post-mating success. Large males sometimes have higher post-mating success than small males, for example in the butterfly *Pieris rapae* (Bissoondath and Wiklund 1997; Wedell and Cook 1998), the beetle *Tribolium castaneum* (Lewis and Austad 1990); the stinkbug *Nezara viridula* (McLain 1985) and the fly *Dryomyza anilis* (Otronen 1994).

It is often clear why large males achieve more matings than small males, but it is sometimes less easy to explain why body size influences post-mating success. However, male size may correlate with other traits that function directly in post-mating competition. In many animals, male success in sperm competition is correlated with ejaculate size, and larger males may produce larger ejaculates than smaller males, as has been demonstrated across species of bushcricket (Wedell 1997). A comparative study has shown that across species of butterfly, males at high risk of sperm competition tend to be larger and have larger testes (Gage 1994). Therefore, adaptations that function directly in post-copulatory competition between males may affect the selection pressures on traits such as body size. Section 1.3.1 describes a number of reproductive traits that function in sperm competition.

In *D. melanogaster*, males vary in their fertilisation success during post-copulatory competition (e.g. Clark et al. 1995). There is considerable interest in why such variation exists, and in identifying its morphological, biochemical and behavioural basis. Again,

correlations between mating and fertilisation success and traits such as body size, accessory gland size and testis size will help illuminate the targets of sexual selection. The relationships between body size, testis size and accessory gland size and pre- and post-mating success of *D. melanogaster* males are examined in Chapter 3.

1.3.1. The consequences of sperm competition

When females mate multiply and store the sperm of more than one male simultaneously, selection will favour traits that improve the displacement of the sperm of previous males (sperm offence). Conversely, if sperm displacement occurs, then selection will favour males possessing mechanisms that help defend their stored sperm, such as prolonged copulation, mate guarding or the production of mating plugs. Male *D. melanogaster* are particularly interesting with respect to sperm competition, as they use seminal fluid proteins to influence female physiology and behaviour. Examination of traits that function in sperm competition requires measurement of the outcome of sperm competition between two males. In this section, I will outline how this is measured and describe several traits that affect and function during post-copulatory competition between males of different species. I will then focus on the functions of seminal fluid proteins in *D. melanogaster.*

1.3.1.1. Measures of sperm competition

The outcome of sperm competition is usually measured by determining sperm precedence. For two males mated to a single female, sperm precedence describes the relative fertilisation success of the first and second males to mate. Variation in sperm precedence across the insects is considerable, ranging from first male precedence, for example in the predatory mite *Macrocheles muscaedomesticae* (Yasui 1995), to almost complete second male precedence, for example in the 2-spot ladybird *Adalia bipunctata* (Dejong et al. 1993).

The relative fertilisation success of two males mated to a single female can be measured empirically in several ways. Offspring paternity can be determined using the sterile male technique, allowing a male that produces non-fertile sperm to compete with one with fertile sperm (e.g. Reinhardt 2000). Paternity may also be determined by offspring

genotyping (Hadrys et al. 1993; Hooper and Siva Jothy 1996), and in some species, it is possible to use males that sire offspring with distinguishable phenotypes (e.g. Clark et al. 1995). For females mated twice to two different males, common indexes of sperm precedence are P_2 (*=number offspring fertilised by the second male/(number offspring sired by the first + second males*) and P_1 (*=number offspring fertilised by the first male/(number offspring sired by the first + second males*). If, when using the sterile male technique, sterility is incomplete, or if the 'fertile' mating results in a proportion of infertile eggs, a modified equation for calculating P_2 can be used (Boorman and Parker 1976; Cook et al. 1997). Sperm displacement ability, *SDA* (*=number offspring fertilised by second male/(number offspring sired by the first male* + 1), is also often used instead of P_2 (Hughes 1997; Gilchrist and Partridge 2000), because the distribution of *SDA* across males is frequently less skewed than that of P_2. Finally, in *D. melanogaster*, the labelling of sperm using green fluorescent protein (GFP) is possible where direct sperm observation is required (Civetta 1999; Price et al. 1999), although it is important to note that males with GFP labelled sperm can have low fertility (Price et al. 1999). For direct observations of stored sperm, the measure S_2 (*number of second male sperm in female sperm storage organ/first+second male sperm in female sperm storage organ*) can be employed (Civetta 1999).

1.3.1.2. Adaptations of sperm and ejaculate size in response to sperm competition

Sperm competition has a number of selective consequences for males. Sperm competition risk is defined as the probability that two male ejaculates overlap in the reproductive tract of a female (Simmons and Siva-Jothy 1998). The conditions under which sperm competition results in selection on sperm size and number have been examined theoretically by Parker and colleagues (e.g. 1970; 1990a, b; 1993; Parker and Begon 1993; Parker et al 1990). Where sperm compete numerically, positive correlations are expected between the risk of sperm competition and the number of sperm passed to a female during mating. Across species of fish, males of polyandrous species produce larger ejaculates than monoandrous species (Stockley et al. 1996). Selection on ejaculate size is, in turn, predicted to result in a correlated response on testis size. Comparative studies have revealed strong interspecific associations between testis size and sperm competition risk, for example across species of *Drosophila* (Pitnick and Markow 1994), butterflies (Gage 1994), primates (Harcourt et al. 1981;

Harvey and Harcourt 1984) and frogs (Byrne et al. 2002). Artificial selection experiments on the yellow dung fly *S. stercoraria* have demonstrated that an increased risk of sperm competition results in an increase in testis size (Hosken and Ward 2001). Indeed, the correlation between testis size and levels of polygamy across species is now considered so robust that testis size is sometimes used as an indirect measure of the risk of sperm competition (Morrow and Gage 2000; Dunn et al. 2001; Hosken et al. 2001b).

There are a number of conditions under which sperm competition may result in selection on sperm size. When larger sperm are more motile than smaller sperm, large sperm may out-compete smaller sperm during competition for eggs (Gomendio and Roldan 1991). In internally fertilising organisms, large sperm may be better able to fill a female's sperm storage organs than small sperm, reducing the access of subsequent male sperm to eggs (Sivinski 1980, 1984; reviewed Simmons and Siva-Jothy 1998). Large sperm may also provide benefits in the form of gamete provisioning, if, for example, the whole of a sperm enters an egg (Markow 1985). Predictions regarding the relationship between the risk of sperm competition and selection on sperm size are made more complicated because increased sperm number can result in a trade off with sperm size (Oppliger et al. 2003; and for further discussion see Arnaud et al. 2001).

Nevertheless, despite some uncertainty as to the predictions regarding the relationship between sperm size and sperm competition risk, some notable correlations do exist. In species of the externally fertilising cichlid fish, polygamous species have significantly longer sperm than their closest monogamous counterparts (Balshine et al. 2001; although see Stockley et al. 1997). Comparative studies also reveal correlations between sperm size and levels of polygamy. This is seen across species of the nematode family *Rhabditidae* (LaMunyon and Ward 1999), butterflies (Gage 1994), birds (Briskie and Montgomerie 1992; Briskie et al. 1997; Johnson and Briskie 1999), rodents and primates (Gomendio and Roldan 1991; although see Gage and Freckleton 2003). In addition, intraspecific variation in sperm size is correlated with success in sperm competition in the bulb mite *Rhizoglyphus robini* (Radwan 1996) and in the nematode *Caenorhabditis elegans* (LaMunyon and Ward 1998). Artificial selection experiments have demonstrated that in *C. elegans*, increased sperm competition risk results in increased sperm size (LaMunyon and Ward 2002).

Fertile sperm are not the only components of the ejaculate that can affect the outcome of sperm competition. In large numbers of invertebrates, particularly in species of Diptera and Lepidoptera, males produce at least two sperm types, only one of which is capable of fertilisation (Silberglied et al. 1984; Swallow and Wilkinson 2002). In most examples of sperm heteromorphism, the function of non-fertilising sperm has not yet been determined. However, in the butterfly *P. napi*, in which the second male to mate has sperm precedence (Bissoondath and Wiklund 1997), the quantity of non-fertilising sperm passed to females during insemination is correlated with the extent to which males inhibit female post-mating sexual receptivity (Cook and Wedell 1999). In this species, the non-fertilising sperm inhibit female receptivity by acting as a sperm storage organ 'filler' (Wedell 2001), reducing the risk of sperm competition. The production of non-fertilising sperm also responds to sperm competition risk in the snail *Viviparus ater*. Here, the number of non-fertilising sperm in the ejaculate increases as the male-female sex ratio is experimentally increased (Oppliger et al. 1998). In *V. ater*, the size of non-fertile sperm is positively correlated with the fertilisation success of males (Oppliger et al. 2003). In the face of sperm competition, non-fertilising sperm may perform functions similar to chemical components of the ejaculate of other species, such as *D. melanogaster*, the details of which are discussed in section 1.4.

1.3.1.3. Adaptations of genitalia in response to sperm competition

Adaptations of the genitalia may also influence post-copulatory competition between males. Males of many species of insect, particularly the Odonata, physically remove sperm of previous mates from the sperm storage organs of females, a mechanism first documented in the damselfly *Calopteryx maculata* (Waage 1979a). The possibility that the genitalia can function in both sperm transfer and removal has also been recognised in a number of other insects, for example in the flour beetle *T. castaneum* (Haubruge et al. 1999;), the earwig *Euborellia plebeja* (Kamimura 2000), the yellow dung fly *S. stercoraria* (Hosken et al. 1999), the rove beetle *Aleochara curtula* (Gack and Peschke 1994), the beetle *Psacothea hilaris* (Yokoi 1990), the tree cricket, *Truljalia hibinonis* (Ono et al. 1989) and in species of the Libellulidae (Siva-Jothy 1987; Artiss 2001). In the water strider *Gerris lateralis*, the shape of the male genitalia is related to relative

paternity success (Arnqvist and Danielsson 1999a). There is also evidence to suggest a relationship between genital morphology and sperm removal in birds. Waterfowl (of the family Anatidae) are unusual among birds in that the males have intromittant organs. Several characteristics of the intromittant organ (e.g. its length and the area covered in ridges) are correlated with frequency of forced extra pair copulations and, in turn, with the intensity of sperm competition (Coker et al. 2002).

1.3.1.4. Mate guarding and prolonged copulation

The numerous ways in which males may prevent rivals from competing with their sperm can include prolonged physical associations with females. Mate guarding can enhance male fitness by preventing rapid female remating, for example in the damselfly *C. maculata* (Waage 1979b) and in the spider mite *Tetranychus urticae* (Satoh et al. 2001), or to deter courtship by potential rivals, for example in the decorated cricket *Gryllodes sigillatus* (Frankino and Sakaluk 1994). In some species, mate guarding represents one of several alternative strategies that males can adopt in the face of sperm competition. For example, males of the Mediterranean Sea wrasse *Symphodus ocellatus* guard their mates after mating and only produce large numbers of sperm if mate guarding is ineffective (Alonzo and Warner 2000; see also Warner et al. 1995; Warner 1997).

Males may also increase their fertilisation success by prolonging the duration of copulation, as observed in a large number of insect orders, for example in the firebug, *Pyrrhocoris apterus* (Schofl and Taborsky 2002), in water striders (Arnqvist and Danielsson 1999a) and in dragonflies (Wolf et al. 1989; reviewed Alcock 1994). In some species, prolonged copulation, or prolonged attachment to the female of a sperm-containing ampulla, may be induced by the presentation of nuptial gifts; the larger the nuptial gift, the longer females mate and the greater the male's fertilisation benefits (Sakaluk 1986; Wedell and Arak 1989). Prolonged copulation may result in greater fertilisation success because it allows more efficient sperm transfer and storage (Sakaluk 1984; Wolf et al. 1989; Campbell and Fairburn 2001; Schofl and Taborsky 2002), or because it results in a greater delay of female remating (Wedell 1993). Prolonged copulation-duration may also function to pass ejaculate components other

than sperm to females. In *D. melanogaster*, sperm transfer is over by the midpoint of copulation. Prolonging copulation beyond this midpoint improves sperm displacement and the inhibition of female receptivity, probably through the effects of seminal fluid proteins, as discussed in section 1.4 (Gilchrist and Partridge 2000).

1.3.1.5. Remote mate guarding

Males of some insects guard their mates 'remotely,' influencing female post-mating physiology and behaviour via components of the seminal fluid (reviewed Leopold 1976; Gillott and Friedel 1977; Simmons and Siva-Jothy 1998; Gillott 2003). In some animals, males temporarily or permanently prevent their mates from remating by introducing a mating plug at the opening of the female reproductive tract (Simmons and Siva-Jothy 1998). Mating plugs are seen, for example, among *Drosophila* species (Patterson 1946; Bairati and Perotti 1970; AlonsoPimentel et al. 1994; Polak et al. 1998, 2001), species of *Coproica* flies (Lachmann 1997, 1998), anopheline mosquitoes (Gilles 1956; Giglioli and Mason 1966), species of *Lepidoptera* (Dickinson and Rutowski 1989; Orr and Rutowski 1991; Matsumoto and Suzuki 1992), the spider *Phidippus johnsoni* (Jackson 1980) and the nematode *C. elegans* (Barker 1994). In some insects, the mating plug improves the efficiency of sperm storage (Polak et al 1998, 2001) and in others, it can increase the length of time before a female remates (Jackson 1980; Dickenson and Rutowski 1989; Orr and Rutowski 1991; Lachmann 1998; Polak et al 1998, 2001). The mating plug produced by males of the bumblebee *Bombus terrestris* is particularly effective in this last respect (Duvoisin et al. 1999). The plug contains a fatty acid component that prevents females from mating more than once per lifetime (Baer and Schmid-Hempel 1999; Baer et al. 2000, 2001).

Protein components of the ejaculate may also mediate remote mate guarding by acting on the reproductive tracts or nervous systems of females to reduce female receptivity and attractiveness to other males (section 1.4). Such seminal fluid components have been identified by transplanting male accessory glands or testes, or by injecting purified male seminal fluid extracts, into virgin females. Seminal fluid proteins that affect female physiology and behaviour are particularly common amongst species of Diptera, for example in the housefly *Musca domestica* (Nelson et al. 1969; Reimann and

Thorson 1969; Bird et al. 1991), the black blow fly *Phormia regina* (Nelson et al 1969), the screw worm fly *Cochliomyia hominivorax* (Nelson et al. 1969) ~~the corn earworm *Heliothis zea* (Bird et al. 1991)~~, the tsetse fly *Glossina morsitans* (Gillott and Langley 1981), the mosquitoes *Aedes aegyptii* (Leahy and Craig 1965; Craig 1967; Fuchs and Hiss 1970; Klowden and Chambers 1991; Dickenson and Klowden 1997) and *Aedes albopictus* (Leahy and Craig 1965; Klowdon 1993) and the fruit flies *D. funebris* (Baumann 1974) and *D. melanogaster* (Garcia-Bellido 1964; Merle 1968; Chen and Bühler 1970). The effects of these seminal fluid proteins have been widely studied in species of mosquito (reviewed by Klowden 1999) and *Drosophila* (reviewed by Kubli 1996; Wolfner 1997; Chapman 2001).

1.3.2. Female sperm storage and the influence of females on male fertilisation success

As described in preceding sections, the production of offspring by multiply mated females is heavily influenced by post-mating competition between males. There has also been speculation, although limited experimental investigation, into the influence of females on sperm use (Walker 1980; Otronen et al. 1997; Qazi et al. 1998; Ward 1998; Hellreigel and Bernasconi 2000; Ward 2000; Knowles and Markow 2001). One reason why females might influence sperm use is to acquire direct benefits, such as an increased longevity of stored sperm. The morphological complexity of many female insect reproductive tracts has also lead to speculation that females gain indirect benefits through post-copulatory sperm choice, or by differential investment in embryos sired by different males (e.g. Walker 1980; Thornhill 1983; Eberhard 1996, 1998). However, sperm choice is difficult to dissect from sperm competition, requiring detailed understanding and manipulation of insemination, sperm storage, displacement and fertilisation mechanisms. Experimental evidence of post-mating sperm choice is therefore currently lacking (for extensive discussion on this subject, see Birkhead 1998; and the subsequent responses of Birkhead 2000; Eberhard 2000; Kempenaurs et al. 2000; Pitnick and Brown 2000).

In *D. melanogaster*, there is extensive opportunity for sperm competition. As in many insect species, females mate repeatedly throughout their lives, even while they retain the

sperm from previous inseminations (Vianen and Bijilsma 1993; Harshman and Clark 1998; Imhof et al. 1998; Marks et al, 1988). Females simultaneously store the sperm from multiple males, both in the wild (Milkman and Zeitler 1974; Marks et al. 1988) and under laboratory conditions (Milkman and Zeitler 1974). They can store sperm for up to two weeks after mating (Kaufman and Demerec 1942; Neubaum and Wolfner 1999) and in the wild, estimates of the numbers of mating partners for females up to the time of capture have ranged from 1.9 (Harshman and Clark 1998) to 6.0 (Imhof et al. 1998).

In *D. melanogaster*, doubly mated females vary in the degree of sperm precedence achieved by their mates. Studies using doubly mated females homozygous for chromosomes extracted from natural populations have shown that female genotype is correlated with variation in P_2 (Clark and Begun 1998), and that interactions between chromosomes I and II are associated with variation in P_1 (Civetta and Clark 2000a). Interactions between the sexes also influence sperm precedence (Clark et al. 1999). Male fertilisation success is influenced by interactions between the sexes in other species too (e.g. in the red flour beetle *T. castaneum*, Nilsson et al. 2003; in the housefly *Musca domestica* Andres and Arnqvist 2001; the beetle *Calloscobruchus maculatus*, Brown and Eady 2001; and in the water strider *Gerris lateralis*, Arnqvist and Danielsson 1999a). In species of an externally fertilising sea urchin (genus *Echinometra*), sperm-egg associations are mediated by the sperm protein bindin. The affinity with which a female's eggs bind to sperm depends on the genotype at the male's bindin locus (Palumbi 1999) and eggs of different females bind with different affinities to sperm of different males. Genetic incompatibility between males and females may also affect male fertilisation success (reviewed Tregenza and Wedell 2000), preventing inbreeding (Markow 1982; Olsson et al. 1996; Bishop et al. 1996; Markow 1997) or fertilisation between closely related species (e.g. Howard et al. 1998; Price et al. 2000; Price et al. 2001).

Pitnick et al. (1999) suggested that female *Drosophila* could affect the outcome of sperm competition through the utilisation of different types of sperm storage organ. Like all other species of *Drosophila* (Pitnick et al. 1999), *D. melanogaster* females possess two distinct types of sperm storage organ: a long coiled seminal receptacle and

two spheroid spermathecae (Fig. 1.2). The seminal receptacle is 2mm long with an opening of 3-5 microns in diameter and a distal half of 20 microns in diameter (Lefevre and Jonsson 1962). The seminal receptacle is generally regarded as the most important site of sperm storage in this species, holding the majority of the stored sperm (Lefevre and Jonsson 1962; Filosi and Perotti 1975; Gilbert 1981; Pitnick et al. 1999). Dissections of sperm storage organs after mating have shown that sperm are released from the seminal receptacle sooner than sperm from the spermathecae (Gilbert 1981). Recent work suggests that the longer a female's seminal receptacle, the greater her sperm storage capacity and the higher her fecundity, although no evidence was found to suggest that seminal receptacle length affected the sperm precedence achieved by her mates (Miller and Pitnick 2003).

The spermathecae of female *D. melanogaster* are similar to the spermathecae of other species of Diptera; mushroom shaped and heavily sclerotized (Filosi and Perotti 1975). The spermathecae are long-term sperm storage organs, releasing sperm later than the seminal receptacle (Gilbert 1981). Studies of the lower Diptera have suggested that the heavily sclerotized spheroid morphology of these organs may be required by the pressures set up by fluid absorption that help sperm into storage (Linley and Simmons 1981). Only 20-40% of stored sperm are retained in the spermathecae (Lefevre and Jonsson 1962; Fowler 1973; Filosi and Perotti 1975; Gilbert 1981). However, in *D. melanogaster*, the role of the spermathecae may not just be one of sperm storage. Similar to the spermathecae of other species, those of *D. melanogaster* include cells that resemble secretory cells, and these produce fluid that may aid the longevity of sperm during storage (Filosi and Perotti 1975). The ablation of one or both spermathecae, and experiments conducted on mutant females that lack one or more spermathecae, support the idea that the spermathecae are required for the sustained longevity of sperm in the seminal receptacle (Anderson 1945; Bouletreau-Merle 1977). Certainly, sperm storage morphology affects the temporal pattern of offspring production (see Chapter 4). The relative functions of these two sperm storage organs in sperm utilisation, and possible reasons why both types of sperm storage organ have been retained in this species, remain unclear (Fowler 1973; Gilbert 1981; Briskie and Montgomerie 1993; Keller and Reeve 1995; Ward 1998).

Sperm 'offence' in *D. melanogaster* occurs through a process of displacement and incapacitation (Price et al. 1999), and is heavily influenced by male accessory gland proteins (section 1.4). However, despite detailed knowledge of the anatomy of the female reproductive tract, the mechanisms of sperm displacement in *D. melanogaster* remain elusive. Lefevre and Jonsson (1962) observed the continual inbound and outbound movement of sperm to and from the seminal receptacle and suggested that observed patterns of sperm displacement could be determined by the mixing of first and second male sperm in the uterus. They proposed the hypothesis that first male sperm from the storage organs enters the uterus and mixes with larger quantities of sperm deposited by the second male. All sperm from the uterus are subsequently restored to the seminal receptacle. This is referred to as an 'indirect' mechanism of sperm displacement, and is similar to the one proposed for *S. stercoraria* (Simmons et al. 1999), but has not been tested.

Naturally occurring morphological variation in the spermathecae of female *D. melanogaster* provides the means to investigate the relationship between female reproductive tract morphology and male fertilisation success. It is likely that the correlation between female genotype and sperm precedence achieved by two males mated to a single female (Clark and Begun 1998) is related to biochemical or morphological variation in the female reproductive tract. Chapter 4 describes an experiment carried out to test the relationship between sperm storage organ morphology and patterns of sperm use, using a stock of *D. melanogaster* with females that varied in spermathecal morphology.

1.4. The accessory gland proteins of *Drosophila melanogaster*

In a large number of insects, seminal fluid proteins dramatically influence the physiology and behaviour of females and the reproductive success of their mates (e.g. Gillott 2003). Amongst insect species, the seminal fluid proteins of *D. melanogaster* have been examined particularly extensively. Directly after mating, female physiology and behaviour changes considerably from that of virgins. Mated females typically become unreceptive to courtship, become unattractive to other males and oviposit at a high rate. These changes are induced by the receipt of male accessory gland proteins

(Acps) (Lefevre and Jonsson 1962; Garcia-Bellido 1964; Manning 1967; Leahy and Lowe 1967; Merle 1968; Hihara 1981; Chen et al. 1988; Kalb et al. 1993; Harshman and Prout 1994; Wolfner 1997; Chapman 2001). The male accessory glands contain two types of cell (Fig. 1.1 b & c): approximately 1000 main-cells in the lumen of the gland and 40-50 secondary cells in the distal part of the gland (reviewed in Kubli 1996). So far, the functions of only a few of the estimated 80 Acps produced by males have been identified. However, as well as their effects on post-mating physiological and behavioural changes in females, their functions include an increase in the efficiency of sperm transfer, the formation of a mating plug, sperm storage, protection against bacterial or fungal infection and possibly the inhibition of proteolysis to protect sperm from degradation. After mating, some Acps localise to specific areas of the female reproductive tract (Fig. 1.2b) (Bertram et al. 1996; Wolfner et al. 1997; Heifetz et al. 2000; Lung and Wolfner 2001) and others pass into the haemolymph (Lung and Wolfner 1999) and are incorporated in other somatic tissues (Pitnick et al. 1997). Some of the effects of Acps on females are regarded as beneficial to both sexes, some as adaptations beneficial for males in the face of sperm competition, and one or more is beneficial to males but costly to females (section 1.5). Understanding the functions of Acps, and their receptors in females, is crucial for understanding natural and sexual selection on the mating system of *D. melanogaster*.

1.4.1. Acps and sperm transfer

In *D. melanogaster*, a typical ejaculate consists of about 4000 2mm long sperm produced in the testes and at least 80 proteins expressed in the ejaculatory duct and accessory glands (Fig. 1.1). The transfer of sperm and ejaculate proteins to females is caused by the action of the ejaculatory bulb. Mating typically takes between 15 and 20 minutes. Sperm transfer takes place rapidly after mating starts and is over by its midpoint (Tram and Wolfner 1998; Gilchrist and Partridge 2000). It is thought that prolonged copulation beyond the time needed to pass sperm to females delivers seminal fluid proteins to females that help to delay female remating (Gilchrist and Partridge 2000). At least one Acp contributes to the efficiency of sperm transfer during mating (Tram and Wolfner 1999), perhaps by guiding or lubricating the passage of sperm in the female reproductive tract (Tram and Wolfner 1999).

1.4.2. Acps, sperm storage and sperm displacement

As discussed in section 1.3.2, *D. melanogaster* females store sperm in two different types of organ (Fig. 1.2), the seminal receptacle and spermathecae. The numbers of sperm passed to females is in vast excess of those stored. Sperm storage by females requires the receipt of the male-derived protein Acp36DE, which localises with stored sperm (Neubaum and Wolfner 1999). Males that lack Acp36DE pass normal quantities of sperm to females, but their mates store only 15% of the normal amount (Neubaum and Wolfner 1999; Tram and Wolfner 1999). Acp36DE also has consequences for sperm competition; males that lack Acp36DE are less able to displace sperm of previous males, because fewer sperm are retained in the sperm storage organs (Chapman et al. 2000).

When females mate multiply and store the sperm of two or more males simultaneously, sperm displacement ability can affect considerably male reproductive success. For doubly mated *D. melanogaster* females under laboratory conditions, the proportion of offspring sired by the second male to mate (P_2) is highly variable, although the most recent male to mate a female tends to sire the majority of her subsequent offspring (Kaufman and Demerec 1942; Prout and Bundgaard 1977; Scott and Richmond 1990; Scott and Williams 1993). *D. melanogaster* males vary in sperm displacement ability, for example, Prout and Bundgaard (1977) found differences among strains for male sperm displacement ability. In addition, males made homozygous for chromosomes extracted from natural populations vary in P_2 values achieved during sperm competition (Clark et al. 1995). Sperm precedence is also affected by interactions between male and female genotypes (Clark et al. 1999), as well as interactions between the genotypes of the pair of males competing (Clark et al. 2000).

In *D. melanogaster*, it is also important for a male to be able to prevent displacement of his own sperm during a female's subsequent copulations. There are indications that Acps have a role in resisting sperm displacement; variation at some Acp loci is correlated with variation in male sperm defence ability (Clark et al. 1995; Civetta and Clark 2000a). It is not known however, how this genetic variation translates into

biochemical or morphological variation between males or how Acps affect sperm precedence (Chapman 2001). Experiments using sterile males, and those deficient in Acps, have suggested that Acps are involved in both displacement and incapacitation of sperm from previous inseminations (Prout and Bundgaard 1977; Scott and Williams 1993; Harshman and Prout 1994; Gilchrist and Partridge 1995). Sperm displacement can occur without the receipt of second male sperm (Harshman and Prout 1994) although evidence suggests that without sperm, displacement is an inefficient process (Gilchrist and Partridge 2000).

1.4.3. Drop in female attractiveness post-mating

Mated female *D. melanogaster* are less sexually attractive than virgins for between 5 and 9 days after mating (Tram and Wolfner 1998). The drop in attractiveness (measured as the percentage of time a male courts a female before mating) occurs in two stages. An initial, short-term, drop is independent of the receipt of both sperm and Acps (Tram and Wolfner 1998). Persistence of the effect beyond 24 hours has been linked to the storage of sperm, as males lacking sperm, or Acps and sperm, do not maintain this reduction in attractiveness. It has been suggested that the relationship between female attractiveness and sperm storage may benefit both males and females, relieving females of the costly rejection of courting males and protecting male sperm from displacement (Tram and Wolfner 1998).

1.4.4. Post-mating inhibition of sexual receptivity and elevation of egg laying

Two of the most dramatic behavioural changes to occur in female *D. melanogaster* after mating are an acute reduction of sexual receptivity and a rapid elevation of egg laying, both of which last for several days. One or more factors originating from the main cells of the male accessory glands contribute to these changes (Kalb et al. 1993). One of the best-characterised Acps, and a candidate for producing these responses, is the small peptide Acp70A, also called the sex-peptide (SP). SP was first isolated using HPLC chromatography (Chen et al. 1988). Injection of the purified SP fraction into virgin females caused a repression of sexual receptivity and stimulated oogenesis (egg maturation), ovulation (release of eggs from the ovaries into the reproductive tract) and egg laying (Chen et al. 1988). Ectopic expression of SP in transgenic virgin females

altered female reproductive behaviour in a similar manner (Aigaki et al. 1991). Purification of the biologically active fraction of accessory gland extract yielded a mature protein of 36 amino acids (Chen et al. 1988).

The effect of SP on oogenesis occurs via a neurosecretory organ called the *corpora allata*. SP acts on the *corpora allata* to increase juvenile hormone production, which, in turn, stimulates the maturation of oocytes (Moshitzky et al. 1996; Soller et al. 1999; Fan et al. 2000). The N-terminal of the SP seems to be important for its effects on oogenesis (Fan et al. 2000). Acp26Aa is a second Acp that stimulates oogenesis (Herndon and Wolfner 1995). Acp26Aa localises to the base of the ovaries and stimulates the release of mature eggs into the lateral oviducts (Fig. 1.2). Experiments carried out using males lacking Acp26Aa show that the effects of this protein last for only 1 day following mating (Herndon and Wolfner 1995) and that Acp26Aa affects oviposition by only 8-18%.

Stimulation of oocyte maturation by SP via juvenile hormone production does not affect female receptivity or translate into higher rates of ovulation or oviposition, and so SP is thought to have an additional, separate, effect on these latter responses (Soller et al. 1999). The targets through which SP acts to cause a repression of receptivity and stimulation of oviposition are not yet known, although ectopic expression of SP in a number of tissues (Nakayama et al. 1997), and functional analysis of fragments of the protein (Schmidt et al. 1993a), suggest that both responses are elicited through the same target molecule or tissue (Kubli 1996). The C-terminal of the protein is thought to be responsible for its effects on female receptivity and oviposition, and this region is conserved across sex-peptides of many other *Drosophila* species (Schmidt et al. 1993b; Cirera and Aguade 1997; Cirera and Aguade 1998a, b). In addition, incubation of adult female *D. melanogaster* with radio-labelled synthetic SP has suggested that the C-terminal of SP is required for the *in vitro* binding of the protein to peripheral nerves, the suboesophageal ganglion, cervical connective tissue, discrete parts of the thoracic ganglion and the parts of the genital tract (Ottiger et al. 2000). These targets may be accessed through the haemolymph (Chen et al. 1988; Aigaki et al. 1991; Schmidt et al. 1993a). It is not clear, however, whether SP reaches these areas *in vivo*, or how SP might bind to its respective receptors. Unpublished data suggests that SP binds to sperm

and is thus localised in the sperm storage organs shortly after mating (S. Busser and E. Kubli, Unpublished data).

Neither injection experiments nor ectopic expression of SP in transgenic females can determine whether SP is the only Acp involved in the inhibition of receptivity and elevation of egg laying, or whether its function overlaps with other seminal fluid components. The ejaculatory duct protein Dup99B, which shares strong C-terminal sequence homology to the SP, also shows remarkable functional similarity to the SP when injected into females (Saudan et al. 2002). Dup99B was isolated from male heads and its injection into females induced oviposition and reduced female receptivity to the same degree as SP (Saudan et al. 2002). Like SP, Dup99B is able to bind to sperm (J. Peng and E. Kubli, Unpublished data) and autoradiography of adult females incubated with radio-labelled Dup99B show that Dup99B has the same *in vitro* binding patterns as SP (Ottiger et al. 2000). Dup99B is unable to stimulate the *corpora allata* to produce juvenile hormone. This is consistent with the lack of homology between Dup99B and SP at their N-termini, as the N-terminal of SP is though to be responsible for the action of SP on the *corpora allata* (Fan et al. 2000). Nevertheless, despite the similarities between Dup99B and SP, females mated to males lacking Dup99B appear to behave no differently post-mating to mates of normal males (H. Liu and E. Kubli, Unpublished data). In addition, females mated to males that transfer Dup99B but no Acps show little or no reduction in receptivity (Kalb et al. 1993; Xue and Noll 2000; Saudan et al. 2002). So far, therefore, despite a strong female response to injected Dup99B, it seems that the importance of Dup99B *in vivo* is unlikely to be very great.

The storage of sperm too has consequences for the post-mating sexual receptivity of females. Prolonged inhibition of female receptivity has long been associated with sperm storage (Manning 1962). Males that lack sperm but express normal levels of Acps and ejaculatory duct proteins induce only a short-term reduction in receptivity in females, before receptivity returns to levels similar to that of virgins (Manning 1967; Xue and Noll 2000). Males lacking SP are necessary to determine whether functional redundancy exists between it and other seminal fluid components. Chapter 5 describes female receptivity and egg-laying following matings to males lacking SP.

1.4.5. The mating plug of *D. melanogaster*

A mating plug consisting of seminal fluid components forms in the uterus of *D. melanogaster* females during copulation (Fig. 1.2) (Bairati 1968; Bairati and Perotti 1970; AlonsoPimentel et al. 1994). The mating plug is formed in two stages in different regions of the uterus: anterior and posterior. An accessory gland protein found in high quantities in the mating plug, Acp76A, has strong sequence similarity to a serpin proteolytic regulator and plays a role in mating plug coagulation (Wolfner et al. 1997). Following direct observations of copulation, Bairati (1968) suggested that a secretion from the ejaculatory bulb was transferred to the female before sperm, aiding the progression and retention of sperm in the female reproductive tract. Indeed, the protein PEB-me, expressed in the ejaculatory bulb (Ludwig et al. 1991), is found in high concentration in the mating plug and has protein sequence similarity to photopolymer proteins in spider silk (Lung and Wolfner 2001). Three minutes after the beginning of mating, PEB-me coagulates into a posterior mating plug, facilitating sperm movement into the uterus. At the end of mating, as yet unidentified Acps, which probably include the protein Acp36DE, then contribute to the formation of the anterior mating plug (Lung and Wolfner 2001).

1.4.6. Anti-microbial ejaculate proteins

Protection of sperm, the female reproductive tract and newly laid eggs from bacteria or fungi is important for both male and female reproductive success. Some seminal fluid proteins function as antibacterial or antifungal agents (reviewed in Chapman 2001). At least three antibacterial proteins are transferred to females during mating; one is produced in the accessory glands and two in the ejaculatory duct (Samakovlis et al. 1991; Lung et al. 2001). One of these important antibacterial seminal fluid proteins is thought to be the ejaculatory duct protein Andropin (Samakovlis et al. 1991; Lung et al. 2001).

1.5. Acps, costs of mating and sexual conflict

In *D. melanogaster*, many seminal fluid proteins benefit males during post-copulatory competition, while others benefit both sexes. At least one Acp, however, is detrimental to female fitness. Using males that expressed low quantities of Acps, or lacked

expression from the main cells of the accessory glands altogether, it was demonstrated that, for multiply mated females, one or more Acps causes a reduction in female survival and lifetime reproductive success (Chapman et al. 1995). This as yet unidentified Acp is thought to carry both benefits for males and costs for females (Chapman et al. 1995; Rice 1996; Holland and Rice 1999; Civetta and Clark 2000b; Lung et al. 2002). Any trait with asymmetric costs and benefits for females and males is interesting in the light of recent theory and empirical research regarding the effect of sexually antagonistic selection on the evolution of mating systems.

A trait that confers asymmetric costs and benefits for the two sexes may result in intra-locus or inter-locus sexual conflict (Partridge and Hurst 1998). Intra-locus conflict will arise when a trait is encoded by alleles subject to different selective pressures according to the sex in which they are present. Males and females may have different optima for certain traits, and this can be resolved by sex limitation. In the absence of complete sex limitation however, selection on a locus in one sex can persistently constrain its evolution away from the optima of the other sex (Rice 1984; Slatkin 1984; Lande 1987).

Inter-locus conflict may also occur. Traits have been identified that are beneficial to one sex but costly to the other. In several species, males behave in a way that damages female reproductive fitness. Male-derived harm is expected to result in selection on female sex-limited traits or behaviours that mitigate these costs. Such counteradaptive traits may, in turn, prove detrimental to male fitness. Theory suggests that traits conferring benefits to one sex and costs to the other are important because they could lead to rapid coevolutionary change between the sexes. Complex evolutionary chases between sex-limited loci were first formally explored by Parker (1979). Parker's (1979) original model considered a male sex-limited allele that had different thresholds of costs for males and females. Under some conditions, this allele would confer benefits to males and costs to females. The model also considered the simultaneous spread of a counteradaptive allele conferring benefits to females. Game theory showed that this could resolve into an evolutionary stable strategy, but that under some conditions, irresolvable evolutionary chases could arise (Parker 1979).

It is these evolutionary chases that are of particular interest, as they suggest that sexual conflict has the potential to influence strongly the evolution of reproductive traits, in a manner analogous to arms races. Conflicts are predicted to arise frequently over whether or not to mate – males and females are likely to have different levels of mating incentive due to anisogamy and differential investment in parental care. However, due to the difficulty in measuring costs and benefits of particular traits in the two sexes, it was initially difficult to determine how important or common sexual conflict might be in shaping mating systems, relative to natural and sexual selection (Parker 1979). However, in some species, male-derived costs do have measurable effects on female longevity and fecundity, as Chapman et al. (1995) demonstrated in *D. melanogaster*. Examination of sexual conflict in *D. melanogaster* is helped by the genetic tools available to probe these traits. Further theory and empirical work has sought to understand the influence of male-derived costs on antagonistic coevolution between the sexes, and whether sexual conflict can explain phenomena that sexual selection alone cannot.

1.5.1. Current evidence for sexual conflict in *D. melanogaster*

Evidence of intra-locus conflict between the sexes in *D. melanogaster* comes from genetic experiments. Chippendale et al. 2001 found a positive genetic correlation between the sexes for juvenile survival, but a strong negative correlation between the reproductive successes of adult males and females, suggesting the existence of intra-locus conflict between the sexes during adulthood. A separate study of *D. melanogaster* suggested the existence of inter-locus conflict over a male trait conferring sperm defence ability in males and survival costs in females (Civetta and Clark 2000b). Females housed with males made homozygous for chromosomes from natural populations were found to vary in the extent to which they suffered early mortality. This variation was correlated with variation in sperm defence ability of these males (Civetta and Clark 2000b), suggesting the presence of a male-derived factor conferring costs to females and sperm competitive benefits for males.

The link between sperm competitive ability in males and the cost of mating suffered by females has also been explored using artificial selection experiments. Holland and Rice (1999) tested the association between the intensity of post-copulatory competition between males and the cost of mating suffered by females. Populations were maintained under either promiscuous conditions, where males were forced to compete with other males for mates and fertilisations, or under monogamous conditions. Males kept under promiscuous conditions became more costly to females than those under monogamous conditions. This supported the idea that post-copulatory competition between males promoted selection (directly or indirectly) for the male-derived cost of mating suffered by females. In addition, females kept under monogamous conditions were more sensitive to male-derived costs than females kept under promiscuous conditions. This suggested that exposure to costly males promoted selection on females to mitigate the male-derived cost. However, as is noted in a recent review of the field (Chapman et al. 2003), inbreeding could confound the outcomes predicted by sexual conflict theory. Females of the monogamous lines could be subject to greater inbreeding than promiscuous females, and inbreeding would predict lower survival and fecundity. An improved selection regime would retain male and female-biased sex ratios, but equalise the effective population size of the monogamous and polygamous lines (Chapman et al. 2003).

In a separate artificial selection experiment, Rice (1996) investigated the idea that sexual conflict promoted rapid coevolution between the sexes. For 41 generations, males were allowed to compete for females that, each generation, were derived afresh from a static population. Males possessed a chromosome translocation, which meant that, each generation, females contributed little genetic material to the evolving male line (see comment by Chapman and Partridge 1996b). Female evolution was therefore arrested, whereas the males were allowed to adapt to the static female phenotype. Males kept under these conditions evolved a more costly ejaculate compared to males that had co-evolved with females (Rice 1996). This supported the idea that in *D. melanogaster*, a costly male trait normally coevolves with a female resistance trait. Although the results of these tests were consistent with predictions of conflict, questions remain over the suitability of the control males used. In order to produce control males with the same genetic background as those kept with static females, the chromosome translocation was

crossed into the control male stock during the last generation of selection. This abrupt change of genetic background may have affected male viability and could explain the weaker post-mating responses induced by control males compared to those from the experimental line.

Different types of selection can leave distinctive patterns on genetic sequence variation, and some effort has been made to identify sequence variation that could provide a 'hallmark' for sexual conflict. However, the influences of sexual conflict and sexual selection on sequence variation are hard to dissect. Firstly, both sexually antagonistic selection and sexual selection are expected to promote rapid evolution of the loci involved. For example, in *D. melanogaster*, some Acp gene loci have high rates of evolution compared to non-reproductive tract genes (Civetta and Singh 1995; Swanson et al. 2001a), and positive selection has been found to act on the genes *Acp26Aa* (Aguade et al. 1992; Aguade 1998; Tsaur et al. 1998), *Acp70A* (Cirera and Aguade 1997) *Acp29AB* (Aguade 1999) and *Acp36DE* (Begun et al. 2000). Rapid evolution may exist because these loci are subject to sexually antagonistic coevolution with female sex-limited loci, but could also result from selection due to sperm competition. Secondly, genetic polymorphism may be maintained by both sexual conflict and sexual selection. The maintenance of genetic polymorphism at some Acp loci in *D. melanogaster* (Coulthart and Singh 1988; Clark et al. 1995; Prout and Clark 1996) is thought to be consistent with selection resulting from sexual conflict. Hughes (1997) suggested that genetic variation in sperm competitive ability can be maintained by antagonistic selection acting on Acps, with pleiotrophic effects on female life history traits (Hughes 1997). Again however, such polymorphism could also be maintained by sexual selection if sperm precedence is affected by the combination of genotypes of the males competing (Prout and Bundgaard 1977; Clark et al. 2000). Thirdly, another prediction of sexually antagonistic selection is rapid coevolution between male and female sex-limited loci. Only one study has examined the rates of sequence divergence among female sex-limited genes in *Drosophila* species. *D. melanogaster-D. virilis* comparisons revealed higher rates of divergence for unidentified ovary proteins than for non-gonadal proteins (Civetta and Singh 1995). Again, such coevolution could also result from sexual selection, if there are female post-copulatory mechanisms of choice (although no evidence has yet been found for such mechanisms).

The influences of sexual selection and sexual conflict on the evolution of Acps and female reproductive traits therefore remain difficult to separate. In order to determine whether conflict might play a selective role in the evolution of particular traits, it is essential to have good understanding of the costs and benefits associated with these traits. In *D. melanogaster*, investigation of the Acp responsible for the cost of mating will help determine why and how males cause females to suffer a cost of mating, and may facilitate the identification of female traits involved in sexual conflict.

1.5.2. Acp62F and the cost of mating

Although the Acp responsible for the cost of mating has not yet been identified, a good candidate is the accessory gland protein Acp62F. Female adults repeatedly exposed to ectopic expression of Acp62F suffered survival costs, whereas ectopic expression of eight other Acp proteins had no such effect (Lung et al. 2002). Acp62F is passed to females during mating and 10% of this protein enters the haemolymph (Lung and Wolfner 1999). The rest of the protein remains in the female reproductive tract and enters the sperm storage organs, although its function there is not yet known. The correlation found between sperm competitive ability of males and the degree to which these males harm females (Civetta and Clark 2000b) suggests that the costly factor also confers sperm competitive benefits in males. Acp62F encodes a protein predicted to have protease inhibitory activity (Lung et al. 2002) and it may therefore protect other Acps or sperm from proteolytic degradation. Acp62F may also regulate post-translational modification of other Acps (Lung et al. 2002). This predicted trypsin inhibitory activity could also explain its toxic effects upon ectopic expression in the haemolymph of females (Lung et al. 2002).

Identifying the male factor responsible for the cost of mating is of paramount importance. Its predicted biochemical function will help us to understand how males cause females to suffer a cost of mating and it may also facilitate the search for its receptor or receptors in the female reproductive tract or nervous system. For example, the yeast two-hybrid system is an efficient way of testing whether a protein coded by a known gene (e.g. the as yet unidentified costly protein) interacts with one of a set of

candidate genes (its putative receptor). Once these genes are identified, it will be possible to examine rates of evolution of both Acp and receptor genes. The costs and benefits associated with these traits could indicate whether sexually antagonistic selection or sexual selection is at play. However, the function of Acp62F has not yet been determined and requires males that lack Acp62F. Using matings with males that lack full expression of Acp62F due to RNA interference (RNAi), Chapter 6 examines the effect of the protein on female survival and fecundity.

1.6. Outline of Thesis

The work in this thesis was funded by the Biotechnology and Biological Sciences Research Council and was performed under the supervision of Linda Partridge and Tracey Chapman. The execution of all experiments was by the author, except where stated here. In Chapter 4, the equation for the two compartment model, and programs for fitting the model to the data, were written using the programming software Matlab (version 6.0.0.88; Release 12), by J. Andrew Bangham (University of East Anglia). All statistical analyses of parameters resulting from the model were carried out by the author. In Chapter 5, males carrying constructs that induced RNA interference (RNAi) of SP (*UAS-SP-IR1, UAS-SP-IR2* and *UAS-SP-IR3*) were created by Beth Seifried (University College London). The Western blot shown in Fig 5.2 was carried out by Giovanna Vinti (University College London). The line carrying the *Gal4* driver, used for RNAi in Chapters 5 and 6 (*Acp26Aa-P-Gal4*), was created by Oliver Lung and Mariana Wolfner (Cornell University). In Chapter 6, males carrying constructs that induced RNAi of Acp62F (*UAS-Acp62F-IR1, UAS-Acp62F-IR3A* and *UAS-Acp62F-IR3C*) were created by Oliver Lung and Mariana Wolfner (Cornell University). The Western blots shown in Fig. 6.2 and 6.3 were carried out by Jacob Mueller (Cornell University).

Chapter 2 describes the General Materials and Methods used in this thesis.

Chapter 3 addresses the relationship between the pre- and post-copulatory success of males and traits such as body size, accessory gland size and testis size. The work of this chapter contributes to our understanding of the consequences of selection on the pre- and post-copulatory success of male *D. melanogaster*. The work described in Chapter 3 has been published in the journal *Animal Behaviour* (2002, Vol. 64, pp 915-921) with co-authors T. Chapman and L. Partridge (see Appendix I).

Females as well as males may influence the outcome of sperm competition, and may do so through the anatomy of their reproductive tracts. Chapter 4 describes an experiment to test whether female reproductive morphology is correlated with the outcome of sperm competition. The results of this experiment were analysed using traditional

measures of sperm competition as well as by a new method that took into account the temporal patterns of offspring production. Analysis of temporal patterns of offspring production may prove useful during future tests, not least during studies of the effects of specific seminal fluid proteins on sperm competition. The work described in Chapter 4 has been published in the journal *Proceedings of the Royal Society, Series B, Biological Sciences* (2003, Vol. 270, 523-530) with co-authors T. Chapman, H.K. Smith and L. Partridge (see Appendix II).

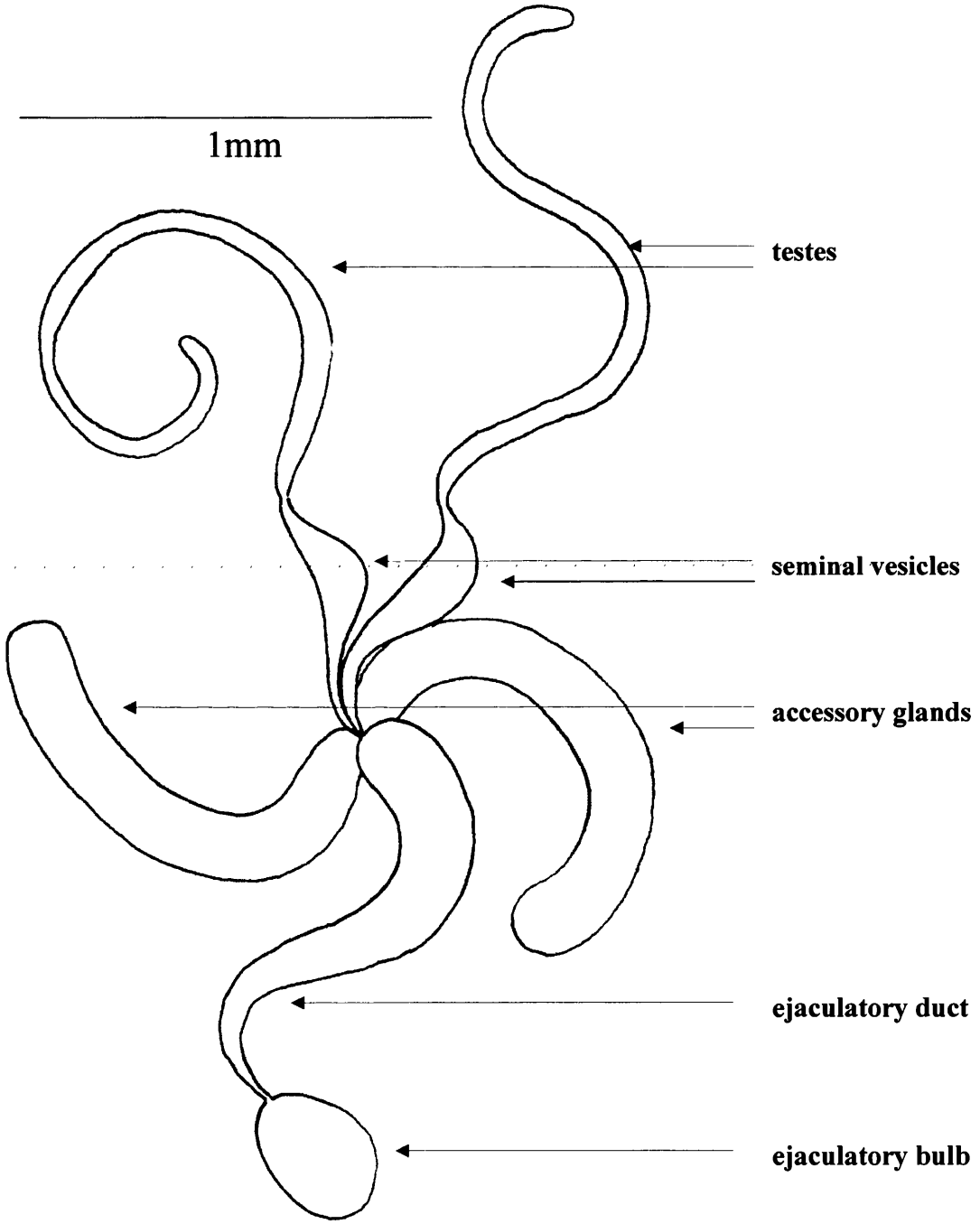
I also examined some of the physiological effects of Acps on females. A new and powerful method of targeted gene suppression, RNAi, was used to investigate the functions of two accessory gland proteins: the sex peptide (SP) and Acp62F. SP has been shown, when injected and when expressed ectopically, to alter female post-mating receptivity, oviposition and ovulation. Chapter 5 describes how males that lack SP were used to examine its *in vivo* effects on females. This work has been accepted for publication by the journal *Proceedings of the National Academy of Sciences, USA* with co-authors T. Chapman, G. Vinti, B. Seifried, O. Lung, M. F. Wolfner, H. K. Smith and L. Partridge (see Appendix III).

Chapter 6 investigates the function of Acp62F, a protein that previous studies have suggested may contribute to the cost of mating suffered by females. It is so far unknown which of the estimated eighty Acps are responsible for a cost of mating suffered by females, although identifying this protein will facilitate the search for its receptor or receptors in the female reproductive tract or nervous system

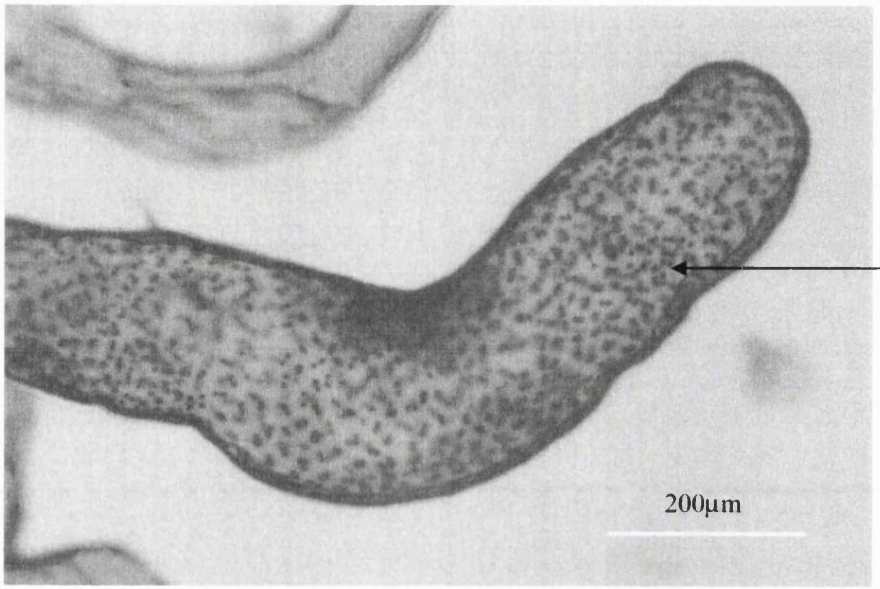
During the General Discussion (Chapter 7), I discuss the wider implications of my findings. In particular, I discuss future work that might be undertaken to increase our understanding of the role of sexual conflict in the evolution of the *D. melanogaster* mating system. These ideas have also been discussed in a recent review by first author T. Chapman and co-authors G. Arnqvist, J. Bangham and L. Rowe, entitled 'Sexual Conflict,' published in *Trends in Ecology and Evolution* (2003, Vol. 18, pp 41-47).

Figure 1.1. (a) The male reproductive tract showing testes, seminal vesicles, accessory glands, ejaculatory duct and ejaculatory bulb. Nuclear lacZ staining highlights the binucleate **(b)** secretory main-cells and **(c)** the secondary cells of the accessory glands.

(a)



(b)



(c)

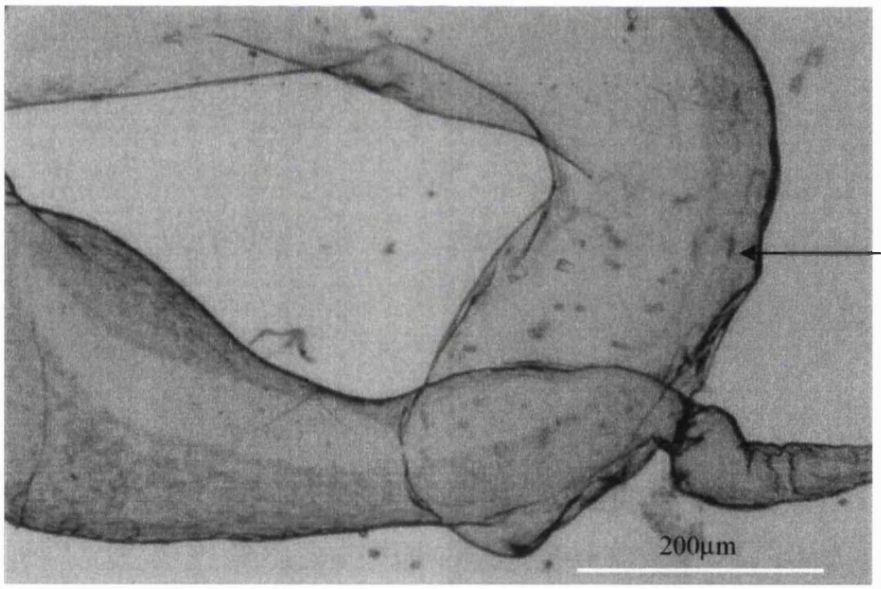
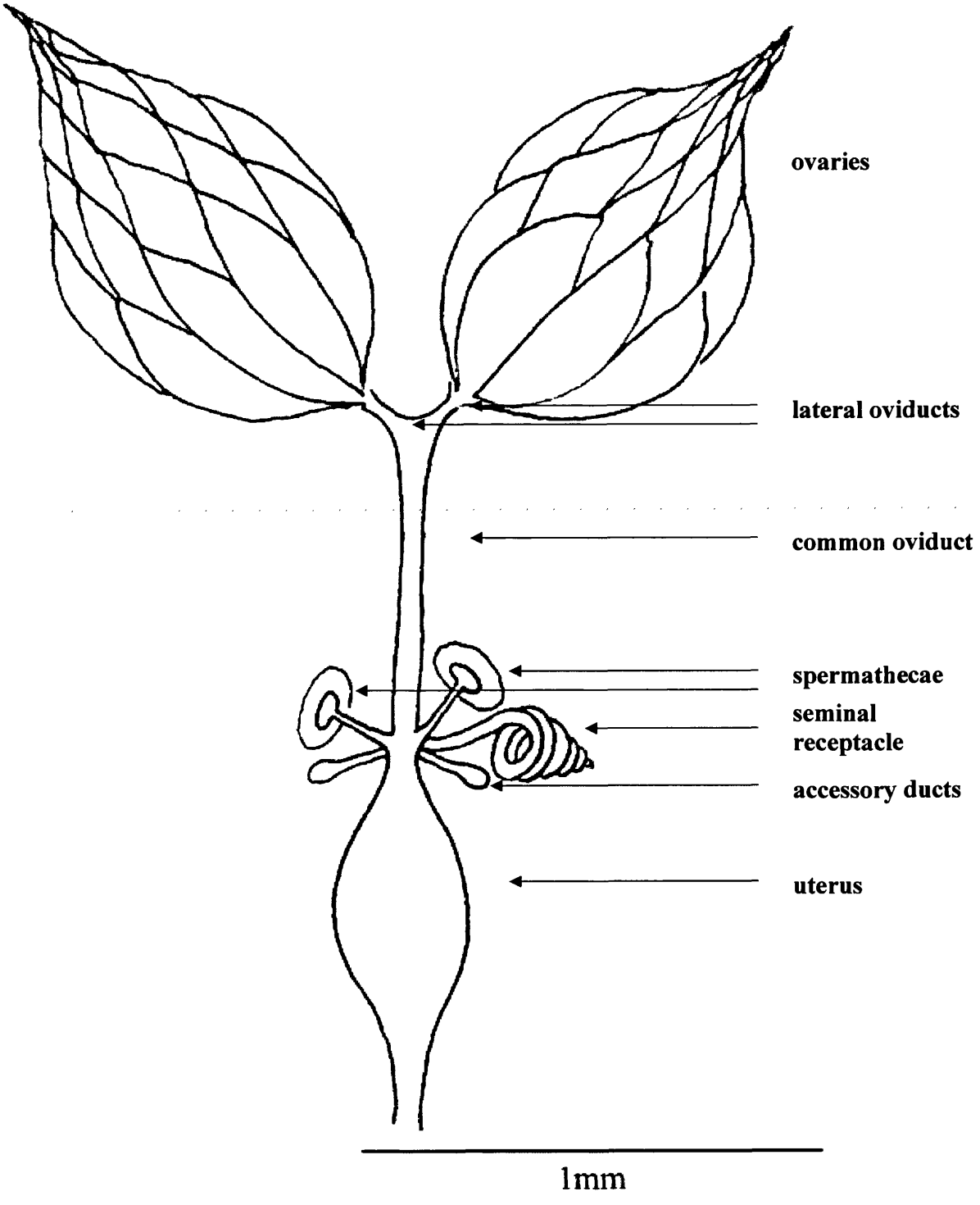
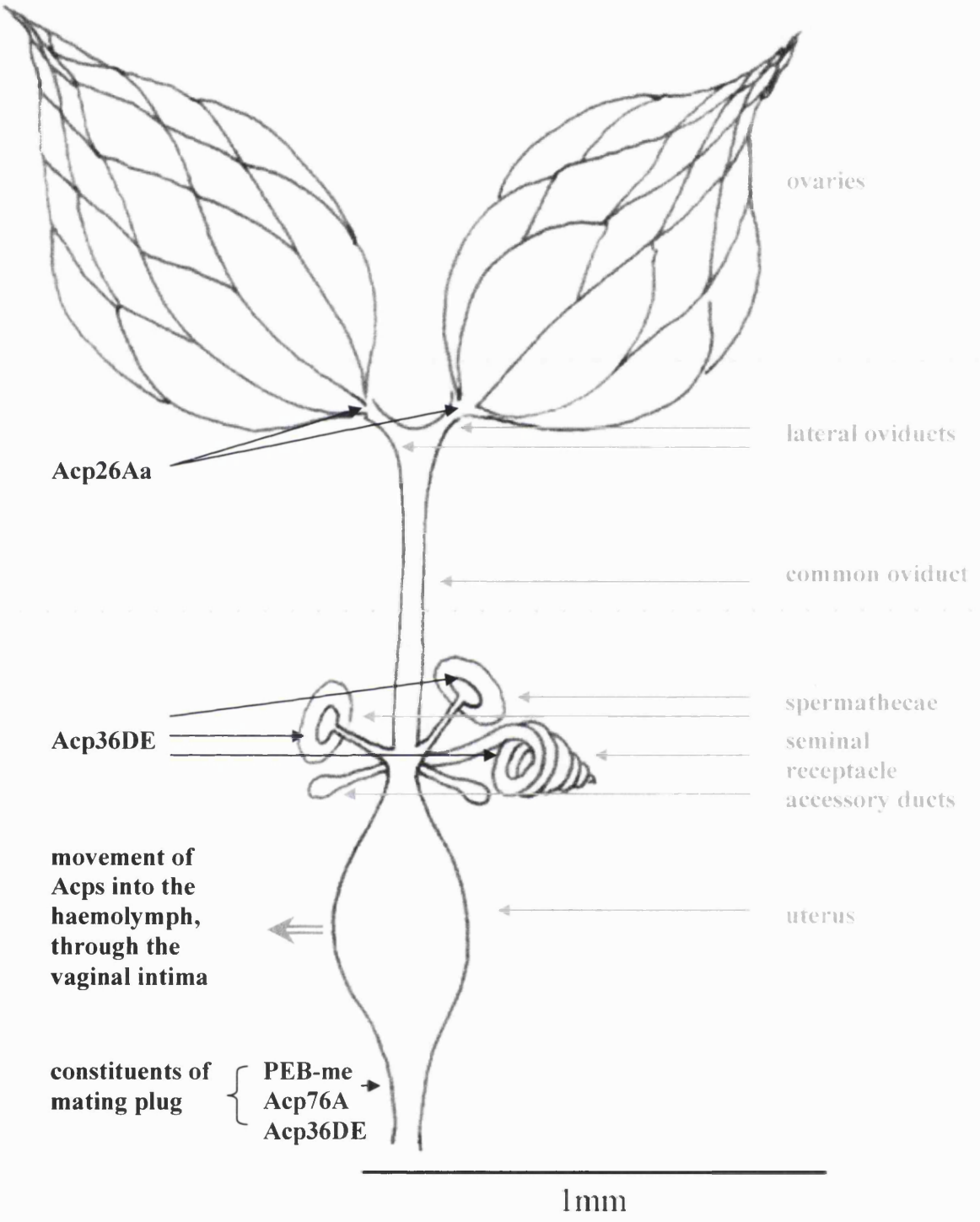


Figure 1.2. (a) The female reproductive tract of *D. melanogaster* and **(b)** post-mating localisation within the female reproductive tract of some male-derived accessory gland proteins.

(a)



(b)


ovaries
lateral oviducts
Acp26Aa
common oviduct
spermathecae
Acp36DE
seminal
receptacle
accessory ducts
movement of
Acps into the
haemolymph,
through the
vaginal intima
uterus
constituents of
mating plug
PEB-me
Acp76A
Acp36DE
1mm

Chapter 2. General Materials and Methods

2.1. Stocks and Cultures

Laboratory stocks of *D. melanogaster* were kept in cage, vial or bottle culture, with one of two types of food, Sugar-Yeast medium or Maize Yeast medium (see below). During the experiments described in this thesis, two types of wild-type stocks were used (French and Dahomey, see below) and two mutant stocks (bw^D and *scarlet*, see below).

2.1.1. Population cage culture

Population cages, dimensions 45x25x25cm, were kept at 25°C and on a 12:12 light: dark regime. Each cage contained 12 bottles of medium. Each week the cages were supplied with three 158ml litre glass bottles containing 70ml food medium, and the three oldest bottles were removed. Bottles were left in the cages for a total of 4 weeks.

2.1.2. Bottle and vial cultures

All experiments were carried out using 23mm by 73mm glass shell vials that contained 7ml food medium. Vials were also frequently used to keep stocks of *D. melanogaster*, as were 158ml litre glass bottles containing 70ml food.

2.1.3. Sugar-Yeast medium

1000ml of water was brought to boiling point, and 100g yeast, 27g agar and 100g sugar was added. The ingredients were left to simmer for several minutes, before the medium was removed from the heat and left to cool. When temperature reached 60°C, 30ml of 10% nipagin in ethanol and 3ml propionic acid was added (both are antifungal agents), and the medium was immediately dispensed.

2.1.4. Maize-Yeast medium

1000ml of water was brought to boiling point, and 20g yeast, 10g agar, 85g sugar and 60g maize was added. The medium was left to simmer for several minutes, before being

removed from the heat and left to cool. When the temperature reached 60°C, 25ml of 10% nipagin in ethanol was added, and the medium was immediately dispensed.

2.1.5. Grape-juice medium

1000ml water was boiled and 50g of agar added. This was left to simmer for approximately five minutes, before the heat was removed and 600ml grape-juice concentrate was added with 100ml of extra cold water, and mixed thoroughly. The mixture was left to cool to 60°C and 42.5ml of 10% nipagin in ethanol was added just before the medium was dispensed.

2.1.6. Dahomey wild-type *D. melanogaster*

Dahomey wild-type *D. melanogaster* flies (used in experiments described in Chapters 5 and 6) were derived from a stock collected in Dahomey (now Benin, West Africa) in 1970. These were subsequently kept in the laboratory in population cages containing Sugar-Yeast medium.

2.1.7. French wild-type *D. melanogaster*

French wild-type *D. melanogaster* (used in experiments described in Chapters 3 and 4) were derived from a collection made at Mas Canet (near Montpellier) in France in 1999. These were subsequently kept in the laboratory in population cages containing Sugar-Yeast medium.

2.1.8. bw^D stocks

Brown dominant (bw^D) D. melanogaster are eye colour mutants that possess a dominant allele that confers dark red eye colour without pseudopupils, in contrast to the wild-type bright red eye colour with pseudopupils. bw^D flies were obtained from the Umea *Drosophila* Stock Centre (stock number 40650) in February 2000, and were cultured in 158ml glass bottles containing 70ml Maize-Yeast medium.

2.1.9. *scarlet* stocks

Homozygous *scarlet* flies were derived from a stock (obtained from Alan Robertson in 1975) that had been backcrossed into a wild-type Dahomey background and subsequently maintained in 158 ml bottles containing 70 ml Sugar-Yeast medium. *D. melanogaster* possessing the scarlet allele have brighter red eye colour than wild-type flies. The *scarlet* recessive mutation occurs spontaneously in wild populations (tenHave et al. 1995) and appears to have a negligible effect on fitness.

2.2. General Methods

2.2.1. Egg collection from population cages

To collect eggs from population cages, Petri-dishes containing grape-juice medium and a small amount of autolysed yeast paste were placed in population cages containing adult flies, for a maximum of 6 hours.

2.2.2. Egg collection from laying pots

Egg collections from adult flies maintained in bottle or vial culture were carried out by placing adults in laying pots (approximately 20mm diameter x 70mm) that contained grape-juice medium in the bottom, and a small quantity of autolysed yeast paste. Flies were left to lay eggs in the laying pots for a maximum of 6 hours.

2.2.3. Standard density culture

Where a standard, uncrowded larval density was required, eggs were collected from population cages or laying pots (sections 2.2.1 and 2.2.2) and then kept for 24 hours at 25°C. The resulting larvae were placed, using metal pins, into vials containing 7ml of the appropriate food medium at a standard density of 50 larvae per vial.

2.2.4. Mating observations

Mating observations were carried out at 25°C by placing vials containing flies on shelves able to carry up to 480 vials. In *D. melanogaster*, mating typically takes between 15 and 20 minutes. In laboratory populations of *D. melanogaster*, mating

frequency tended to be highest between about 30 minutes before dawn (10am) and midday.

2.2.5. Egg counts

Egg counts were carried out using a dissection microscope at a magnification of approximately x25. If estimates of egg fertility were not required, vials containing eggs were placed in a -80°C freezer for preservation to await egg counts. Freezing in this manner also improved the visibility of eggs. Egg visibility could also be enhanced by making the food black using charcoal-food medium. Charcoal-food was made by adding 4g powdered charcoal per 1000ml of food medium just before dispensing.

2.2.6. Morphological measurements of accessory gland, testis and body size.

Measurements of the testes and the accessory glands of males were made by dissecting individuals in phosphate-buffer solution (PBS: 75.97g NaCl, 12.4g Na_2HPO_4, 4.8g NaH_2PO_4, dissolved in 800ml H_2O, pH adjusted to 7, volume brought to 1 litre and autoclaved) on a glass slide under a dissection microscope. Due to the fragility of the samples, coverslips were not used. Samples were placed in 250µl PBS, which was sufficient to ensure they were free floating. It was occasionally necessary to add additional PBS due to excessive evaporation during measurement. Images of the accessory glands and testes were captured from a compound microscope (magnification: x100) using a video camera connected to a Macintosh computer. The accessory gland (*A*) and testis (*T*) perimeters of each male (Fig. 3.1) were measured using a polygon tool in the NIH Object Image program, version 1.62n3 (by Norbert Vischer, based on the public domain NIH Image and available at http://simon.bio.uva.nl/object-image.html). Where possible, each of the pair of accessory glands and testes were measured and the mean of the two measurements calculated. Otherwise, only a single measurement was made.

Body size was estimated from the wing perimeter of an individual (e.g. see Gilchrist et al. 2000). The right wing of each male was removed and mounted on a slide using Aquamount (BDH). Images of wings were captured from the compound microscope (magnification: x100) in the same way as for the accessory glands and testes. Wing

perimeter (*W*) was measured (Fig. 3.1) as described in Gilchrist and Partridge (1999), using the NIH Object Image program.

2.2.7. Protein extraction for Western blots

Groups of 5 males were transferred, using ice as an anaesthetic, into chilled Eppendorf tubes. 40µl of homogenisation buffer (25mM Tris-HCl, pH 7.5, 5mM EDTA, pH 8) was added to each tube and the flies were partially homogenised. An equal volume of 2xSDS sample buffer (62.5mM Tris-HCl, pH 6.8, 10% Glycerol, 2% SDS, 0.0005% Bromophenol Blue, adding 1/10 of the volume of β-mercaptoethanol to aliquots of the buffer before use) was then added, and the sample was homogenised. Samples were then boiled for 4 minutes, transferred onto ice for 2 minutes, centrifuged at 10k rpm for 5 minutes at 4 °C and snap-frozen in liquid nitrogen.

2.2.8. Statistical Analysis

Statistical analyses were performed using JMP 4.0.5 for the PC and Microsoft Excel 97-2000 for the PC.

Chapter 3: Relationship between body size, accessory gland and testis size and pre- and post-copulatory success in *Drosophila melanogaster*

3.1. Abstract

The reproductive success of males is affected by events that occur both before and after mating. *D. melanogaster* males vary in mating success and post-mating fertilisation success, and this chapter examines the associations between these and body size, accessory gland and testis size. Males with larger accessory glands were found to mate at higher frequency than males with smaller accessory glands, and this association was over and above allometry of accessory gland size with body size. As accessory gland proteins influence the post-copulatory success of males, it could be that males with larger accessory glands have greater resources for mating frequently than those with smaller accessory glands. In addition, larger males were found to have higher post-copulatory success than smaller males, which may be because they pass seminal fluid proteins to females faster than small males. Taken together with previous literature, these findings indicate that larger males have both higher pre-copulatory and post-copulatory success than smaller males.

3.2. Introduction

Since Darwin's discussion of sexual selection in 1871, there has been considerable interest in the morphological traits and behaviours that influence the mating success of males. In some species of insect, males compete with one another physically or through agonistic displays in order to obtain access to females. Males may also obtain higher reproductive success by attracting or coercing females to mate. Male body size is a trait that is correlated with the outcome of pre-copulatory competition between males in a number of species (Otronen 1984a; Crespi 1986, 1988; Alcock 1996). In some insects, large males are better able to attract, stimulate or coerce potential mates than smaller males (Davidson 1982; Hughes and Hughes 1985; Simmons 1986, 1988). In contrast, smaller males are favoured in species where agility is required during scramble competition for mates (Goldsmith and Alcock 1993; Vencl and Carlson 1998), during courtship (Steele and Partridge 1988) and during flight in male-male competition (Marshall 1988).

Mating success alone is not enough to ensure that a male sires a large number of offspring. The reproductive success of males is affected by both pre- and post-copulatory events. Females of most insect species mate multiply and store sperm in storage organs inside the reproductive tract. Where this occurs, scope for male-male competition is extended beyond the point of copulation. Parker's work on sperm competition in the 1970s stimulated extensive exploration of the mechanisms of post-copulatory competition among males (Parker 1970). It also led to widespread reinterpretation of the selection pressures on a number of traits.

In several species of insect, correlations exist between male post-copulatory success and traits such as body size (McLain 1985; Lewis and Austad 1990; Otronen 1994; Bissoondath and Wiklund 1997; Wedell and Cook 1998) and genital morphology (Arnqvist and Danielsson 1999a; Danielsson and Askenmo 1999). Comparative studies show that testis size is associated with male post-copulatory success in a wide range of animals (Harcourt et al. 1981; Harvey and Harcourt 1984; Gage 1994; Pitnick and Markow 1994), and in many species of Diptera, post-copulatory success is strongly influenced by proteins produced in the male accessory glands (Nelson et al. 1969;

Reimann and Thorson 1969; Chen and Bühler 1970; Fuchs and Hiss 1970; Baumann 1974; Bird et al. 1991; Wolfner 1997; Cordero 1998; Klowden 1999; Chapman 2001).

The relationships between pre- and post-copulatory success and traits such as body size will indicate whether that trait is subject to similar selection pressures at different stages of the reproductive process. For example, body size may be positively associated with both the mating and the fertilisation success of males. Large males of the stinkbug *N. viridula* both obtain more mates and have better per-mating fertilisation success than smaller males (McLain 1985, 1991). Large males of the fly *D. anilis* are more successful during take-over contests for mates than small males (Otronen 1984a, b), and in doubly mated females, large second males achieve higher fertilisation success than small males (Otronen 1994). Females of the arctiid moth *U. ornatrix* favour larger males over smaller males for mating and, of two males that mate with a single female, the larger male usually sires all of the resultant offspring (Conner et al. 1981; LaMunyon and Eisner 1993).

In contrast, body size may be positively associated with mating success and negatively associated with post-mating success. In the water strider *Gerris lacustris*, large males mate at higher frequency than small males, but small males mate for longer and sire a greater number of offspring per mating, leading to a similar overall reproductive success in males of all sizes (Danielsson 2001). In the yellow dung fly *S. stercoraria*, large males achieve a higher fertilisation success per unit time copulation because they pass ejaculate to their mates faster than do small males (Simmons et al. 1996). Small male *S. stercoraria* tend to copulate for longer and, overall, gain the same fertilisation success as large males (Simmons and Parker 1992; Parker and Simmons 1994; Simmons et al. 1996).

The correlations between mating frequency and body size are often easily explained by an advantage of large body size in male-male competition, or the ability of larger males to better stimulate or coerce potential mates than small males. However, the mechanisms behind associations between post-copulatory success and body size are sometimes less clear, partly because post-copulatory events occur inside the female reproductive tract. One possibility is that larger males possess larger accessory glands

and testes and produce larger quantities of Acps and sperm than smaller males. This could translate into a positive correlation between post-copulatory success and body size if males producing larger quantities of Acps and sperm were to elicit greater post-mating responses in females.

In *D. melanogaster* there is variation between males in their pre- and post-copulatory success. Pre-copulatory success is positively correlated with male size in a number of *Drosophila* species, including *D. hydei* (Markow 1985), *D. nigrospiracula* (Markow 1988), *D. buzzatii* (Santos et al. 1988) and *D. melanogaster* (Partridge and Farquhar 1983; Partridge et al. 1987a, b; Markow 1988). Post-copulatory competition is an important factor in male reproductive success. In *D. melanogaster*; females mate multiply (Harshman and Clark 1998; Imhof et al. 1998), can store sperm for at least 14 days after mating (Kaufman and Demerec 1942; Patterson 1954; Neubaum and Wolfner 1999) and store the sperm of more than one male simultaneously (Milkman and Zeitler 1974; Marks et al. 1988). Therefore, as well as mating at high frequency, it is important for a male to be able to displace the sperm from a previous male from the storage organs of his mate and to prevent his own sperm from being displaced during the female's subsequent copulations.

In many insects, the quantity of sperm passed to females during mating influences male post-copulatory success (e.g. Parker et al. 1990; Arnqvist and Danielsson 1999b; section 1.3.1). However, in *D. melanogaster*, the number of sperm delivered to females is not the only factor to affect male post-copulatory success. Males made temporarily sterile through serial matings still possessed mature sperm in their seminal vesicles, but had deflated accessory glands (Lefevre and Jonsson 1962; Hihara 1981). Lefevre and Jonsson (1962) postulated that sterility resulting from these successive matings was a consequence not of lack of sperm, but from a deficiency of accessory gland proteins. The mechanisms by which males displace sperm and defend their own stored sperm are unknown but strongly influenced by accessory gland proteins (Acps) (Harshman and Prout 1994; Gilchrist and Partridge 1995). Acps affect sperm transfer, sperm storage and sperm displacement, as well as inducing a number of female post-mating responses: a reduction in sexually receptivity and attractiveness to males and an elevation of oogenesis and oviposition rate. Males that produce reduced quantities of Acps have

greatly reduced reproductive success (Kalb et al. 1993). Females mated to males with progressively more depleted accessory glands due to serial matings, exhibit progressively less intense post-mating reactions (Hihara 1981). It is therefore possible that the quantities or effectiveness of accessory gland proteins produced by males affects the magnitude of the female post-mating responses.

Although the details of post-copulatory events are probably better known in *D. melanogaster* than in any other insect, the relationships between pre- and post-copulatory success and traits such as body size have not been investigated. I explored the associations between body size and pre- and post-copulatory success in males. In addition, I considered whether such associations are explained by allometric relationships between body size and testis or accessory gland size. In this chapter, I will describe two separate experiments. The first examined the relationship between mating frequency, body size, accessory gland and testis size. The second investigated the association between post-copulatory success, body size and accessory gland size.

3.3. Methods

3.3.1. Male mating frequency, body size, accessory gland size and testis size

French wild-type flies (section 2.1.7) were derived from population cages and cultured at standard density on Sugar-Yeast medium. Males and females emerging from standard density cultures were collected within eight hours of eclosion, using ice as an anaesthetic, and kept in single-sex groups of 20 in vials containing Sugar-Yeast medium with approximately 1.5mg live yeast, which was subsequently throughout the experiment.

Male mating frequency was recorded by observing single males in vials together with five females during three observation periods over 3 separate days. Approximately 100 8-day-old wild-type males were placed singly in vials with 5 wild-type females per vial. CO_2 anaesthetic was used throughout except where stated. *D. melanogaster* are more active at 27°C than at 25°C. Therefore, during observation periods, flies were transferred to 27°C to increase the number of matings observed. The number of matings observed for each male (M_{ij} where i=1 to N and N is the number of males in the cohort,

and j is the observation period index number, j=1 to 3) was recorded over 6 – 8 hours. At the end of each observation period, all individuals were returned to 25°C. Mating observations took place over two weeks. At the end of each mating period, females were removed from the vials and pooled into groups of 20 to 40 per vial. Before the next mating observation period, females were again immobilised and redistributed amongst males in groups of 5. This minimised the risk that males particularly effective at repressing receptivity in females would encounter the same females during different observation periods.

Matings were observed over three such observations periods, each on a separate day. Because observation periods were not identical in length and the intensity of mating activity varied on different days, the index of mating frequency was based on the fraction of the total numbers of matings observed during each period. The number of matings observed for each male during each mating observation period (M_{ij}) was expressed as a fraction of the total number of matings by all males during that period, j:

$$F_{i,j} = \frac{M_{i,j}}{\sum_{i=1}^{i=N} M_{i,j}}$$

The fraction $f_{i,j}$ for each male was calculated for each of the three mating periods, and the average fraction over the three mating periods was calculated for each male, resulting in an index of mating frequency referred to as:

$$f_{i,j} = \frac{1}{3}\sum_{j=1}^{j=3} F_{i,j}$$

Before obtaining measures of body size, accessory gland size and testis size, males were subsequently maintained individually in fresh vials for 10-12 days after the last mating observation period, to ensure sufficient time for full replenishment of the contents of the testes and accessory glands. Males were then immobilised using ice, and accessory gland size, testis size and body size (Fig. 3.1) were estimated using the techniques described in section 2.2.6.

3.3.2. Male sperm displacement ability, body size, accessory gland size and testis size

The relationships between male body size, accessory gland size and post-copulatory reproductive success were examined. Wild-type flies were collected as above. Eggs were collected from the *scarlet* stock (section 2.1.9) from laying pots, and larvae resulting from these eggs were cultured at a standard density on Sugar-Yeast medium. Virgin males and females were collected from these vials within 8 hours of eclosion and kept in single-sex groups of 20.

Evidence suggests that females mate multiply in the wild (Harshman and Clark 1998; Imhof et al. 1998), and therefore males are most likely to encounter non-virgin females. Thus, during estimation of male post-copulatory success, double matings were conducted so that the target male was in post-copulatory competition with the female's previous mate. 300 virgin *scarlet* females and 300 virgin *scarlet* males were anaesthetised on ice and aspirated in pairs into vials. The pairs were observed for 7 hours. 38 pairs did not mate during this time and were therefore discarded. After this first mating, which resulted in *scarlet* offspring, males were removed. Three days later, 1 virgin wild-type male was paired with each female, the pairs were again observed for about 7 hours. During this second mating, copulation duration was measured. 93 pairs did not remate and were discarded. After this second mating, which resulted in offspring with a wild-type phenotype, males were transferred to fresh vials and stored for 11-13 days, again to replenish the contents of the testes and accessory glands before size measurements of these organs were taken.

The twice-mated females were transferred every 2 days to fresh vials to avoid excessive larval competition due to overcrowding. This was repeated until females ceased producing fertilised eggs, between 12 and 16 days later. After accounting for female deaths, sterility and escapes, the sample size for females from which offspring were collected was 112. Vials were stored at 25°C during larval development. Progeny counts and eye colour scores were made 12 and 15 days after the adult female had been introduced into each vial. Progeny counts were carried out in two stages to avoid deaths amongst the adult progeny due to overcrowding. Offspring sired by *scarlet* males (the first males to mate) had bright red eyes, and offspring sired by wild-type males (the

second males to mate) had wild-type eyes. This provided a measure of the number of offspring produced by each female that were sired by the first male (P_F), and the number sired by the second male (P_S). I assessed post-copulatory competition between males by calculating sperm displacement ability, *SDA* (section 1.3.1.1) (Hughes 1997).

$$SDA_i = {}^{P_{Si}}\!/\!_{(P_{Fi}+1)}$$

Since the association between *SDA* and the three independent variables was not linear, the fifth root transformation of *SDA* was used (SDA_T) for the multiple regression analysis (see also Gilchrist and Partridge 1997). The number of offspring that emerged from eggs produced after the first mating but before the second mating was counted (P_B). This provided an indication of variation in first male post-copulatory success in the absence of any post-copulatory competition. Females that laid sterile eggs after the first mating were omitted from the data set. Body size, accessory gland size and testis size of the second (wild-type) males to mate were determined by dissection 11-13 days after mating. This meant that the males measured in this experiment were of comparable age to those measured in the mating frequency experiment and measurements were again carried out according to the method described in section 2.2.6. After dissection, the sample size was approximately 90. However, for some individuals, I was unable to obtain accurate measurements for one or two of the three traits measured. The sample sizes for the three measurements therefore differed slightly, and the degrees of freedom for each regression analysis are indicated in the results.

3.3.3. Data analysis

Correlations between independent variables (body size, accessory gland and testis size) were examined by calculating correlation coefficients. Where variables were normally distributed, the Product-Moment correlation coefficient, r, was used and where variables were not normally distributed, the Spearman Rank correlation coefficient, r_s, was used. Normality of variables was tested using the Shapiro-Wilk method (Shapiro and Wilk 1965). Simple linear regression was used to determine which of the independent variables significantly accounted for variation in mating frequency (f) for the first experiment and variation in paternity (SDA_T) for the second experiment. The independent variables of interest were body size (W), accessory gland size (A) and testis size (T). In the second experiment, two additional factors were considered: the number

of offspring sired by the first male before the second mating (P_B) and copulation duration of the second mating. The proportion of the variation in the dependent variable accounted for by the independent variable was tested by calculating the coefficient of determination (R^2). This describes the sum of squares explained by the independent variable, relative to the total sum of squares of the dependent variable.

$$R^2 = \text{sum of squares for model} / \text{total sum of squares}$$

To test the significance of the relationships, F-tests were used.

Regression was also used to examine whether any significant effects of accessory gland or testis size on mating frequency or sperm displacement ability were due to allometry with body size, or whether there were effects over and above body size. One assumption of regression is that the unexplained residuals resulting from the regression models are normally distributed. Normality of the residuals was tested using the Shapiro-Wilk method (Shapiro and Wilk 1965).

3.4. Results

3.4.1. Male mating frequency, body size, accessory gland size and testis size

First, simple linear regression was used to examine the relationship between variance in mating frequency (f) and variation in the separate independent variables: body size (W), accessory gland size (A) and testis size (T). The regression of f onto W (Fig. 3.2) showed that body size alone explained a marginally non-significant proportion of the variation in mating frequency (R^2=0.0425, $d.f.$=1, 78, P=0.0665). The regression of f onto A (Fig. 3.3) showed that accessory gland size explained a significant proportion of the variation in mating frequency (R^2=0.1224, $d.f.$=1, 73, P=0.0021), before taking into account the allometric relationship between accessory gland size and body size. The regression of f onto T (Fig. 3.4) showed that testis size did not explain any variation in mating frequency (R^2=0.0002, $d.f.$=1, 75, P=0.9043).

As the distributions of all three variables were normal, the Product-Moment correlation coefficient was used to examine correlations between body size, accessory gland and testis size. There was a positive allometric relationship between body size and accessory

gland perimeter (r=0.2542, $d.f.$=69, P=0.0345) and a non-significant relationship between body size and testis perimeter (r=0.2018, $d.f.$=70, P=0.0891). I wanted to examine the variation in mating frequency explained by accessory gland size and testis size, over and above variation explained body size. To do this, the unexplained residuals that resulted from regression of f onto W were, in turn, regressed onto A, and separately onto T. This showed that accessory gland size explained a significant proportion of the variation in mating frequency, over and above the variation explained by body size (Fig. 3.5; R^2=0.0865, $d.f.$=1, 69, P=0.0128). There was no such relationship between testis size and mating frequency, over and above variation explained by body size (Fig. 3.6; R^2= 0.0069, $d.f.$=1, 70, P=0.4888).

Previous studies have shown that male mating frequency is correlated with male body size (Partridge and Farquhar 1983; Partridge et al. 1987a, b). I therefore wanted to determine whether body size accounted for any significant proportion of the variation in mating frequency, over and above that explained by accessory gland size. To do this, the unexplained residuals that resulted from regression of f onto A were, in turn, regressed onto W (Fig. 3.7). This showed that body size did not account for a significant proportion of the variation in mating frequency over and above the variation accounted for by accessory gland size (R^2=0.0274, $d.f.$=1, 69, P=0.1679). All unexplained residuals resulting from these regression models were found to be normally distributed.

3.4.2. Male sperm displacement ability, body size, accessory gland size and testis size

Given that males with relatively large accessory glands tended to have a higher mating success than those with small accessory glands, I next examined the success of males during post-copulatory competition. The associations between second male sperm displacement ability (SDA_T), body size (W), accessory gland size (A) and testis size (T) were again examined using simple linear regression. SDA is a measure of second male fertilisation success relative to first male fertilisation success. As I was interested in variation in second male sperm competitive ability, I wanted to minimise variation in SDA_T that was due to variation amongst the first males to mate. Therefore, an additional factor P_B (number of offspring sired by the first male before the second mating) was

also included in this analysis. This meant it was possible to factor out potential variation in first male fertilisation success from the estimation of second male sperm displacement ability.

A negative relationship between SDA_T and P_B (Fig. 3.8) meant that P_B explained a significant proportion of the variation in SDA_T (R^2= 0.1914, *d.f.*=1, 92, P<0.0001). This was consistent with the idea that first male post-copulatory success before a second mating is related to the ability of the first male to resist second male sperm displacement. Since the proportion of variance in SDA_T explained by P_B was highly significant, I wanted to examine the effects of W, A and T on SDA_T, over and above variation explained by P_B. I therefore took the unexplained residuals resulting from regression of SDA_T onto P_B, and regressed them, separately, onto W, A and T. This showed that body size accounted for a significant proportion of the variation in sperm displacement ability (Fig. 3.9; R^2=0.0735, *d.f.*=1, 81, P=0.0132). However, it also showed that neither accessory gland size (Fig. 3.10; R^2=0.0016, *d.f.*=1, 87, P=0.7062) nor testis size accounted for any variation in sperm displacement ability (Fig. 3.11; R^2=0.0009, *d.f.*=1, 84, P=0.7865).

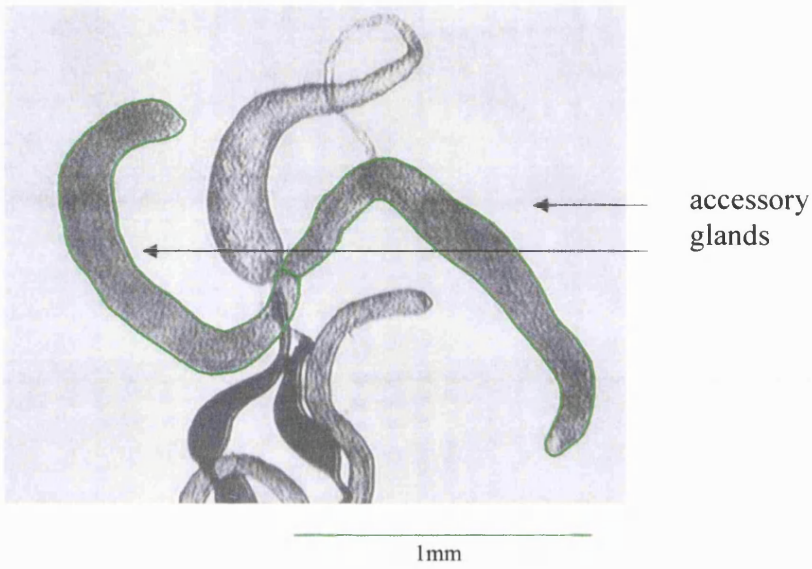
As, in this experiment, wing perimeter was not normally distributed, the Spearman Rank correlation coefficient was used to examine correlations between body size, accessory gland and testis size. There was a significant positive allometric relationship between accessory gland perimeter and body size (r_s=0.2426, *d.f.*=79, P=0.0291) and no significant relationship between testis perimeter and body size (r_s=0.1395, *d.f.*=77, P=0.2263). I tested whether any variation in SDA_T was explained by variation in accessory gland or testis size, over and above the variation in SDA_T explained by body size and P_B. To do this, the unexplained residuals that resulted from regressing SDA_T onto P_B and W were, in turn, regressed onto A, and separately onto T. This showed that neither accessory gland size (R^2=0.0001, *d.f.*=1, 79, P=0.9447) nor testis size (R^2=0.0003, *d.f.*=1,75, P=0.8736) explained any additional variance in sperm displacement ability over and above that accounted for by body size.

Finally, regression was also used to test for associations between the duration of the female's second mating, second male sperm displacement ability, and second male body

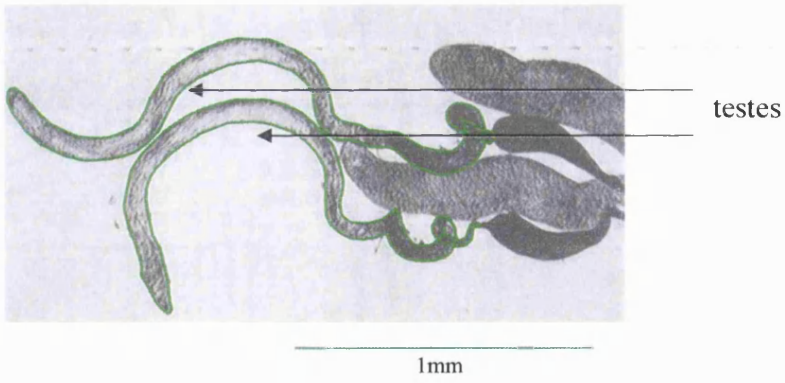
size, accessory gland size and testis size. There were no associations between copulation duration and SDA_T, body size, accessory gland, or testis size (Table 3.1).

Figure 3.1. The **(a)** accessory glands, **(b)** testes and **(c)** wings of males. Green line indicates the measurements made using NIH Object Image software (version 1.62n3).

(a)



(b)



(c)

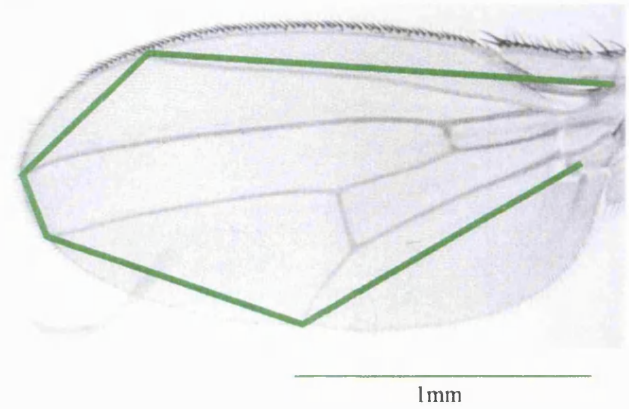


Figure 3.2. Regression of mating frequency (f) (as defined in section 3.3.1) onto wing perimeter, (W) (measured in mm). Variation in body size (represented by wing perimeter) accounted for a marginally non-significant proportion of the variation in mating frequency amongst males (R^2=0.0425, $d.f.$=1, 78, P=0.0665).

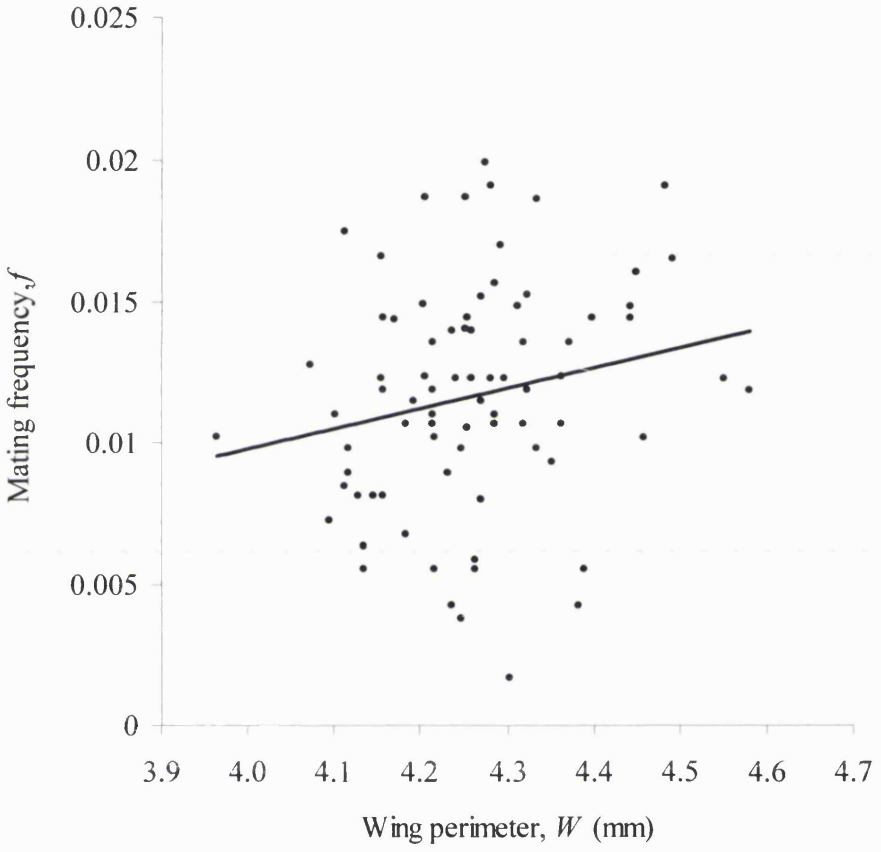


Figure 3.3. Regression of mating frequency (f) onto accessory gland perimeter (A) (measured in mm). Variation in accessory gland size accounted for a significant proportion of variation in mating frequency (R^2=0.1224, $d.f.$=1, 73, P=0.0021).

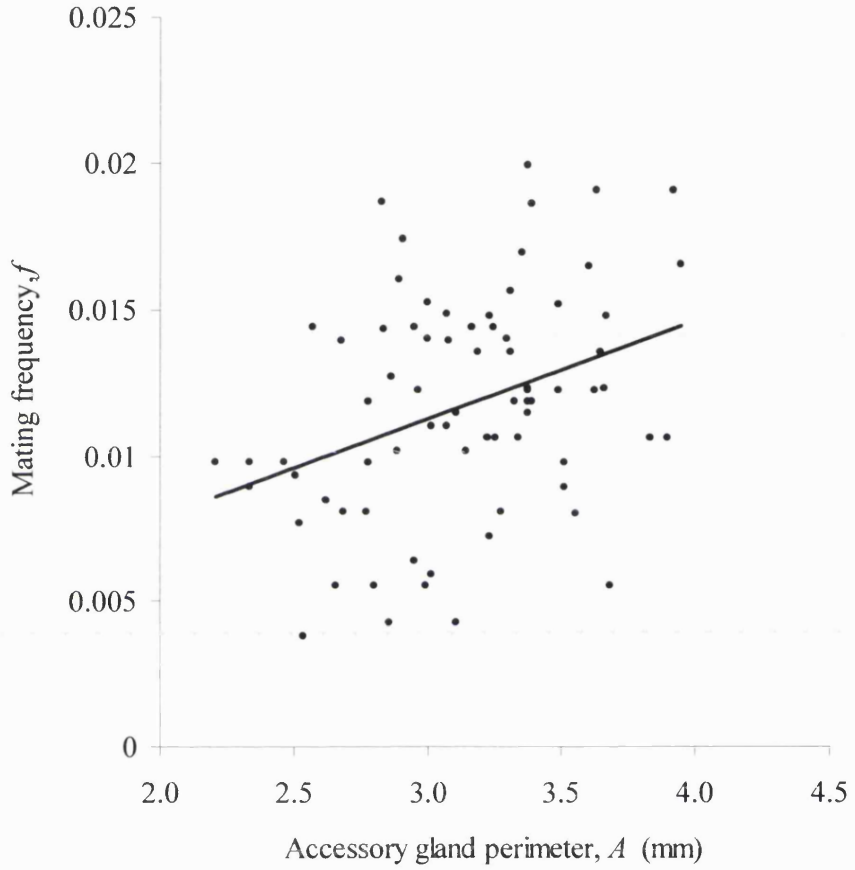


Figure 3.4. Regression of mating frequency (f) onto testis perimeter (T) (measured in mm). Variation in testis perimeter did not explain a significant proportion of variation in mating frequency (R^2=0.0002, $d.f.$=1, 75, P=0.9043).

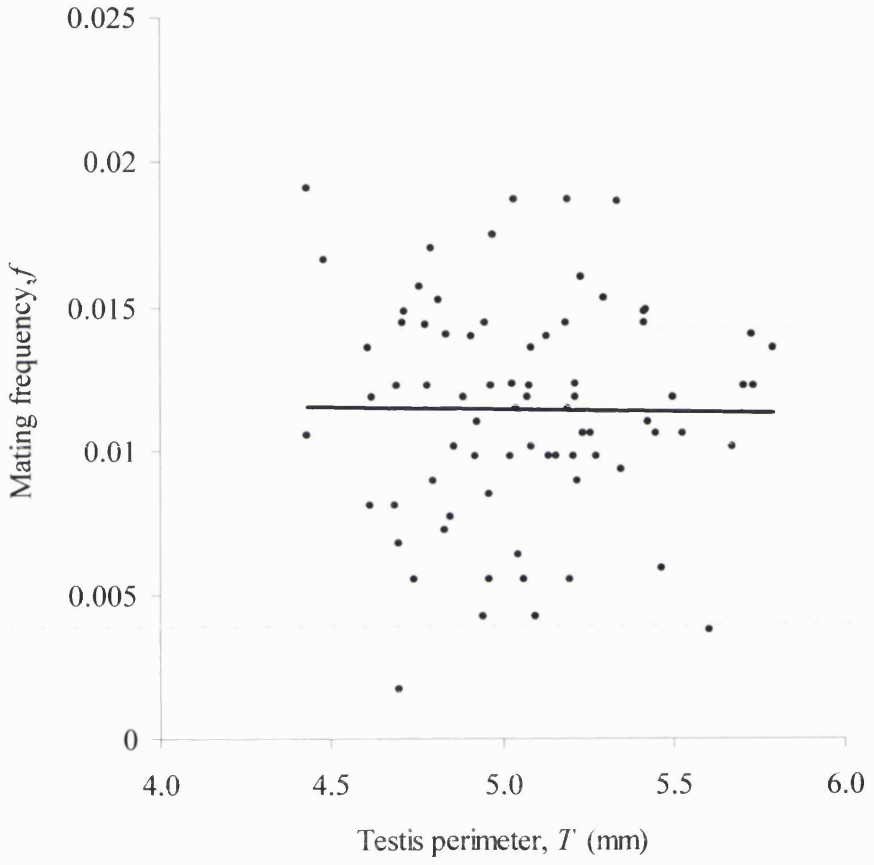


Figure 3.5. The unexplained residuals resulting from the regression of mating frequency (f) onto wing perimeter (W) (measured in mm), in turn regressed onto accessory gland perimeter (A) (measured in mm). Variation in accessory gland size accounted for a significant proportion of variation in mating frequency, over and above the allometric relationship between accessory gland size and body size. (R^2=0.0865, $d.f.$=1, 69, P=0.0128).

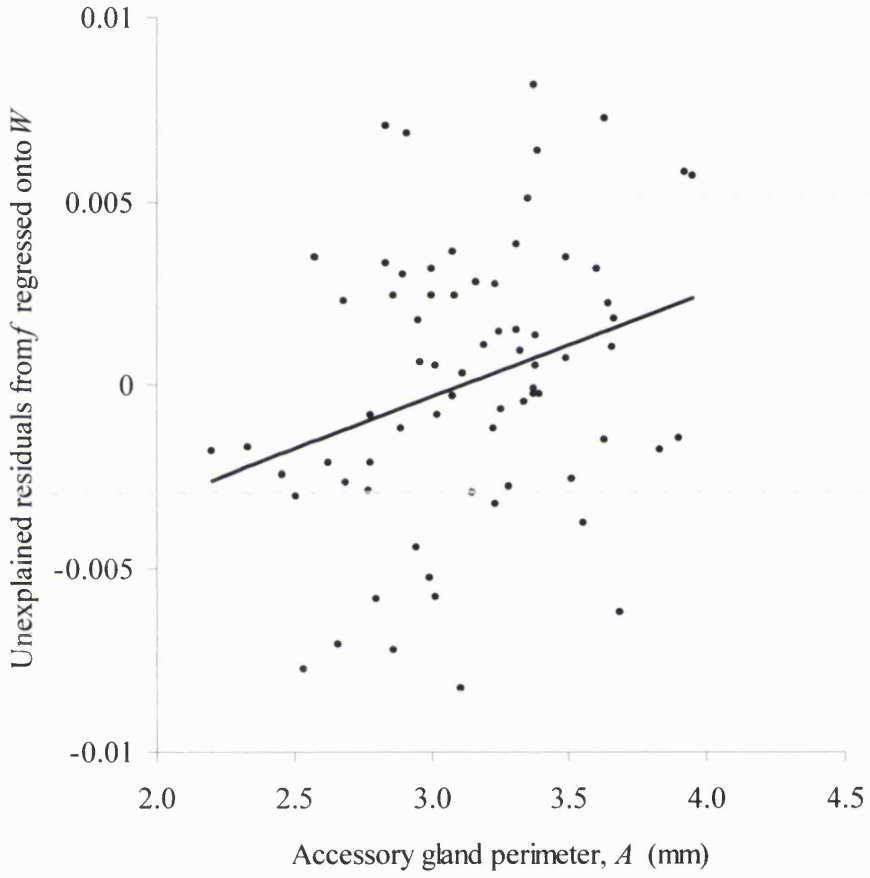


Figure 3.6. The unexplained residuals resulting from the regression of mating frequency (f) onto wing perimeter (W) (measured in mm), in turn regressed onto testis perimeter (T) (measured in mm). Variation in testis size did not account for a significant proportion of variation in mating frequency, over and above the allometric relationship between accessory gland size and body size. (R^2=0.0.0069, $d.f.$=1, 70, P=0.4888).

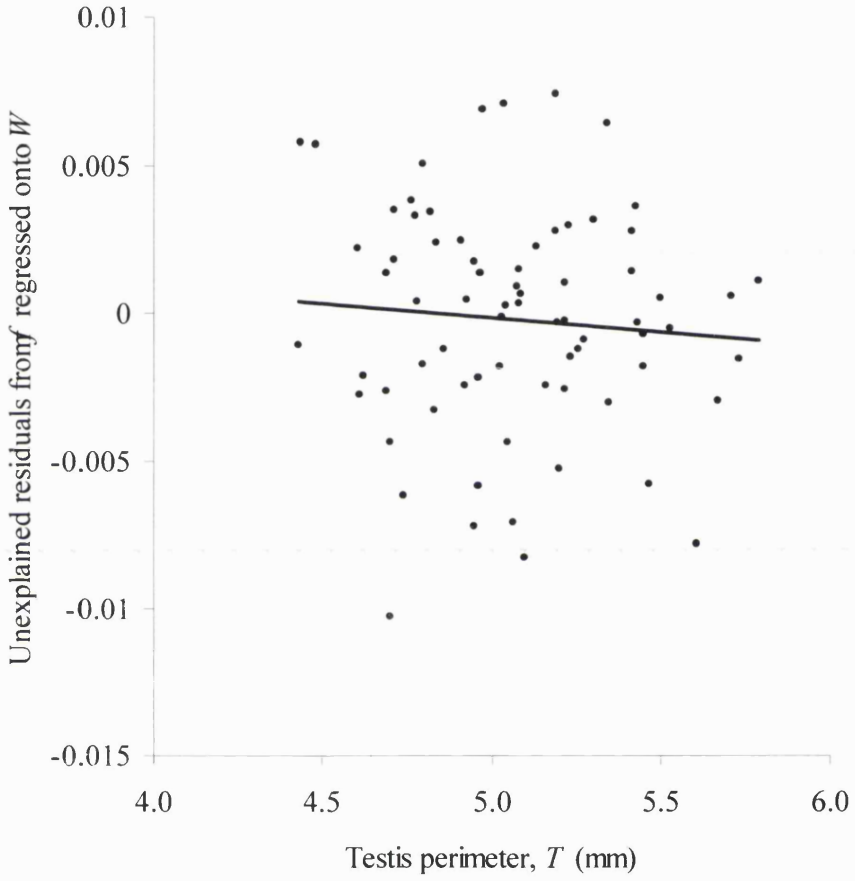


Figure 3.7. The unexplained residuals resulting from the regression of mating frequency (f) onto accessory gland perimeter (A) (measured in mm), in turn regressed onto wing perimeter (W) (measured in mm). Variation in body size did not account for a significant proportion of variation in mating frequency, over and above the variation in mating frequency accounted for by accessory gland size (R^2=0.0274, $d.f.$=1, 69, P=0.1679).

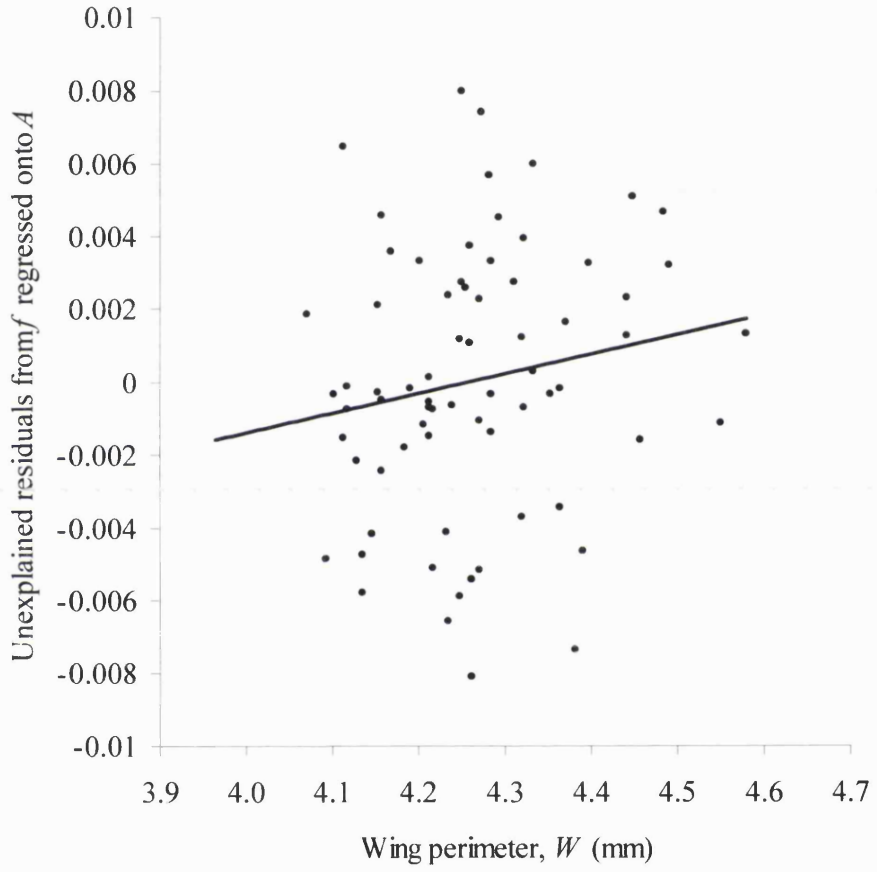


Figure 3.8. Regression of sperm displacement ability (fifth root transformed) (SDA_T) onto the number of offspring sired by the first male before the second mating (P_B). Variation in the number of offspring sired by the first male before the second mating accounted for a significant proportion of the variation in sperm displacement ability (R^2=0.1914, *d.f.*=1, 92, P<0.0001).

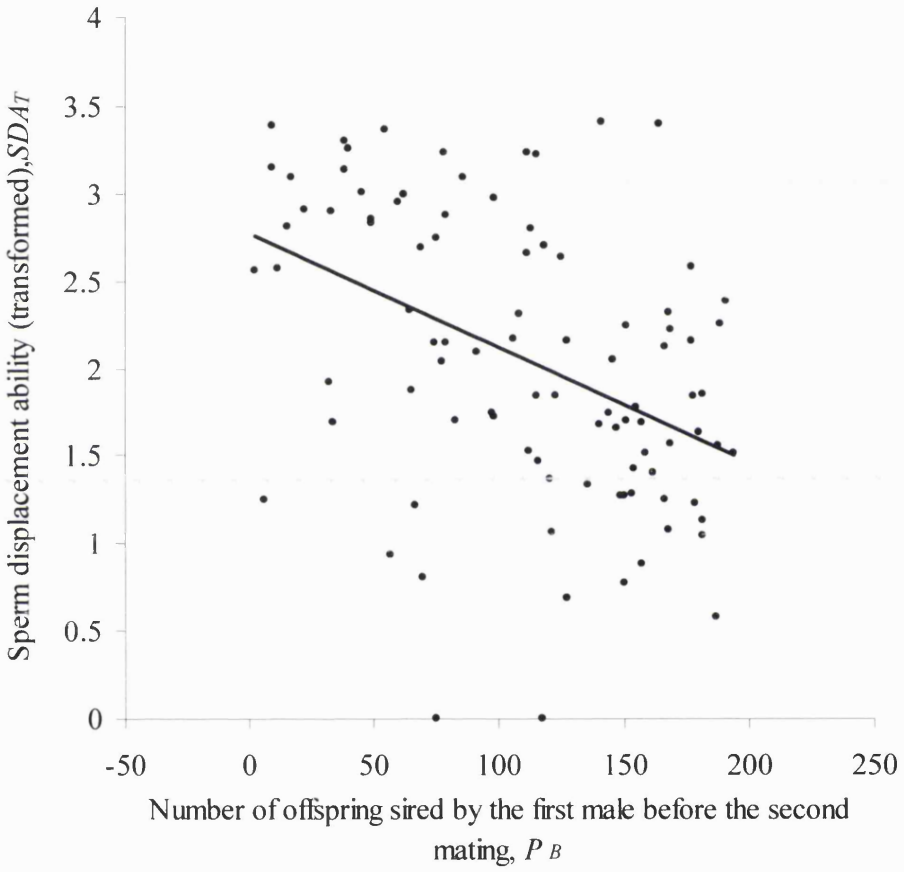


Figure 3.9. The unexplained residuals resulting from regression of sperm displacement ability (fifth root transformed) (SDA_T) on the number of offspring sired by the first male before the second mating (P_B), regressed in turn onto wing perimeter (W) (measured in mm). Variation in body size (represented by wing perimeter) accounted for a significant proportion of the variation in sperm displacement ability (R^2=0.0735, $d.f.$=1, 81, P=0.0132).

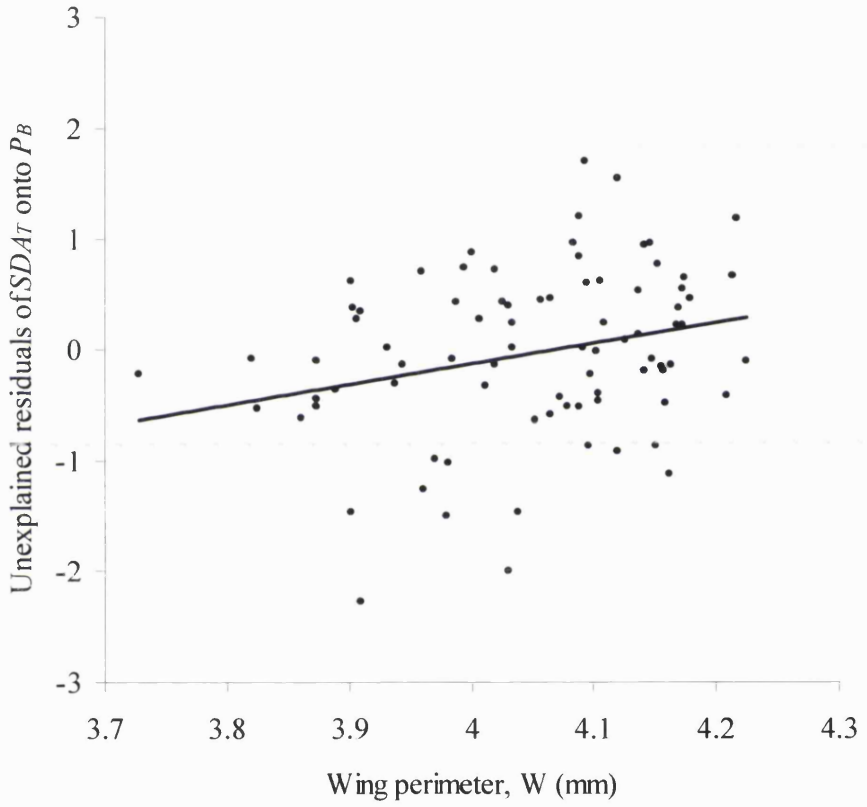


Figure 3.10. The unexplained residuals resulting from regression of sperm displacement ability (fifth root transformed) (SDA_T) on the number of offspring sired by the first male before the second mating (P_B), then regressed in turn onto accessory gland perimeter (A) (measured in mm). Variation in accessory gland perimeter did not account for any significant proportion of the variation in sperm displacement ability (R^2=0.0016, $d.f.$=1, 87, P=0.7062).

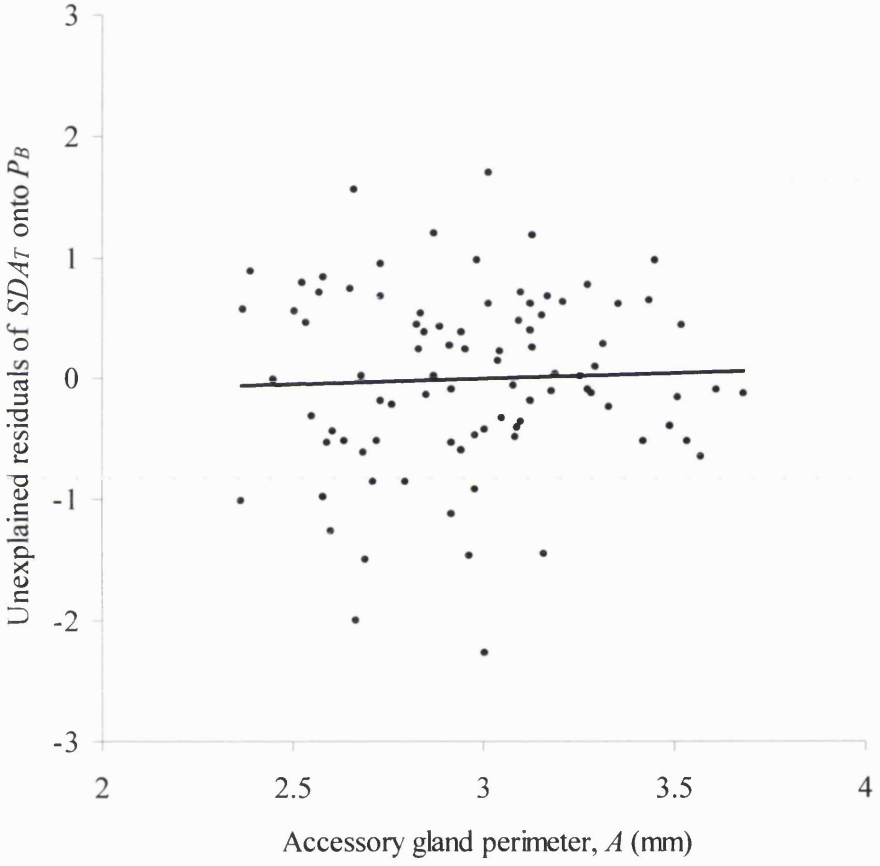


Figure 3.11. The unexplained residuals resulting from regression of sperm displacement ability (fifth root transformed) (SDA_T) on the number of offspring sired by the first male before the second mating (P_B), regressed in turn onto testis perimeter (T) (measured in mm). Variation in accessory gland perimeter did not account for any significant proportion of the variation in sperm displacement ability (R^2=0.0009, $d.f.$= 1, 84, P=0.7865).

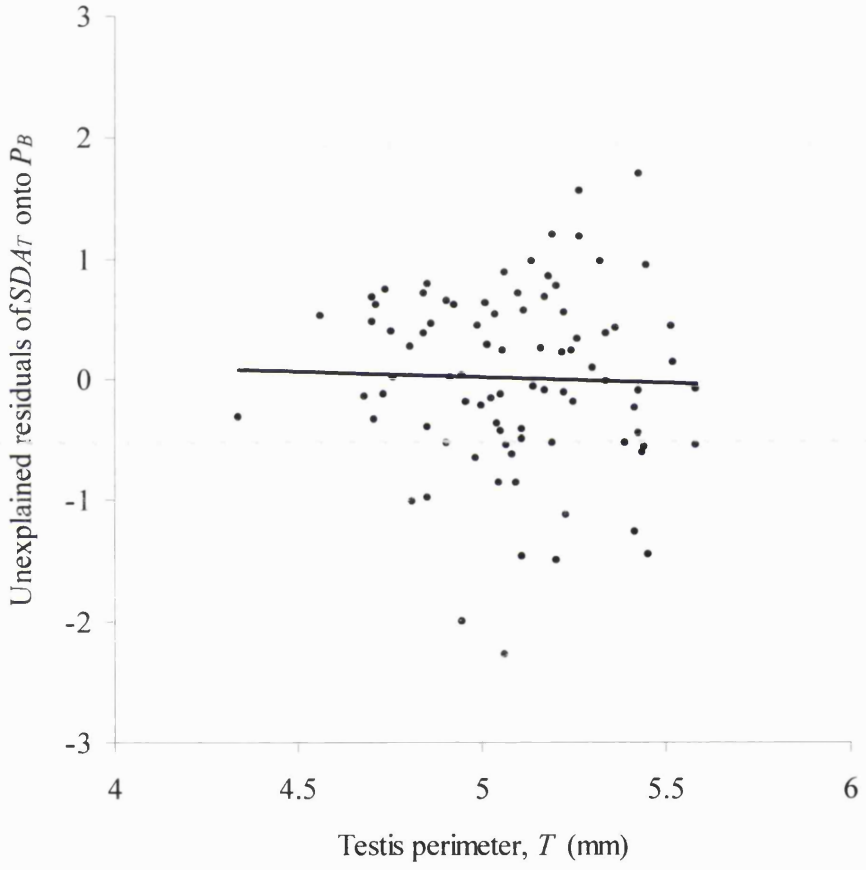


Table 3.1. Results of the regression of copulation duration onto sperm displacement ability, body size, accessory gland size and testis size. The coefficient of determination (R_2) shows the degree to which the independent variables account for variation in copulation duration. *P*-values result from *F*-tests employed to test the significance of the relationships.

	R^2	*d.f.*	*P*-value
Copulation duration regressed onto SDA_T	0.0079	1, 91	0.3966
Copulation duration regressed onto W	0.0008	1, 80	0.8068
Copulation duration regressed onto A	0.0149	1, 86	0.2567
Copulation duration regressed onto T	0.0014	1, 83	0.7320

3.5. Discussion

The most important finding of this chapter was the positive relationship between mating frequency and accessory gland size in males. There was no significant association between mating frequency and body size over and above effects due to accessory gland size. The second experiment showed that larger males had significantly higher post-copulatory success than smaller males, and there was no evidence that this association was due to the size of the accessory glands, over and above allometry with body size. Testis size was correlated neither with male mating success nor with male post-copulatory success.

Previous studies have shown that large males deliver more courtship to females, and have a higher lifetime mating success, than small males, both in the lab (Partridge & Farquhar 1983; Partridge et al. 1987a), and in the wild (Partridge et al. 1987b). In contrast, the experiment described in this chapter revealed only a marginally significant association between male body size and mating frequency, and there are a number of possible explanations for this. The experiment here was designed to examine correlations between accessory gland size and testis size and pre- and post-copulatory success, over and above variation in body size. This meant that standard density cultures were used to keep variation in body size deliberately low, and may have reduced the likelihood of observing a correlation between body size and mating frequency. Dow and von Schilcher (1975) suggest that in *D. melanogaster*, size is related to mating success partly through the greater aggression of larger males. Therefore, the absence of male-male competition for matings during observations of mating frequency in the experiment described here could have contributed to the weaker than expected association between body size and mating frequency. The female biased conditions under which this experiment was carried out could also have reduced any influence of female choice on male mating frequency. However, past evidence suggests that the correlation between male size and mating success is not due to female discrimination but, again, rather to behaviour determined by the male (Partridge et al. 1987a).

Males with larger accessory glands mated at higher frequency than males with smaller glands, regardless of effects due to body size. Male size did not account for a significant

amount of variation in mating success once accessory gland size had been taken into account. Therefore it is likely that accessory gland size is at least partly responsible for the well-established correlation between body size and mating frequency found in earlier studies (Partridge and Farquhar 1983; Partridge et al. 1987a, b).

The associations between mating success and body size, accessory gland and testes size seen in this chapter are not restricted to *D. melanogaster* but are consistent with observations made in a distantly related species of Diptera, the stalk-eyed fly *Cyrtodiopsis dalmanni*. In this species, there is a significant association between male mating rate and accessory gland size, but not between male mating rate and testis or body size (Baker et al. In press). Males with relatively larger glands may be able to retain the Acp resources to achieve high post-copulatory success for a greater number of successive matings than males with smaller glands. Proteins produced in the accessory glands strongly influence male success during post-copulatory competition with other males. Although mating triggers the rapid replenishment of proteins in the accessory glands, Hihara (1981) showed that mating males repeatedly in quick succession depleted the accessory glands (see also Lefevre and Jonsson 1962). Furthermore, serial matings resulted in males progressively less able to elicit post-mating in females (Hihara 1981). Therefore, males with smaller glands may become depleted of Acps after fewer sequential matings than males with larger glands, suffering the costs of courtship (Cordts and Partridge 1996) without obtaining the fertilisation benefits of mating. This hypothesis will need to be confirmed by the manipulation of accessory gland and body size, and examination of the effects of this manipulation on mating success. Body size can be manipulated by altering levels of nutrition in larval cultures, or by growing larvae in vials at different densities. Alternatively, body size, accessory gland size or testis size could be manipulated using artificial selection.

The significant negative correlation between second male sperm displacement ability and P_B (number of offspring sired by the first male, before the female remated) suggested that when first male fertilisation success is high, second males are less able to displace first male sperm. This is consistent with the results of Gilchrist & Partridge (1997). Over and above this association, I found a positive association between post-copulatory success and male body size, similar to studies on other species (e.g. the red

flour beetle *T. castaneum*, Lewis and Austad 1990; the moth *U. ornatrix* LaMunyon and Eisner 1993; and the butterfly *P. napi*, Wedell and Cook 1998). However, the biological mechanism underlying the correlation between body size and sperm displacement ability in *D. melanogaster* is not clear. It is possible that larger males pass greater quantities of seminal fluid proteins to females than small males, where they elicit stronger post-mating responses. However, my results suggest that the higher sperm displacement ability of larger males is not due to the size of their accessory glands, as accessory gland size was not correlated with sperm displacement ability, before or after body size was taken into account. Furthermore, although accessory glands become deflated after mating, they are not exhausted (Lefevre and Jonsson 1962); a male transfers only approximately one third of the contents of the accessory gland to the female during each mating, suggesting that, within a single mating, males (big or small) are not likely to be limited by accessory gland size. In addition, consistent with previous literature, I found no evidence that copulation duration was correlated with sperm displacement ability (Lefranc and Bundgaard 2000) or body size, suggesting that large males do not mate for longer than small males. Nevertheless, instead of accessory gland size or copulation duration, a more important factor determining the quantities of ejaculate reaching females during a single mating, may be the rate at which seminal fluid is passed to females. It is possible that large males have a higher rate of ejaculate transfer and pass greater quantities of seminal fluid protein to females than small males. This is the case in the yellow dung fly *S. stercoraria*, where large individuals pump ejaculate faster and more efficiently during copulation than smaller males (Simmons et al. 1996).

This study found no association between testis size and mating frequency or post-copulatory success in *D. melanogaster*. Comparative studies have shown that, in a number of other animals, levels of polygamy within a species are associated with the testis size of males. This implies that males with larger testes are often more successful during sperm competition than males with smaller testes (Harcourt et al. 1981; Harvey and Harcourt 1984; Gage 1994; Pitnick and Markow 1994). However, in *D. melanogaster*, males appear not to be limited by the quantities of sperm they produced. Males pass greater quantities of sperm during copulation (approximately 5000) than the numbers stored (approximately 1100) in the female sperm storage organs (Gilbert

1981). Furthermore, males made sterile as a consequence of repeated matings do not lack sperm in the seminal vesicles (Lefevre and Jonsson 1962). This sterility is instead correlated with the depletion of the accessory glands. The lack of correlation between testis size and sperm displacement ability in *D. melanogaster* is consistent with the observation that during single matings, males tend not to be limited by sperm quantities.

The conclusions of the experiments of this chapter, together with previous studies in *D. melanogaster*, indicate that larger males are likely to mate at higher frequency and have a higher post-copulatory success than smaller males. The association between body size and mating frequency appears to be at least partly due to accessory gland size; males with larger accessory glands mate at higher frequency, over and above effects due to body size. This suggests that pre-copulatory selection on body size in males is reinforced at the post-copulatory stage of the reproductive process.

Chapter 4. Influence of female reproductive tract morphology on the outcome of sperm competition

4.1. Abstract

Females as well as males can influence the outcome of sperm competition, and may do so through the anatomy of their reproductive tracts. Female *Drosophila melanogaster* store sperm in two morphologically distinct organs: a single seminal receptacle and two spermathecae. These organs have different temporal roles in sperm storage. I used a population of *D. melanogaster* possessing variation in spermathecal morphology (two and three spermathecae) to examine the association between sperm storage organ morphology and sperm use. Although the common measure of sperm precedence, P_2, did not differ between females with two and three spermathecae, the pattern of sperm use over time indicated that female morphology did affect male reproductive success. The rate of offspring production by females with three spermathecae rose and fell more rapidly than by females with two. This difference in the temporal pattern of offspring production suggests that if females remate or die before using up second male sperm, then second male reproductive success will be higher when they mate with females with three spermathecae. Variation in the patterns of offspring production was also correlated with male genotype. Males of genotype bw^D were less able to displace first male sperm than were wild-type males. However, bw^D males also sired offspring for longer and the total number of offspring they sired was not compromised. The results suggest that temporal patterns of sperm use as well as P_2 should be taken into account when measuring the outcome of sperm competition.

4.2. Introduction

Females of many insects mate multiply and simultaneously store the sperm of two or more males in their reproductive tracts. Since the inception of work on sperm competition (Parker 1970), studies of post-copulatory events have concentrated on interactions between competing males. It has also been suggested that females too can influence the post-copulatory success of males (Walker 1980; Otronen et al. 1997; Qazi et al. 1998; Ward 1998; Hellreigel and Bernasconi 2000; Ward 2000; Knowles and Markow 2001). Females might influence sperm use in order to acquire direct benefits, such as the increased longevity of stored sperm. The morphological complexity of many female insect reproductive tracts has also lead to speculation that females gain indirect benefits through post-copulatory sperm choice, or by differential investment in embryos sired by different males (e.g. Thornhill 1983; Eberhard 1996; Eberhard et al. 1998), although evidence for this is still lacking (Birkhead 1998, 2000).

In *D. melanogaster*, females mate multiply (Marks et al. 1988; Vianen and Bijilsma 1993; Harshman and Clark 1998; Imhof et al. 1998) and can store sperm for up to two weeks after mating (Gilbert et al. 1981; Neubaum & Wolfner 1999). They can also store the sperm of multiple males simultaneously (Milkman and Zeitler 1974; Marks et al. 1988). Genetic variation among females is correlated with variation in the degree of sperm precedence achieved by their mates (Civetta and Clark 2000a). Clark and Begun (1998) showed that variation in P_2 was correlated with the genotypes of doubly mated females made homozygous for chromosomes extracted from natural populations. In *D. melanogaster*, sperm precedence is also affected by an interaction between male and female genotypes (Clark et al. 1999).

Male fertilisation success is influenced by interactions between the sexes in other species too, for example in the red flour beetle, *T. castaneum* (Nilsson et al. 2003), in the housefly *Musca domestica* (Andres and Arnqvist 2001), in the beetle *Calloscobruchus maculatus* (Wilson et al. 1997), in the water strider *Gerris lateralis* (Arnqvist and Danielsson 1999a) and species of sea urchin, genus *Echinometra* (Palumbi 1999). Male fertilisation success may also be affected by genetic incompatibility between males and females (reviewed by Tregenza and Wedell 2000);

perhaps to avoid inbreeding (Markow 1982; Olsson et al 1996; Bishop et al. 1996; Markow 1997) or to avoid fertilisation between species or allopatric populations (e.g. Howard et al. 1998; Price et al. 2000; Brown and Eady 2001; Price et al. 2001). These studies suggest that the female reproductive tract is more than just a constant environment in which sperm competition occurs. However in most instances, it remains to be seen how genetic variation between females or mating pairs corresponds to biochemical, physiological and morphological variation in the female reproductive tract, and why it might be correlated with the fertilisation success of males.

It has been suggested that female *Drosophila* could affect the outcome of sperm competition through the utilisation of different types of sperm storage organ (Pitnick et al. 1999). Female *D. melanogaster* possess two morphologically distinct sperm storage organs: a 2 mm long seminal receptacle and two mushroom-shaped, sclerotized spermathecae. These have different temporal roles; the seminal receptacle is commonly regarded as the primary sperm storage organ, as it has a larger sperm storage capacity (holding approximately 60% of the total stored sperm) than the two spermathecae (Gilbert 1981). It has also been suggested that the spermathecae have a secretory role, producing fluid that enhances the longevity of stored sperm (Anderson 1945; Filosi and Perotti 1975; Bouletreau-Merle 1977), although this has not be determined experimentally. Direct observations show that sperm from the seminal receptacle are released sooner than sperm from the spermathecae (Gilbert et al. 1981). A recent study used artificial selection to show that seminal receptacle length affected female fecundity but not the sperm precedence achieved by two males mated to that female (Miller and Pitnick 2003). Nevertheless, it is possible that the genetic variation between females for the degree of sperm precedence achieved by their mates corresponds to morphological variation inside the reproductive tract. The functions of the sperm storage organs in sperm utilisation, and the reasons why both types of sperm storage organ have been retained in many *Drosophila* species, remain unclear (Fowler 1973; Gilbert 1981; Briskie and Montgomerie 1993; Keller and Reeve 1995; Ward 1998; Pitnick et al. 1999).

Naturally occurring variation in spermathecal morphology among female *D. melanogaster* provides the means to investigate the relationship between female

reproductive tract morphology and sperm use. The first aim of the experiments described in this chapter was to examine the association between sperm storage organ morphology and sperm use, using a stock of *D. melanogaster* that had a small number of females with three spermathecae, rather than two (Fig. 4.1). Females were each mated to two males, and these males sired offspring with distinguishable genotypes. Sperm precedence was estimated by counting the numbers of offspring sired by each male. For two males mated to a single female, sperm precedence is typically measured using the index P_2 (*=number offspring fertilised by the second male/(number offspring sired by the first + second males*).

However, this measure of sperm precedence is calculated using the total numbers of offspring sired by two males mating a female and therefore ignores the dynamics of sperm use. If a female mates more than once, first male reproductive success will be affected by the rate at which first male sperm are used for fertilisation (Prout and Bundgaard 1977; Gilchrist and Partridge 1995). Therefore, I also monitored sperm use over time. If females die or remate before all the sperm of a previous male is used, those males whose sperm is used very quickly will be at an advantage over males whose sperm is used slowly. Temporal patterns of sperm use provide an additional source of variation in male success during sperm competition. The experiments described in this chapter describe correlations between the temporal patterns of male fertilisation success and female spermathecal morphology.

4.3. Methods

4.3.1. Stocks

Females were derived from a line created by the mobilisation of the *PlacW* transgene in the line *P{w+mC=lacW}l(3)j10B6^{j10B6}/ TM3*, from the Bloomington Stock Centre (stock number: 10175) during a mutagenesis screen. This line was cultured in vials containing Maize-Yeast medium, which was subsequently used throughout the experiment.

Dissection of females from this line revealed that 30% possessed three spermathecae (3SP) rather than the normal two (2SP) (Fig. 4.1). However, as 2SP and 3SP females

were found in the original *PlacW* line, it is unlikely that this rare spermathecal morphology was caused by mobilisation of the *PlacW* to a new position in the genome. The genetic basis for this variation in spermathecal morphology was therefore unknown and naturally occurring. Dissection of mated females in phosphate buffered saline (PBS) and examination under a compound microscope (magnification x250) confirmed that the spermathecae of 2SP and 3SP females all contained sperm. 2SP and 3SP females were obtained from the same vials and experienced the same culture conditions, which meant that 2SP females were excellent controls for the 3SP females.

4.3.2. Mating females twice to males of different genotypes

To determine the effect of spermathecal number on the outcome of sperm competition, 2SP and 3SP females were mated to either (i) *brown dominant* (bw^D; section 2.1.8) males then French wild-type males (section 2.1.7), or to (ii) wild-type then bw^D males. When mated to 2SP and 3SP females, these males sired phenotypically different female offspring. The female offspring of wild-type males had wild-type eye colour – bright red with pseudopupils. In contrast, female offspring sired by bw^D males had dark red eyes and no pseudopupil. Because the parental females used in the experiment were homozygous for the *white* allele on the X-chromosome (although partially rescued by the *PLacW* insertion), male offspring sired by wild-type or bw^D males all carried only the maternally derived *white* allele and were thus indistinguishable with pale orange eyes. These were therefore excluded from the data set. The sex ratio was assumed to be 1:1 and estimates of bw^D and wild-type sperm use were made using female offspring only.

All parental flies were reared at standard density of 50 larvae per vial (section 2.2.3). Virgin males and females were collected within 8 hours of eclosion and placed in vials in single sex groups of 20. Approximately 260, 7-day-old, virgin females were anaesthetised using ice and aspirated into individual vials, and each was paired with one 4-day-old bw^D or wild-type male. Pairs were observed for the next 6 hours and any that had not mated during this time were discarded. After a single mating, males were removed to ensure that females did not mate twice. 72 hours later, females that had previously mated with a wild-type male were given a 7-day-old bw^D male, and each

female that had previously mated with a bw^D male was given a 7-day-old wild-type male. Pairs were observed for the next 9 hours and any females that did not remate during this time were discarded. After remating, females were aspirated into fresh vials with about 1.5mg of fresh yeast. 104 females were mated to bw^D then to wild-type males, and 82 were mated to wild-type then to bw^D males. Significantly fewer females whose first mates were wild-type males remated, compared to females whose first mates were bw^D males ($\chi_{[1]}$=8.061; P=0.0045). This difference in time to remating could be because wild-type males were better at eliciting post-mating inhibition of receptivity than bw^D males. Alternatively, this could have been because bw^D males had lower courtship ability than wild-type males.

4.3.3. Offspring collection

After mating twice, females were repeatedly transferred to new vials with 1.5mg fresh yeast every 2 days until they ceased producing offspring. This prevented crowding-induced mortality by restricting the numbers of growing offspring to no more than 125 per vial. Offspring resulting from these vials were counted and scored for eye colour on days 11 and 14 after the female parent had been introduced into each vial. This resulted in offspring counts (female offspring only) from each 2-day sample vial (n_r), which represented the rate of use of bw^D and wild type male sperm per two-day period.

Parental females were transferred to Eppendorf tubes and frozen at –84°C until dissection in PBS, to determine whether they were 2SP or 3SP females. A small proportion of females resembled 3SP females but had two spermathecae that were fused or shared a single duct. For simplicity, only females with two distinct or three distinct spermathecae were included in the data set. The final sample sizes were 89 for females mated first to bw^D then to wild-type males, and 58 for females mated first to wild type then to bw^D males.

4.4. Results

4.4.1. Associations between total numbers of offspring, P_2, spermathecal morphology and male genotype

(i) Total number of offspring produced and spermathecal morphology

The association between spermathecal morphology and the total numbers (n_t) of bw^D and wild type female offspring produced (Table 4.1a) was examined. For females mated first to bw^D then to wild-type males, the total numbers of offspring sired by the first male to mate (first male n_t) produced by 2SP females were compared to first male n_t produced by 3SP females. Similar comparisons between 2SP and 3SP females were made for second male n_t and for females mated in the opposite order. Wilcoxon tests were used throughout, since n_t was distributed non-normally.

Only one of these tests revealed a significant difference in n_t between 2SP and 3SP females. For second male n_t produced by females mated first to bw^D and then to wild-type males, 3SP females produced significantly fewer offspring than did 2SP females (Table 4.1a; P=0.0283). However, for several independent tests whose results are in a consistent direction, the P-values resulting from these tests can be combined using a method devised by R.A. Fisher, as described in Sokal and Rohlf (1995). This is a form of meta-analysis that can allow overall conclusions to be made from a number of different independent tests. Combining probabilities from 4 independent tests of significance showed that overall, 3SP females tended to produce fewer offspring than did 2SP females ($\chi_{[8]}$=15.90; P=0.0438).

(ii) Total number of offspring produced and male genotype

Separate Wilcoxon tests were also carried out to compare n_t sired by first and second males of different genotypes. For 2SP females, first male n_t produced by females mated first to bw^D then to wild-type males was compared to first male n_t produced by females mated first to wild type then to bw^D males. Similar comparisons were carried out for second male n_t and for 3SP females. Females mated first to bw^D then wild-type males tended to produce fewer first and second male offspring than females mated in the opposite order (Table 4.1a). However, these tests were only significant for first male n_t

produced by 2SP females (P=0.0371), and combining probabilities from all four independent tests suggested that there were no consistent significant differences ($\chi_{[8]}$=11.99; P=0.1514).

(iii) Sperm precedence and spermathecal morphology

Comparisons were also made between the P_2 values resulting from the double matings of 2SP and 3SP females (Table 4.1b). These tests revealed no significant differences in P_2 values for 2SP and 3SP females (result of combining probabilities, $\chi_{[4]}$=4.14; P=0.3866).

(iv) Sperm precedence and male genotype

Comparisons were also made between P_2 values achieved by bw^D and wild type males (Table 4.1b). This revealed no associations between P_2 and the genotype of the second male to mate (result of combining probabilities, $\chi_{[4]}$=4.82; P=0.3061).

4.4.2. Associations between offspring production over time, spermathecal morphology and male genotype

All offspring were collected in 2-day samples and the number of female offspring per two-day sample is henceforth referred to as n_r. Fig. 4.2 show examples of first and second male n_r over time produced by eight individual parental females in the experiment. Fig. 4.3 shows bw^D and wild-type n_r over time for all 147 parental females in a single graph. These examples show that there is considerable variation between females for the temporal patterns of offspring production. Tests were carried out to determine whether any of this variation was accounted for by female spermathecal morphology or male genotype. In Fig. 4.4, graphs of n_r over time have been categorised by whether the parental female had 2SP or 3SP, and whether offspring were sired by the first (column a) or second (column b) male to mate. Additional graphs (Fig. 4.4, column c) show the total offspring production for each female over time. Females were either mated first to wild type and then to bw^D males or to bw^D then wild type males. On the graphs in Fig. 4.4, median numbers of offspring produced within each two-day sample are indicated for clarity.

(a) Analysis of time of maximum rate of offspring production

I examined the time (the day on which the two-day sample was collected) at which n_r reached a maximum (t_{nmax}) and used Wilcoxon tests to determine whether variation in t_{nmax} among females was associated with sperm morphology or male genotype.

i. Maximum rate of offspring production and spermathecal morphology

These tests (Table 4.2a) showed that for females mated first to bw^D and then to wild-type males, 3SP females had a lower t_{nmax} than did 2SP females, both for offspring sired by the first (P=0.0357) and second (P=0.0387) males. This shows that n_r rose more rapidly for 3SP than for 2SP females. For females mated in the opposite order, no significant differences in t_{nmax} were seen between 2SP and 3SP females.

ii. Maximum rate of offspring production and male genotype

Comparing t_{nmax} for offspring sired by different genotypes showed that t_{nmax} was lower for wild type offspring than for bw^D offspring (Table 4.2b). This was the case for offspring sired by both first (2SP females P=0.0001; 3SP females P=0.0055) and second males to mate (2SP females P=0.0001; 3SP females P=0.0231). The results of these tests show that maximum offspring production occurred later for those sired by bw^D males compared to those sired by wild-type males, suggesting that the use of bw^D sperm was delayed relative to use of wild type sperm.

(b) Analysis of 2-day samples

Any differences between 2SP and 3SP females in the numbers of offspring produced during any 2-day sample will indicate a difference in the temporal patterns of n_r. Wilcoxon tests were carried out on n_r (each 2-day sample separately), comparing the same groups as for tests carried out on n_t. For example, for females mated first to bw^D then to wild-type males, offspring produced on day 2 by 2SP females were compared to offspring produced on day 2 by 3SP females. This was carried out for each day that offspring were produced. After correcting for multiple comparisons, the critical P-value for these tests was 0.0071.

i. Analysis of 2-day samples and spermathecal morphology

For second male offspring produced by females mated first to bw^D and then to wild-type males, n_r on days 6 and 8 was significantly lower for 3SP females than for 2SP females (Table 4.3a). This suggests that the falling phase of n_r over time was more rapid for 3SP compared to 2SP females (illustrated by graphs in Fig. 4.4). There were similar patterns for first male offspring, although the main difference (for n_r collected on day 8) was marginally non-significant (P=0.0081; not shown in Table). Differences in n_r between 2SP and 3SP females mated to males in the opposite order were not significant (Table 4.3a).

ii. Analysis of 2-day samples and male genotype

The analysis also revealed some differences in temporal patterns of n_r associated with male genotype. 2SP females mated to bw^D and wild-type males in different orders differed significantly in first male n_r (collected on days 2 and 4) and second male n_r (collected on day 2) (Table 4.3b; Fig. 4.4). These differences suggest that bw^D offspring production was delayed relative to wild-type offspring production. Differences in n_r for bw^D and wild-type males mated to 3SP females were non-significant.

(c) Analysis of temporal patterns of offspring production using a non-linear parametric model

Although differences in the temporal patterns of offspring production have been analysed statistically by carrying out tests on n_r and t_{nmax}, a model was fitted to the patterns of offspring production to illustrate how temporal data of this type might be analysed in greater detail. Using a program constructed by J. A. Bangham (Matlab, version 6.0.0.88; Release 12), a non-linear parametric model was fitted to temporal patterns of n_r for each female, bw^D and wild type males separately. Differences in the temporal patterns of sperm use between 2SP and 3SP females and between offspring sired by bw^D or wild-type males were then determined by statistical analysis of the best-fit parameters of the model.

In a previous study, passive sperm loss in the zebra finch *Taeniopygia guttata* was adequately described using a 'one-compartment' model (Colegrave et al. 1995; Lessells

and Birkhead 2002), which described a single exponential decay curve. In contrast, the rising and falling phases of n_r over time for females in the present experiment (Figs. 4.2 & 4.3) meant that a one-compartment model was not adequate to fit the data. First and second male offspring production by *D. melanogaster* females typically contained both a rising and falling phase, so instead, a 'two-compartment' model was used.

The differential equations that describe the model are as follows:

$$\frac{dv}{dt}(1) = (v_2 - v_1)/\tau_1$$

$$\frac{dv}{dt}(2) = -\left(v_2/\tau_2\right) - \left((v_2 - v_1)/\tau_3\right)$$

Although the two-compartment model is unnecessarily complex for fitting n_r over time in the example in Fig. 4.2 (iiia), it is able to fit the rising and falling phases of n_r over time seen in, for example, Fig. 4.2 (iiib). The model was fitted to the temporal patterns of offspring production of each individual female, first and second male offspring separately. Fig. 4.2 provides examples of these patterns. In Fig. 4.2, the empirical values of n_r are indicated by large dots and the fitted model is illustrated by the fine dotted line. The model was integrated to produce model estimates of n_r (open circles), and these model estimates can be compared to the empirical values of n_r.

A fitting program was used to search for the values of parameters of the two-compartment model (v_1, v_2, τ_1, τ_2, τ_3) that minimised the residuals (using Matlab function *fminsearch*) between the model estimates of n_r and the empirical values of n_r. Having fitted the model to the temporal patterns of offspring production from each individual female, first and second male offspring separately, statistical analysis was carried out on the fitted parameters of the model τ_1, τ_2, τ_3 and v_1, to test whether there were significant differences in the temporal patterns between groups.

Fig. 4.5 shows how changes in the fitted parameters τ_1, τ_2 and τ_3 represent the shape of the function in a qualitatively comprehensible way. In the present study, the existence of differences in temporal patterns has already been established using independent tests.

Before such a model is used as the sole analysis of this type of data however, future work will use maximum likelihood methods to establish which of a range of likely models, including the two-compartment model used here, best describes the data.

Parameter τ_1 (Fig. 4.5a) was strongly associated with the initial falling phase of the curve, where a lower value of τ_1 corresponded to a sharper initial falling phase. Parameter τ_2 (Fig. 4.5b) was strongly associated with the long-term falling phase of the curve, where the higher the value of this parameter the longer the falling phase and flatter the overall appearance of the curve. Both τ_1 and τ_2 described a difference in the time at which maximum offspring production occurred. As they therefore corresponded to both the rising and falling phases, tests carried out on τ_1 and τ_2 should not necessary be taken as evidence of significant differences in the rising phase without supporting evidence from independent tests (such as those used in this experiment). Parameter τ_3 (Fig. 4.5c) was not related to the time at which maximum offspring production occurred but was strongly influenced the magnitude of this maximum: a higher value of τ_3 corresponded to a lower maximum. Parameter v_1 affected only the magnitude and not the shape of the curves. v_1 is therefore not of interest in the present analysis and is not included in the tables. Finally, parameter v_2 affected the 'offset' of the curves. This variable was required in order to achieve a close fit for some individual patterns of offspring production. v_2 varies only very slightly, and a negative value of v_2 described a delay for the whole curve. Again, if a model of this type is used in isolation, care must be taken during the interpretation of the results when two or more parameters affect similar aspects of the curves. Wilcoxon tests were used to determine if the model parameters were significantly affected by spermathecal morphology and male genotype.

i. Analysis of model parameters and spermathecal morphology

For females mated first to bw^D then to wild-type males, parameters τ_1 and τ_2 were significantly lower for 3SP than for 2SP females for both first and second male n_r over time (Fig. 4.4, rows i & ii). Table 4.4a shows the results of these tests, but for simplicity, shows the results for second male offspring only. The same trend was seen for females mated in the opposite order (Fig. 4.4, rows iii & iv), although the differences here were non-significant (Table 4.4a). Combining probabilities from each

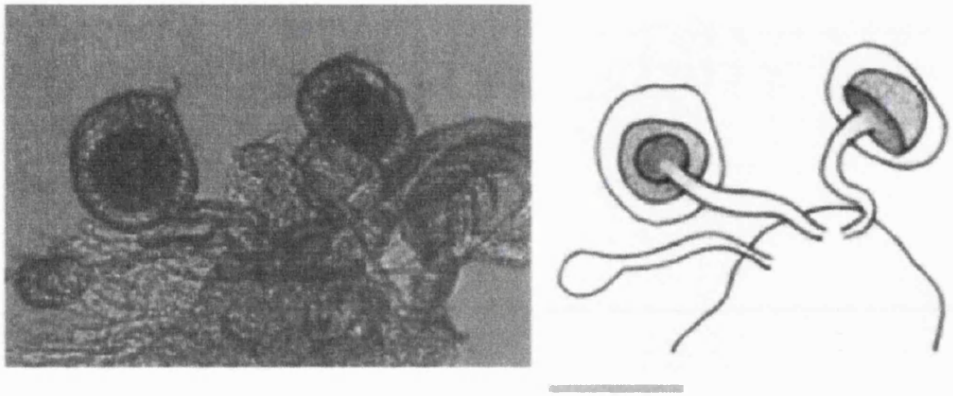
of the four independent Wilcoxon tests showed that overall, τ_1 was consistently lower for 3SP females than for 2SP females ($\chi_{[8]}$=22.74; P=0.0037). Similarly, combining probabilities across all tests carried out on τ_2 (tests carried out on both first and second male offspring), showed that overall, τ_2 was lower for 3SP females than for 2SP females ($\chi_{[8]}$=19.46; P=0.0126). These results are consistent with results of statistical analysis carried out on t_{nmax}. They confirm that the rising and falling phases of offspring production were more rapid for 3SP than for 2SP females.

ii. Analysis of model parameters and male genotype

There were also significant differences in the temporal patterns of bw^D and wild type offspring production. Table 4.4b shows, for simplicity, the results for offspring produced by 2SP females only. Each of the four tests showed that τ_1 was significantly higher for bw^D offspring than for wild-type offspring. This was confirmed by combining probabilities resulting from all four tests, and this showed that parameter τ_1 was significantly higher for offspring sired by bw^D males than for offspring sired by wild-type males ($\chi_{[8]}$=50.21; P<0.0001). The same was true for parameter τ_2 ($\chi_{[8]}$=48.60; P<0.0001). This again confirmed statistical analysis carried out on t_{nmax} showing that for first and second male offspring, bw^D n_r over time was delayed relative to wild-type n_r over time. In addition, for first male offspring, parameter τ_3 was higher for bw^D n_r over time as compared to wild-type n_r over time (Table 4.4b), suggesting that for first male offspring, the curve was smaller in magnitude for bw^D n_r over time compared to wild-type n_r over time. The same was not true for second male offspring (Table 4.4b). Finally, v_2 was significantly lower (and negative) for bw^D first male offspring compared to wild-type first male offspring. This again supported the finding that the pattern of bw^D first male offspring production was delayed overall relative to wild type first male offspring production (Fig. 4.4, column a, rows i & iii), and is consistent with analysis of t_{nmax}.

Figure 4.1. The darkly pigmented cuticle of the spermathecae of *D. melanogaster* in **(a)** 2SP female, **(b)** 3SP female. Samples were dissected on glass slide in PBS. Images were captured from a compound microscope (magnification: x250) using a video camera connected to a Macintosh computer.

(a)



(b)

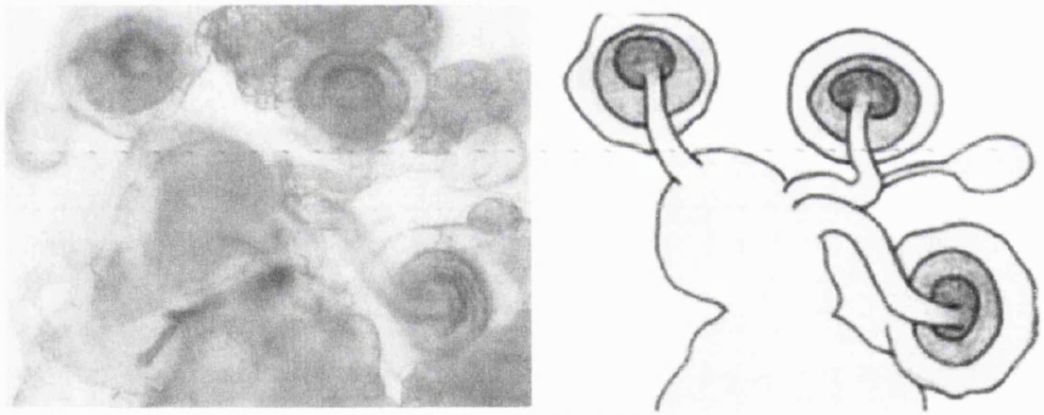


Figure 4.2. Eight examples of numbers of **(a)** first or **(b)** second male offspring (indicated by dots) produced during each two-day sample (n_r) over time (in days), by individual females. Dotted lines represent the best-fit two-compartment model to n_r over time (see Appendix). Open circles represent the integral of the dotted line over two days, indicating the model's estimation of n_r from the best-fit parameters. The large dots indicate empirical values of n_r. Graphs show n_r over time for:

(i) 2SP females mated first to bw^D then to wild-type males

(ii) 3SP females mated first to bw^D then to wild-type males

(iii) 2SP females mated first to wild-type then to bw^D males

(iv) 3SP females mated first to wild-type then to bw^D males

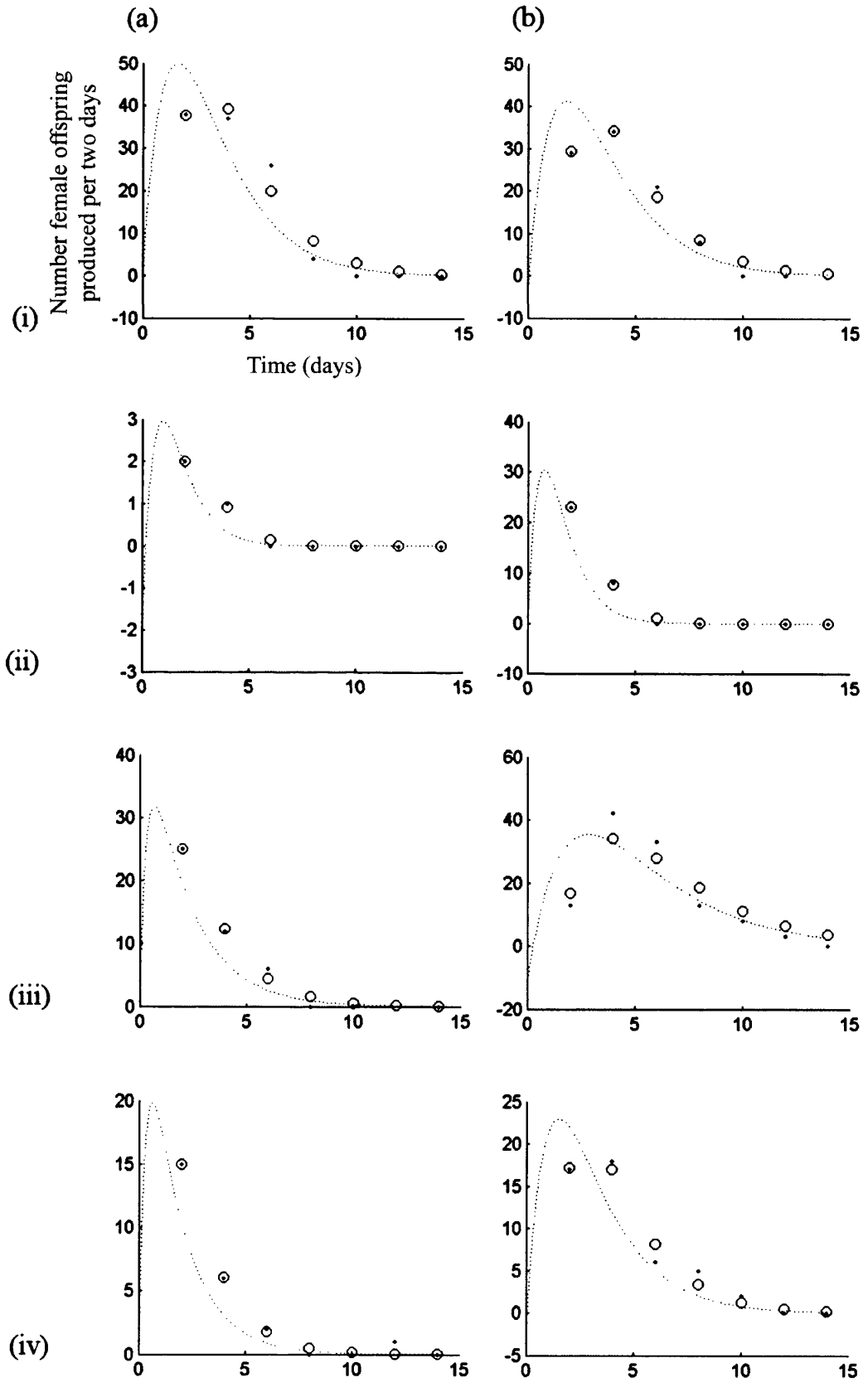

(a)
(b)
(i)
(ii)
(iii)
(iv)
Number female offspring produced per two days
Time (days)

Figure 4.3. Numbers of offspring (crosses) produced during each two-day sample (n_r) over time (in days) by each female, bw^D and wild-type offspring shown as separate data points. Open squares indicate the numbers of first male offspring produced by each female during the equivalent time interval before the second mating occurred. The model was fitted to n_r for each individual female. The median parameter values for each of these fits were taken and the model was replotted from these median parameter values. Closed squares indicate the re-estimated values of n_r from the model using the median best-fit parameters.

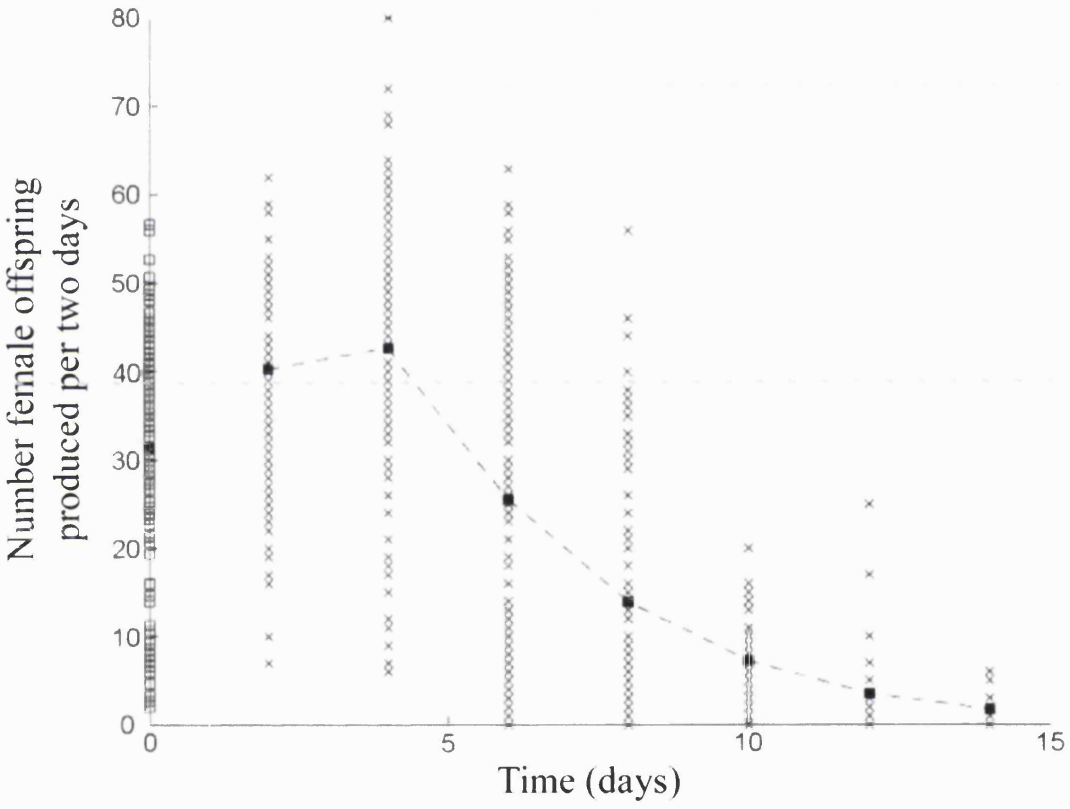


Figure 4.4. Numbers of offspring (crosses) produced during each two-day sample (n_r) over time (in days) by each female. Graphs show n_r sired by **(a)** the first male **(b)** the second male to mate, and **(c)** the offspring sired by both first and second males. On each graph, filled circles indicate the median n_r. Graphs in column (a) also include the numbers of offspring sired by the first male before the second mating (time 0 on X-axes) during the equivalent time sample. Graphs show n_r over time for:

(i) 2SP females mated first to bw^D then to wild-type males

(ii) 3SP females mated first to bw^D then to wild-type males

(iii) 2SP females mated first to wild-type then to bw^D males

(iv) 3SP females mated first to wild-type then to bw^D males

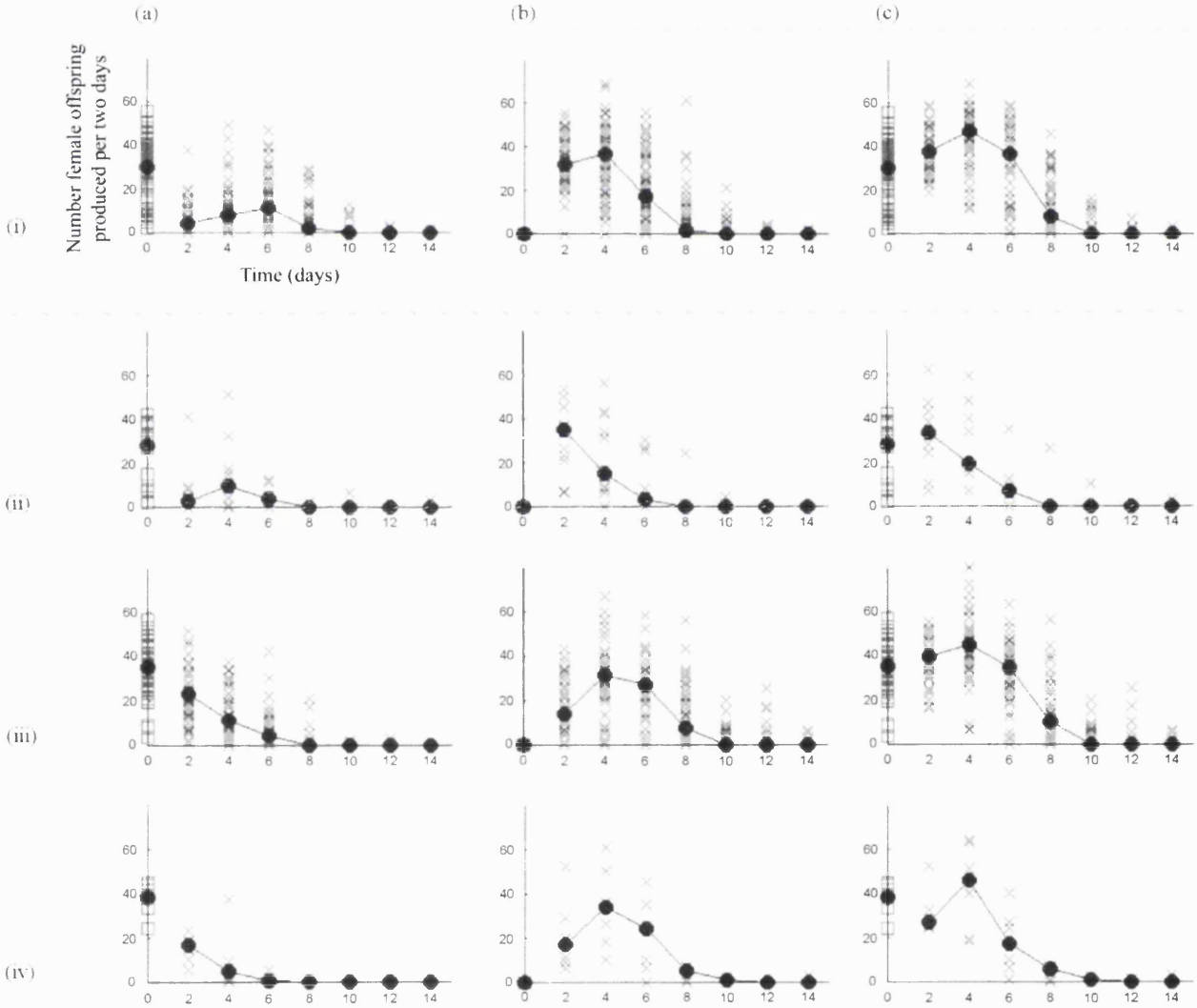


Figure 4.5. Parameters τ_1, τ_2 and τ_3 relate to different aspects of the curves of offspring production. Parameter **(a)** τ_1 is strongly associated with the initial falling phase of the curve, where a lower value of τ_1 corresponded to a sharper initial falling phase. Both τ_1 and τ_2 described a difference in the time at which maximum offspring production occurred, but parameter τ_2 **(b)** was most strongly associated with the long-term falling phase of the curve, where the higher the value of this parameter the longer the falling phase and flatter the overall appearance of the curve. Parameter τ_3 **(c)** was not related to the time at which maximum offspring production occurred but was strongly associated with the magnitude of this maximum: a higher value of τ_3 corresponded to a lower maximum.

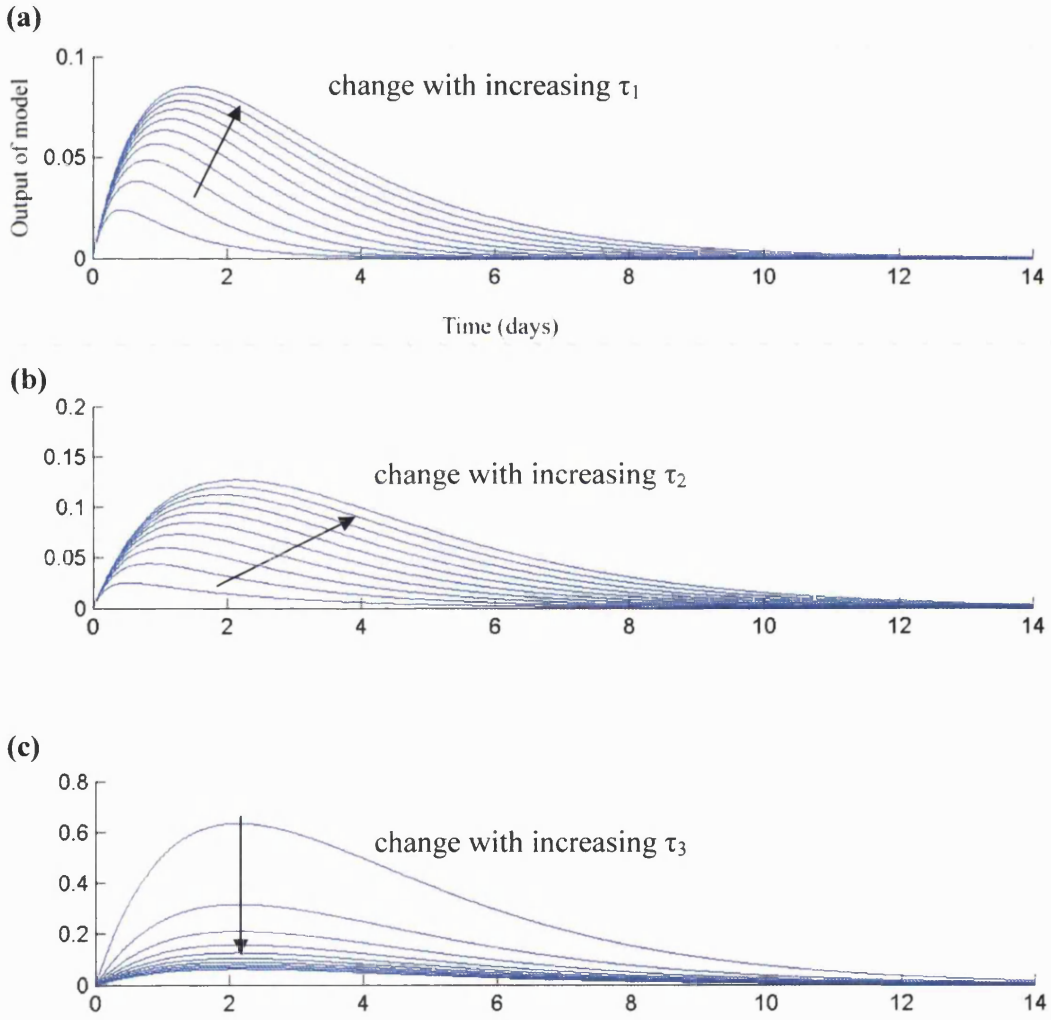


Table 4.1. (a) Median total number of female offspring (n_t) sired by the first and second males to mate 2SP and 3SP females, from the time of the second mating until female sterility. Females were mated first to bw^D and then to wild-type males or first to wild-type and then to bw^D males. **(b)** Median P_2 values (first male n_t/(first male n_t + second male n_t)) for 2SP and 3SP females mated first to bw^D and then to wild-type males or to wild-type, then to bw^D males.

(a)

mating order	sired by:	spermathecae	median n_t
bwD, wild-type	first male	2	29
		3	13
	second male	2	91
		3	59
wild-type, *bwD*	first male	2	42
		3	24
	second male	2	94
		3	82

(b)

mating order	spermathecae	median P_2
bwD, wild-type	2	0.749
	3	0.867
wild-type, *bwD*	2	0.723
	3	0.806

Table 4.2. (a) Comparisons between maximum time of offspring production (t_{nmax}) between 2SP and 3SP females. *P*-values show the results of Wilcoxon tests carried out on t_{nmax} within first and second male offspring and within mating order. **(b)** Comparisons of t_{nmax} for the two different mating orders adopted. Separate comparisons are made for females with 2SP and 3SP and for first and second male offspring.

(a)

offspring genotype	**mating order**	**spermathecal morphology**	**median t_{nmax} (day)**	***P*-value**
first male offspring	bw^D then wild-type	2SP	6	0.0357
		3SP	4	
second male offspring	bw^D then wild-type	2SP	4	0.0387
		3SP	2	
first male offspring	wild-type then bw^D	2SP	2	0.5527
		3SP	2	
second male offspring	wild-type then bw^D	2SP	4	0.3195
		3SP	4	

(b)

offspring genotype	spermathecae	mating order	median t_{nmax} (day)	*P*-value
first male offspring	2SP	$\underline{bw^D}$ then wild-type	6	0.0001
		wild-type then bw^D	2	
second male offspring	2SP	bw^D then wild-type	4	0.0001
		wild-type then $\underline{bw^D}$	4	
first male offspring	3SP	$\underline{bw^D}$ then wild-type	4	0.0055
		wild-type then bw^D	2	
second male offspring	3SP	bw^D then wild-type	2	0.0231
		wild-type then $\underline{bw^D}$	4	

Table 4.3. Median n_r produced during two-day samples from time of the second mating until female sterility. After correcting for multiple corrections, the critical *P*-value was 0.0071). *P*-values resulted from Wilcoxon tests carried out on each measurement of n_r separately to compare:

(a) offspring production by 2SP and 3SP females. Results for second male offspring only are shown here.

(b) offspring sired by the first and second males of bw^D or wild-type genotype. Offspring produced by 2SP females only are shown here.

(a)

sired by	mating order	spermathecae	days 2	4	6	8	10	12	14
second male	*bwD*, wild-type	2SP	32	35	16	1	0	0	0
		3SP	34	13.5	3	0	0	0	0
		P-value	0.9681	0.0098	0.0013	0.0030	0.3428	0.0864	0.2818
second male	wild-type, *bwD*	2SP	13	29	26	4	0	0	0
		3SP	17	34	24	5	1	0	0
		P-value	0.4506	0.4722	0.7419	0.3642	0.8194	0.1785	0.5382

(b)

spermathecae	sired by	mating order	days 2	4	6	8	10	12	14
2SP	first male	*bwD*, wild-type	2	5	4	0	0	0	0
		wild-type, *bwD*	22	10.5	3.5	0	0	0	0
		P-value	0.0001	0.0055	0.4415	0.0969	0.2852	0.9505	0.4058
2SP	second male	*bwD*, wild-type	32	35.5	16.5	1	0	0	0
		wild-type, *bwD*	13	29	26	4	0	0	0
		P-value	0.0001	0.0396	0.3087	0.0664	0.0843	0.2283	0.5149

Table 4.4. Median values for best-fit parameters of the two-compartment model (see Appendix) to n_r over time. *P*-values are the result of Wilcoxon tests carried out on the best-fit parameters to compare n_r over time for:

(a) 2SP and 3SP females. Results for second males only are shown here.

(b) offspring sired by the first and second males of different genotypes. Results for 2SP only are shown here.

(a)

sired by	mating order	spermathecae	parameters v_2	τ_1	τ_2	τ_3
2nd male	*bwD*, wild-type	median for 2SP	0.0001	1.590	1.560	5420
		median for 3SP	0.0031	1.050	1.150	8510
		P-value	0.4847	0.0031	0.0334	0.9556
2nd male	wild-type, *bwD*	median for 2SP	-0.0006	2.250	2.240	4430
		median for 3SP	-2.2700	2.050	1.780	368
		P-value	0.8202	0.6862	0.3247	0.3372

(b)

spermathecae	sired by	mating order	parameters v_2	τ_1	τ_2	τ_3
2SP	first male	$\underline{bw^D}$, wild-type	-0.2230	2.590	2.410	134000
		wild-type, $\underline{bw^D}$	0.0010	1.145	1.005	21.95
		P-value	0.0002	0.0001	0.0001	0.0001
2SP	second male	wild-type, $\underline{bw^D}$	-0.0006	2.250	2.240	4430
		bw^D, wild-type	0.0001	1.590	1.560	5420
		P-value	0.1466	0.0001	0.0002	0.8837

4.5. Discussion

Three important conclusions can be drawn from these experiments. First, the rate of offspring production by females with three spermathecae (3SP) increased and decreased more rapidly than for females with two (2SP), although this difference was significant only for females mated first to bw^D then to wild-type males. Second, and consistent with previous literature, there were dramatic differences in sperm displacement ability between males of different genotypes. Third, the results show that analysis of temporal patterns of offspring production can reveal features of sperm competition that are missed by tests carried out on P_2 or on the total numbers of offspring produced. These temporal patterns are important because, if a female dies or remates with a third male, the reproductive success of the second male to mate will be higher if his sperm is used faster (Anderson 1945; Prout and Bundgaard 1977; Gilbert et al. 1981; Gilchrist and Partridge 1995).

The rapid rise and fall of the rate of offspring production for 3SP compared to 2SP females may have been due to the faster release of sperm from three spermathecae with three ducts compared to two spermathecae and two ducts. This could have resulted in the loss of co-ordination between sperm release, ovulation and fertilisation. The present results are therefore consistent with two alternative hypotheses. The first is that a careful balance is required between sperm storage, release and fertilisation mechanisms, reflecting a more co-ordinated system than previously thought. Females with unusual spermathecal morphology may rely more on the seminal receptacle for sperm storage, an organ that fills and releases sperm more rapidly than do the spermathecae (Gilbert 1981). A second explanation is that 3SP females had reproductive tract defects. Past experiments have shown that females possessing only one spermathecae, either due to the *lozenge* mutation (Anderson 1945) or due to the ablation of one spermathecae (Bouletreau-Merle 1977), produce fertile eggs for a shorter duration than females with two spermathecae. This could be due to a reduced sperm storage capacity in ablated or mutant females, or to a reduction of fluid secreted by the spermathecae, a fluid postulated to maintain sperm longevity (Anderson 1945; Filosi and Perotti 1975). In the present experiment, 3SP females may have been less able to maintain viable sperm for fertilisation compared to 2SP females.

There is widespread interest in the selection pressures that maintain the complexity of female reproductive tracts in the insects. In *D. melanogaster*, genetic variation amongst females is correlated with the sperm precedence achieved by their mates (Clark and Begun 1998; Civetta and Clark 2000a), but it is not yet known how this genetic variation translates to physical variation amongst females. In a recent study, Miller and Pitnick (2003) showed that artificially selected differences in seminal receptacle length did not affect patterns of sperm precedence. However, this chapter shows that important variation in sperm storage organ morphology does exist in females and that it can affect sperm use. The variation in spermathecal morphology seen here provides only a blunt probe with which to investigate the influence of female reproductive morphology on sperm use. However, the principle that the sperm storage organs vary in size and shape could apply to females from natural populations and may be one mechanism by which genetic variation among females is correlated with variation in the degree of sperm precedence achieved by their mates (Clark and Begun 1998; Civetta and Clark 2000a).

Male genotype was also correlated with differences in the temporal patterns of sperm use. bw^D males were less able to displace first male sperm than were wild-type males. This is illustrated by the graphs in Fig. 4.4, where the graphs shown in column (a) show offspring sired by the first male to mate a female, both before and after the female mated for a second time. Just as the sperm of wild-type first males was not displaced by bw^D second males, maximum offspring production occurred significantly later for bw^D compared to wild-type offspring, and this finding was supported by analysis of the non-linear model parameters. The finding that bw^D males have poor sperm displacement ability is consistent with previous studies (Clark and Begun 1998), including direct observations of sperm storage in doubly mated females. Civetta (1999) made transgenic *D. melanogaster* with sperm expressing green fluorescent protein (GFP) in order to track labelled sperm inside the female reproductive tract. bw^D second males were less able to displace resident GFP-labelled sperm from the seminal receptacle compared to control males, although interestingly, the same study showed that both bw^D and control males displaced GFP-labelled sperm equally efficiently from the spermathecae (Civetta 1999). The differences in the sperm displacement abilities of bw^D and wild-type males could be an effect of genetic background or could be due to the bw^D allele itself. It is

possible that genetic differences are manifested as differences in the quantity or quality of Acps produced. Indeed, another observation described in this chapter was that females mated first to wild-type males remated significantly more slowly to a second male than did females mated first to bw^D males (section 4.3.2). This may have been due to differences in the relative abilities of wild-type and bw^D males to reduce female sexual receptivity or attractiveness – both influenced by Acps (Chen et al. 1988; Tram and Wolfner 1998).

For second male offspring too, analysis of the timing of maximum offspring production showed that the rising phase of bw^D offspring production was slower than for wild-type offspring, a delay that could be associated with the reduced ability of bw^D males to displace first male sperm. Despite this, bw^D second males sired similar total number of offspring as did wild-type males, siring offspring for longer than wild-type males. This delay in the temporal pattern of sperm use could be important for male reproductive success. It suggests that the reproductive success of bw^D compared to wild-type males would have been reduced had females mated a third time.

The important findings of this chapter result from analysis of simple features of the temporal patterns of offspring production (n_r and t_{nmax}), revealing details of sperm use over time that P_2 alone cannot. This chapter also illustrates how a function fitted to the pattern of offspring production can be used to examine such curves. The conclusions drawn from the analysis of model parameters are robust, as they are substantiated by independent tests on simple features of the curves (n_r and t_{nmax}). Before such a model is used as the sole analysis of temporal patterns, maximum likelihood methods should be used to establish which of a range of likely models (with 2, 3, 4 and 5 parameters, including the two-compartment model used here) might best describe the data. For example, an alternative model comprising two half-Gaussian curves is better at describing some of the patterns of offspring production in this data set, and analysis on this latter model yields similar results to those presented here (data not shown). For the experiment described here, a further assessment of the suitability of the model to fit the data may be possible if offspring counts were to be made at a finer resolution time intervals, particularly in the days immediately following remating.

Sperm use by females, and the extent to which males affect female post-mating physiology, are areas of intensive current interest. This study shows that the temporal pattern of sperm use is potentially an important determinant of success in sperm competition. I would not have seen such a complete picture of events had my analysis been restricted to the total numbers of offspring produced. Temporal patterns of sperm use have been examined before (Anderson 1945; Prout and Bundgaard 1977; Gilbert et al. 1981; Gilchrist and Partridge 1995), but this chapter presents several ways in which statistical analysis of such patterns can be carried out. Analysis of this type may, in the future, facilitate examination of the influence of male accessory gland proteins on sperm storage, patterns of offspring production and mechanisms of sperm competition.

Chapter 5. The Sex Peptide of *Drosophila melanogaster*: investigation of post-mating responses of females using RNA interference

5.1. Abstract

The Acps produced by *D. melanogaster* males induce a number of dramatic post-mating changes in females. For example, female sexual receptivity is reduced and egg laying is increased for several days after mating. Previous work has shown that injection of the Acp sex-peptide (SP) into the virgin female reproductive tract elicits both of these post-mating responses, as does ectopic expression of SP in the female. However, the female's responses to SP transferred during a normal mating have not yet been explored. A number of other components of the male seminal fluid, such as sperm, Dup99B and Acp26Aa, also contribute to the post-mating physiological and behavioural changes of females. It is impossible to determine the level of functional redundancy between components of the seminal fluid without males that lack SP, Dup99B, Acp26Aa or sperm. Here, I show that females mated to males subject to RNA interference to suppress SP expression had higher receptivity and laid fewer eggs post-mating than females mated to normal males. The changes in receptivity and egg laying of females mated to males lacking SP were short-lived compared to females mated to normal males. The results show that SP is required for the normal post-mating responses of females. The post-mating responses of females mated to males lacking SP resembled those of females mated to spermless males, and this suggests that the well documented and prolonged 'sperm effect' on female receptivity, is, in fact, a response to the SP.

5.2. Introduction

Female *D. melanogaster* undergo several physiological and behavioural changes after mating. Two of the most dramatic of these changes are a sharp reduction in sexual receptivity and a rapid elevation of egg laying which lasts for 5-7 days after mating. Female post-mating responses are triggered by the receipt of seminal fluid proteins produced in the male accessory glands (Kalb et al. 1993). By inhibiting female receptivity and by augmenting oviposition, males reduce the opportunity for rivals to compete for fertilisations. The first Acp identified in *D. melanogaster* was Acp70A, also called the sex-peptide (SP). Injection of SP into virgin females represses sexual receptivity and stimulates egg development and the rate of egg laying (Chen et al. 1988). Similar effects are seen during ectopic expression of SP in transgenic virgin females (Aigaki et al. 1991). SP is secreted by the main-cells of the accessory glands (Kubli 1992). It is coded by a single copy gene on the third chromosome and is translated as a 55 amino acid precursor. The precursor peptide then undergoes post-translational modification to cleave its 19 amino acid secretory signal sequence (Chen et al. 1988), resulting in the mature 36 amino acid peptide.

Sex-peptide-like effects have been demonstrated in a large number of fly species (Miller et al. 1994). Peptides homologous to the *D. melanogaster* SP are found in a number of other species in the melanogaster subgroup: *D. simulans*, *D. mauritiana* and *D. sechellia*. Injection of sex-peptides from *D. melanogaster* and *D. sechellia* are equally efficient in repressing female sexual receptivity in virgins of each of these four sibling species, demonstrating remarkable conservation of function (Chen et al. 1988). In *D. biarmipes*, a protein expressed in the ejaculatory duct of males has sequence homology to the *D. melanogaster* SP (Imamura et al. 1998). There is also homology between SP of *D. melanogaster* and the sex-peptide of *D. suzukii*, species that are more distantly related, and here too, the sex-peptides are functional in both species (Schmidt et al. 1993b). In contrast, reciprocal injections of accessory gland secretions between *D. melanogaster* and a more distantly related species, *D. funebris*, do not result in post-mating responses (Chen et al. 1985; Chen and Balmer 1989). The SP of *D. melanogaster* does however, have startling *in vitro* effects on the *corpora allata* of the moth *Helicoverpa armigera*, stimulating the production of juvenile hormone (Fan et al.

1999, 2000). Although the SP of *D. melanogaster* is the most extensively studied of accessory gland proteins in the insects, males lacking SP have never been created. It is therefore not yet known whether SP is necessary for these post-mating responses during normal matings.

Oocyte maturation in *D. melanogaster* progresses through a number of stages along ovarioles. The progression of oocytes beyond a putative control point at stage nine is stimulated by juvenile hormone production by the *corpora allata* (Soller et al.1999; Moshitzky et al 1996; Fan et al. 2000). SP increases juvenile hormone production in the *corpora allata* and in so doing, accelerates the maturation of oocytes (Moshitzky et al. 1996; Soller et al. 1999; Fan et al. 2000). The N-terminal of SP appears to be essential for it effect on oocyte maturation (Fan et al. 2000).

The receptors through which SP affects egg laying and sexual receptivity are not yet known (Soller et al. 1999). Fragments of SP elicit either both or neither of these responses, suggesting that both are triggered by the same active region: the C-terminus, of the protein (Schmidt et al. 1993a). In addition, ectopic expression of SP in a variety of tissues results in either both or none of the post-mating responses, indicating that both are elicited through the same target molecular or tissue (Nakayama et al. 1997). The idea that changes in both receptivity and egg laying are triggered through the same receptors is also supported by the finding that both responses require the same physiological quantities of SP (Schmidt et al. 1993a). Unpublished data suggests that SP binds to sperm and is thus localised in the sperm storage organs shortly after mating (S. Busser and E. Kubli, Unpublished data). Incubation of adult females with radio-labelled synthetic SP has shown that *in vitro*, SP binds to tissues of the female genital tract and nervous system (Ottiger et al. 2000), probably via the haemolymph (Chen et al. 1988; Aigaki et al. 1991; Schmidt et al. 1993a). It is not clear however, whether this *in vitro* picture reflects the true *in vivo* binding patterns.

SP is only one of an estimated 80 Acps passed to females during mating, many of which also affect female physiology and behaviour, and whose functions may overlap with SP. In *D. melanogaster*, Acp26Aa is a second Acp that stimulates egg-laying (Herndon and Wolfner 1995), although it does so through a different mechanism to that of SP and

during a different stage of ovulation. The effect of Acp26Aa on egg-laying is small; females mated to males lacking Acp26Aa lay 8-18% fewer eggs than normal, and this effect persists for only one day after mating (Herndon and Wolfner 1995). It is thought that other Acps also affect female receptivity and egg laying. The seminal fluid protein Dup99B is a 31 amino acid peptide expressed in the ejaculatory duct of males, which shares strong sequence homology to SP at its C-terminus. Injection of Dup99B into females induces oviposition and inhibition of receptivity to the same degree as SP (Saudan et al. 2002). However, unlike SP, Dup99B does not stimulate the *corpora allata* to produce juvenile hormone *in vitro*, and is not likely therefore to affect oogenesis (Fan et al. 2000). Dup99B also shares the *in vitro* binding patterns of SP (Ottiger et al. 2000), including binding to sperm (J. Peng and E. Kubli, Unpublished data), although it is not yet clear if these binding patterns accurately represent the *in vivo* situation. No differences in the sexual receptivity of females mated with males lacking Dup99B and those mated to normal males have been detected (H. Liu and E. Kubli, Unpublished data). Furthermore, females mated to males that transfer Dup99B but no Acps show no or little reduction in receptivity (Kalb et al. 1993; Xue and Noll 2000; Saudan et al. 2002). These experiments currently suggest that Dup99B is not important in the post-mating responses of females *in vivo*.

Sperm is also reported to affect female receptivity. Prolonged inhibition of female receptivity has long been associated with sperm storage (Manning 1962). The normal post-mating repression of receptivity lasts for 5-7 days. Matings to spermless males result in female post-mating responses lasting for only 1-2 days (Manning 1967; Kalb et al. 1993; Xue and Noll 2000), similar to the response elicited by injection of SP into virgin females (Chen et al. 1988). Manning (1962) hypothesised that the 'sperm effect' on female receptivity could either be due to the storage of sperm itself or to a chemical component associated with sperm.

Without males that lack SP, it is impossible to determine whether SP alone, or a number of different proteins, are required to elicit normal post-mating responses in females. RNA interference (RNAi) is a powerful technique for targeted gene suppression (Fire et al. 1998). RNAi of SP can be induced in transgenic males that possess a construct containing an inverted repeat segment of SP sequence. This inverted repeat SP DNA is

transcribed into inverted repeat SP RNA, which adopts a hairpin loop conformation. The presence of SP hairpin loop RNA induces the destruction of endogenous SP mRNA, preventing SP protein from being expressed. Using male flies that lacked any detectable SP expression, I show in this chapter that the normal female post-mating changes in receptivity and oviposition require the receipt of SP. Females mated to males lacking SP laid fewer eggs and had higher receptivity than females mated to normal males, and rapidly returned to virgin levels of receptivity and egg laying. Females mated to males lacking SP produced eggs of normal fertility. SP knock-down males were therefore thought to pass normal quantities of sperm to females, sperm that were stored and used in numbers comparable to that of normal males. This experiment demonstrates the efficiency of RNAi and its potential in determining the phenotype of other Acps in the future.

5.3. Materials and Methods

5.3.1. Stocks

5.3.1.1. *UAS-SP-IR* stocks

In order to induce targeted suppression of SP using RNAi, stocks were produced that possessed a SP sequence in an inverted repeat orientation adjacent to an upstream activating sequence. Stocks possessing these elements were produced as described in Chapman et al. (In press, see Appendix III). A vector (pSP-IR), marked with *white*$^{+}$, was created, and this vector contained an upstream activating sequence (*UAS*) and 305bp of sequence of the SP coding region in inverted repeat orientation (*SP-IR*). The vector was injected into a *white*^{1} genetic background and lines with three separate *SP-IR* transgenic insertions were recovered. These stocks were made homozygous by single pair crossing and this resulted in three transgenic stocks called *UAS-SP-IR1*, *UAS-SP-IR2* and *UAS-SP-IR3*.

5.3.1.2. *Acp26Aa-P-Gal4* stocks

Gal4 is a yeast transcriptional activator that drives expression of *UAS*. Tissue-specific RNAi is induced if the individual possesses the *UAS-SP-IR* construct and tissue-specific expression of *Gal4*. A stock possessing X-linked *Gal4* was produced as described in

Chapman et al. (In press, see Appendix III). *Gal4* was fused to the promotor of *Acp26Aa*, a gene expressed exclusively in the male accessory glands. This resulted in the transgenic stock *Acp26Aa-P-Gal4*. *Acp26Aa-P-Gal4* males express *Gal4* only in the male accessory glands (Fig. 5.3).

5.3.1.3. Generation of SP knock-down and control males

Putative SP knock-down males used for the experiment were obtained by crossing homozygous males from each of the three transgenic inverted repeat insert lines recovered (*UAS-SP-IR1, UAS-SP-IR*2 and *UAS-SP-IR3*) to virgin females from the *Acp26Aa-P-Gal4* line (Fig. 5.1; Chapman et al. In press, see Appendix III). This resulted in 3 putative SP knock-down male lines *Acp26Aa-P-Gal4;UAS-SP-IR1*, *Acp26Aa-P-Gal4;UAS-SP-IR2* and *Acp26Aa-P-Gal4;UAS-SP-IR3*. As the *Gal4* driver was X-linked, positive controls were the male offspring of the reciprocals of these crosses (Fig. 5.1): homozygous females from each of the three *UAS-SP-IR* lines crossed to *Acp26Aa-P-Gal4* males. The resulting control males (*+;UAS-SP-IR1*, *+;UAS-SP-IR2* and *+;UAS-SP-IR3*) shared the same genetic background as the putative knock-down males, except for the X-chromosome.

The *UAS-SP-IR3* insertion appeared to be unstable, as *white*^{1} revertants were often observed in the stock. For this reason, experiments on post-mating responses of females were conducted using two of the transgenic stocks only (i.e. *UAS-SR-IR1* and *UAS-SP-IR2*). During the receptivity, oviposition, ovulation and fertility tests, matched pairs of experimental and control lines were used: *+;UAS-SP-IR1* and *+;UAS-SP-IR2* males acted as controls for *Acp26Aa-P-Gal4;UAS-SP-IR1* and *Acp26Aa-P-Gal4;UAS-SP-IR2* males respectively. Males for the experiments were generated by placing 5 virgin male and 5 virgin female parents of each cross into vials for three days before transferring into fresh vials for a further 3 days. Virgin male offspring were collected from these vials over 4 days.

5.3.1.4. Wild-type strain culturing

Dahomey wild-type females (section 2.1.6) were collected from population cages and raised at standard density. Females were collected within 7 hours of eclosion and kept in

vials on Maize-Yeast medium supplemented with live yeast, which was used throughout the experiment.

5.3.1.5. Testing levels of SP expression using Western blots

The levels of SP protein expressed by the putative SP knock-down (*Acp26Aa-P-Gal4;UAS-SP-IR1*, *Acp26Aa-P-Gal4;UAS-SP-IR2* and *Acp26Aa-P-Gal4;UAS-SP-IR3*) and control (*+;UAS-SP-IR1*, *+;UAS-SP-IR2* and *+;UAS-SP-IR3*) males were determined using Western blots. Additional controls used in the Western blots were homozygous males from each *UAS-SP-IR* insert lines and the *Acp26Aa-P-Gal4* line. Both virgin and mated males were tested for expression of SP protein. Mating induces the expression of Acps (DiBenedetto et al. 1990; Herndon et al. 1997; Wolfner et al. 1997) and RNAi efficiency could therefore potentially be increased by mating young males to stimulate the transcription of Acps and RNAi transgenes. Previous optimization experiments (Lung 2000) using an RNAi transgene designed to knock-down levels of another Acp, Acp62F, suggested that mated males had more efficient RNAi of the target gene than unmated males. To test mated males for levels of SP protein expressed, two-day old virgin male progeny from all experimental and control crosses were mated *en masse* to females from their own lines. Before carrying out Western blots, mated males and virgin males of the same age and genotype were transferred in groups of 20 to fresh food vials. 24 hours later, protein was extracted from groups of 5 males (section 2.2.7) and Western blots were then carried out by G. Vinti (Chapman et al. In press, see Appendix III).

5.3.2. Effect of SP on female receptivity

For all experimental and control crosses, 1 to 3-day-old virgin male offspring were mated *en masse* to females from their own vials and stored in single sex groups of 10 for 1-2 days before the start of the experiment. To determine the effect of SP on female receptivity, wild-type females were mated once to SP knock-down or control males, then exposed to wild-type males in a receptivity assay 24 and 48 hours later. Virgin wild-type females were raised at standard density (section 2.2.3), collected from these cultures on a single day within 7 hours of eclosion, and stored in groups of 10 flies per vial. Two days later, pairs consisting of a single 4 to 5-day-old knock-down or control

male, and a single 4-day-old wild-type virgin female, were put into individual vials using CO_2 as an anaesthetic. Approximately 130 pairs of each cross and control were set up. Pairs were observed for about 8 hours and 120-150 pairs from each line mated within this time. Immediately after mating, males were removed from the vials. After 1 day, half of these once-mated females were tested for remating propensity. A 5-day-old wild type male was introduced into each of the vials, and these pairs were observed for remating. The number of females remating within 1 hour was recorded. Using the remaining females, this test was repeated two days after the initial mating, with 6-day-old wild type males.

5.3.3. Effect of SP on oviposition

2-day-old wild-type virgin females were mated in single pairs to 4 to 5-day-old SP knock-down (*UAS-SR-IR1*, *UAS-SP-IR2*) or control (*+;UAS-SP-IR1* and *+;UAS-SP-IR2*) males. This was carried out using Maize-Yeast medium with added powered charcoal to make the food black and the eggs more visible (section 2.2.5). 60 pairs of each cross and control were set up. In addition, 60 unmated females were included as a virgin control. Females were transferred to fresh food vials every 24 hours for 5 days and the number of eggs laid every day was counted.

5.3.4. Effect of SP on ovulation

3-day-old virgin wild-type females were mated in single pairs to 5 to 6-day-old SP knock-down (*UAS-SR-IR1*, *UAS-SP-IR2*) or control (*+;UAS-SP-IR1* and *+;UAS-SP-IR2*) males. 70 pairs of each knock-down and control treatment were set up. In addition, 70 unmated females were included as a virgin control. At 6, 24 and 48 hours after the first mating, subsets of approximately 25 females from each group were anaesthetised using ice and dissected in PBS to determine the percentage of females in each group with an egg in the uterus (after Chen et al. 1988).

5.3.5. Effect of SP on egg fertility

In order to determine egg fertility (number of offspring / number of eggs laid x 100), food vials from days 1,3 and 5 of the oviposition assay (section 5.3.3) were retained to count progeny. Offspring were counted 7-8 days after the eggs were laid. At the time of

counting, offspring were at the pupal stage and were consequently easily visible on the sides of the glass vial.

5.4. Results

5.4.1. Recovery of SP RNAi and Gal4 transgenic stocks

The recovery of the three *UAS* SP transgenic stocks (described in Chapman et al, In press, Appendix III) was made by identifying flies carrying the *white*$^{+}$ marker among the progeny of injected embryos. The *UAS-SP-IR1*, *UAS-SP-IR2* and *UAS-SP-IR3* lines contained insertions on chromosomes III, II and II respectively. The *UAS-SP-IR3* insertion appeared to be unstable, as *white*^{1} revertants were often observed in the stock. For this reason, experiments on post-mating responses of females were conducted using two of the transgenic stocks only (i.e. *UAS-SR-IR1*, *UAS-SP-IR2*).

5.4.2. Levels of SP expression as determined by Western blots

Western blotting showed that males carrying the *Acp26Aa-P-Gal4* driver and each of the three *UAS-SP-IR* transgenes produced no detectable SP (Fig. 5.2, lanes 6, 8 and 10). This was the case for both mated and virgin males. All control males produced detectable SP (Fig. 5.2, lanes 5, 7 and 9), as did the four parental lines (Fig. 5.2, lanes 1 to 4).

5.4.3. Effect of SP on female receptivity

The receptivity of females mated to knock-down males (*Acp26Aa-P-Gal4;UAS-SP-IR1* and *Acp26Aa-P-Gal4;UAS*-SP-IR2) was compared to the receptivity of females mated to their respective controls (*+;UAS-SP-IR1* and *+;UAS-SP-IR2*) at 24 and 48 hours after mating (Fig. 5.4). The numbers of females that did and did not mate within 1 hour of being placed with a wild-type male following each type of initial mating, were analysed in 2 x 2 contingency tables using Fisher exact tests. The results (Table 5.1) show that females mated to males deficient in SP were significantly more receptive than were mates of control males. Females mated to both types of SP knock-down males (*Acp26Aa-P-Gal4;UAS-SP-IR1* and *Acp26Aa-P-Gal4;UAS-SP-IR2*) were significantly more receptive than their respective controls (females mated to *+;UAS-SP-IR1* and

+;UAS-SR-IR2 males; Table 5.1). At 24 hours, the receptivity of females mated to SP knock-down males was intermediate between that of females mated to control males and that of virgin females. Females mated to SP knock-down males were significantly less receptive than virgin females at 24 hours. By 48 hours, the receptivity of females mated to SP knock-down males was similar to that of virgins (Table 5.1). Mates of SP knock-down males therefore did not have virgin levels of sexual receptivity until 48 hours after mating; there was some residual reduction in receptivity caused by matings to SP knock-down males.

5.4.4. Effect of SP on oviposition and ovulation

The numbers of eggs laid over 5 days post-mating were analysed using Wilcoxon tests. On each of the five successive days after mating, females mated to SP knock-down males laid significantly fewer eggs than did females mated to control males (with one exception) (Fig. 5.5; Table 5.2). After 1 day, females mated to *Acp26Aa-P-Gal4;UAS-SP-IR1* SP knock-down males did not differ in egg-production from mates of +; *UAS-SP-IR1* males, but on days 2-5 laid significantly fewer eggs than their mated controls. Females mated to the other SP knock-down line males (*Acp26Aa-P-Gal4;UAS-SP-IR2*) laid significantly fewer eggs than their controls (mates of *+;UAS-SP-IR2* males) on all days. On days 1-2, mates of SP knock-down males laid eggs at an intermediate level between that of females mated to control males and that of virgin females. Thereafter, egg-production of females mated to SP knock-down males was similar to that of virgins (Table 5.2).

The numbers of females that did and did not have an egg in the uterus were analysed in 2 x 2 contingency tables using Fisher exact tests. Females mated to SP knock-down males were significantly less likely to have an egg in the uterus than were their respective control females (Fig. 5.6, Table 5.3). At 6, 24 and 48 hours after mating, females mated to SP knock-down males from both lines showed significantly lower ovulation than mates of control males (with one exception). Females mated to SP knock-down males were also significantly more likely (in all comparisons except one) to have an egg in the uterus than were virgin females (Table 5.3).

5.4.5. Effect of SP on egg fertility

The fertility of eggs laid during the oviposition assay (section 5.3.3) was monitored to check that SP knock-down males were as fertile as control males. The fertility and offspring production data were analysed using Wilcoxon tests. On days 1 and 3, the fertility of eggs produced by females mated to SP knock-down males was not significantly different from the fertility of eggs produced by females mated to control males (Fig. 5.7; Table 5.4). On day 5, the fertility of eggs laid by mates of control males was significantly lower than that of females mated to SP knock-down males. On day 5, females of all lines produced non-significantly different absolute numbers of offspring (Table 5.5). This suggests that there were no significant differences in the numbers of sperm stored across treatments despite enormously elevated rates of egg laying by females mated to control males. Lower fertility in the control lines on day 5 (Fig. 5.7) may also have been due to the high levels of egg production by females mated to control males during the preceding days. This would have led them to run out of sperm more quickly than females mated to the SP knock-down males (Trevitt et al. 1988).

On days 1 and 3, the absolute number of offspring produced by mates of control males was significantly higher than those produced by mates of SP knock-down males on days 1 and 3 (Fig. 5.8; Table 5.5). This was due to the differences in egg laying rates between these lines. On day 5, the numbers of offspring produced by all groups of females were almost identical, despite significantly larger numbers of eggs being produced by females mated to control males than by females mated to normal males. The results of the egg fertility and offspring production tests indicate that SP knock-down males transferred sperm and that females stored and used these sperm normally and in numbers comparable to females mated to knock-down males.

Figure 5.1. Crossing scheme describing the generation of SP knock-down and control males. SP knock-down males were obtained by crossing homozygous males from each of the transgenic inverted repeat insert lines recovered (*UAS-SP-IR1* and *UAS-SP-IR2*) to virgin females from the *Acp26Aa-P-Gal4* line, whose *Gal4* expression is accessory gland specific. These crosses (**i** and **iii**) resulted in males lacking SP (*Acp26Aa-P-Gal4;UAS-SP-IR1*, *Acp26Aa-P-Gal4;UAS-SP-IR2*). Positive controls were the male offspring of the reciprocals of these crosses (**ii** and **iv**): homozygous females from each of the three *UAS-SP-IR* lines crossed to *Acp26Aa-P-Gal4* males. The resulting control males (*+;UAS-SP-IR1*, *+;UAS-SP-IR2* and *+;UAS-SP-IR3*) shared the genetic background of the knock-down males, except for the X-chromosome.

(i)

UAS-SP-IR1/UAS-SP-IR1 ♂ x *Acp26Aa-P-Gal4/Acp26Aa-P-Gal4* ♀

↓

Acp26Aa-P-Gal4;UAS-SP-IR1 ♂offspring, producing **knocked-down** levels of SP

(ii)

Acp26Aa-P-Gal4/Acp26Aa-P-Gal4 ♂ x *UAS-SP-IR1/UAS-SP-IR1* ♀

↓

+;UAS-SP-IR1 ♂offspring, producing **control** levels of SP

(iii)

UAS-SP-IR2/UAS-SP-IR2 ♂ x *Acp26Aa-P-Gal4/Acp26Aa-P-Gal4* ♀

↓

Acp26Aa-P-Gal4;UAS-SP-IR2; ♂offspring, producing **knocked-down** levels of SP

(iv)

Acp26Aa-P-Gal4/Acp26Aa-P-Gal4 ♂ x *UAS-SP-IR2/UAS-SP-IR2* ♀

↓

+;UAS-SP-IR2 ♂offspring, producing **control** levels of SP

Figure 5.2. Western blot showing levels of SP in SP knock-down and control males, when the 3-day-old males were **(a)** virgins and **(b)** mated at 2 days old. From left, in lanes 1-4 homozygous *UAS-SP-IR1*, *UAS-SP-IR2*, *UAS-SP-IR3* and *Acp26Aa-P-Gal4* control males produced SP. In lanes 5, 7 and 9, control males carrying the inverted repeat insert without the Gal4 driver (*+;UAS-SP-IR1, +;UAS-SP-IR2* and *+;UAS-SP-IR3*) produced SP. In lanes 6, 8 and 10, males with the inverted repeat and the Gal4 driver (*Acp26Aa-P-Gal4;UAS-SP-IR1*, *Acp26Aa-P-Gal4;UAS-SP-IR2* and *Acp26Aa-P-Gal4;UAS-SP-IR3*) produced no detectable SP. The sex-peptide has a predicted molecular weight of 4428Da.

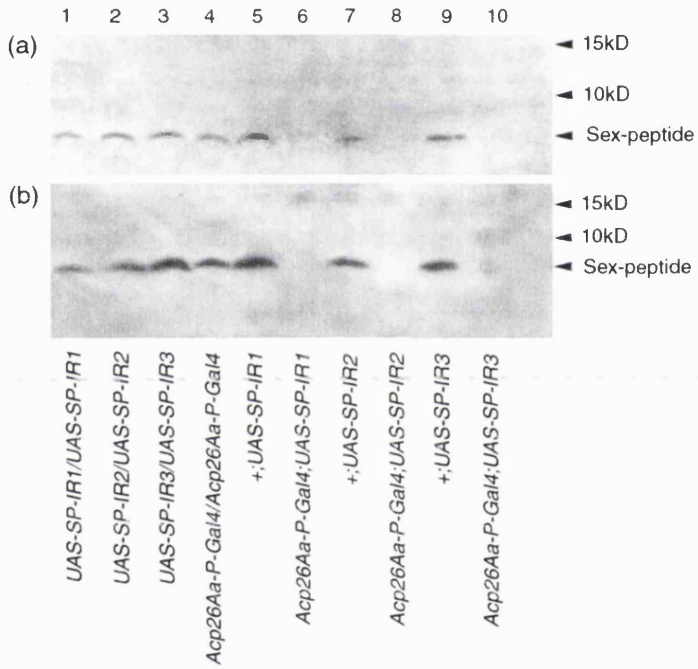
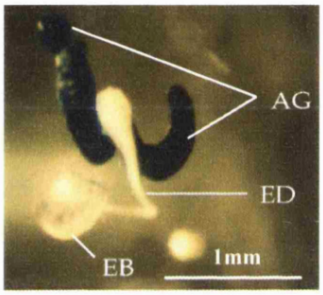


Figure 5.3. Specificity of expression driven by the *Acp26Aa-P-Gal4* transgene, as demonstrated by lacZ expression in *Acp26Aa-P-Gal4;UAS-lacZ* males (Lung 2000). **(a)** The *Acp26Aa-P-Gal4* transgene drives expression *of UAS-lacZ* specifically in male accessory glands (AG) but not the ejaculatory duct (ED) or ejaculatory bulb (EB). **(b)** No lacZ expression is detected in control males, which are homozygous for *UAS-lacZ* but lack the Gal4 driver (picture: Lung 2000; Chapman et al. In press).

(a)



(b)

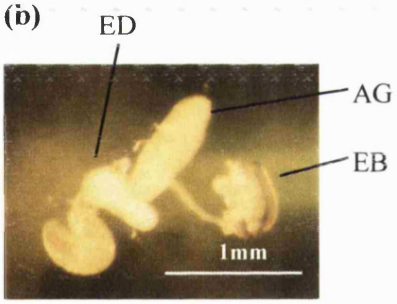


Figure 5.4. Effect of SP on female receptivity. The percentage of females remating within 1 hour in a receptivity test with wild-type males, 24 and 48 hours after initial matings to (from left to right) *Acp26Aa-P-Gal4;UAS-SP-IR1* males, *+;UAS-SP-IR1* males, *Acp26Aa-P-Gal4;UAS-SP-IR2* males or *+;UAS-SP-IR2* males. The receptivity of virgin females is also shown.

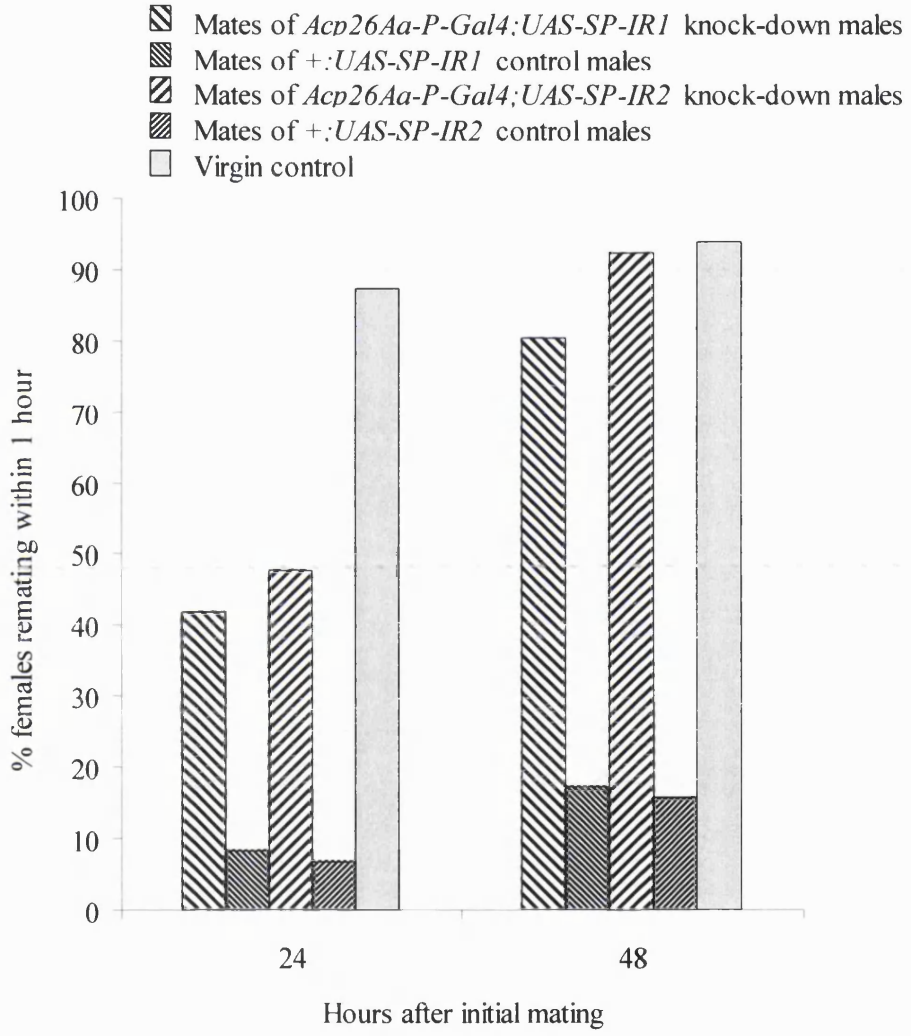


Figure 5.5. Effect of SP on oviposition. The median (± interquartile range) numbers of eggs laid per 24 hours by females over 5 days after mating to (from left to right) *Acp26Aa-P-Gal4;UAS-SP-IR1* males, *+;UAS-SP-IR1* males, *Acp26Aa-P-Gal4;UAS-SP-IR2* males, or *+;UAS-SP-IR2* males. Data for unmated virgin females are also shown.

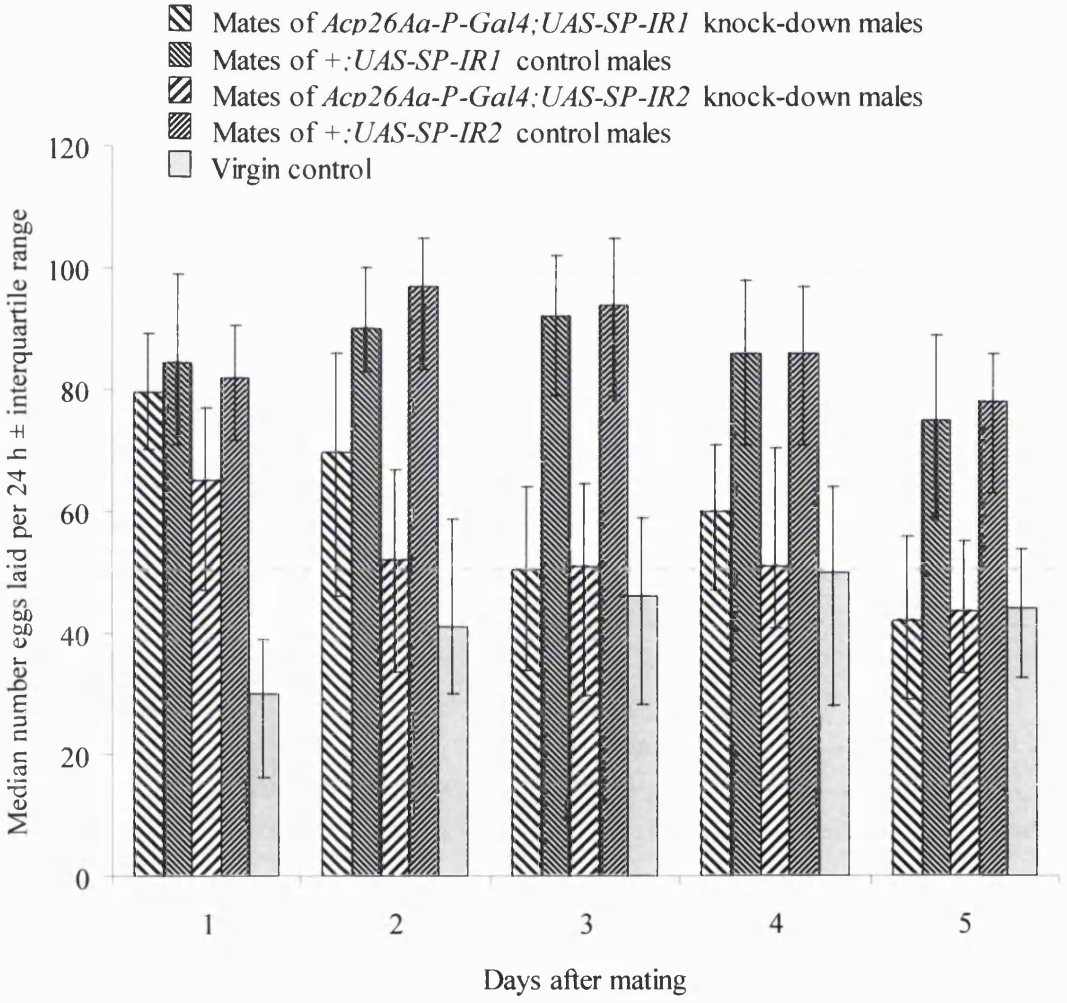


Figure 5.6. Effect of SP on ovulation. The percentage of females with a egg in the uterus 6, 24 and 48 hours after mating to (from left to right) *Acp26Aa-P-Gal4;UAS-SP-IR1* males, *+;UAS-SP-IR1* males, *Acp26Aa-P-Gal4;UAS-SP-IR2* males or *+;UAS-SP-IR2* males. Data for unmated virgin females are also shown.

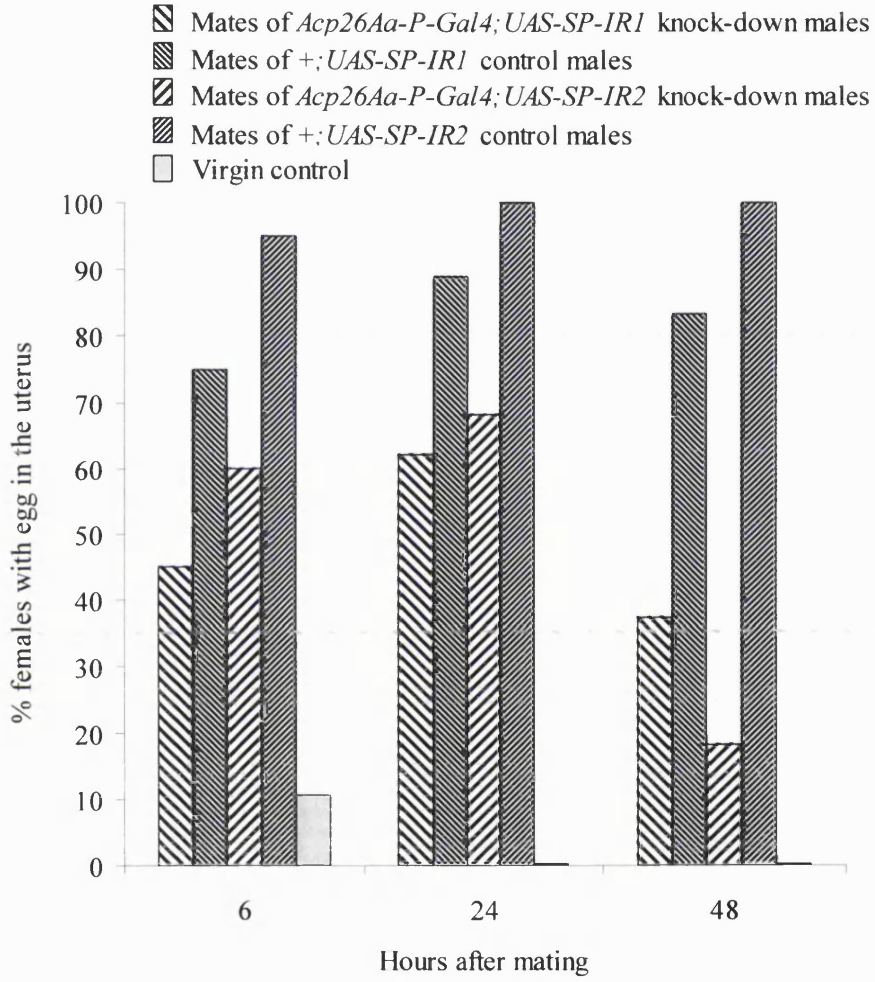


Figure 5.7. Effect of SP on egg fertility. Egg fertility (number of offspring / number of eggs laid x 100, ± interquartile range) of females 1, 3 and 5 days after mating to (from left to right) *Acp26Aa-P-Gal4;UAS-SP-IR1* males, *+;UAS-SP-IR1* males, *Acp26Aa-P-Gal4;UAS-SP-IR2* males or*+;UAS-SP-IR2* males.

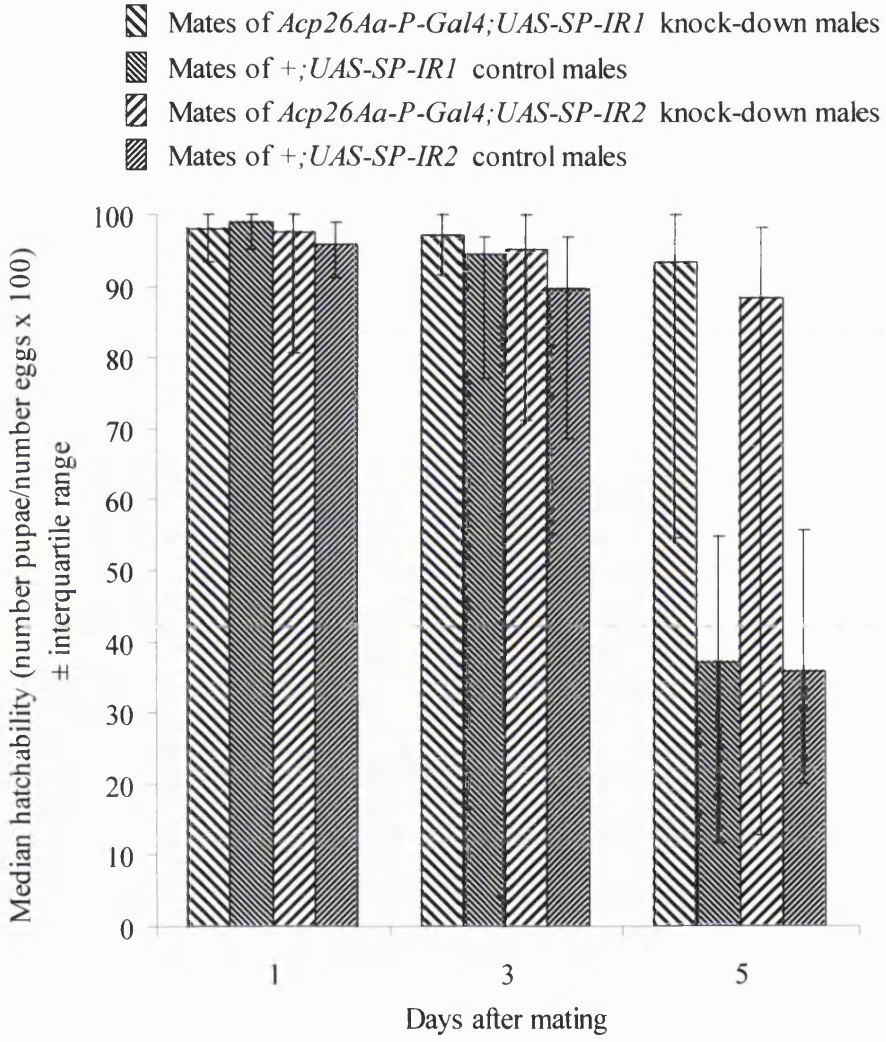


Figure 5.8. Effect of SP on offspring production. Numbers of offspring produced per female 1, 3 and 5 days after mating to *Acp26Aa-P-Gal4;UAS-SP-IR1* males, *+;UAS-SP-IR1* males, *Acp26Aa-P-Gal4;UAS-SP-IR2* males or*+;UAS-SP-IR2* males.

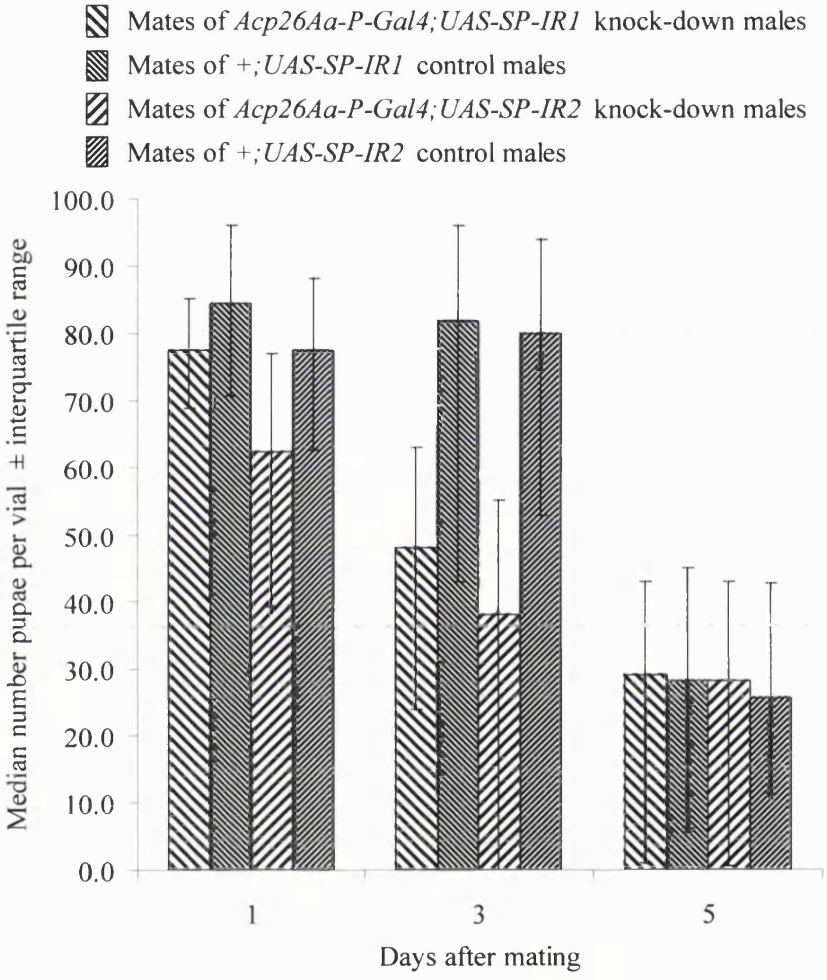


Table 5.1: *P*-values resulting from Fisher exact tests carried out to compare the percentage of females remating within 1 hour, 24 and 48 hours after mating to SP knock-down (*Acp26Aa-P-Gal4;UAS-SP-IR1*, *Acp26Aa-P-Gal4;UAS-SP-IR2*) and control (*+;UAS-SP-IR1* and *+;UAS-SP-IR2*) males. Also shown are comparisons between the percentage of virgin females remating within 1 hour, and females remating within one hour, 24 and 48 hours after mating to SP knock-down males.

% females remating within one hour, after first mating to:	Time after initial mating: **24 hours**	**48 hours**
***Acp26Aa-P-Gal4;UAS-SP-IR1* and *+;UAS-SP-IR1* males**	<0.0001	<0.0001
***Acp26Aa-P-Gal4;UAS-SP-IR2* and *+;UAS-SP-IR2* males**	<0.0001	<0.0001
***Acp26Aa-P-Gal4;UAS-SP-IR1* and virgin females**	<0.0001	0.0282
***Acp26Aa-P-Gal4;UAS-SP-IR2* and virgin females**	<0.0001	1.0000

Table 5.2: Test statistics and *P*-values resulting from Wilcoxon tests carried out to compare the numbers of eggs laid by females on 5 consecutive days after mating to SP knock-down (*Acp26Aa-P-Gal4;UAS-SP-IR1*, *Acp26Aa-P-Gal4;UAS-SP-IR2*) and control (*+;UAS-SP-IR1* and *+;UAS-SP-IR2*) males. Also shown are comparisons between the numbers of eggs laid by virgin females and the numbers of eggs laid by females mated to SP knock-down (*Acp26Aa-P-Gal4;UAS-SP-IR1*, *Acp26Aa-P-Gal4;UAS-SP-IR2*) males.

	Days after initial mating:				
Eggs laid by females mated to:	**1**	**2**	**3**	**4**	**5**
Acp26Aa-P-Gal4;UAS-SP-IR1 **and** ***+;UAS-SP-IR1*** **males**	χ^2=2.55 *P*=0.1102	χ^2=27.20 *P*<0.0001	χ^2=51.26 *P*<0.0001	χ^2=30.48 *P*<0.0001	χ^2=36.82 *P*<0.0001
Acp26Aa-P-Gal4;UAS-SP-IR2 **and** ***+;UAS-SP-IR2*** **males**	χ^2=17.05 *P*<0.0001	χ^2=51.39 *P*<0.0001	χ^2=48.77 *P*<0.0001	χ^2=38.25 *P*<0.0001	χ^2=35.56 *P*<0.0001
Acp26Aa-P-Gal4;UAS-SP-IR1 **and virgin females**	χ^2=70.53 *P*<0.0001	χ^2=18.29 *P*<0.0001	χ^2=0.9300 *P*=0.3349	χ^2=5.602 *P*=0.0179	χ^2=0.1878 *P*=0.6647
Acp26Aa-P-Gal4;UAS-SP-IR2 **and virgin females**	χ^2=45.55 *P*<0.0001	χ^2=2.35 *P*=0.1251	χ^2=0.6103 *P*=0.4347	χ^2=1.4209 *P*=0.2333	χ^2=0.0296 *P*=0.8633

Table 5.3: *P*-values resulting from Fisher exact tests carried out to compare the percentage of females with an egg in the uterus 6, 24 and 48 hours after mating to SP knock-down (*Acp26Aa-P-Gal4;UAS-SP-IR1*, *Acp26Aa-P-Gal4;UAS-SP-IR2*) and control (*+;UAS-SP-IR1* and *+;UAS-SP-IR2*) males. Also shown are comparisons between the percentage of virgin females with an egg in the uterus, and females mated to SP knock-down (*Acp26Aa-P-Gal4;UAS-SP-IR1*, *Acp26Aa-P-Gal4;UAS-SP-IR2*) males.

Comparing % females with egg in the uterus for females mated to:	Hours after initial mating: 6	24	48
Acp26Aa-P-Gal4;UAS-SP-IR1* and *+;UAS-SP-IR1 males	0.1053	0.0304	0.0003
Acp26Aa-P-Gal4;UAS-SP-IR2* and *+;UAS-SP-IR2 males	0.0197	0.0032	<0.0001
***Acp26Aa-P-Gal4;UAS-SP-IR1* and virgin females**	0.0309	<0.0001	0.0020
***Acp26Aa-P-Gal4;UAS-SP-IR2* and virgin females**	0.0022	<0.0000	0.1135

Table 5.4: Test statistics and *P*-values resulting from Wilcoxon tests carried out to compare the fertility of eggs laid by females on 5 consecutive days after mating to SP knock-down (*Acp26Aa-P-Gal4;UAS-SP-IR1*, *Acp26Aa-P-Gal4;UAS-SP-IR2*) and control (*+;UAS-SP-IR1* and *+;UAS-SP-IR2*) males.

Comparing fertility of eggs produced by females mated to:	Day after initial mating: 1	3	5
***Acp26Aa-P-Gal4;UAS-SP-IR1* and *+;UAS-SP-IR1* males**	χ^2=1.8223 *P*=0.1770	χ^2=3.8856 *P*=0.0487	χ^2=21.27 *P*<0.0001
***Acp26Aa-P-Gal4;UAS-SP-IR2* and *+;UAS-SP-IR2* males**	χ^2=0.4540 *P*=0.5005	χ^2=1.8621 *P*=0.1736	χ^2=11.60 *P*=0.0007

Table 5.5: Test statistics and *P*-values resulting from Wilcoxon tests carried out to compare the numbers of offspring resulting from eggs laid by females on 5 consecutive days after mating to SP knock-down (*Acp26Aa-P-Gal4;UAS-SP-IR1*, *Acp26Aa-P-Gal4;UAS-SP-IR2*) and control (*+;UAS-SP-IR1* and *+;UAS-SP-IR2*) males.

Comparing offspring resulting from eggs produced by females mated to:	Days after initial mating: 1	3	5
***Acp26Aa-P-Gal4;UAS-SP-IR1* and *+;UAS-SP-IR1* males**	χ^2=4.6957 *P*=0.0305	χ^2=23.8364 *P*<0.0001	χ^2=0.0685 *P*=0.7936
***Acp26Aa-P-Gal4;UAS-SP-IR2* and *+;UAS-SP-IR2* males**	χ^2=13.01 *P*=0.0003	χ^2=29.18 *P*<0.0001	χ^2=0.3553 *P*=0.5112

5.5. Discussion

The experiments of this chapter show that receipt of SP is necessary for the full post-mating changes in female receptivity and egg laying. Following matings to males with suppressed levels of SP expression, females were more receptive than females mated to normal males at both 24 and 48 hours after mating. By 48 hours after mating, the receptivity of females mated to males lacking SP returned to a level similar to that of virgins. Mates of SP knock-down males also ovulated and oviposited fewer eggs than females mated to normal males, and by three days post-mating, oviposition rate was similar to that of virgins. However, there was some short-term residual stimulation of egg-production and ovulation following matings to SP knock-down males. These residual, short-term effects must be caused by the transfer of Acps other than the SP, as females mated to males lacking all Acps have virgin levels of sexual receptivity and egg laying immediately after mating (Kalb et al. 1993).

The results described here are quantitatively similar to those of a concurrent study carried out by Liu and Kubli (In press). These authors used homologous recombination to produce males that possessed a mutant SP gene and therefore produced no SP. Similar to the experiments described in this chapter, mates of males lacking SP showed some residual reduction in receptivity and stimulation of egg-production. The residual effects seen in Liu and Kubli (In press) were smaller in magnitude than those observed in this chapter. However, any differences between the two studies are likely attributable to differences in the fly strains and culturing techniques used; the virgin levels of egg laying for the two studies were markedly different from one another. A past study has shown that egg laying shows substantial natural genetic variation between strains as well as clinal, geographic variation (Bouletreau-Merle et al. 1992).

This chapter demonstrates the efficiency with which RNAi can suppress gene expression. It suggests that the same approach will provide a useful strategy for examining the functions of other Acps. Western blots showed that neither virgin or mated SP knock-down males expressed any ~~Acp62F~~ SP. Previous optimisation experiments, testing the efficiency of RNAi of the gene Acp62F (Lung 2000), supported the idea that young mated males would exhibit more efficient RNAi compared to virgin

males. However, this was not the case for RNAi of SP using the constructs described here. The tests described in this chapter show that neither virgin nor mated SP knock-down males show any detectable SP protein expression.

Despite an apparent absence of protein, SP expression was explored quantitatively by measuring the levels of SP mRNA using real-time PCR (carried out by G. Vinti, results not shown here). Real-time PCR test confirmed that RNAi knocked down levels of SP mRNA considerably (to 10.5% of normal levels for *Acp26Aa-P-Gal4;UAS-SP-IR1* males and 1.2% of normal levels for *Acp26Aa-P-Gal4;UAS-SP-IR2* males), but that detectable levels of mRNA were still apparent. This may mean that, although undetected by the Western blots, small quantities of SP continue to be expressed in the SP knock-down lines. Low levels of SP expression could explain the residual short term post-mating effects seen in females mated to SP knock-down males. However, this is unlikely because these residual post-mating effects of females were also observed by Liu and Kubli (In press), who used mutagenesis to knock out SP gene expression. Alternatively, the small quantities of SP mRNA detected by real-time PCR analysis could be due to a time-lag between the production of endogenous mRNA and its destruction by RNAi. In this case, the presence of SP mRNA would not necessarily indicate residual levels of SP protein expression.

The real-time PCR method was also used to detect levels of Dup99B mRNA produced in the same samples of males (carried out by G. Vinti, results not shown here). Dup99B is expressed in the ejaculatory duct of males and, when injected into females, elicits similar post-mating responses to SP (Saudan et al. 2002). Although the *Gal4* driver used to drive RNAi of SP was expressed exclusively in the accessory glands (Lung 2000) and Dup99B is expressed only in the ejaculatory duct (Saudan et al. 2002), there was some concern that the SP RNAi construct could 'leak' and, due to the strong homology between SP and Dup99B, affect Dup99B expression. Previous experiments have shown that females mated to males lacking Dup99B did not differ in their post-mating responses compared to those mated to normal males. Nevertheless, it was still important to test for levels of Dup99B expression in order to show that RNAi is Acp-specific and to exclude the possibility that Dup99B was affected by RNAi for SP. Quantification of Dup99B confirmed that levels of Dup99B in the SP knock-down lines remained high

and at a level comparable to that in the control lines (94.9% of normal levels for *Acp26Aa-P-Gal4;UAS-SP-IR1* males and 74.1% of normal levels for *Acp26Aa-P-Gal4;UAS-SP-IR2* males). Therefore the inhibition of receptivity and elevation in oviposition cannot be attributed to lowered levels of Dup99B. Similarly, experiments by O. Lung and M.F. Wolfner (Unpublished data) showed that RNAi of another Acp (Acp62F) using the same *Gal4* driver (Acp26Aa-P-Gal4), did not affect expression of proteins Acp36DE or Acp26Aa, verifying that RNAi by this method is Acp-specific.

The fertility of eggs oviposited over a 5 day period showed that SP knock-down males transferred sperm in numbers comparable to control males, and that these sperm were stored and used to fertilise eggs as normal. The effects of SP on receptivity and egg laying seen in the present study strikingly resemble those seen for females mated to males producing normal levels of Acps but no sperm (Kalb et al. 1993; Xue and Noll 2000). The full post-mating changes in female receptivity and egg laying are associated with the storage of sperm, the 'sperm effect.' Manning (1962) postulated that the sperm effect could be due to an effect of sperm storage itself, or due to some chemical associated with sperm. As females mated to males lacking SP resemble females mated to males lacking sperm, the experiment described in this chapter suggests that the 'sperm effect' is in fact an effect of SP. Recent evidence suggests that SP binds to sperm (S. Busser and E. Kubli, Unpublished data), anchoring it in the sperm storage organs and prolonging its effects on females.

Chapter 6. Testing whether Acp62F contributes to the cost of mating suffered by females

6.1. Abstract

The accessory glands of male *D. melanogaster* express at least one protein that has a measurable detrimental effect on female longevity and lifetime reproductive success. Theory suggests that traits conferring benefits to one sex and costs to the other are important because they could lead to rapid coevolutionary change between the sexes. Male-derived harm is expected to result in selection on female sex-limited traits or behaviours that mitigate these costs. Such female traits may, in turn, prove detrimental to male fitness. However, the identity of the Acp that causes the cost of mating in *D. melanogaster* is unknown. A candidate protein, Acp62F, has recently been identified. This protein has been shown to be toxic to females upon ectopic expression in the female haemolymph, where 8 other Acps were not. Using males lacking Acp62F, the experiments of this chapter investigated the effect of the accessory gland protein Acp62F on female survival following multiple matings. These experiments revealed no differences in survival or reproductive success of females kept with males expressing Acp62F, compared to those kept with males expressing reduced levels of the protein. Therefore, the results of this chapter do not support the idea that Acp62F is responsible for the cost of mating in *D. melanogaster*.

6.2. Introduction

Among the many effects that male seminal fluid proteins have on female post-mating physiology and behaviour in *D. melanogaster*, at least one Acp causes females to suffer a cost of mating. Using males with decreased or no gene expression from the main cells of the accessory glands, Chapman et al. (1995) demonstrated that females kept with males lacking Acp gene expression had higher survival and lifetime reproductive success than normal. It has been suggested that the as yet unidentified Acp conferring this cost of mating on females could simultaneously carry benefits to males, perhaps sperm competitive benefits (Rice 1996; Holland and Rice 1999; Lung et al. 2002). Indeed, in *D. melanogaster*, there is evidence of a correlation between male success during sperm displacement and the ability of males to induce early mortality in their mates (Civetta and Clark 2000b). In addition, genetic evidence supports the idea that some alleles increase male fitness and decrease female fitness, and *vice versa* (Chippendale et al. 2001). In *D. melanogaster* there is a positive genetic correlation between male and female larval survival, but a negative correlation between the reproductive successes of male and female adults (Chippendale et al. 2001). Traits that confer different costs and benefits for males and females are interesting because theory suggests that they could be affected by sexually antagonistic selection leading to rapid evolutionary change in these traits (Parker 1979).

Costly traits that function in sperm competition have been identified in other species too. For example, males of the bed bug *Cimex lectularius* perforate the female's abdomen and inseminate into the body cavity (Stutt and Siva-Jothy 2001). This provides males with high sperm precedence but also causes reduced longevity and reproductive success in females, and does not provide females with any indirect benefits (Stutt and Siva-Jothy 2001). The consequences of male-derived harm were first explored theoretically by Parker (1979), who showed that a trait carrying asymmetric costs for males and females had the potential to precipitate evolutionary 'arms races' between the sexes, and hence rapid evolutionary change. Male-derived harm is expected to result in selection on traits or behaviours in females that reduce the magnitudes of these costs. This may have selective consequences for female mating frequency and female mate choice. In *D. melanogaster,* the costly male trait could result in selection

on female mating frequency or it could result in selection on a female protein to mitigate the cost of mating.

The possible influence of sexual conflict on the rapid evolution of the *D. melanogaster* mating system has been further explored using artificial selection. Holland and Rice (1999) tested the association between the intensity of post-copulatory competition between males and the cost of mating suffered by females. Their results showed that males kept under promiscuous conditions became more costly to females than those kept under monogamous conditions. Conversely, females from monogamous lines were more sensitive to male derived costs than were females from promiscuous lines. This supported the idea that post-copulatory competition between males selected for the male-derived cost and, in turn, selection on females to mitigate this cost. In another experiment, Rice (1996) examined the consequences for female longevity and lifetime fecundity of keeping *D. melanogaster* free from coevolution between the sexes. In lines where female evolution was arrested, males rapidly became more costly to females compared to the lines in which males had coevolved with females (Rice 1996).

It is not yet known which Acp is responsible for the cost of mating in *D. melanogaster.* However, a candidate protein has recently been identified. Acp62F is passed to females during mating and 10% of this protein enters the haemolymph (Lung and Wolfner 1999). The remaining protein remains in the reproductive tract and enters the sperm storage organs. Ectopic expression of Acp62F in the haemolymph of females is toxic, whereas ectopic expression of seven other Acps is not (Lung et al. 2002). The genetic sequence of Acp62F predicts that the protein has trypsin inhibitory activity, and this has been confirmed *in vitro* (Lung et al. 2002). Its biochemical activity could be responsible for its toxic effects upon ectopic expression in the haemolymph of females (Lung et al. 2002). Its trypsin inhibitory activity and its localisation to the sperm storage organs also suggest that Acp62F might serve to protect sperm or other seminal fluid proteins from degradation by proteolysis. Acp62F may also regulate post-transcriptional modification of other Acps (Lung et al. 2002). This provides a mechanism by which Acp62F could simultaneously confer benefits to the males in which it is expressed and costs to their mates.

The only way to test whether Acp62F is responsible for the cost of mating suffered by females is to produce males lacking normal Acp62F expression. This chapter describes an experiment carried out to test the effect of male Acp62F gene expression on female longevity and fecundity. The longevity and fecundity of females housed with males that expressed Acp62F was compared to that of females housed with males expressing reduced or greatly reduced quantities of Acp62F. Similar to the experiments described in Chapter 5, these tests were carried out using males subject to post-transcriptional gene silencing by RNAi. Males from 3 separate transgenic lines were used, two lines whose males were previously shown to express 3% of the control levels of Acp62F, and one whose males expressed 80% of normal levels (Lung 2000). If receipt of Acp62F during mating is responsible for a reduction in female reproductive success, then we would expect females housed with males lacking Acp62F to have a higher survival and/or daily fecundity than females housed with control males.

In a previous study in *D. melanogaster*, the efficiency of post-transcriptional gene silencing by inverted repeat transgenes was found to be temperature-sensitive. Repression of the sex-differentiation gene *transformer-2* by RNAi occurred at 29°C but not 22°C (Fortier and Belote 2000). Improved efficiency of RNAi would improve the power of the tests carried out on Acp62F described here. Therefore, I tested whether the RNAi of Acp62F expression was more efficient at 27°C and 29°C than at 25°C.

6.3. Materials and Methods

6.3.1. Stocks

6.3.1.1. *UAS-Acp62F* stocks

In order to induce targeted suppression of Acp62F using RNAi, stocks were produced that possessed the Acp62F sequence in inverted repeat orientation, adjacent to an upstream activating sequence. Production of transgenic stocks was carried out by O. Lung (Cornell University). A vector was produced that contained an upstream activating sequence (*UAS*), the Acp62F coding region in the sense followed by antisense orientation (*Acp62F-IR*) and a w^+ gene. Transgenic lines were produced by injecting the vector containing *UAS-Acp62F-IR* into a *w'(d2,3)/TM2Sb* background. Three separate *UAS-Acp62F-IR* transgenic progeny were recovered, which were used to

generate three homozygous transgenic stocks (*UAS-Acp62F-IR1*, *UAS-Acp62F-IR3A* and *UAS-Acp62F-IR3C*). Non-transgenic siblings of these progeny were also recovered, and these had the same genetic background as the transgenic progeny. A line of flies derived from these non-transgenic siblings was used to generate a control for the tests carried out to examine the effect of Acp62F function on females.

6.3.1.2. *Acp26Aa-P-Gal4* stocks

Activation of *UAS* and expression of *Acp62F-IR* was driven, in turn, by *Gal4* expression (see also Chapter 5, section 5.3.1.2). A stock possessing X-linked *Gal4* expressed exclusively in the male accessory glands was produced by fusing *Gal4* to the promotor of the gene *Acp26Aa*, as described in Chapman et al. (In press, see Appendix III). This resulted in the transgenic stock *Acp26Aa-P-Gal4*.

6.3.1.3. Generation of Acp62F knock-down and control males

Fig. 6.1 shows the crossing scheme used to obtain males with knock-down and control levels of Acp62F. 5 virgin males from each of the three transgenic inverted repeat insert lines (*UAS-Acp62F-IR1, UAS-Acp62F-IR3A* and *UAS-Acp62F-IR3C*) were mated to 5 virgin females from the *Acp26Aa-P-Gal4* line. These parents were placed into vials for three days and transferred to fresh vials for a further 3 days. Virgin male offspring were collected from these vials over 4 days. The resulting males (*Acp26Aa-P-Gal4;UAS-Acp62F-IR1*, *Acp26Aa-P-Gal4;UAS-Acp62F-IR3A* and *Acp26Aa-P-Gal4;UAS-Acp62F-IR3C*) were used in the longevity and fecundity tests.

The positive control consisted of male offspring resulting from crossing virgin *Acp26Aa-P-Gal4* males to virgin females derived from a stock of non-transgenic flies with the same genetic background as those carrying the *UAS*. The resulting control males (*Acp26Aa-P-Gal4;+*) shared the genetic background of the putative knock-down males, except for the *UAS* transgene.

6.3.1.4. Wild-type strain culturing

Dahomey wild-type females (section 2.1.6) were collected from population cages and raised at standard density. Females were collected within 7 hours of eclosion and kept in vials on Maize-Yeast medium.

6.3.2. Efficiency of RNAi of Acp62F expression at different temperatures

To test levels of Acp62F expression by males cultured at different temperatures, three sets of identical crosses were performed and the resulting developing larvae were kept at 25°C, 27°C and 29°C respectively. Mated males were used throughout the tests carried out in this chapter because previous experiments carried out on males of the same genotype showed that young mated males had increased knock-down of Acp62F relative to older or unmated males (Lung 2000). Therefore, 2-day-old virgin males were mated *en masse*, one-day before protein extraction. 2-day-old transgenic virgin males emerging from these crosses (*Acp26Aa-P-Gal4;UAS-Acp62F-IR1*, *Acp26Aa-P-Gal4;UAS-Acp62F-IR3A*, *Acp26Aa-P-Gal4;UAS-Acp62F-IR3C* and *Acp26Aa-P-Gal4;+*) were mated *en masse* to females of the same type in 1/3 pint glass bottles containing Maize-Yeast medium. After approximately 8 hours, males were transferred in groups of 20 to new vials. One day later, males were subjected to protein extraction ready for Western blots carried out by J. Mueller (Cornell University).

6.3.3. Measuring Acp62F levels before and during longevity experiment

Levels of Acp62F expressed by Acp62F knock-down and control males were tested on a small subset of males before the beginning of the longevity experiment. Two-day old *Acp26Aa-P-Gal4;UAS-Acp62F-IR1*, *Acp26Aa-P-Gal4;UAS-Acp62F-IR3A, Acp26Aa-P-Gal4;UAS-Acp62F-IR3C* and *Acp26Aa-P-Gal4;+* mated males were anaesthetised using ice and homogenised for protein extraction (section 2.2.7). Western blots of Acp62F were carried out by J. Mueller (Cornell University).

Acp62F levels in Acp62F knock-down and control males were also monitored throughout the experiment. Small subsets of males were removed 10 and 20 days into the longevity experiment before being homogenised and subjected to protein extraction (section 2.2.7) ready for Western blots carried out by J. Mueller (Cornell University).

6.3.4. Effect of Acp62F on female longevity

Virgin wild-type females were housed for approximately eight hours every 2-3 days with either Acp62F knock-down males (*Acp26Aa-P-Gal4;UAS-Acp62F-IR1*, *Acp26Aa-P-Gal4;UAS-Acp62F-IR3A*, *Acp26Aa-P-Gal4;UAS-Acp62F-IR3C*) or with control males (*Acp26Aa-P-Gal4;+*). All matings were observed to ensure that no one line mated more than any other and, therefore, that any differences in survival were due to Acp62F and not due to differences in mating frequency between lines. 800 five-day-old virgin wild-type females were anesthetised using ice and placed in groups of five with five males in individually labelled vials containing Maize-Yeast medium. After eight hours, the males were removed using CO_2, which was subsequently used as an anaesthetic throughout. Females were left in the vials for two to three days, before the males were reintroduced for another eight hours. This procedure was repeated every two to three days for 37 days and female deaths were scored every day. The number of males added to each vial during each mating period was altered to match the number of surviving females, except where sex-ratio alterations were made (as described in section 6.3.6). The vials in which females were housed were changed every two to three days. On day 24 after the start of the experiment, new 2-5 day old males were introduced to replace the old.

6.3.5. Effect of Acp62F on fecundity

Fecundity tests were carried out on females from the longevity test on days 2, 8 and 15. Egg laying was also measured on day 25, to monitor fecundity following the introduction of new males into the experiment. Females were transferred to new vials for between 20 and 27 hours. The number of hours that females were left in the vials during fecundity tests was noted and the vials were stored at -80°C until eggs were counted. Fecundity was measured as the numbers of eggs counted per vial, divided by the numbers of females remaining in each vial and the numbers of hours females were left in the vial. This was then multiplied by 24 to provide a measure of egg laying per female per day.

6.3.6. Mating frequency across treatments

Mating frequency was monitored and all matings that occurred were observed. Sex-ratio alterations were carried out to reduce variation in the cumulative number of matings per female per vial. At the end of each mating observation period, the number of matings observed per vial was divided by the remaining number of females per vial. The cumulative number of matings per vial per female was calculated, and the overall mean cumulative mating number was determined. Vials that had a cumulative number of matings per vial per female greater than 1 standard deviation above this overall mean had 1 male removed. Vials that had a cumulative number of matings per vial per female less than 1 standard deviation than this overall mean, had 1 male added. In addition, after each mating period, three separate *t*-tests were carried out on the cumulative number of matings per vial per female between each of the three Acp62F knock-down lines and the control line. These tests were carried out to identify any significant differences in the mean cumulative matings between lines. *t*-tests were used because Shapiro-Wilk tests showed that the distributions of the cumulative number of matings were normal for each line on most of the days that matings were observed. Any significant differences between lines resulted in alterations of the sex-ratio to ensure that significant differences in mean cumulative number of matings between lines did not persist. Lines that mated significantly more than the control had two males removed and lines that mated significantly less than the control had two males added. Despite these sex-ratio alterations, at least one male was left in each vial of all lines during each mating observation period, in order to give all females an opportunity to mate.

6.3.7. Estimation of courtship delivered by Acp62F knock-down males and control males

In order to estimate the level of courtship received by females across the four treatments, several snapshots of courtship activity were carried out as follows. Vials were scanned for courtship, identified as wing vibration or the licking of female genitalia. The number of males courting in each vial was counted. This was divided by the number of females in each vial, thereby providing a measure of the average number of females per vial courted during the snapshot. Snapshots were carried out twice in a single day, and the mean of two snapshots was calculated. This procedure was carried

out on day 11 of the experiment and day 26 of the experiment (day 26 was 2 days after new males were introduced into the vials).

6.4. Results

6.4.1. Efficiency of RNAi of Acp62F expression at different temperatures

There was no evidence of temperature-dependent Acp62F suppression. Males cultured at 25°C (Fig. 6.2, lanes 1-4), 27°C (Fig. 6.2, lanes 5-8) and 29°C (Fig. 6.2, lanes 9-12) appeared to show no differences in Acp62F expression. At each of these temperatures, expression levels of Acp62F differed between the four types of male in the same manner as was seen at 25°C.

6.4.2. Acp62F expression before and during longevity experiment

Levels of Acp62F expression were estimated by the intensity of the bands on Western blots. The results confirmed that levels of Acp62F protein expressed by *Acp26Aa-P-Gal4;UAS-Acp62F-IR1* and *Acp26Aa-P-Gal4;UAS-Acp62F-IR3A* males were knocked-down relative to the control, both at the beginning of (Fig. 6.2, lanes 2 and 4), and during (Fig. 6.3, day 10: lanes 2 and 3, day 20: lanes 6 and 7) the longevity test. However, faint bands of Acp62F of were present, suggesting that RNAi of Acp62F was not as efficient as RNAi of SP seen in Chapter 5. Levels of Acp62F in lines *Acp26Aa-P-Gal4;UAS-Acp62F-IR3C* appeared similar to the control (*Acp26Aa-P-Gal4;+*), both before (Fig. 6.2, compare lanes 1 and 3) and during the experiment (Fig. 6.3, day 10: compare lanes 1 and 4, day 20: compare lanes 5 and 8).

6.4.3. Effect of Acp62F on female survival

Log-rank tests were used to determine differences in female survival between females mated to control males and those mated to *Acp26Aa-P-Gal4;UAS-Acp62F-IR1*, *Acp26Aa-P-Gal4;UAS-Acp62F-IR3A* and *Acp26Aa-P-Gal4;UAS-Acp62F-IR3C* males. Univariate survival plots for each of the four treatments are shown in Fig. 6.4. The survival of females housed with control (*Acp26Aa-P-Gal4;+*) males was no different from the survival of females mated either to *Acp26Aa-P-Gal4;UAS-Acp62F-IR1* males (P=0.1009), *Acp26Aa-P-Gal4;UAS-Acp62F-IR3A* males (P=0.1116) or *Acp26Aa-P-*

Gal4;UAS-Acp62F-IR3C males (P=0.3130). This suggests that receipt of Acp62F does not affect female survival.

Western blots from both during and after the experiment (section 6.4.2) showed that Acp62F expression was reduced in *Acp26Aa-P-Gal4;UAS-Acp62F-IR1* and *Acp26Aa-P-Gal4;UAS-Acp62F-IR3A* males, but was not apparently different in *Acp26Aa-P-Gal4;UAS-Acp62F-IR3C* males, compared to the control. Thus, males from the *Acp26Aa-P-Gal4;UAS-Acp62F-IR3C* line had no reduction in Acp62F and can be considered an extra control line. This finding changed slightly the predictions regarding the results of the survival experiment. If male-expression of Acp62F affected female survival, we would expect the survival of females mated to *Acp26Aa-P-Gal4;UAS-Acp62F-IR3C* males to be significantly lower to that of females mated to *Acp26Aa-P-Gal4;UAS-Acp62F-IR1* and *Acp26Aa-P-Gal4;UAS-Acp62F-IR3A* males. Instead, females mated to *Acp26Aa-P-Gal4;UAS-Acp62F-IR3C* males had higher survival than females mated to *Acp26Aa-P-Gal4;UAS-Acp62F-IR1* (P= 0.0065) and *Acp26Aa-P-Gal4;UAS-Acp62F-IR3A* (P=0.0077) males. This is the opposite to that expected if Acp62F contributed to the cost of mating. It is possible that the different transgenic insertion sites in the three knock-down lines result in different male fitnesses between these lines. If males of different fitness affect female survival in different ways, this may account for some heterogeneity of female survival between the experimental lines (log-rank test on all four lines shown together in Fig. 6.4, P=0.0164).

6.4.4. Effect of Acp62F on fecundity

Analysis was carried out to test whether female fecundity was affected by receipt of Acp62F. Across days 2, 8 and 15, the fecundity of females steadily dropped (Fig. 6.5). On day 25, the same day that new males were introduced into the vials, fecundity increased across all lines (Fig. 6.5).

Wilcoxon tests were carried out to compare fecundity of females mated to control males and females mated to *Acp26Aa-P-Gal4;UAS-Acp62F-IR1*, *Acp26Aa-P-Gal4;UAS-Acp62F-IR3A* and *Acp26Aa-P-Gal4;UAS-Acp62F-IR3C* males. The only significant differences in fecundity that arose from these tests were on day 15 of the experiment.

On this day, females mated to control males had significantly lower fecundity than females mated to *Acp26Aa-P-Gal4;UAS-Acp62F-IR1* males (P=0.0075), females mated to *Acp26Aa-P-Gal4;UAS-Acp62F-IR3A* males (P=0.0151) and females mated to *Acp26Aa-P-Gal4;UAS-Acp62F-IR3C* males (P=0.0151). As three Wilcoxon tests were carried out for each day that fecundity was measured, this result was checked using a more stringent approach. A Bonferroni correction was adopted and the critical value for significant differences was changed to 0.05/3=0.0167. After this correction, the differences in fecundity between females mated to control males and females mated to males of the three knockdown lines remained significant.

The differences in fecundity on day 15 for females mated to the control line and females mated to the knockdown lines, was explored further. Again, Western blots from both during and after the experiment (section 6.4.2) changed slightly the predictions regarding the results of the fecundity experiment. If male-expression of Acp62F affected female fecundity, we would expect the fecundity of females mated to *Acp26Aa-P-Gal4;UAS-Acp62F-IR3C* males to be significantly different to the fecundity of females mated to *Acp26Aa-P-Gal4;UAS-Acp62F-IR1* and *Acp26Aa-P-Gal4;UAS-Acp62F-IR3A* males. However, for the data from day 15 of the experiment, no such significant differences were found (for comparisons between females mated to *Acp26Aa-P-Gal4;UAS-Acp62F-IR3C* males and *Acp26Aa-P-Gal4;UAS-Acp62F-IR1* males, P=1.000; for comparisons between females mated to *Acp26Aa-P-Gal4;UAS-Acp62F-IR3C* males and *Acp26Aa-P-Gal4;UAS-Acp62F-IR3A* males, P=0.8477). Furthermore, an association between Acp62F levels and female fecundity would not be able to account for the significantly lower fecundity of females mated to control males compared to females mated to *Acp26Aa-P-Gal4;UAS-Acp62F-IR3C* males (P=0.0151). In addition, on day 1, females mated to *Acp26Aa-P-Gal4;UAS-Acp62F-IR3C* males had significantly lower fecundity to females mated to *Acp26Aa-P-Gal4;UAS-Acp62F-IR1* males (P=0.0029), and again, this difference was not reflected in the same comparisons carried out between females mated to control and *Acp26Aa-P-Gal4;UAS-Acp62F-IR1* males (P=0.8342).

Overall therefore, although there were some differences in the fecundity of females mated to males with reduced quantities of Acp62F and those mated to males with

normal quantities of Acp62F, these differences were not consistent across each of the days on which fecundity was measured. Furthermore, any differences in fecundity between females housed with males with reduced and normal levels of Acp62F were not consistent across the three insertion lines. This experiment therefore provides no evidence that Acp62F expression in males affects female fecundity.

6.4.5. Mating frequencies amongst females mated to knock-down and control males

In total, females had 16 opportunities to mate, and the mean cumulative number of matings per vial per female across treatments is shown in Fig. 6.6. *t*-tests were carried out after each mating observation period to test whether females of the four treatments experienced similar numbers of matings. Significant differences in mating frequency occurred very rarely and sustained differences in the cumulative numbers of matings within treatments were avoided by altering the sex ratios within vials. Of the 16 mating opportunities, females mated to *Acp26Aa-P-Gal4;+* males had mated significantly more than females mated to *Acp26Aa-P-Gal4;UAS-Acp62F-IR3A* by day 11 (P=0.0468), and had mated significantly less than females mated to *Acp26Aa-P-Gal4;UAS-Acp62F-IR3C* by day 15 (P=0.0378). On day 21, females kept with *Acp26Aa-P-Gal4;UAS-Acp62F-IR1* males and females kept with *Acp26Aa-P-Gal4;UAS-Acp62F-IR3C* males were found to have mated significantly more frequently than females exposed to *Acp26Aa-P-Gal4;UAS-Acp62F-IR3A* and control males. In order to avoid persistence of these differences, in addition to the normal adjustment of sex-ratios, females housed with *Acp26Aa-P-Gal4;UAS-Acp62F-IR3A* and *Acp26Aa-P-Gal4;+* males were allowed to mate during a single extra observation period on day 23, for half the time normally allowed. This added measure successfully helped resolve the differences in cumulative mating frequency seen between the lines. Overall therefore, there were no persistent significant differences in mating frequency.

6.4.6. Estimation of courtship delivered by Acp62F knock-down males and control males

Wilcoxon tests carried out on the numbers of females courted per vial during courtship snapshots showed that there were no significant differences in the courtship activity

measured between females mated to control males and females mated to males of the other four treatments (Table 6.1).

Figure 6.1. The crosses used to produce Acp62F knock-down and control males. To induce RNA interference of Acp62F, homozygous females carrying 3 constructs (*UAS-Acp62F-IR1*, *UAS-Acp62F-IR3A,* and *UAS-Acp62F-IR3C*) were crossed to males carrying the X-linked transgene *Acp26Aa-P-Gal4*. The resulting male offspring possessed the *Gal4* driver and *UAS-Acp62F* transgenes. This induced RNAi in lines **(i)** *Acp26Aa-P-Gal4;UAS-Acp62F-IR1*, **(ii)** *Acp26Aa-P-Gal4;UAS-Acp62F-IR3A,* and **(iii)** *Acp26Aa-P-Gal4;UAS-Acp62F-IR3C*). As a control, non-transgenic females with the same genetic background as those carrying *UAS-Acp62F* were crossed to the same stock, *Acp26Aa-P-Gal4*. The male offspring resulting from this cross **(iv)** have the genotype *Acp26Aa-P-Gal4;+* and produce wild-type levels of Acp62F expression.

(i)
UAS-Acp62F-1 ♂x *Acp26Aa-P-Gal4* ♀
↓
Acp26Aa-P-Gal4;UAS-Acp62F-IR1 ♂offspring, with **knocked-down** levels of Acp62F

(ii)
UAS-Acp62F-IR3A ♂x *Acp26Aa-P-Gal4* ♀
↓
Acp26Aa-P-Gal4;UAS-Acp62F-IR3A ♂offspring, with **knocked-down** levels of Acp62F.

(iii)
UAS-Acp62F-IR3C ♂x *Acp26Aa-P-Gal4* ♀
↓
Acp26Aa-P-Gal4;UAS-Acp62F-IR3C ♂offspring, with **knocked-down** levels of Acp62F

(iv)
Non-transgenic sibling ♂x *Acp26Aa-P-Gal4* ♀
↓
Acp26Aa-P-Gal4;+ ♂offspring, with **control** levels of Acp62F

Figure 6.2. Western blot (carried out by J. Mueller, Cornell University) showing levels of Acp62F in *Acp26Aa-P-Gal4;+*, *Acp26Aa-P-Gal4;UAS-Acp62F-IR3A, Acp26Aa-P-Gal4;UAS-Acp62F-IR3C* and *Acp26Aa-P-Gal4;UAS-Acp62F-IR1* males before start of experiment. From left to right: lanes 1-4 show homozygous males, cultured at 25°C. Lanes 5-8 show males of the four genotypes in the same order, cultured at 27°C. Lanes 9-12 show males of the four genotypes in the same order, cultured at 29°C. Lane 13 shows males of the homozygous *Acp26Aa-P-Gal4* parental line.

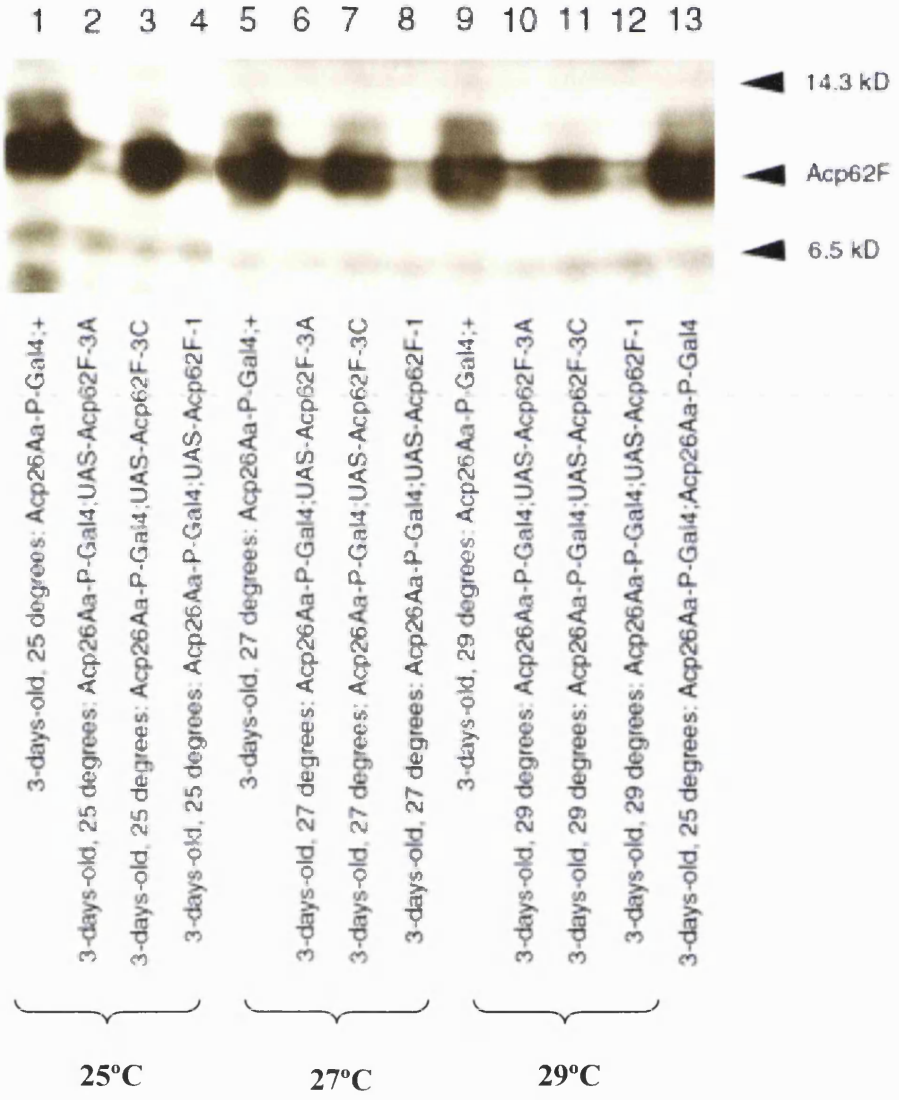


Figure 6.3. Western blot (carried out by J. Mueller, Cornell University) showing levels of Acp62F expression in Acp62F knock-down and control males, 10 and 20 days into the experiment. From left to right: lanes 1-4 show homozygous *Acp26Aa-P-Gal4;+*, *Acp26Aa-P-Gal4;UAS-Acp62F-IR1*, *Acp26Aa-P-Gal4;UAS-Acp62F-IR3A* and *Acp26Aa-P-Gal4;UAS-Acp62F-IR3C* males, taken from day 10 of the longevity experiment. Lanes 5-8 show males of the four genotypes in the same order, taken from day 20 of the longevity experiment.

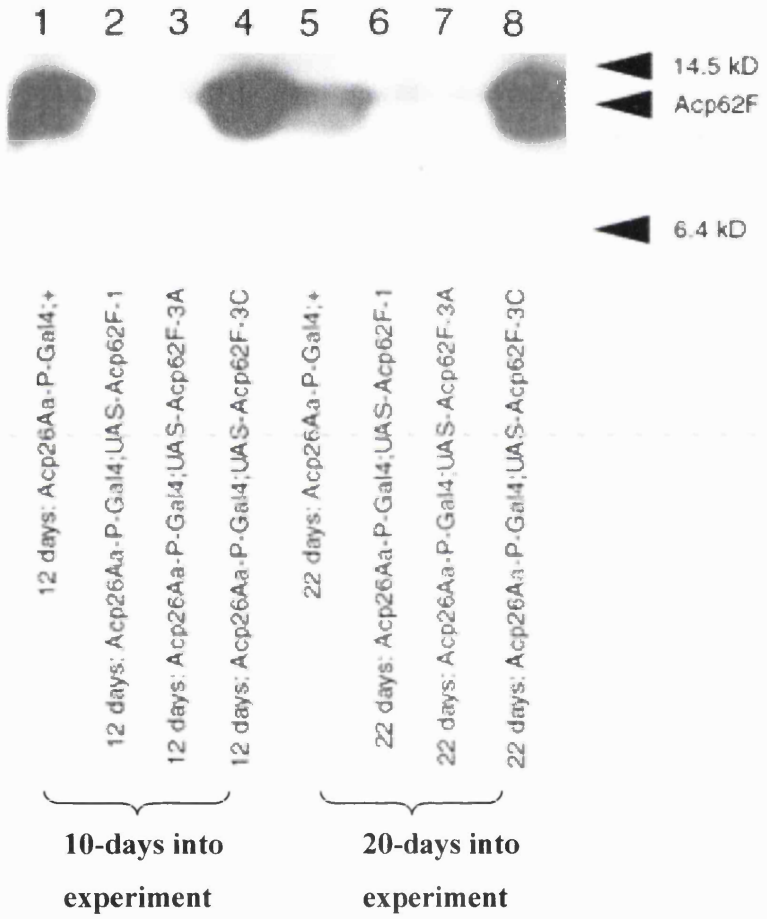


Figure 6.4. Effect of Acp62F on female lifespan. Cumulative survival of females against time in days for females mated to *Acp26Aa-P-Gal4;+* males (black), *Acp26Aa-P-Gal4;UAS-Acp62F-IR1* males (red), *Acp26Aa-P-Gal4;UAS-Acp62F-IR3A* males (green) and *Acp26Aa-P-Gal4;UAS-Acp62F-IR3C* (blue).

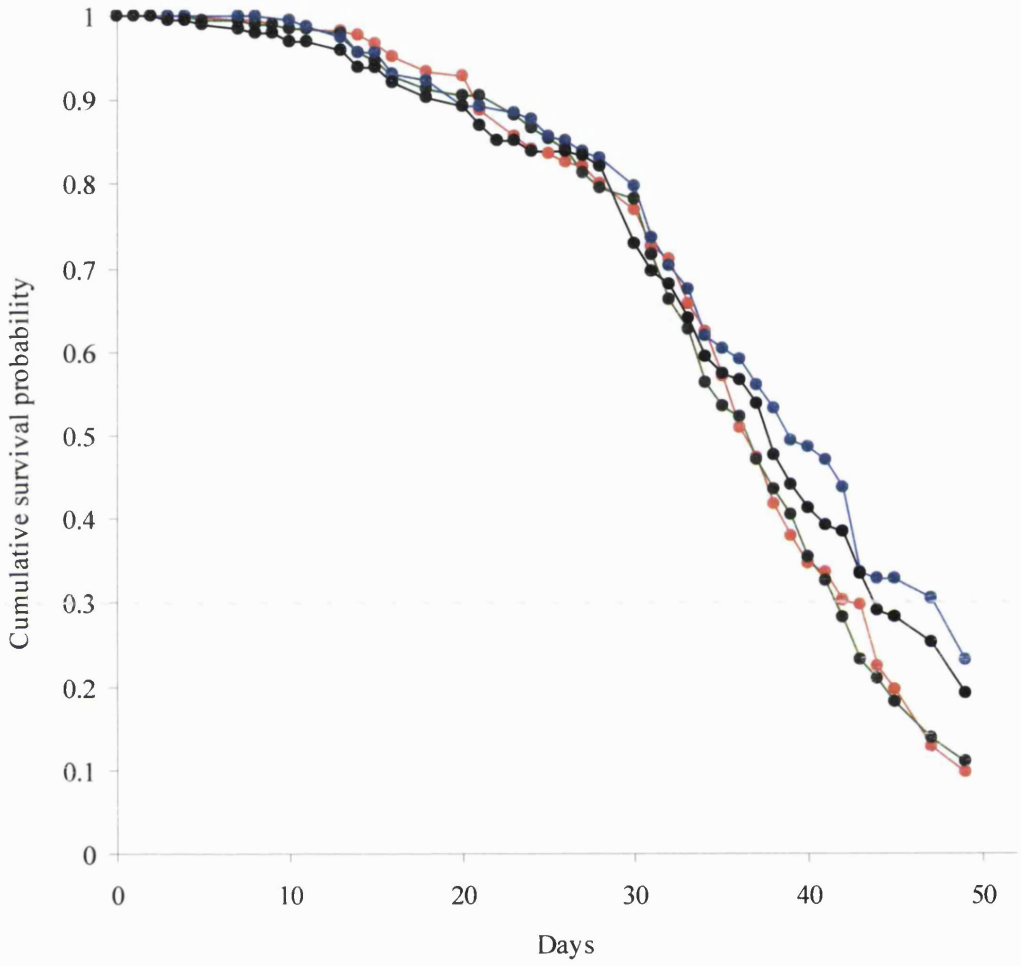


Figure 6.5. Effect of Acp62F on female fecundity. The median number of eggs (± interquartile range) laid per 24 hours by females mated to *Acp26Aa-P-Gal4;+* (black), *Acp26Aa-P-Gal4;UAS-Acp62F-IR1* males (red), *Acp26Aa-P-Gal4;UAS-Acp62F-IR3A* males (green) and *Acp26Aa-P-Gal4;UAS-Acp62F-IR3C* males (blue), during four different time points after mating.

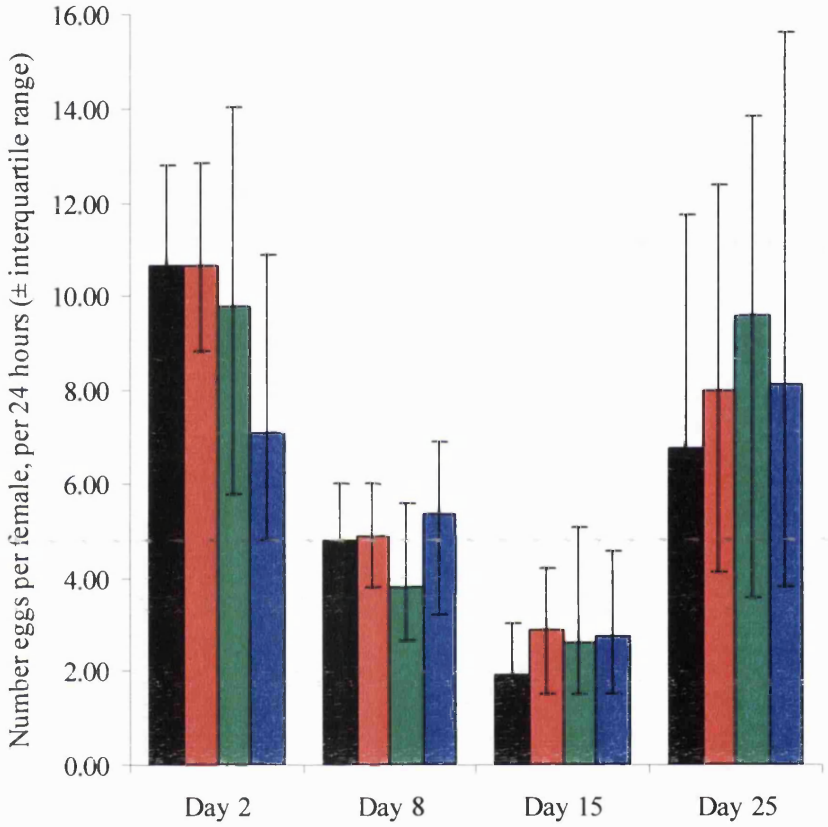


Figure 6.6. The cumulative number of matings per female (±95% confidence intervals) over each observation period for females mated to *Acp26Aa-P-Gal4;+* males (black), *Acp26Aa-P-Gal4;UAS-Acp62F-IR1* males (red), *Acp26Aa-P-Gal4;UAS-Acp62F-IR3A* males (green) and *Acp26Aa-P-Gal4;UAS-Acp62F-IR3C* (blue).

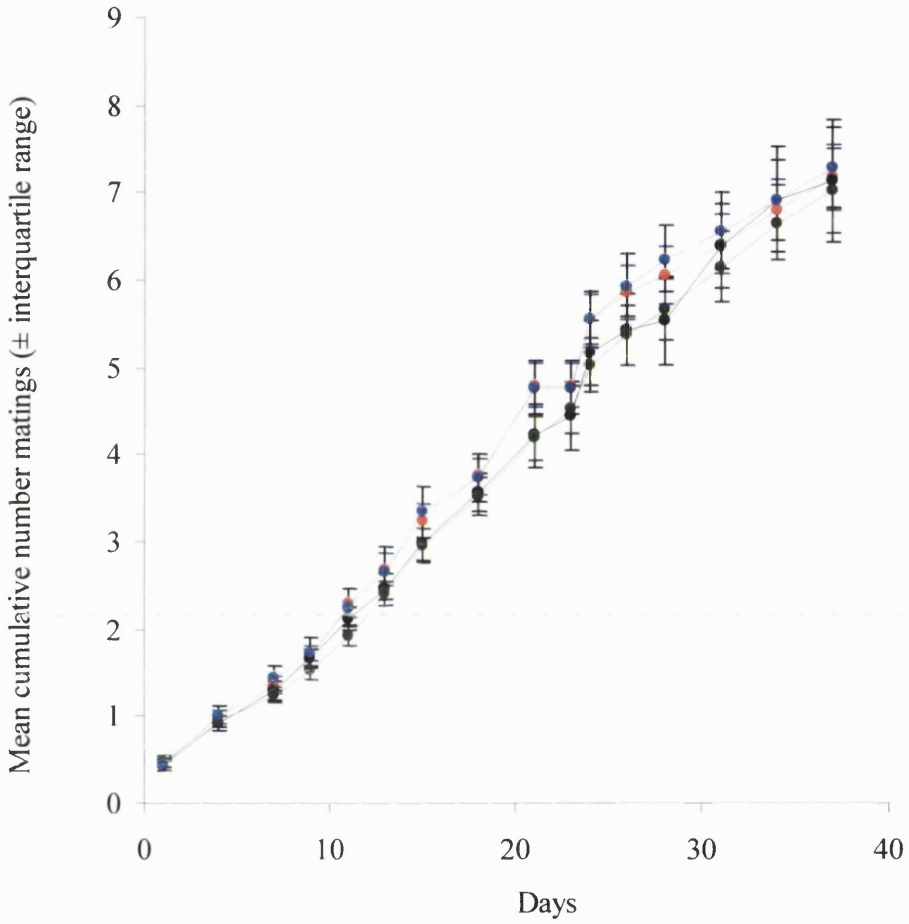


Table 6.1. Test statistics and *P*-values resulting from Wilcoxon tests carried out on number of females courted on days 11 and 26 of the experiment, by *Acp26Aa-P-Gal4;+* control males and **(a)** *Acp26Aa-P-Gal4;UAS-Acp62F-IR1* males **(b)** *Acp26Aa-P-Gal4;UAS-Acp62F-IR3A* males and **(c)** *Acp26Aa-P-Gal4;UAS-Acp62F-IR3C* males.

(a)	**Mean number of females being courted during snapshots**	
Comparing numbers of female being courted by males of genotype:	**Day 11**	**Day 26**
Acp26Aa-P-Gal4;+* and *Acp26Aa-P-Gal4;UAS-Acp62F-IR1	χ^2=1.3809 *P*=0.2400	χ^2=0.0921 *P*=0.7615

(b)	**Mean number of females being courted during snapshots**	
Comparing numbers of female being courted by males of genotype:	**Day 11**	**Day 26**
Acp26Aa-P-Gal4;+* and *Acp26Aa-P-Gal4;UAS-Acp62F-IR3A	χ^2=1.0063 *P*=0.3158	χ^2=0.5544 *P*=0.4565

(c)	**Mean number of females being courted during snapshots**	
Comparing numbers of female being courted by males of genotype:	**Day 11**	**Day 26**
Acp26Aa-P-Gal4;+* and *Acp26Aa-P-Gal4;UAS-Acp62F-IR3C	χ^2=0.0905 *P*=0.9005	χ^2=0.6300 *P*=0.4273

6.5. Discussion

The results of the longevity and fecundity tests suggest that Acp62F is not responsible for the cost of mating suffered by *D. melanogaster* females. Qualitative examination of the Western blots shows that *Acp26Aa-P-Gal4;UAS-Acp62F-IR1* and *Acp26Aa-P-Gal4;UAS-Acp62F-IR3A* males produced less Acp62F than the control males and that *Acp26Aa-P-Gal4;UAS-Acp62F-IR3C* males produced similar quantities of Acp62F to the controls. There were no differences in the survival of females housed with *Acp26Aa-P-Gal4;UAS-Acp62F-IR1* and *Acp26Aa-P-Gal4;UAS-Acp62F-IR3A* males compared to those housed with control males. Moreover, there were no consistent differences in the fecundity of females housed with *Acp26Aa-P-Gal4;UAS-Acp62F-IR1* and *Acp26Aa-P-Gal4;UAS-Acp62F-IR3A* males compared to those housed with control males. This experiment therefore provided no evidence that receipt of Acp62F affected female fecundity or survival.

In contrast to previous studies, tests described in this chapter showed that suppression of Acp62F expression by RNAi was no more efficient at 29°C than at 25°C. In the first published study using *in vivo* transcription of an inverted repeat transgene to produce RNAi in *D. melanogaster*, suppression of gene expression was temperature sensitive (Fortier and Belote 2000). For RNAi of Acp62F described in this chapter, there appeared to be no differences Acp62F expression between males cultured at 25°C, 27°C and 29°C.

All matings that occurred during the longevity test were observed, and significant differences in the cumulative number of matings of females housed with Acp62F knock-down and control males were observed only very infrequently and were non-significant overall. Any sustained differences in the number of matings experienced by females were prevented by manipulation of the within-vial sex ratio. Female survival and fecundity was therefore not likely to have been confounded by differences in mating frequency.

The results presented here contrast with previous findings that suggested that the receipt of Acp62F during mating was toxic to females. Approximately 10% of the Acp62F

received by females during mating passes into the female haemolymph (Lung and Wolfner 1999). Lung et al. (2002) found that ectopic expression of Acp62F in the haemolymph of females was costly where the ectopic expression of 8 other Acps was not. Following this experiment, it was suggested that here in the female haemolymph, the protease inhibitory activity of Acp62F could interfere with essential proteolytic processes, resulting in this toxic effect (Lung et al. 2002). However, a significant drawback with the ectopic expression experiment was that the females expressed Acp62F at levels more than 50 times higher than that experienced after a single normal mating (Lung and Wolfner 1999). The experiment described in this chapter instead examines the effect on females of physiological quantities of Acp62F delivered through the normal route.

Although the results of this chapter suggest that Acp62F is not responsible for the cost of mating, two alternative explanations for these results are possible. It might be that that the reduction in Acp62F expression in the knock-down lines was not sufficient to provoke differences in survival at the mating rates observed. Another possibility is that the numbers of matings obtained by females in this experiment were not high enough for cost to be reflected in the control females. Females remaining in the experiment during the final mating observation period (day 37) had mated an average of 7.1 times. In the wild, estimates of the numbers of mating partners for females up to the time of capture have ranged from 1.9 (Harshman and Clark 1998) to 6.0 (Imhof et al. 1998), initially suggesting that the mating frequencies in the laboratory adequately reflected those in the wild. However, the first demonstration of a cost of mating caused by Acps (Chapman et al. 1995) was carried out using high levels of nutrition. Subsequently, Chapman and Partridge (1996a) measured the costs of mating suffered by females at different levels of nutrition and concluded that female mating frequency in laboratory cultures could have evolved to a level where the cost of mating was small. During the experiment described in this chapter, females were kept on media without live yeast. Females mate at higher frequency at higher nutrition (Chapman and Partridge 1996a), and higher mating frequencies are likely to augment any costs of mating. Therefore, during future tests of this type, levels of mating could be increased by adding live yeast to vials that house the females.

In order to determine whether control females are exposed to a significant cost of mating at a given level of mating frequency, future experiments should include a control group of females housed with non-mating males. Females housed with non-mating males will not suffer a cost of mating, and against this comparison, it would be possible to tell if a cost of mating is expressed in the control females. Only when it is known that a cost of mating is expressed in the control females, will it be possible to confirm whether the cost of mating is reduced or absent in groups of females mated to males with reduced levels of Acp62F. Non-mating males could be microcauterised so that they are unable to pass seminal fluid to females (e.g. Chapman 1992). Alternatively, non-mating mutants could be used.

Chapter 7. General Discussion

This thesis investigated some of the selection pressures that shape the evolution of the *D. melanogaster* mating system. The identification of correlations between pre- and post-copulatory success and male traits such as body size or reproductive tract morphology will help identify targets of sexual selection at different stages of the reproductive process. Chapter 3 showed that males with larger accessory glands have the potential to mate at a higher frequency than males with smaller accessory glands. In addition, large males were found to be better at sperm displacement than small males. Females also vary in the post-mating success achieved by their mates, but the biochemical and morphological basis of this variation is unknown. Chapter 4 showed that variation in female sperm storage organ morphology was correlated with the variation in the temporal pattern of offspring production. This could influence the outcome of sperm competition between males.

Male *D. melanogaster* produce large numbers of seminal fluid proteins that heavily influence post-mating events by inducing a range of physiological and behavioural post-mating changes in females. Chapter 5 showed that the accessory gland protein (Acp) sex-peptide (SP) is necessary for the prolonged inhibition of female receptivity and the elevation of egg laying after mating. Previously, sperm storage was thought to be necessary for the prolonged inhibition of female receptivity, and this was referred to as the 'sperm effect' on female receptivity. However, the sperm effect seems instead to be an effect of SP. Chapter 6 described experiments carried out to determine whether the protein Acp62F affects female survival. This protein was previously shown to be toxic when ectopically expressed in females and thus represented a candidate for the cost of mating in *D. melanogaster*. However, the results of Chapter 6 suggest that Acp62F is not responsible for this cost.

The functions and targets of the Acps SP and Acp62F are of special interest in the light of recent research that suggests a role for sexually antagonistic selection on the evolution of reproductive traits in *D. melanogaster*. In the following sections, I will describe the results of each of the experiments in Chapter 3 to 6, discuss further

questions that arise from this work, and outline some other approaches currently being explored to probe the influence of sexual conflict on the evolution of mating systems.

7.1. Relationship between body size, accessory gland size and testis size and pre- and post-copulatory success (Chapter 3)

When given the opportunity, males with larger accessory glands were found to mate at higher frequency than males with smaller accessory glands. Once accessory gland size was taken into account, there was no significant association between mating frequency and body size, suggesting that accessory gland size may be partly responsible for the association between mating frequency and body size in males of this species (Partridge and Farquhar 1983; Partridge et al. 1987a, b). Previous studies have shown that males mated repeatedly in quick succession have progressively more depleted accessory glands and become increasingly less able to elicit post-mating responses in females (Hihara 1981). It is possible that males with smaller accessory glands will become depleted of Acps after fewer matings than males with larger accessory glands, suffering the costs of courtship (Cordts and Partridge 1996) without the fertilisation benefits of mating. Future work in this direction could examine the effect on male mating success of manipulating accessory gland size using artificial selection.

Males with larger accessory glands did not have higher sperm displacement ability than males with smaller glands, even before body size was taken into account. This is probably because, during a single mating, males are estimated to pass only one third of the contents of their accessory glands to females (Herndon et al. 1997), so males are not likely to be limited by Acp quantities after one mating. In addition, the experiments of Chapter 3 showed that there was no association between testis size and mating frequency or post-copulatory success in *D. melanogaster*. Similarly, this may be because during single matings, males tend to pass quantities of sperm to females in vast excess of those stored (Lefevre and Jonsson 1962; Gilbert 1981). After one or a few matings, males are not limited by the quantities of sperm stored in their testes.

However, larger males achieve higher success during post-copulatory competition than smaller males. Acps influence the sperm displacement ability of males (Harshman and

Prout 1994; Gilchrist and Partridge 1995; Chapman et al. 2000). Success during post-copulatory competition may be influenced by the quantities of Acps passed to females (Hihara 1981). If so, then the correlation between body size and sperm displacement ability may exist because larger males pass greater quantities of Acps to females than small males. However, large males do not mate for longer than small males; copulation duration was not found to be associated with either body size or post-copulatory success. Instead, it is possible that the quantities of Acps that reach females during a single mating are determined by the rate at which seminal fluid is passed to females. Large males may pass greater quantities of seminal fluid proteins to females per unit time than small males.

A recent experiment has demonstrated that environmentally induced variation in male body size is correlated with the extent to which males harm females during mating (Pitnick and Garcia-Gonzalez 2002). Females mated repeatedly to large males had lower survival and fecundity than females mated repeatedly to small males. The results described by Pitnick and Garcia-Gonzalez (2002) and those described in Chapter 3 both suggest that male body size is correlated with the magnitude of female post-mating responses. Again, this could be because large males pass greater quantities of Acps to females than small males.

It is not yet clear whether the receipt of greater quantities of Acps elicits greater post-mating responses in females. Past experiments using males mated in quick succession (Hihara 1981), and males producing greatly reduced quantities of Acps (Kalb et al. 1993), have suggested that the quantities of Acps passed during mating do affect the post-mating responses of females. In contrast, the results of experiments that have measured the magnitude of the post-mating responses following injection of varying quantities of SP into virgin females, provide no conclusions to this question. Above a critical level of injected SP, the numbers of eggs laid by virgin females was found to be positively correlated with the amount of SP injected (Kubli 1996). In contrast, above this same critical concentration of injected SP, low sexual receptivity and high ovulation remained at a constant level, regardless of the quantities of SP injected (Kubli 1996). It is still unclear, therefore, whether we expect the receipt of greater quantities of Acps to elicit greater post-mating responses in females.

However, it should now be possible to use quantitative Western blot analysis to test the quantities of Acps in the mated female reproductive tract and examine whether these quantities are correlated with the magnitude of the female post-mating responses. Antibodies for use in Western blots are now available for a number of Acps, such as SP (Chapman et al. In press; Liu and Kubli In press), Acp26Aa (Heifetz et al. 2000) and Acp62F (Chapter 6). Quantitative Western blots could also be used to determine whether larger males pass greater quantities of Acps to females during mating than small males, and hence whether large males pass seminal fluid more rapidly to females than small males.

In conclusion, the results of these experiments suggest that sexual selection may act on male body size because large males can both mate at higher frequency than small males and have greater sperm displacement ability than small males. This could be confirmed by carrying out similar experiments to the two described here, but by examining both pre- and post-copulatory success in the same individuals.

7.2. Influence of female reproductive tract morphology on the outcome of sperm competition (Chapter 4)

Although studies of post-copulatory events have tended to concentrate on males, the data presented in Chapter 4 confirm that the outcome of sperm competition may also vary with female reproductive tract morphology. Chapter 4 illustrates several ways in which the temporal patterns of sperm use can be analysed statistically. Naturally occurring variation in spermathecal morphology was found to be correlated with variation in the pattern of offspring production over time by doubly mated females. Females with three spermathecae had more rapid rising and falling phases of offspring production than females with the normal two spermathecae. Differences in the temporal patterns of offspring production between females with two and three spermathecae may exist because a careful balance is required between sperm storage, release and fertilisation, and this is disrupted in 3SP females. Alternatively, these differences could be due to reproductive tract defects of 3SP females, undetected under a microscope.

The variation in spermathecal morphology described in Chapter 4 provided an unrefined tool with which to investigate the influence of female reproductive tract morphology on sperm use. The principle that sperm storage organs vary in size and shape may apply to females from natural populations. If so, then variation in sperm storage organ shape could provide a mechanism by which genetic variation among females (Clark and Begun 1998; Civetta and Clark 2000a) is correlated with variation in the degree of sperm precedence achieved by their mates.

The results of Chapter 4 also showed that males of different genotypes differ significantly in their post-copulatory reproductive success. Males of a stock commonly used for studies of sperm competition (bw^D) were unable to displace sperm, and this is consistent with direct observations of the displacement of GFP-labelled sperm (Civetta 1999). Once again, variation between male genotypes was again observed in the patterns of offspring production over time; the traditional measure of sperm competition, P_2 was not sufficient for detecting these correlations.

As males lacking specific Acp components become available (perhaps using genetic tools such as RNAi described in Chapters 5 and 6), the analysis of their effects on sperm storage, defence and displacement will be facilitated by statistical tests on the temporal patterns of sperm use. Acps heavily influence sperm displacement and incapacitation, although the precise mechanisms by which first and second male ejaculates interact to produce the observed patterns of sperm use are unknown. It is possible that the differences in sperm displacement ability seen between bw^D and wild type males used in Chapter 4 were due to differences in the quality or quantity of the accessory gland proteins produced by these males. bw^D males used in the experiment were unable to displace wild-type sperm, although the numbers of offspring sired by bw^D males were not compromised overall. A further observation consistent with differences in the efficiency of bw^D and wild-type Acps, was that females mated first to wild-type males remated significantly more slowly to a second male than did females mated first to bw^D males. This may have been due to the relative abilities of first male wild type and bw^D males to reduce female sexual receptivity or attractiveness post-mating, and both of these are influenced by the receipt of Acps (Chen et al. 1988; Tram and Wolfner 1998).

7.3. The sex-peptide of *Drosophila melanogaster*: investigation of post-mating responses of females using RNA interference (Chapter 5)

The results of Chapter 5 show that the accessory gland protein SP is necessary for the full post-mating reduction in female sexual receptivity and elevation in egg laying that follows a normal mating. SP is one of an estimated 80 Acps passed to females during mating. The experiments described here demonstrate the efficiency of RNAi in suppressing SP function, and the results suggest that RNAi has considerable potential to facilitate investigation of other Acps in the future. A number of questions can only be answered using males that lack specific components of the seminal fluid. First, and unlike injection or ectopic expression experiments, it is possible to confirm the function of an Acp while it is delivered through the normal route. Secondly, males that lack expression of a single protein make it possible to tell whether an Acp is necessary for a particular effect, or whether there is functional redundancy with other Acps. The experiments of Chapter 5 show that males lacking SP are able to induce residual, short-term changes in female receptivity and oviposition rate. These residual, short-term effects must be caused by the transfer of one or more other Acps, as females mated to males lacking all Acp expression have virgin levels of receptivity and egg laying (Kalb et al. 1993).

The changes in receptivity and egg laying in females mated to males lacking SP resembled the post-mating responses of females mated to males lacking sperm. The results suggest that the 'sperm effect' on female receptivity (Manning 1962), is in fact a SP effect. Recent results suggest that SP binds to sperm (S. Busser and E. Kubli, Unpublished data) so sperm may act to anchor the SP, leading to its prolonged effects on females.

Now that males lacking SP expression have been created using RNAi, the roles of SP in other post-mating responses can be explored. Females normally remain unattractive to males for up to 9 days after mating. The act of mating itself is thought to trigger the initial drop in attractiveness, but this post-mating response has two components to it (Tram and Wolfner 1998). The maintenance of the reduction in attractiveness beyond 24 hours after mating does not occur if a female's mate lacks sperm or lacks both sperm and Acps. The maintenance of female attractiveness beyond 24 hours to up to 9 days

post mating was therefore linked to the storage of sperm (Tram and Wolfner 1998). However, this experiment did not include a test using males that produced sperm but lacked Acp expression. It is possible that, rather than sperm itself, a factor that binds to sperm is responsible for the prolonged drop in attractiveness. Furthermore, it is possible that this factor is SP. Males lacking SP and sperm will make it possible to test this idea. For example, four different types of males could be compared: those that lack both sperm and SP, those that produce sperm but no SP, those that produce SP but no sperm and those that produce both sperm and SP. If SP bound to sperm is responsible for the drop in attractiveness of females for several days post-mating, then we should see a lack of response in females mated to the first three types of male and a normal response for females mated to the latter type of male.

7.4. Testing whether Acp62F contributes to the cost of mating suffered by females (Chapter 6)

The results of Chapter 6 suggest that Acp62F is not responsible for the cost of mating suffered by *D. melanogaster* females, despite past studies that proposed this protein as a candidate (Lung et al. 2002). Chapter 6 compared the longevity and fecundity of females housed with normal males and females housed with transgenic males expressing reduced levels Acp62F and no consistent significant differences in survival or fecundity were seen.

A disadvantage of RNAi in studies of protein function, is that, for some constructs, small quantities of protein 'escape' from the RNAi and these small quantities may still have significant physiological effects. In the experiment described in Chapter 6, transgenic males subject to RNAi expressed reduced, but nevertheless detectable, levels of Acp62F. It is possible therefore that Acp62F does contribute to the cost of mating, but that the levels of Acp62F protein expression in the RNAi knock-down lines were not low enough for the effects of Acp62F to be apparent at the mating rates seen in this experiment. The mating rates of females in this experiment may have been too low to see a cost of mating. The experiment was carried out under conditions of low nutrition, but at higher levels of nutrition, mating frequency will be higher and differences in female survival may become apparent.

It will be interesting to test other potential functions for Acp62F. After mating, approximately 10% of Acp62F passes from the uterus through the vaginal intima into the female haemolymph, while the remaining protein is localised to the sperm storage organs of females (Lung and Wolfner 1999). Its predicted biochemical function as a trypsin inhibitor suggests that Acp62F can regulate proteolysis, possibly protecting sperm or other Acps from degradation or regulating post-mating modification of proteins such as Acp26Aa (Lung et al. 2002). It will be interesting to use males lacking Acp62F to examine the effect of Acp62F on sperm longevity, male fertilisation success and egg fertility. The effect of Acp62F on sperm motility could be examined by dissecting females mated to males expressing reduced quantities of Acp62F, then directly observing sperm in storage at fixed times after mating.

7.5. Current and future work to investigate sexual conflict in *D. melanogaster*

The detection of sexual conflict in action requires a clear understanding of the functions of the traits involved. The identification of the costly Acp in *D. melanogaster* remains very important. Understanding of its biochemical function will help explain how males harm females and could facilitate the identification of female receptor proteins, for example using the yeast-two hybrid system for detecting protein interactions. Only when both male and female traits are identified, and the costs and benefits associated with these traits are known, will it be possible to determine whether sexual conflict has an important influence on the evolution of such traits.

Females are found to suffer from male-derived harm in other species too and, below, I describe some examples of these. I also describe some of the work currently underway to identify whether sequence characteristics exist that are specific to loci under sexually antagonistic selection. Using an alternative approach to assess the importance of sexually antagonistic selection on the evolution of reproductive traits, several studies have examined the outcomes of matings between different allopatric populations. Later in this section, I illustrate one way in which inter-population crosses could provide a useful tool for examining the influence of sexually antagonistic selection on specific traits.

7.5.1. Examples of costly traits in other species

Adaptations that give the bearer an advantage at the expense of members of the other sex have the potential to precipitate evolutionary arms races between the sexes. For example, males of the bed bug *Cimex lectularius* engage in traumatic insemination, during which they perforate the female's abdomen and inseminate into the body cavity (Stutt and Siva-Jothy 2001). This provides males with high sperm precedence, but also results in reduced longevity and reproductive success of females, and does not to provide females with any indirect benefits (Stutt and Siva-Jothy 2001). Costs to females may also be indirect. Females of the bumblebee *B. terrestris* suffer decreased offspring fitness from male behaviour (Baer and Schmid-Hempel 1999). Under experimental conditions, females benefit from multiple inseminations through increased parasite resistance of their offspring (Baer and Schmid-Hempel 1999). However, a fatty acid component of the mating plug that forms in the female reproductive tract (Duvoisin et al. 1999) allows males to successfully prevent females from mating more than once (Baer et al. 2000; Baer et al. 2001; Sauter and al. 2001). Therefore, the production of this mating plug appears to be both advantageous to males and costly to females.

Male water striders of many species frequently engage females in a costly struggle, which, if males win, results in mating (Rowe et al. 1994). The outcome of this pre-mating struggle is related to the abdominal morphology of the two sexes (Rowe et al. 1994). Male abdominal adaptations facilitate the grasping of females during the pre-mating struggle, forcing females to mate at a higher than optimal mating frequency. Female abdominal counter-adaptations help to resist the male during this struggle (Arnqvist and Rowe 1995). Sexual conflict over male and female abdominal structures is expected to result in antagonistic coevolution of these traits. Recent studies examined the abdominal morphology and the outcome of the premating struggle in 15 closely related species of water striders. As predicted, a tight coevolution of male and female abdominal structures was found, and this coevolution appeared to be rapid and bidirectional (Arnqvist and Rowe 2002). A general evolutionary trajectory of abdominal morphology was identified across these 15 species. Along this trajectory, males and females had matching levels of armament and the outcome of the struggle was constant

(Arnqvist and Rowe 2002b; Rowe and Arnqvist 2002). Where species deviated from this trajectory, one sex was able to gain a relative advantage over the other (Arnqvist and Rowe 2002a).

7.5.2. Why might males harm females?

It is not yet known why males might harm females. A reduction in female fitness caused by male sex-limited traits such as those seen in *D. melanogaster* is not necessarily considered the strategic 'intent' of males (reviewed Chapman et al. 2003). Such costs may result as a side effect of other traits and behaviours involved in male-male competition. This is the likely scenario in *D. melanogaster*, where the costly factor could provide benefits for males, such as sperm protection by proteolysis regulation, but confer biochemically toxic side effects on females. In *C. lectularius* too, traumatic insemination appears to be a particularly aggressive adaptation to sperm competition (Stutt and Siva-Jothy 2001). Female reproductive tract damage caused by armoured genitalia, such as that seen in the bean weevil, *Calloscobruchus maculatus* (Crudgington and Siva-Jothy, 2000), could also confer post-copulatory benefits to males. Such damage could augment the release into the haemolymph of male derived chemicals that affect oviposition and receptivity, providing males with greater success in competition with other males.

Nevertheless, under some conditions, costly damage by males may constitute a deliberate attempt to shift the optimal life history strategies of females towards the optimum for males. Costs of mating may cause females to increase their investment in immediate reproduction and oviposit at a faster rate, rather than 'saving' investment for future matings. This is the suggested function of reproductive tract damage in *S. cynipsea* (Blankenhorn et al. 2002). Damage or the introduction of costly toxins into the female reproductive tract may cause females to refrain from remating so that they avoid damage suffered during subsequent copulations (Johnstone and Keller 2000).

7.5.3. Genetic evidence of conflict

Section 1.5 outlined some genetic evidence consistent with sexually antagonistic coevolution between male and female sex-limited traits in *D. melanogaster*.

Identification of sequence characteristics resulting from sexually antagonistic selection could provide 'genetic hallmarks' of sexual conflict. However, predictions resulting from sexual selection and expectations under sexual conflict still prove hard to separate. Both sexually antagonistic selection (Hughes 1997) and sexual selection (Prout and Bundgaard 1977; Clark et al. 2000) are predicted to maintain high levels of polymorphism. It has been suggested that sexual conflict could be responsible for rapid evolution of proteins involved in egg-sperm interactions in externally fertilising organisms (Palumbi 1998) and for positive selection on female mammalian egg-coat proteins (Swanson et al. 2001b). However, rapid evolution of male and female sex-limited traits is also expected to result from coevolution between male traits and female post-copulatory mechanisms of sperm choice. Again, therefore, many of the predictions that result from sexual conflict are also consistent with other models of sexual selection. The dissection of influences from sexual selection and sexual conflict on the evolution of Acps and female reproductive traits remains a huge challenge.

7.5.4. Heterospecific and inter-population crosses

It has been suggested that antagonistic arms races between the sexes could result in evolutionary trajectories between male and female adaptations, such as the observed trajectory of abdominal structures across species of male and female water striders (Arnqvist and Rowe 2002a). However, these evolutionary trajectories can be difficult to detect because within populations, males and females are expected to have matching levels of armament. One possible approach is to observe mating interactions between males and females of different allopatric populations, revealing subtle differences in reproductive behaviour or physiology. Predicting the outcomes of allopatric male-female interactions specific to sexually antagonistic selection, and distinct from those expected under sexual selection alone, is difficult. Several early studies undertook to examine the effects of interstrain crosses on several mating characteristics, such as sperm storage and sperm migration (Yanders 1963; DeVries 1964). Differences between inter- and intra-strain matings of *D. melanogaster* were found in the numbers of sperm stored and the rates of sperm storage by females. The results of such studies have recently been cited as evidence of sexually antagonistic coevolution (Rice 1998)

although, without explicit predictions as to the outcome of these interstrain crosses, this point is equivocal.

More recently, Knowles and Markow (2001) examined the outcome of interactions between males and females of two closely related species of *Drosophila*: *D. mojavensis* and *D. arizonae*. Males of these species leave a mating plug inside females after mating (Patterson 1946). The size of this plug is consistently larger and lasts for longer after reciprocal (inter-species) crosses than after matings between individuals of the same species. From this observation, it was suggested that the size and duration of the mating plug depended on the qualitative interaction between the male proteins make the plug and the female proteins that break it down. When males and females 'match' (during conspecific crosses), it is suggested that females are able to rapidly break the plug down; when males and females do not match (during inter-species crosses), the plug persists. It was suggested that there is sexual conflict over the ability of females to break down the plug. Rapid evolution due to sexually antagonistic selection on these proteins is suggested to result in a mismatch between closely related species, and hence the prolonged persistence of the insemination reaction in inter-species matings.

Other interpopulation studies, conducted on the housefly *Musca domestica* (Andres and Arnqvist 2001), the bean weevil *C. maculatus* (Brown and Eady 2001) and the flour beetle *T. castaneum* (Nilsson et al. 2002), have also examined the outcome of crossing two or more different populations in an orthogonal design. The results of these studies were mixed, although all claimed to provide evidence of sexually antagonistic conflict. Without specific *a priori* predictions as to the expectations of such inter-population studies under conditions of sexually antagonistic selection and sexual selection, such studies are unlikely to be very informative as to the existence or nature of sexual conflict. Together, these experiments emphasise a need for stronger *a priori* predictions based on conflict theory (Chapman et al. 2003).

To identify sexual conflict as the force driving the rapid evolution of reproductive traits, a clear functional understanding of the traits involved is required. Nevertheless, an approach using interpopulation crosses may prove to be a valuable tool for testing explicit predictions about the influence of sexual conflict on specific traits. I provide

here an illustration of predictions that might be made in response to a specific question regarding the nature of two traits: one expressed in the male, the other in the female. This scenario relates to putative traits thought to be involved in conflict in *D. melanogaster*. The putative male protein takes the form of an Acp that simultaneously confers benefits to males and costs to females, and the female protein confers resistance to this cost, to the detriment of male fitness. Does selection on these putative male and female traits cause changes in the 'quantities' of the proteins made, or the 'quality' of the protein expressed? I provide two alternative hypotheses (Fig. 7.1) as to the outcome of allopatric male-female matings (a) where selection acts on the quantities of protein involved and (b) where selection acts on the qualities of the proteins involved. These predictions illustrate that inter-population crosses, coupled with tight predictions based on sexual conflict, could prove a useful approach for examining specific questions about the selective influences on male and female reproductive traits (Fig. 7.1).

7.6. Summary

In summary, the data described in this thesis show that males with larger accessory glands mate at a higher frequency than males with smaller accessory glands when given the opportunity to express maximum mating frequency. I also present evidence to show that large males are better at sperm displacement than small males. I have considered the influence of females on post-mating events and have found that variation in female sperm storage organ morphology affects the temporal pattern of offspring production and potentially influences the outcome of sperm competition between two or more males. This experiment also confirmed that males of different genotypes vary in their sperm displacement abilities.

Male *D. melanogaster* produce a large number of seminal fluid proteins that have important consequences for male and female fitness. The identities and functions of only a few of these proteins are so far known, but Chapter 5 shows that the accessory gland protein SP is responsible for full expression of two of the most dramatic post-mating responses: the inhibition of female receptivity and the elevation of egg laying. I also examined the function of another seminal fluid protein, Acp62F, because it was previously shown to be toxic to females when ectopically expressed in the female

haemolymph, and thus represented a candidate for the cost of mating in *D. melanogaster*. The results of my experiment however, provide no evidence to suggest that Acp62F is responsible for the cost of mating.

Recent work suggests that sexually antagonistic selection influences the evolution of reproductive traits in *D. melanogaster*, and this makes the phenotypes and targets of Acps particularly interesting. The experiments described in Chapter 5 and 6 both use the novel technique of RNAi to suppress expression of genes of interest. Sexual selection has been used for over a century to describe the evolution of traits that affect mating success and fertilisation success. The genetic tools now available for *D. melanogaster* promise to help determine whether sexual conflict also has an important influence on the evolution of mating systems.

Figure 7.1. Predicted outcomes of mating interactions within and between three allopatric populations under sexually antagonistic selection. Sexually antagonistic selection acts on both the costly accessory gland protein(s) produced by male *D. melanogaster*, and the corresponding putative female counteradaptation. In **(a)** selection acts on the *quantity* and in **(b)** selection acts on the *quality* of these proteins. The predicted outcomes of these crosses are different under (a) and (b).

(a) Quantitative model for predicting the outcome of within- and between-population crosses.

		Male population		
		A: High quantities of costly Acp	**B: Medium quantities of costly Acp**	**C: Low quantities of costly Acp**
Female population	**A: High quantities of 'resistance' protein**	Base-line	↑ increased female longevity	↑↑ increased female longevity
	B: Medium quantities of 'resistance' protein	↓ decreased female longevity	Base-line	↑ increased female longevity
	C: Low quantities of 'resistance' protein	↓↓ decreased female longevity	↓ decreased female longevity	Base-line

The focus of selection is the effort that each sex invests in the conflict, e.g. the quantities of protein produced by males verses quantities of (the putative) 'resistance' proteins produced by females. Three populations are crossed in all combinations. The predictions rely on the assumption that there are differences in the absolute level of investment in a sexually antagonistic adaptation in each sex across populations. Female harm might be measured by observing female longevity, so in this example, the arrows indicate a predicted increase or decrease in female longevity relative to the base line (where the quantities of male and female proteins are matching). The results of

between-population crosses are expressed relative to the 'base-line' outcomes from within-population crosses. For example, an outcome biased towards male interests would indicate that males cause greater female harm than they do to females of their own population. Similarly, the opposite outcome indicates that females are able to resist males better than they are males of their own population. It is also predicted that pairings between A and C populations (those with largest difference in escalation levels) would result in stronger effects than other combinations. The predictions can be based on the relative quantities of harm and resistance in each of the crosses or on fitness. The model also predicts that reciprocal crosses should produce opposite results (e.g. A males x C females would differ from C males x A females). Males and females have consistent, predictable roles, for example, a male capable of inflicting high levels of harm on females would do so across all lines of females with weaker resistance. Here, manipulation-resistance can evolve only along a single dimension, which means that investment can either increase or decrease. The crucial prediction for this situation is that there is directional symmetry across the population crosses and that there are no interactions between the sexes in their effects. Interactions between the sexes would indicate that the outcome of male-female interactions is determined either by several interacting multidimensional quantitative traits or by responses in a 'state-matching' fashion (see (b) below).

(b) Qualitative model for predicting the outcome of within and between-population crosses.

		Male population		
		State A	**State B**	**State C**
Female population	**State A**	Match	No match	No match
	State B	No match	Match	No match
	State C	No match	No match	Match

The focus of selection is the quality, or 'state' of proteins produced by males and females, e.g. selection acts on males and females to change the quality rather than the quantity of the proteins (toxic, resistance or otherwise) that they produce. Predictions

are made based upon 'state matching' between males and females. All crosses other than within-population crosses result in a 'no-match' between reproductive state in males and females. Under this set of predictions, we expect males and females always to have a 'no-match' wherever they are not paired with mates from their own line. Crucially, however, this outcome differs from the quantitative predictions because of the lack of directional symmetry across the population crosses.

However, unlike in (a), it is not yet known whether, in the 'no-match' situation, the outcome would benefit females or males. It is not straightforward to predict which sex should do better until it is known whether matching is in the interest of one sex but not the other. Outcomes for males and females in crosses between populations are not consistent, and we expect strong interactions between the sexes in their effects. Males that inflict high levels of harm in one between-population cross would not necessarily do so in another. The signature of such a qualitative model would therefore be inconsistency of harmful or resistant females in different crosses. Significant interactions between the sexes have been observed in several studies (Yanders 1963; DeVries 1964; Andres and Arnqvist 2001; Knowles and Markow 2001; Nilsson et al 2002), which suggests that the outcome of male-female interactions are determined either by several interacting multidimensional quantitative traits in both sexes, or by responses in a 'state-matching' fashion.

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Appendix I

Effects of body size, accessory gland and testis size on pre- and postcopulatory success in *Drosophila melanogaster*

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Reproductive success in males is affected by events that occur both before and after mating. We used multiple regression to examine the relation between male pre- and postcopulatory success and body size, accessory gland and testis size in *Drosophila melanogaster*. Males with larger accessory glands mated at higher frequency than did males with smaller accessory glands. This association was over and above allometry of accessory gland size with body size. Larger males had higher postcopulatory success than smaller males. We found no evidence for any associations between pre- or postcopulatory success and testes size. Taken together with previous literature, our results suggest that larger males have both higher pre- and postcopulatory reproductive success than smaller males.

The reproductive success of males is affected by both pre- and postcopulatory events. Since Darwin's (1871) discussion of sexual selection, there has been considerable interest in morphological traits and behaviours that influence the mating frequency of males. In some species of insects, males compete physically or through agonistic displays to obtain access to females, and large male body size is often associated with success during precopulatory competition (Otronen 1984a; Crespi 1986, 1988; Alcock 1996). Large males may also be better able to attract, stimulate or coerce potential mates (Davidson 1982; Hughes & Hughes 1985; Simmons 1986, 1988; Crean et al. 2000). In contrast, smaller males are sometimes favoured in species where agility is required during scramble competition (Goldsmith & Alcock 1993; Vencl & Carlson 1998), for certain aspects of courtship (Steele & Partridge 1988), and during flight in male–male competition (Marshall 1988).

However, mating success alone is not enough to ensure that males sire many offspring. Females of most insect species mate multiply and store sperm; hence, variation in reproductive success and male–male competition among males is extended beyond the point of copulation (Parker 1970). Postcopulatory success of insect males is associated with traits such as genital morphology (Arnqvist & Danielsson 1999; Danielsson & Askenmo 1999) and body size (McLain 1985; Lewis & Austad 1990; Otronen 1994; Bissoondath & Wikland 1997; Wedell & Cook 1998).

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The relation between pre- and postcopulatory success and traits such as body size are important, because they will determine whether that trait is subject to similar selection pressures at different stages of the reproductive process. In some insect species, body size is positively related to both pre- and postcopulatory success (Conner et al. 1981; Otronen 1984a, b, 1994; McLain 1985, 1991; Lewis & Austad 1990, 1994; LaMunyon & Eisner 1993). In contrast, in other species, males of different sizes may adopt different mating strategies. For example, in the waterstrider *Gerris lacustris*, large males mate more often than do small males, but small males mate for longer and sire more offspring per mating, leading to similar overall reproductive success in males of all sizes (Danielsson 2001). Similarly, in the yellow dung fly, *Scatophaga* (=*Scathophaga*) *stercoraria*, large males achieve a higher fertilization success per unit time of copulation, because they pass ejaculate to their mates more rapidly than do small males (Simmons et al. 1996), but small males tend to copulate longer, for similar overall postcopulatory success (Simmons & Parker 1992; Parker & Simmons 1994).

Thus, the mechanisms underlying correlations between precopulatory success and body size may often be due to the advantage of large body size in male–male competition, or the greater ability of larger males to stimulate or coerce potential mates. However, the mechanisms behind associations between postcopulatory success and body size are less clear. One suggestion is that males with high postcopulatory success produce larger quantities of protein or sperm in their accessory glands or testes. This could translate into a positive correlation between

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postcopulatory success and body size if there were allometric relations between body size and the sizes of the accessory glands and testes. To distinguish between these associations, we examine the relation between body size, accessory gland and testis size and aspects of pre- and postcopulatory success in male *Drosophila melanogaster*.

In *D. melanogaster*, males vary in both pre- and postcopulatory success. Male size is positively correlated with mating frequency (Partridge & Farquhar 1983; Partridge et al. 1987a, b; Markow 1988), similar to other species of *Drosophila* (Markow 1985; Santos et al. 1988). However, postcopulatory competition between males is also important to male *D. melanogaster* reproductive success. Females mate multiply (Harshman & Clark 1998; Imhof et al. 1998) and can store sperm for up to 2 weeks after mating (Neubaum & Wolfner 1999). Therefore, besides mating at high frequency, a male must also be able to displace the sperm of a previous male from the storage organs of his mate and prevent his own sperm from being displaced during the female's subsequent copulations. The mechanisms by which males displace sperm and defend their own stored sperm are influenced by ejaculate proteins produced in the accessory glands (Harshman & Prout 1994; Gilchrist & Partridge 1995; Chapman et al. 2000). These proteins strongly influence the postcopulatory success of males (reviewed in Wolfner 1997), and evidence suggests that the amounts produced are important (Kalb et al. 1993).

Although the details of postcopulatory events are probably better known in this species than in any other insect, the relations between pre- and postcopulatory success and traits such as body size in *D. melanogaster* have not been investigated. We explored the associations between male body size and pre- and postcopulatory success. We also considered whether such associations are due to allometric relations between body size and testis or accessory gland size. We conducted two experiments. The first examined the relation between mating frequency, body size, accessory gland and testis size, and the second investigated the association between postcopulatory success, body size and accessory gland size.

METHODS

Maintenance of Stocks

Wild-type flies were derived from a collection made at Mas Canet, France in 1999. After collection, flies were kept in the laboratory in population cages at 25°C and on a 12:12 h light:dark photoperiod. Each cage was supplied with three 158-ml bottles containing 70 ml of Sugar-Yeast (SY) medium (100 g of yeast, 100 g of sugar, 27 g of agar, 30 ml of nipagin and 3 ml of propionic acid per litre of water) per week. These were left in the cage for 4 weeks.

Homozygous *scarlet* flies were derived from a stock (obtained from Alan Robertson in 1975) that had been backcrossed into a wild-type Dahomey background and subsequently maintained in 158-ml bottles containing 70 ml of SY medium as above. The *scarlet* recessive mutation occurs spontaneously in wild populations (tenHave et al. 1995) and appears to have a negligible effect on fitness.

Mating Frequency, Body, Accessory Gland and Testis Size

We examined the associations between male mating frequency, body size, accessory gland size and testis size. Eggs were collected from agar plates containing grape juice and a small quantity of live yeast paste, which were placed in cages containing adult wild-type flies for 6 h. The agar plates were then stored at 25°C for 24 h, and the resulting larvae were placed into vials containing SY medium at a standard density of 50 larvae/vial. SY medium was used throughout the experiment. We collected males and females from these cultures within 8 h of eclosion, using ice to anaesthetize them, and kept them in single-sex groups of 20.

We recorded male mating frequency by observing single males in vials together with five females during three observation periods on 3 separate days. Approximately 100 8-day-old wild-type males were placed singly in vials with five wild-type females per vial. We used CO_2 anaesthetic throughout except where stated. During observation periods, the temperature of these vials was kept at 27°C to increase the number of matings observed. We recorded the number of matings observed for each male (M_{ij}, where $i=1$ to N and N is the number of males in the cohort, and j is the observation period index number, $j=1$ to 3) over 6–8 h. At the end of each observation period, all individuals were returned to 25°C. Mating observations took place over 2 weeks. Data from only the second week were used in the analysis, because only during this week were the nonvirgin females randomized between males, to reduce the risk that males particularly effective at repressing receptivity in females would encounter the same females during different observation periods. At the end of each mating period, females were removed from the vials and pooled into groups of 20–40 per vial. Before the next mating observation period, females were again immobilized and redistributed among males in groups of five. This procedure minimized variation between vials in female remating propensity.

Because observation periods on each day were not identical in length, and the intensity of mating activity varied on different days, we based the index of mating frequency on the fraction of the total numbers of matings in each period. The number of matings observed for each male during each mating observation period (M_{ij}) was expressed as a fraction of the total number of matings by all males during that period, j:

$$F_{i,j}=\frac{M_{i,j}}{\sum_{i=1}^{i=N} M_{i,j}}$$

The fraction $f_{i,j}$ for each male was calculated for each mating period, and the average fraction over the three mating periods was calculated for each male, resulting in an index of mating frequency referred to as:

$$f_{i,j}=\frac{1}{3}\sum_{j=1}^{j=3}F_{i,j}$$

To obtain measures of body size, accessory gland size and testis size, we subsequently maintained males individually in fresh vials for 10–12 days after the last mating observation period to ensure sufficient time for full replenishment of the contents of the testes and accessory glands. Males were then immobilized with ice, and their reproductive tracts dissected in phosphate buffer solution (PBS) on a glass slide under a dissection microscope. Owing to the fragility of the samples, coverslips were not used. Samples were placed in 250 µl of PBS, which was sufficient to ensure they were free floating. We occasionally had to add PBS as it evaporated during measurement. Images of the accessory glands and testes were captured from a compound microscope (100 ×) using a video camera connected to a Macintosh computer. The accessory gland (A) and testis (T) perimeters of each male were measured with a polygon tool in the NIH Object Image program (version 1.62n3). Where possible, we measured each pair of accessory glands and testes and calculated the mean of the two measurements. Otherwise, we made only a single measurement. Body size was estimated from the perimeter of the wing. The right wing of each male was removed and mounted on a slide with Aquamount (BDH). Images of wings were captured from the compound microscope (100 ×) in the same way as the accessory glands and testes. The wing perimeter (W) was measured with the NIH Object Image program (Gilchrist & Partridge 1999).

Paternity, Body and Accessory Gland Size

To examine the relations between body size, accessory gland size and postcopulatory reproductive success, we collected wild-type flies as before. We collected eggs from the *scarlet* stock by placing adults in laying pots with plates that contained grape juice medium with a small quantity of autolysed yeast paste. Flies were left in the laying pots for about 6 h, then removed, and the plates were stored at 25°C. Twenty-four hours later larvae were placed into vials containing SY medium at a standard density of 50 larvae/vial. Virgin males and females were collected within 8 h of eclosion and kept in single-sex groups of 20.

Evidence suggests that females mate multiply in the wild (Harshman & Clark 1998; Imhof et al. 1998), and males are most likely to encounter nonvirgin females. Thus, we conducted double matings so that the target male was in postcopulatory competition with the female's previous mate. We anaesthetized 300 virgin *scarlet* females and 300 virgin *scarlet* males on ice and aspirated them in pairs into vials. The pairs were observed for 7 h; 38 pairs did not mate during that time and were therefore not used. After this first mating, resulting in *scarlet* offspring, males were removed. Three days later, we paired one virgin wild-type male with each female and observed the pairs again for about 7 h. Ninety-three pairs did not remate and were not used. After this second mating, which resulted in wild-type offspring, males were transferred to fresh vials and stored for 11–13 days, again to replenish the contents of the testes and accessory glands before measuring.

The twice-mated females were transferred every 2 days to fresh vials to avoid excessive larval competition from overcrowding. This was repeated until females ceased producing fertilized eggs, between 12 and 16 days later. After accounting for female deaths, sterility and escapes, the sample size for females from which we collected offspring was 112. Vials were stored at 25°C during larval development. Progeny counts and eye colour scores were made 12 and 15 days after the adult female had been introduced into each vial. Progeny counts were carried out in two stages to avoid deaths among the adult progeny from overcrowding. This provided a measure of the number of offspring produced by each female that were sired by the first male (P_F), and the number sired by the second male (P_S). We assessed postcopulatory competition between males by calculating sperm displacement ability, *SDA* (Hughes 1997) as

$$SDA_i=P_{Si}/(P_{Fi}+1)$$

Since the association between *SDA* and the three independent variables was not linear, the fifth root transformation of *SDA* was used (SDA_1) for the multiple regression analysis. The number of offspring that emerged from eggs produced after the first mating but before the second mating (P_u) provided an indication of variation in first male postcopulatory success in the absence of any postcopulatory competition. Females that did not produce fertilized eggs after the first mating were excluded, because this implies that the female, the male, or the mating was sterile. Body size, accessory gland size and testis size of the second (wild-type) males to mate were determined by dissection 11–13 days after mating, at the same stage as comparable measurements were made in the previous experiment. The perimeters of the wings, accessory glands and testes were determined with the methods described earlier. After dissection, the sample size was ca. 90. However, for some individuals, we were unable to obtain accurate measurements for one or two of the three traits measured. The samples sizes for the three measurements therefore differed slightly, and the degrees of freedom for each regression analysis are indicated in the results.

Data Analysis

We examined correlations between the independent variables by calculating correlation coefficients. Where variables were normally distributed, we used the Pearson correlation coefficient, r. For variables that were not normally distributed, we used the Spearman rank correlation coefficient, r_s. Normality of variables was tested with the Shapiro–Wilk method (Shapiro & Wilk 1965).

We used simple linear regression to determine which of the independent variables significantly accounted for variation in mating frequency for the first experiment

and variation in paternity (defined as second-male sperm displacement ability) for the second experiment. The independent variables of interest were body size, accessory gland size and testis size. In the second experiment, we added the number of offspring sired by the first male before the second mating (P_B). The strengths of the associations between the dependent and independent variables were tested by calculating the adjusted coefficient of determination, which described the total variance in the dependent variable that was accounted for by the independent variable, $R^2_a = 1 - (var_{unexplained}/var_{total})$. Negative values of R^2_a do not necessarily indicate that the relationship is negative, but imply that the population coefficient of determination is near zero. To test the significance of the relationships, we used F tests.

When there were correlations between body size, accessory gland size and testis size, multiple linear regression was used to examine whether any significant effects from accessory gland size and testis size were due to allometry with body size, or whether there were associations with these variables over and above body size. Wherever a variable, such as P_B, had a significant effect on the model, we used multiple regression to retain the significant variable in the model during examination of the other variables. Where multiple regression was used, R^2_a described the variance that had been left unexplained by one variable, which was subsequently accounted for by a new variable. Again, significance was tested with F tests. The normality of unexplained residuals resulting from the regression models was tested as before with the Shapiro–Wilk method (Shapiro & Wilk 1965).

RESULTS

Mating Frequency, Body, Accessory Gland and Testis Size

First, we used simple linear regression to examine the relationship between variance in mating frequency and variance in the separate independent variables, body size, accessory gland size and testis size. Body size showed a nonsignificant trend towards explaining a proportion of the variance in mating frequency ($R^2_{a1,78}=0.03$, $P=0.067$). Accessory gland size explained a significant proportion of the variance in mating frequency ($R^2_{a1,73}=0.11$, $P=0.002$; Fig. 1b), before taking into account the allometric relationship between accessory gland size and body size. Testis size did not explain a significant proportion of the variance in mating frequency ($R^2_{a1,75}=-0.013$, $P=0.904$).

Because the distributions of all three variables were normal, we used the Pearson correlation coefficient to examine correlations between body size, accessory gland and testis size. Since there were positive allometric relationships between accessory gland perimeter and body size ($r_{69}=0.254$, $P=0.035$) and testis perimeter and body size ($r_{70}=0.202$, $P=0.089$), multiple linear regression was used to retain the parameter body size in the model during examination of the effects of accessory gland size and testis size. The addition of accessory gland size into a regression model that already contained body size showed that variation in accessory gland size explained a significant proportion of the variance in mating frequency on top of that explained by body size ($R^2_{a1,69}=0.073$, $P=0.01$; Fig. 1c). When, in a separate model, testis size was added to the model already containing body size, no significant proportion of the variance in mating frequency was accounted for by testis size on top of variance that had been explained by body size ($R^2_{a1,70}=-0.007$, $P=0.489$). Conversely, adding body size into a regression model that already contained accessory gland size indicated that body size did not account for a significant proportion of the variation in mating frequency over and above the variation accounted for by accessory gland size ($R^2_{a1,69}=0.013$, $P=0.168$). All unexplained residuals resulting from these regression models were normally distributed.

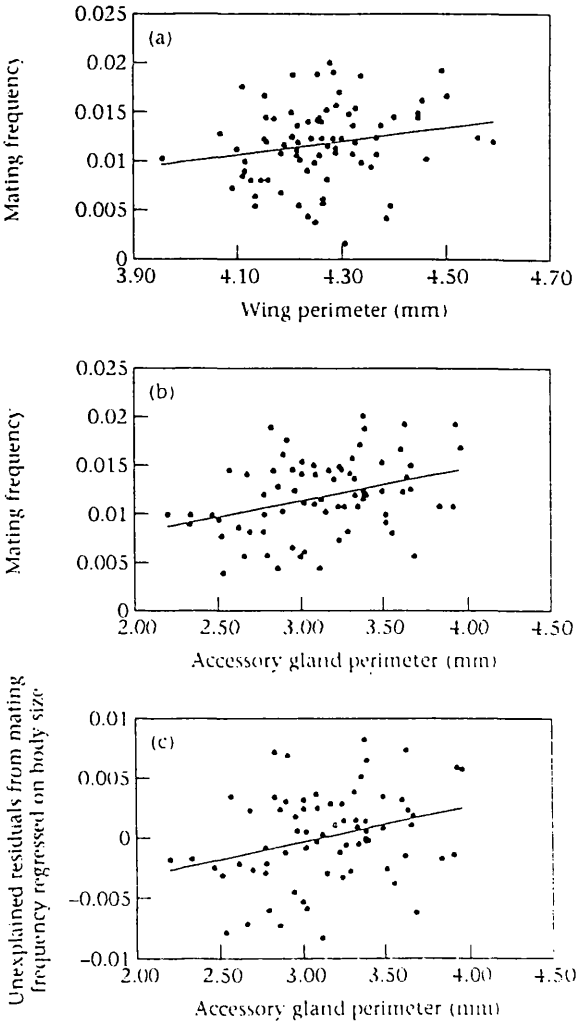

Figure 1. (a) Regression of mating frequency on wing perimeter (mm). (b) Regression of mating frequency on accessory gland perimeter (mm). (c) The unexplained residuals resulting from the regression of mating frequency on wing perimeter (mm), in turn regressed on accessory gland perimeter (mm).

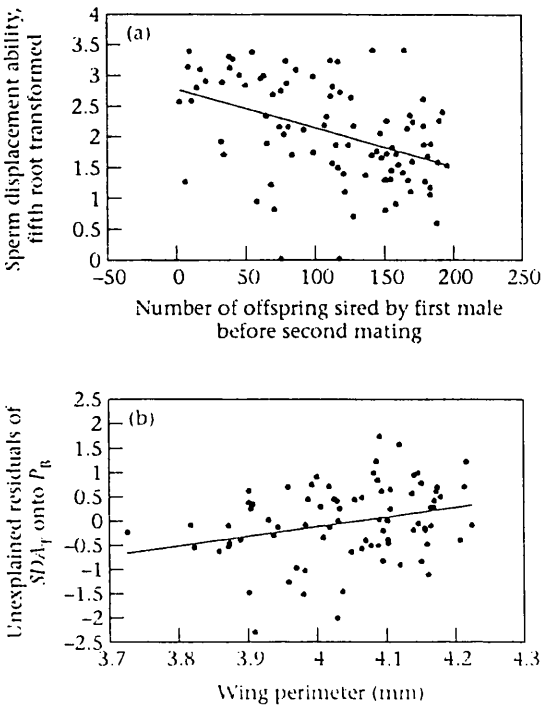

Figure 2. (a) Regression of sperm displacement ability (fifth root transformed, SDA_T) on the number of offspring sired by the first male before the second mating (P_B). (b) The unexplained residuals of SDA_T on the number of offspring sired by the first male before the second mating (P_B), regressed in turn on wing perimeter (mm).

Paternity, Body Size and Accessory Gland Size

Given that males with relatively large accessory glands tended to have higher mating success than those with small accessory glands, we next examined how successfully they engaged in postcopulatory competition. The associations between second-male sperm displacement ability (SDA_1), body size and accessory gland size were examined with multiple linear regression. Because we were interested only in variation in SDA_1, we included an additional factor, P_B (the number of offspring sired by the first male before the second mating), in the analysis.

The result of regressing SDA_1 on P_B explained a significant proportion of the variance in SDA_1 ($R^2_{a1,92}$=0.183, P<0.0001; Fig. 2a). This result was consistent with the expectation that first male postcopulatory success before a second mating is related to the ability of the first male to resist second-male sperm displacement. Since the proportion of variance in SDA_T explained by P_B was highly significant, we retained this parameter in the multiple regression model during examination of the effects of body size, accessory gland size and testis size. The addition of body size into a regression model that already contained the parameter P_B showed that second-male body size also accounted for a significant proportion of variance in second-male sperm displacement ability ($R^2_{a1,81}$=0.062, P=0.013; Fig. 2b). The addition of accessory gland size into a regression model that previously contained only the parameter P_B indicated that accessory gland size did not account for a significant proportion of the variance in sperm displacement ability ($R^2_{a1,87}$= −0.01, P=0.706).

Because wing perimeter was not normally distributed, we used the Spearman rank correlation coefficient to examine correlations between body size, accessory gland and testis size. Since there was a significant positive allometric relationship between accessory gland perimeter and body size (r_S=0.243, N=81, P=0.029), we used multiple regression to retain body size as well as P_B in the model during examination of the effect of accessory gland size. The result of adding accessory gland size into a model that already contained the parameters P_B and body size indicated that accessory gland size did not explain additional variance in sperm displacement ability over and above that accounted for by body size ($R^2_{a1,79}$= −0.013, P=0.945). All unexplained residuals resulting from the regression models were normally distributed.

DISCUSSION

An important finding of this study was the positive relationship between mating frequency and accessory gland size. The additional result that there was no significant association between mating frequency and body size over and above the effects of accessory gland size suggests that accessory gland size could be at least partly responsible for the well-established correlation between body size and mating frequency in *D. melanogaster* (Partridge & Farquhar 1983; Partridge et al. 1987a, b). Furthermore, larger males had a higher postcopulatory success than smaller males, although we found no evidence that this association was due to the size of the accessory glands.

Although we found only a nonsignificant trend for an association between body size and mating frequency, other studies have shown that large males court females more and have a higher lifetime mating success than do small males under laboratory conditions (Partridge & Farquhar 1983; Partridge et al. 1987a). In the wild, too, males captured while mating were larger than were nonmating males (Partridge et al. 1987b). If body size is related to mating success partly through greater aggression by larger males, as suggested by Dow & von Schilcher (1975), the absence of male–male competition for matings in the present study could have resulted in the weaker-than-expected association between body size and mating frequency.

Males with larger accessory glands mated more often than did males with smaller glands, regardless of the effects of body size. There was a significant allometric relationship between accessory gland size and body size. The association between body size and mating frequency found in earlier studies (Partridge & Farquhar 1983; Partridge et al. 1987a, b) could therefore be partly because larger males have larger accessory glands. This view is supported by the absence of a significant association between mating frequency and body size, over and above accessory gland size. One possible explanation for this result is that males with relatively larger glands are able to mate more frequently and retain the resources to achieve

high postcopulatory success for longer. We might therefore expect males with smaller accessory gland resources to refrain from mating frequently, because they could suffer the costs of courtship (Cordts & Partridge 1996) without the fertilization benefits. This hypothesis is also consistent with evidence that the relatively high mating frequency of larger males is because of behaviour determined by the male, rather than female discrimination (Partridge et al. 1987a). However, this hypothesis will need to be evaluated by manipulating accessory gland size and body size, and examining the effects on mating success.

Our results suggest that there is no association between testis size and mating frequency in *D. melanogaster*. This is consistent with the finding that males tend to pass a quantity of sperm during copulation that is in excess of that stored in the female sperm storage organs (Lefevre & Jonsson 1962), suggesting that males are not limited by sperm quantities. Our results are similar to those obtained from the stalk-eyed fly, *Cyrtodiopsis dalmanni*, where there was a significant association between mating rate and accessory gland size, but not between mating rate and testis or body size (R. Baker, M. Denniff, K. Fowler, A. Pomiankowski & T. Chapman, unpublished data).

The number of offspring sired by the first male before the second mating was negatively associated with success in second-male postcopulatory competition. This finding is consistent with the results of Gilchrist & Partridge (1997), and suggests that the higher a male's fertilization success in the absence of postcopulatory competition, the more difficult it is for a second male to displace his sperm.

We found a positive association between postcopulatory success and male body size in *D. melanogaster*, similar to results of studies on other species (Lewis & Austed 1990; LaMunyon & Eisner 1993; Bissoondath & Wikland 1997; Wedell & Cook 1998). However, the biological mechanism underlying this correlation is not clear. One explanation could be that large individuals pump ejaculate faster and more efficiently during copulation than do smaller males, which is the case in the yellow dung fly (Simmons et al. 1996). Under this hypothesis, accessory gland size need not reflect postcopulatory success. Future work should quantify the amount of ejaculate passed to females by males of different sizes, and test the relation between ejaculate size and postcopulatory success. An important question is whether males with high precopulatory success and large accessory glands also have larger body size and higher postcopulatory success. Although the results of this study support this hypothesis, confirmation will require comparison of pre- and postcopulatory success in the same individuals.

Thus, our conclusions together with results of previous studies indicate that larger males both mate at high frequency and have higher postcopulatory success than do smaller males. However, the association between body size and mating frequency appears to be at least partly due to accessory gland size. Males with larger accessory glands mate more often, over and above the effects of body size. These results suggest that premating selection on body size in males is reinforced at the postcopulatory stage of the reproductive process.

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THE ROYAL SOCIETY

Appendix II

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Influence of female reproductive anatomy on the outcome of sperm competition in *Drosophila melanogaster*

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Females as well as males can influence the outcome of sperm competition, and may do so through the anatomy of their reproductive tracts. Female *Drosophila melanogaster* store sperm in two morphologically distinct organs: a single seminal receptacle and, normally, two spermathecae. These organs have different temporal roles in sperm storage. To examine the association between sperm storage organ morphology and sperm competition, we used a mutant type of female with three spermathecae. Although the common measure of sperm competition, P_2, did not differ between females with two and three spermathecae, the pattern of sperm use over time indicated that female morphology did affect male reproductive success. The rate of offspring production by females with three spermathecae rose and fell more rapidly than by females with two spermathecae. If females remate or die before using up second male sperm, then second male reproductive success will be higher when they mate with females with three spermathecae. The results indicate that temporal patterns of sperm use as well as P_2 should be taken into account when measuring the outcome of sperm competition.

Keywords: spermathecae; sperm storage; temporal sperm use

1. INTRODUCTION

Since the inception of work on sperm competition (Parker 1970), studies of post-copulatory events have tended to concentrate on interactions between competing males. However, females too may influence patterns of sperm use (Walker 1980; Otronen *et al.* 1997; Ward 1998, 2000; Hellreigel & Bernasconi 2000). Various mechanisms have been suggested for ways in which multiply mated females may bias paternity in favour of particular males (Birkhead *et al.* 1993; Keller & Reeve 1995; Eberhard 1996). The complex morphology of female reproductive tracts in many species may reflect selection on females to influence the use of sperm from different males for fertilization (Walker 1980; Linley & Simmons 1981).

Females may affect the outcome of sperm competition through the utilization of different types of sperm storage organ (Pitnick *et al.* 1999). Female *Drosophila melanogaster* mate multiply (Imhof *et al.* 1998) and there is natural genetic variation between females for the degree of last-male sperm precedence (Clark & Begun 1998; Civetta & Clark 2000). Female flies can store sperm for up to two weeks after mating (Gilbert *et al.* 1981) and commonly store the sperm of two or more males simultaneously (e.g. Marks *et al.* 1988). They possess two morphologically distinct sperm storage organs: a seminal receptacle and, normally, two spermathecae. These organs have different temporal roles: sperm from the spermathecae are released sooner than are sperm from the seminal receptacle (Gilbert 1981).

The main aim of the study was to examine how female reproductive tract morphology affects sperm use, using mutant female *D. melanogaster* with three spermathecae rather than the normal two (figure 1). Sperm use can be estimated by counting the numbers of offspring sired by a male and, for two males mated to a single female, the outcome of sperm competition is generally measured using the index P_2 (second-male offspring/(first + second-male offspring)). However, this measure ignores the dynamics of sperm use. If a female mates more than twice, the second male's reproductive success will be affected by the rate at which second male's sperm are used for fertilization (Prout & Bundgaard 1977; Gilchrist & Partridge 1995). The seminal receptacle and spermathecae are thought to have different temporal roles in sperm storage (Gilbert 1981), so sperm use over time was also monitored. Temporal patterns of sperm use will provide an additional source of variation in male success during sperm competition if females die or remate before all the sperm of a previous male are used.

2. METHODS

(a) *Stocks and cultures*

(i) *Females with two and three spermathecae*

Females were derived from a line created by the mobilization of the *PlacW* insertion in the line *P{w + mC = lacW} l(3)j10B6*^{10B6}*/TM3*, from the Bloomington stock centre (stock number: 10175) during a mutagenesis screen. This line was cultured in vials containing 7 ml of maize–yeast medium (10 g agar, 85 g sugar, 60 g maize, 20 g yeast and 25 ml nipagin per litre of water) which was subsequently used throughout the experiment.

Dissection of the females revealed that about 30% of females possessed three spermathecae (3SP) rather than the normal two (2SP) (figure 1). However, as 2SP and 3SP females were also found in the original *PlacW* line, it is unlikely that the 3SP morphology was caused by mobilization of the *PlacW* to a new pos-

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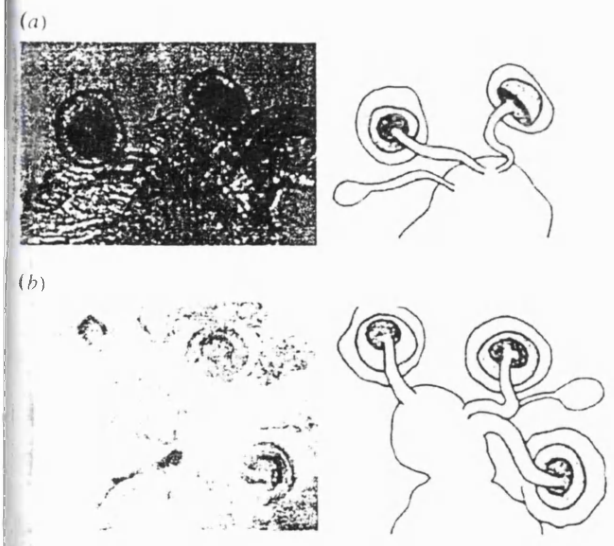

Figure 1. The spheroid, darkly pigmented cuticle of *Drosophila melanogaster* spermathecae from (*a*) a 2SP female and (*b*) a 3SP female. Samples were dissected on glass slide in PBS. Images were captured from a compound microscope (magnification × 250) using a video camera connected to a Macintosh computer.

ition in the genome. The genetic basis for this variation in spermathecal morphology was therefore unknown and naturally occurring. Dissection of mated females in phosphate-buffered saline (PBS) and examination under a compound microscope (magnification × 250) confirmed that the spermathecae of 2SP and 3SP females all contained sperm. 2SP and 3SP females were obtained from the same vials and experienced the same culture conditions.

(ii) *Wild-type*

Wild-type flies were derived from a collection made at Mas Canet (near Montpellier, France) in 1999. Flies were kept in the laboratory in population cages at 25 °C on a 12 L : 12 D regime. Each cage was supplied with three one-third of a pint glass bottles containing 70 ml sugar–yeast medium (100 g yeast, 100 g sugar, 27 g agar, 30 ml nipagin and 3 ml propionic acid per litre of water) per week, which were left in the cage for a total of four weeks.

(iii) *Brown dominant (bw^D)*

bw^D flies were obtained from the Umeå *Drosophila* stock centre (stock number: 40650) (February 2000), and were cultured in one-third of a pint glass bottles containing maize–yeast medium.

(iv) *Offspring phenotypes*

When mated to females described in § 2a(i), bw^D males sire phenotypically bw^D female offspring, distinguishable from wild-type offspring by their dark red eye colour. Because the females used in the experiment were homozygous for the *white* allele on the X chromosome (although partially rescued by the *PLacW* insertion), male offspring sired by wild-type or bw^D males all carried only the maternally derived *white* allele and were thus indistinguishable. The sex ratio was assumed to be 1 : 1 and estimates of bw^D and wild-type sperm use were made using female offspring only.

(b) ***Experimental methods***

To determine the effect of spermathecal number on the outcome of sperm competition, 2SP and 3SP females were mated to either (i) bw^D then wild-type males, or to (ii) wild-type then bw^D males. All parental flies were reared at a standard density of 50 larvae per vial. Virgins were collected within 8 h of eclosion and placed in vials in single sex groups of 20. Approximately 260 7-day-old virgin females were anaesthetized using ice and aspirated into individual vials, and each was paired with one 4-day-old bw^D or wild-type male. Pairs were observed for the next 6 h and any that had not mated during this time were discarded. After each mating, males were removed. Seventy-two hours later, females that had previously mated with a wild-type male were given a 7-day-old bw^D male, and each female that had previously mated with a bw^D male was given a 7-day-old wild-type male. Pairs were observed for the next 9 h and any females that did not remate were again discarded. After each successful remating, the males were again removed and females were aspirated into fresh vials. After remating, 104 females had been mated first to bw^D then to wild-type males, and 82 had been mated first to wild-type then to bw^D males. Significantly fewer females mated first to wild-type males remated in the time allowed, compared with females mated in the opposite order ($\chi_{[1]} = 8.061$; $p = 0.0045$), probably because of a lower courtship ability among bw^D males.

After mating, the twice-mated females were transferred to new food with *ca.* 1.5 mg of live yeast. This was repeated every 2 days until the females ceased to produce offspring. To prevent crowding-induced mortality, the numbers of growing offspring were restricted to no more than 125 per vial. The offspring were counted and scored for eye colour on days 11 and 14 after the female parent had been introduced into each vial. This resulted in female offspring counts from each 2-day sample vial (n_t) representing the rate of use of bw^D and wild-type male sperm per 2-day period by parental females.

Parental females were transferred to Eppendorf tubes and frozen at −84 °C until dissection in PBS to determine whether they were 2SP or 3SP females. A small proportion of females resembled 3SP females but had two spermathecae that were fused or shared a single duct. For simplicity, only females with three distinct spermathecae were included in the dataset. The sample size was further reduced by deaths of the female parents before dissection, bringing the final sample sizes to 89 for females mated first to bw^D then to wild-type males, and 58 for females mated first to wild-type then to bw^D males.

3. RESULTS

(a) ***Associations between total numbers of offspring, P_2, spermathecal morphology and male genotype***

Associations between total numbers (n_t) of bw^D and wild-type female offspring produced and spermathecal morphology were examined. For females mated first to bw^D then to wild-type males, total numbers of offspring sired by the first male to mate (first-male n_t) produced by 2SP females were compared with first-male n_t produced by 3SP females. Similar comparisons between 2SP and 3SP females were made for second-male n_t and for females mated in the opposite order. Kruskal–Wallis tests were used throughout, because n_t was distributed non-normally. For second-male n_t produced by females mated first to bw^D and then to wild-type males, 3SP females produced

Table 1. (*a*) Median total number of female offspring (n_t) sired by the first and second males to mate 2SP and 3SP females, from the time of the second mating until female sterility. Females were mated first to bw^D and then to wild-type males or first to wild-type and then to bw^D males. (*b*) Median P_2 values (first-male n_t/(first-male n_t + second-male n_t)) for 2SP and 3SP females mated first to bw^D and then to wild-type males or in the opposite order.

(*a*)

mating order	sired by	spermathecae	median n_t
bw^D, wild-type	first male	2	29
		3	13
	second male	2	91
		3	59
wild-type, bw^D	first male	2	42
		3	24
	second male	2	94
		3	82

(*b*)

mating order	spermathecae	median P_2
bw^D, wild-type	2	0.749
	3	0.867
wild-type, bw^D	2	0.723
	3	0.806

significantly fewer offspring than did 2SP females ($p = 0.0283$). Moreover, combining probabilities from all tests of significance (Sokal & Rohlf 1995) showed that, overall, 3SP females produced significantly fewer offspring than did 2SP females ($\chi^2_8 = 15.90$; $p = 0.0438$).

Kruskal–Wallis tests to examine differences in n_t sired by first and second males of different genotypes were carried out on another four comparisons. For 2SP females, first-male n_t produced by females mated first to bw^D then to wild-type males was compared with first-male n_t produced by females mated first to wild-type then to bw^D males. Similar comparisons were carried out for second-male n_t and for 3SP females. Females mated first to wild-type then bw^D males tended to produce more first- and second-male offspring than females mated in the opposite order (table 1*a*). However, this was only significant for first-male n_t produced by 2SP females ($p = 0.0371$) and combining probabilities from all four tests yielded no significant differences ($\chi^2_8 = 11.99$; $p = 0.1514$). Similar tests, and tests on their combined probabilities, were carried out on P_2 values (table 1*b*). This revealed no significant differences between 2SP and 3SP females (for combined probabilities, $\chi^2_4 = 4.14$; $p = 0.3866$), and no associations between P_2 and the genotype of the second male to mate (for combined probabilities, $\chi^2_4 = 4.82$; $p = 0.3061$).

(b) *Associations between offspring produced in 2-day samples, spermathecal morphology and male genotype*

All offspring were collected in 2-day samples and the number of female offspring per 2-day sample is referred to as n_r. The examples in figure 2 show first-male (figure 2*a*) and second-male (figure 2*b*) n_r over time produced by eight individual parental females in the experiment. Figure 3 shows bw^D and wild-type n_r production over time for all 147 parental females. We tested for an effect on these temporal patterns of spermathecal number and genotype of the first and second males to mate. In figure 4, n_r over time has been categorized by whether the parental female had 2SP (figure 4, columns *a–c*, rows (i) and (iii)) or 3SP (figure 4, columns *a–c*, rows (ii) and (iv)), and whether offspring were sired by the first (figure 4*a*) or second male (figure 4*b*) to mate. Females were either mated first to wild-type and then to bw^D males (figure 4*a–c*, (i) and (ii)) or to bw^D then wild-type males (figure 4*a–c*, (iii) and (iv)). Median numbers of offspring produced within each 2-day sample have been illustrated for clarity.

We examined the time (the 2-day sample) at which n_r reached a maximum (t_{nrmax}). Kruskal–Wallis tests showed that for females mated first to bw^D and then to wild-type males, 3SP females had a lower t_{nrmax} than did 2SP females, both for offspring sired by the first ($p = 0.0357$) and second ($p = 0.0387$) males. n_r therefore rose more rapidly for 3SP compared with 2SP females. In addition, t_{nrmax} was lower for wild-type than for bw^D offspring, both for offspring sired by the first (2SP females: $p = 0.0001$; 3SP females: $p = 0.0055$) and second (2SP females: $p = 0.0001$; 3SP females: $p = 0.0231$) males to mate. Use of bw^D sperm was therefore delayed relative to wild-type.

Any differences between 2SP and 3SP females in the numbers of offspring produced during any 2-day sample will indicate a difference in the temporal patterns of n_r. Kruskal–Wallis tests were carried out on n_r (each 2-day sample separately), comparing the same groups as for tests carried out on n_t. After correcting for multiple comparisons, the critical p-value for these tests was 0.0071. For second-male offspring produced by females mated first to bw^D and then to wild-type males, n_r on days 6 and 8 was significantly lower for 3SP females than for 2SP females (table 2*a*; figure 4*b*(i) and (ii)). There were similar patterns for first-male offspring, although the main difference (for n_r collected on day 8) was marginally non-significant ($p = 0.0081$; figure 4*a*(i) and (ii)). Similar trends for females mated to males in the opposite order were not significant (table 2*a*; figure 4*a–c* (iii) and (iv)). These results indicate that the falling phase of n_r over time is

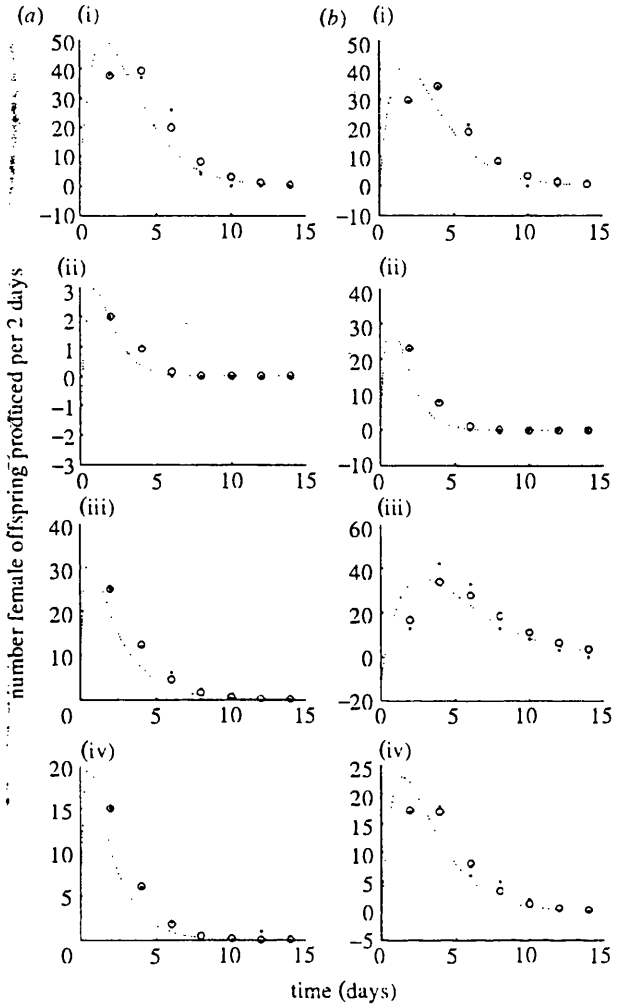

Figure 2. Numbers of female offspring (indicated by dots) produced per 2-day sample (n_r) over time (in days), by eight different parental females, sired by the (*a*) first or (*b*) second male to mate. Dotted lines represent the best-fit two-compartment model to n_r over time (see Appendix A). Open circles represent the integral of the dotted line over 2 days, indicating the model's estimation of n_r from the best-fit parameters. Graphs show n_r over time for: (i) 2SP females mated first to bw^D then to wild-type males; (ii) 3SP females mated first to bw^D then to wild-type males; (iii) 2SP females mated first to wild-type then to bw^D males; and (iv) 3SP females mated first to wild-type then to bw^D males.

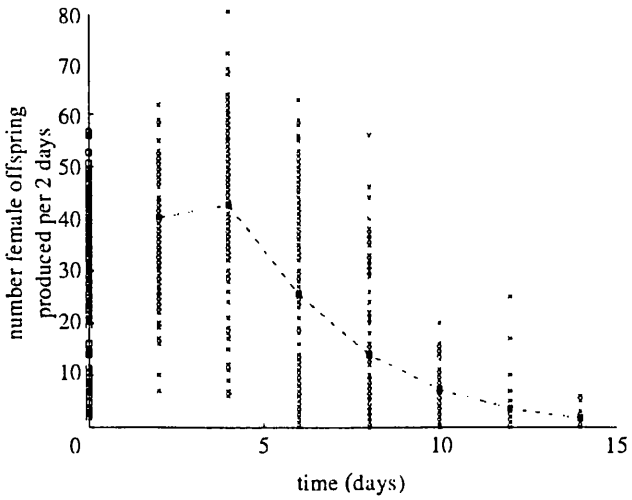

Figure 3. Numbers of female offspring (crosses) produced per 2-day sample (n_r) over time (in days) by each female parent, bw^D and wild-type offspring shown as separate data-points. Open squares (at day 0) indicate the numbers of first-male offspring produced by each female parent during the equivalent time interval before the second mating occurred. The model was fitted to n_r over time for each individual female parent, bw^D and wild-type males separately. The median parameter values from these fits were taken and used in the model to produce re-estimated 'model' values of n_r for each 2-day sample (closed squares).

more rapid for 3SP compared with 2SP females. The analysis also revealed differences in temporal patterns of n_r associated with male genotype. 2SP females mated to males in different orders differed significantly in first-male n_r (collected on days 2 and 4) and second-male n_r (collected on day 2) (table 2*b*; figure 4*a–c* (i) and (iii)). Although similar trends for 3SP females were non-significant, these differences indicate that bw^D n_r over time was delayed relative to wild-type n_r over time.

(c) *Associations between the temporal patterns of offspring production, spermathecal morphology and male genotype*

To summarize temporal patterns of sperm use, a model was fitted to n_r over time for each parental female separately (see examples in figure 2). Differences in temporal patterns of sperm use between groups (figure 4) can be determined by statistical analysis of the best-fit parameters of the model. In a previous study, passive sperm loss in the zebra finch *Taeniopygia guttata* was adequately described using a one-compartment model (Lessells & Birkhead 1990; Colegrave *et al.* 1995). By contrast, the rising and falling phases of n_r over time for females in the present experiment (figures 2 and 3) mean that a one-compartment model was not adequate (see Appendix A). A two-compartment model was therefore used to describe the temporal patterns of first- and second-male n_r for each individual female (see Appendix A). Kruskal–Wallis tests were used to determine if the model parameters were significantly affected by spermathecal morphology and male genotype.

A low value of parameter τ_1 corresponds to a rapid initial falling phase of n_r over time (see Appendix A). For females mated first to bw^D then to wild-type males, parameter τ_1 was significantly lower for 3SP than for 2SP females (table 3*a*) for both first- (not shown) and second-male n_r over time (figure 4*a*,*b* (i) and (ii)). The same trend was seen for females mated in the opposite order (figure 4*a–c* (iii) and (iv)), although the differences here were non-significant (table 3*a*). Combining probabilities from each of the independent tests of significance, showed that, overall, τ_1 was consistently significantly lower for 3SP females than for 2SP females ($\chi^2_8 = 20.82$; $p = 0.0076$). Parameter τ_2 is associated with both the rising phase and (less strongly) with the long-term falling phase of n_r over time (see Appendix A). For females mated first to bw^D and then to wild-type males, parameter τ_2 was lower for 3SP compared to 2SP females (table 3*a*). Moreover, combining probabilities across all Kruskal–Wallis tests carried out on τ_2 (second-male offspring shown in table 3*a*),

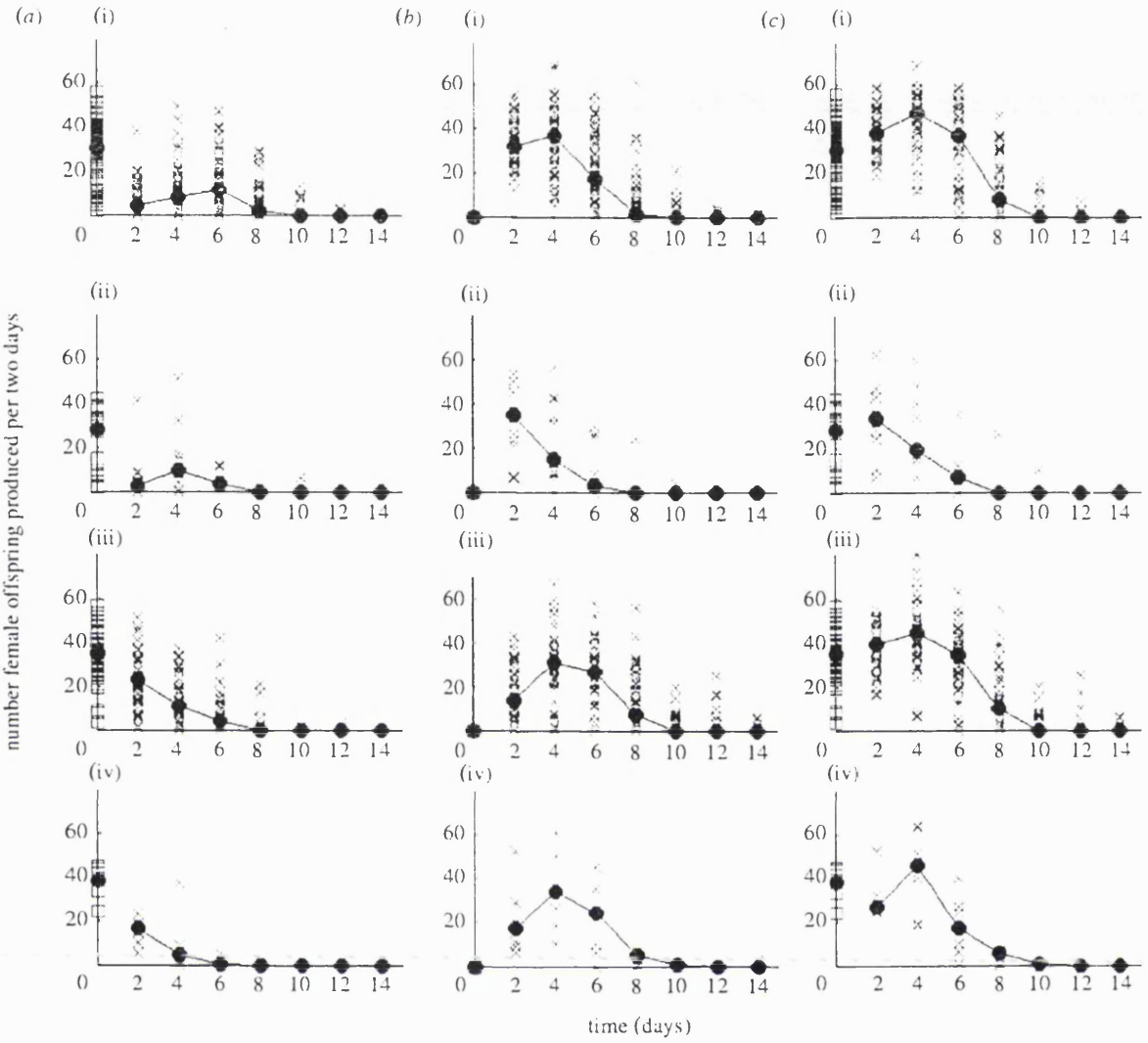

Figure 4. Numbers of female offspring (crosses) produced per 2-day sample (n_r) over time (in days) by each female parent. Graphs show n_r over time sired by (*a*) the first male to mate, (*b*) the second male to mate, and (*c*) the offspring sired by both first and second males. On each graph, filled circles indicate the median n_r across females within time samples. Lines joining the filled circles represent the median n_r over time. Graphs in (*a*) also include the numbers of offspring sired by the first male before the second mating occurred (filled squares) during the equivalent time sample. Graphs show n_r over time for: (i) 2SP females mated first to bw^D then to wild-type males; (ii) 3SP females mated first to bw^D then to wild-type males; (iii) 2SP females mated first to wild-type then to bw^D males; and (iv) 3SP females mated first to wild-type then to bw^D males.

showed that, overall, τ_2 was significantly lower for 3SP females than for 2SP females ($\chi^2_8 = 16.60$; $p = 0.0346$). Thus, these results support the finding that 3SP females have faster rising and falling phases of n_r over time than females with 2SP (figure 4).

There were also significant differences in the temporal patterns of bw^D and wild-type offspring production. Combining probabilities resulting from tests carried out on first- and second-male offspring and across 2SP and 3SP females (table 3*b*), showed that parameter τ_1 was significantly higher for bw^D than for wild-type males ($\chi^2_8 = 54.02$; $p < 0.0001$). The same was true for parameter τ_2 ($\chi^2_8 = 49.05$; $p < 0.0001$). This confirms that for first- and second-male offspring, bw^D n_r over time was delayed relative to wild-type n_r over time (figure 4). Parameter τ_3 also relates to the rising phase, but corresponds most strongly to the overall magnitude of the curves (see Appendix A), where a higher value of τ_3 corresponds to a lower overall magnitude of the curve. Again, combining probabilities from independent tests of significance (table 3*b*), showed that parameter τ_3 was significantly higher for bw^D as compared with wild-type n_r over time ($\chi^2_8 = 28.20$; $p = 0.0004$). This is therefore consistent with previous tests carried out on n_t, where for 2SP females bw^D first males sired fewer total offspring than did wild-type first males.

4. DISCUSSION

(a) *Associations between sperm use and spermathecal morphology*

The results show that analysis of temporal patterns of offspring production can reveal features of sperm competition that are missed by tests carried out on P_2 or on the total numbers of offspring produced. One of the main findings was that the rate of offspring production by females with three spermathecae (3SP) increased and decreased

Table 2. Median n_r produced during 2-day samples from time of remating until female sterility.
(Significant p-values (after correcting for multiple comparisons, critical value $p = 0.0071$) are marked with *. p-values result from Kruskal–Wallis tests carried out on each measurement of n_r separately to compare (*a*) offspring production by 2SP and 3SP females (results for second-male offspring only are shown here) and (*b*) offspring sired by the first and second males of bw^D or wild-type genotype (offspring produced by 2SP females only are shown here).)

(*a*)

			days						
sired by	mating order	spermathecae	2	4	6	8	10	12	14
second male	bw^D, wild-type	2SP	32	35	16	1	0	0	0
		3SP	34	13.5	3	0	0	0	0
		p-value	0.9681	0.0098	0.0013*	0.0030*	0.3428	0.0864	0.2818
second male	wild-type, bw^D	2SP	13	29	26	4	0	0	0
		3SP	17	34	24	5	1	0	0
		p-value	0.4506	0.4722	0.7419	0.3642	0.8194	0.1785	0.5382

(*b*)

			days						
spermathecae	sired by	mating order	2	4	6	8	10	12	14
2SP	first male	bw^D, wild-type	2	5	4	0	0	0	0
		wild-type, bw^D	22	10.5	3.5	0	0	0	0
		p-value	0.0001*	0.0055*	0.4415	0.0969	0.2852	0.9505	0.4058
2SP	second male	bw^D, wild-type	32	35.5	16.5	1	0	0	0
		wild-type, bw^D	13	29	26	4	0	0	0
		p-value	0.0001*	0.0396	0.3087	0.0664	0.0843	0.2283	0.5149

Table 3. Median values for best-fit parameters of the two-compartment model (see Appendix A) to n_r over time.
(p-values are the result of Kruskal–Wallis tests carried out on the best-fit parameters to compare n_r over time for: (*a*) 2SP and 3SP females (results for second-male n_r only are shown here); and (*b*) offspring sired by the first and second males of different genotypes (results for 2SP females only are shown here). Significant p-values are marked with *.)

(*a*)

			parameters				
sired by	mating order	spermathecae	ν_1	ν_2	τ_1	τ_2	τ_3
second male	bw^D, wild-type	median for 2SP	410 000	5×10^{-5}	1.590	1.560	5420
		median for 3SP	922 000	0.0031	1.050	1.150	8510
		p-value	0.9359	0.4847	0.0031*	0.0334*	0.9556
second male	wild-type, bw^D	median for 2SP	157 500	−0.0006	2.250	2.240	4430
		median for 3SP	124 000	−2.27	2.050	1.780	368
		p-value	0.6494	0.8202	0.6862	0.3247	0.3372

(*b*)

			parameters				
spermathecae	sired by	mating order	ν_1	ν_2	τ_1	τ_2	τ_3
2SP	first male	bw^D	2×10^6	−0.223	2.59	2.41	134 000
		wild-type	21 600	0.0007	1.145	1.005	21.95
		p-value	0.0003	0.0002*	0.0001*	0.0001*	0.0001*
2SP	second male	bw^D	157 500	−0.0006	2.25	2.24	4430
		wild-type	410 000	0.0005	1.59	1.56	5420
		p-value	0.6336	0.1466*	0.0001*	0.0002*	0.8837

more rapidly than for females with two (2SP), although this difference was significant only for females mated first to bw^D then to wild-type males. These temporal patterns are important because, if a female dies or remates with a third male, second male reproductive success will be higher if his sperm is used faster (Anderson 1945; Prout & Bundgaard 1977; Gilchrist & Partridge 1995). Thus, analysis of temporal patterns of sperm use can provide insights into the potential effects of traits on male reproductive success.

The rapid rise and fall of the rate of offspring production for 3SP compared with 2SP females may have been due to the faster release of sperm from three spermathecae with three ducts compared with two spermathecae and two ducts. This could result in the loss of coordination between sperm release, ovulation and fertilization. An earlier study (Bouletreau-Merle 1977) showed that the removal of one spermatheca from 2SP females also resulted in a rapid falling phase of offspring production. The present results are therefore consistent with two alternative hypotheses. The first is that 3SP females had reproductive tract defects that could not be detected under the microscope but represented a system functionally equivalent to the one-spermathecal system. This would result in a reduction of sperm storage capacity and perhaps a reduction in the ability to maintain viable sperm for fertilization longer than 4 days (Anderson 1945; Filosi & Perotti 1975). A second idea is that a careful balance is required between sperm storage, release and fertilization mechanisms, reflecting a more coordinated system than previously thought. Under both hypotheses, females with unusual spermathecal morphology may rely more for sperm storage on the seminal receptacle, which fills and releases sperm sooner than do the spermathecae (Gilbert 1981).

(b) *Associations between sperm use and first and second male genotype*

Male genotype also affected the temporal pattern of sperm use. Comparisons across mating orders of the numbers of first-male offspring produced before the second mating with those produced after the second mating indicates that bw^D males were less able to displace first-male sperm. The reduced displacement of wild-type first-male sperm meant that maximum first-male offspring production occurred significantly later for bw^D compared with wild-type offspring, and this was consistent with the significantly higher values of the model parameter τ_2 in this group. Poor displacement by bw^D males is consistent with direct observations of sperm storage in doubly mated females, and may be an effect either of genetic background or of bw^D itself (Civetta 1999).

For second-male offspring too, the rising phase of bw^D offspring production was slower than for wild-type offspring, a delay that could be associated with the reduced ability of bw^D males to displace first-male sperm. However, values of parameter τ_1, describing the falling phase of offspring production, indicated that bw^D males continued to sire offspring for longer than did wild-type males (also see figure 4). Although bw^D second males did not suffer in total offspring numbers produced (table 1*a*), this delay in the temporal pattern of sperm use is important. It indicates that the reproductive success of bw^D compared with wild-type males could have been compromised had females mated a third time.

Sperm use by females and the extent to which males affect female post-mating physiology are areas of intensive current interest. This study shows that the temporal pattern of sperm use is potentially an important determinant of success in sperm competition. We would not have seen such a complete picture of events had our analysis been restricted to the total numbers of offspring produced.

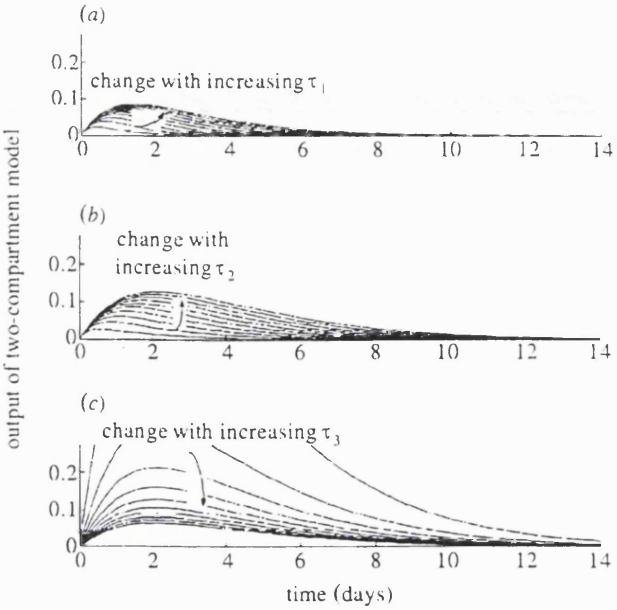

Figure 5. The shape of the curve defined by the two-compartment model over time undergoes changes according to small changes in parameters (*a*) τ_1, (*b*) τ_2 and (*c*) τ_3. Arrows indicate the change in the curve as the value of each of the parameters is increased.

Methods for carrying out analysis of the temporal patterns of sperm use will facilitate future investigation in this area.

The two-compartment model and all programs for fitting the model to the data were constructed by J. Andrew Bangham. We are grateful for help with analytical techniques from J. Andrew Bangham, Scott Fletcher and Jason Kennington and received valuable comments on the manuscript from Andy Barnes. BBSRC, NERC and The Royal Society provided financial support for the work.

APPENDIX A

Graphs of n_r over time produced by eight individual females are shown in figure 2. A two-compartment model was fitted to these temporal patterns of n_r for each female, bw^D and wild-type males separately. In figure 2, the model is illustrated by the dotted line and was integrated to produce re-estimated values of n_r (open circles). Although the two-compartment model is unnecessarily complex for fitting n_r in the example in figure 2*a*(iii), it is able to fit the rising and falling phases seen in, for example, figure 2*b*(i). Figure 3 shows bw^D and wild-type n_r over time for all 147 females in a single graph.

The differential equations that describe the two-compartment model are as follows:

$$\frac{dv}{dt}(1) = \frac{(v_2 - v_1)}{\tau_1}, \qquad \text{(A 1)}$$

$$\frac{dv}{dt}(2) = -\left(\frac{v_2}{\tau_2}\right) - \left(\frac{(v_2 - v_1)}{\tau_3}\right). \qquad \text{(A 2)}$$

A fitting program (MATLAB, v. 6.0.0.88; release 12) was used to search for the values of parameters of the two-compartment model (v_1, v_2, τ_1, τ_2, τ_3) that minimized the residuals (using MATLAB function *fminsearch*) between empirical values of n_r and model estimates of n_r. Parameter τ_1 (figure 5) is a time constant strongly associated

with the initial falling phase of the curve, where a higher value of τ_1 corresponds to a longer initial falling phase. Parameter τ_2 (figure 5) is associated with both the rising and long-term falling phases, and again, the higher the value of this parameter the longer these phases and flatter the overall appearance of the curve. Because it corresponds to both the rising and falling phases, τ_2 should not necessarily be taken as evidence of significant differences in the rising phase without supporting evidence from independent tests. Parameter τ_3 (figure 5) also relates to the rising phase of the curve, but more strongly influences the overall magnitude of the curves. Thus, a higher value of τ_3 corresponds to a longer rising phase, but more strongly corresponds to a lower overall magnitude of the curve.

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Appendix III

The Sex Peptide of *Drosophila melanogaster*: investigation of post-mating responses of females using RNA interference.

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Abbreviations: SP, sex peptide, Acps, accessory gland proteins.

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Mating induces profound changes in female insect behaviour and physiology. In *Drosophila melanogaster*, mating causes a reduction in sexual receptivity and an elevation in egg-production for at least 5 days. Injection of the seminal fluid sex peptide (SP) induces both responses in virgin females, but only for 1-2 days. The role of SP in eliciting the responses to mating remains to be elucidated. Functional redundancy between seminal fluid components may occur. In addition, mating with spermless males results in brief (1-2 day) post-mating responses, indicating either that there is a 'sperm effect', or that sperm act as carriers for SP or other seminal fluid components. Here we used RNA interference to suppress SP expression, to determine if SP is required to elicit full post-mating responses, the magnitude of responses due to other seminal fluid components and whether SP accounts for the 'sperm effect'. Receptivity was higher and egg-production lower in females mated to SP knock-down males than in controls. Comparison with virgins showed that the responses were brief. SP is therefore required for normal magnitude and persistence of post-mating responses. Sperm-transfer and use were normal in mates of SP knock-down males, yet their post-mating responses were briefer than after normal matings, and similar to those reported in mates of spermless *son-of-tudor* males. The prolonged 'sperm effect' on female receptivity and egg-production is therefore entirely attributable to SP, but sperm are necessary for its occurrence.

In many insects, mating induces striking changes in the behaviour and physiology of females (1, 2). These post-mating responses of females are of interest because of their potential utility in insect pest control (3, 4) and because they appear to be subject to unusually strong natural selection (5-9). After mating, female insects can become temporarily unattractive, they can show reduced receptivity to mating and they can increase their rate of egg-production (e.g. 10-17). In *Drosophila melanogaster* females, post-mating responses are elicited by pheromones, sperm and male ejaculate proteins (reviewed by 18, 19). The accessory glands of the male were originally implicated in inducing post-mating responses of *D. melanogaster* females because transplantation of whole glands and injection of gland extracts into females increased egg-production and reduced receptivity (13, 20, 21). Injection of HPLC fractions of accessory gland extracts led to the isolation of the 36 amino acid sex peptide (SP or Acp70A). Injection of physiological amounts of purified or synthetic SP caused virgin females to become unreceptive to mating and stimulated egg-production, for a period of 1-2 days (22). Transfer of SP may, therefore, be at least in part responsible for the reduced receptivity and increased egg-production in *D. melanogaster* females after mating. However, the role of SP after a normal mating remains to be elucidated.

Although SP injection affects female receptivity and egg-laying, SP may not be necessary at mating for these responses to occur. Functional redundancy between SP and other accessory fluid proteins (Acps) in determining post-mating receptivity and egg-production could involve at least two other ejaculate components: Acp26Aa (ovulin) and Dup99B (23, 24). Females mated to males that are null for the *Acp26Aa* gene produce approximately 18% fewer eggs on the first day after mating, and ovulate at a far lower rate, than do mates of wild-type mates (23,25). Dup99B, produced in the ejaculatory duct of the male, and the SP exhibit strong sequence similarity in their C terminal regions and the genes that encode them may have arisen by gene duplication (24). Injection of purified Dup99B causes SP-like effects on female receptivity and egg-production (24). However, females mated to males that transfer Dup99B but no SP or other Acps show no or very brief (1-2h) reduction in receptivity (24, 26, 27) and only a slight increase in egg-production (27) and none in the absence of sperm (26). Other, as yet unidentified, male-derived factors may also affect subsequent female receptivity and rate of egg-production. Matings with males that lack the SP are necessary to determine its unique role in female post-mating responses.

SP may or may not interact with sperm in its effects on receptivity and egg-production. After a normal mating, both responses persist for at least 5 days (28), rather than the 1-2 days seen after SP injection or after mating with males that do not transfer sperm (26-29). The more rapid return of female receptivity and egg-laying to virgin levels after a spermless mating led to the idea of a 'sperm effect'

(28). Alternatively, since SP binds to sperm, sperm may be necessary at mating in order for the response to SP to exceed 1-2 days (24). A further possibility is that an interaction between sperm and some other seminal fluid component is responsible for the 'sperm effect'. Matings with males that lack SP but that transfer sperm are needed to elucidate the role of SP in the 'sperm effect'.

Examination of genetic variation within and between species has shown that Acps evolve rapidly as a result of strong natural selection (5, 7-9). To understand this rapid evolution, it is necessary to elucidate the role of Acps in determining female and male reproductive success. Acps could enhance offspring-production of both sexes if, for instance, they caused egg-production to be stimulated and co-ordinated only after mating, when sperm are available for fertilisation. Seminal fluid proteins could enhance male reproductive success only. For instance, particular Acps are essential for normal sperm storage (30) and success in sperm competition (31). Acps can increase male success in competition with other males, for instance by disabling or removing the sperm of previous mates (32) or by delaying the interval until the female mates again (22), thus increasing the time for which eggs are fertilised by the sperm of the first male (32-34). Acps can also increase male reproductive success at the expense of that of females. As yet unidentified Acps (although see (35)) can increase mortality rate of females and hence reduce their lifetime reproductive success, presumably as an unselected side-effect of male-beneficial functions of the same Acps ((33, but see (36)). The role of Acps in competition between males for mating and in sexual conflict between males and females may account for their rapid evolutionary change under selection (5-9). To determine the way that selection acts on Acps, it is necessary to determine the effects of their removal on female and male reproductive success.

We have produced males that lack detectable SP in their seminal fluid, using RNA interference (RNAi) (37). RNAi is a powerful technique for inducing targeted suppression of gene expression in *D. melanogaster* (e.g. (38)). We produced 3 independent lines carrying a SP sense-antisense transgene, and drove the expression of this construct in the normal site of expression for the SP gene, the male accessory glands. Females mated to these SP knock-down males were significantly more willing to remate and had significantly lower ovulation and egg-production than mates of control males. SP is thus necessary for normal expression of these post-mating responses. Mates of SP knock-down males initially showed significantly lower receptivity and higher egg-production than virgin females, but these responses were of smaller magnitude than normal, particularly for receptivity. Other Acps, or Acp interactions with sperm, must produce these additional short-term post-mating effects. Sperm transfer and use was normal in SP knock-down males. Despite this, the responses of females mated to knock-down males were similar to those reported for mates of males that transfer full ejaculate

components but no sperm (*son-of-tudor* males, (26, 27)). This suggests that sperm are necessary for full SP activity after mating, and that the 'sperm effect' (28) is in fact an SP effect.

Materials and Methods

Wild-type strain and fly culturing. Wild-type flies were from a stock collected in Dahomey (now Benin) in 1970. All experiments were conducted at 25°C on a 12/12 h light/dark cycle. Maize-Yeast medium (10g agar, 85g sugar, 60g maize, 20g autolysed yeast, and 25 ml Nipagin per 1000ml water) supplemented with live yeast was used throughout. Flies were reared in vials (23mm by 73mm) containing 7ml of medium. Wild-type experimental flies were obtained by placing first instar larvae in groups of 50 each into vials. Virgin flies were collected from these standard density cultures within 7 h of eclosion.

Generation of SP RNAi and Gal4 driver transgenic stocks. SP knock-down males were produced by generating flies with SP RNAi transgenes. We produced stocks with a *pUAST* transgene (vector donated by Andrea Brand) with *UAS* upstream of a 305bp portion of the *SP* gene in the sense-antisense orientation. A 305bp fragment covering the entire SP coding region was amplified from genomic DNA (forward primer GAAGATCTGGTGTAAAATGAAAACTCTAGC, reverse CGGGATCCGATTTTAAGACATTTTGGTGGG). Amplified *SP* sequence was digested with *BglII* and cloned between the *BglII* site and the (blunted) *XhoI* site in *pUAST* to give a vector, named pSPsense, carrying a single copy of the *SP* gene. Amplified SP sequence, digested with *BglII* and *BamHI* was cloned into pSPsense, which had been digested with *BglII* and treated with CIAP, using the *E. coli* SURE strain (Stratagene). The resulting vector, named pSP-IR, was marked with w^-, and carried two copies of the SP sequence in an inverted repeat orientation. Clone structure of pSPsense and pSP-IR was verified by multiple restriction digests. Transcription of this transgene is predicted to produce a hairpin loop with sense 5' to the antisense sequence.

pSP-IR transgenic flies were constructed as previously described (39, 40) by injection of pSP-IR into a w^1 genetic background. Homozygous viable and fertile stocks were obtained by backcrossing w^- individuals to the w^1 injection stock and then crossing w^+ individuals *inter se.* Thus the genetic background of the lines remained purely that of the injection stock. Balancer chromosomes were subsequently used purely for mapping the inserts.

An accessory-gland-specific *Gal4* driver line was established by generating transgenic flies carrying the *Acp26Aa* promoter (~1.4kb upstream of the coding region (41) fused to *Gal4,* or *Acp26Aa-P-Gal4*). The construct was injected into a z^1w^{11e4} background, and transgenic individuals recovered as w^+ progeny. A homozygous viable stock was obtained by crossing transgenic individuals to the z^1w^{11e4} background used for injections, followed by crossing the progeny *inter se.* We confirmed that this

construct drives accessory gland-specific expression of a UAS-transgene by staining reproductive tracts of 3-day-old virgin males (*Acp26Aa-P-Gal4;UAS-lacZ* or homozygous *UAS-lacZ* controls) for lacZ using minor modifications of the method in (42).

Western blot. The level of SP in males carrying the *Acp26Aa-P-Gal4* and *UAS-SP-IR* (inverted repeat) constructs was determined by Western blotting. Putative SP knock-down males were obtained by crossing homozygous males from each of the 3 inverted repeat lines obtained (*UAS-SP-IR1, UAS-SP-IR2* and *UAS-SP-IR3*) to virgin females from the *Acp26Aa-P-Gal4* line. The resulting males (*Acp26Aa-P-Gal4;UAS-SP-IR1*, *Acp26Aa-P-Gal4;UAS-SP-IR2* and *Acp26Aa-P-Gal4;UAS-SP-IR3*) were analysed. Positive controls were the male offspring of the reciprocal crosses, i.e. homozygous females from each of the 3 *UAS-SP-IR* lines crossed to *Acp26Aa-P-Gal4* males. The resulting control males (*+;UAS-SP-IR1*, *+;UAS-SP-IR2* and *+;UAS-SP-IR3*) shared the genetic background of the putative knock-down males, except for the X chromosome. Additional controls were homozygous males from each *UAS-SP-IR* insert line and homozygous *Acp26Aa-P-Gal4* males.

We used young, mated males because optimization experiments predicted they would have maximal SP knock-down. Quantification of Acp expression in flies carrying an *Acp62F-IR* RNAi transgene and the *Acp26Aa-Gal4* driver, showed that young mated males had increased knock-down (to <3% of control levels, O.L. and M.W. unpublished) relative to older, or unmated, males. This is likely to be due to the combined effects of mating on the depletion of existing Acps (by 75% in 1-day-old males, O.L. and M.W. unpublished), and mating-induced transcription from the Acp26Aa promoter (43) used in the driver construct. Levels of Acps 26Aa and 36DE were unaffected by RNAi of Acp62F, verifying that knock-down by this method is Acp-specific.

Two-day old virgin male progeny from all experimental and control crosses were mated *en masse* to females from their own lines. These mated males, and virgin males of the same age, were then transferred in groups of 20 to fresh vials. 24 h later, groups of 5 males were transferred into chilled Eppendorf tubes with 40µl of homogenisation buffer (25mM Tris-HCl, pH 7.5, 5mM EDTA, pH 8) and partially homogenised. 40µl sample buffer (62.5mM Tris-HCl, pH 6.8 10% Glycerol, 2% SDS, 0.0005% Bromophenol Blue, 10% β-mercaptoethanol) was then added, the samples fully homogenised, boiled for 4 min, transferred to ice for 2 min, centrifuged at 10k rpm for 5 min at 4 °C and snap-frozen in liquid N_2. An equal amount of protein extract for each line was loaded on a SDS/PAGE gel (15% Acrylamide bisacrylamide) and run at 120 volts for approximately 1 h. The gel was blotted with Towbin buffer on hybond ECL Nitrocellulose membrane (Amersham). The membrane was washed in blocking solution (5% low-fat dry milk in PBS-T) for 1 h and incubated for 1.5 h with the primary

antibody (alpha SP rabbit antibodies, donated by Eric Kubli). After washing with PBS-T solution, the membrane was incubated with peroxidase labelled anti-rabbit secondary antibody (Amersham) for 1 h, then treated with the ECL Western blotting detection system (Amersham), according to the manufacturer's instructions.

Real-Time PCR. We also measured the levels of SP and Dup99B RNA in *Acp26Aa-P-Gal4;UAS-SP-IR1* and *Acp26Aa-P-Gal4;UAS-SP-IR2* males and their controls (males were obtained as described above). Primers were designed using ABI Prism Primer Express Version 2.0 (SP forward primer GAATGGCCGTGGAATAGGAA, reverse GGCACCACTTATCACGAGGATT; Dup99B forward CGCTATTTCTCCTCTTGGTCGTA, reverse TCTCACGATCCTTCTGACTTTGG). We used the SuperScript First-Strand Synthesis System for RT-PCR (Invitrogen) for cDNA synthesis. Real-time PCR was performed using an ABI Prism 7000 Sequence Detection System, and SYBR Green (Molecular Probes), ROX Reference Dye (Invitrogen) and HotStar Taq (Qiagene). Quantitification of transcript levels in SP knock-down males relative to their controls (all normalised to Actin 5C) was performed using a standard curve method (following Applied Biosystems protocols).

Generation of experimental males. For the receptivity, ovulation and oviposition assays, two lines of males carrying the SP inverted repeat and *Gal4* transgenes, (*Acp26Aa-P-Gal4;UAS-SP-IR1* and *Acp26Aa-P-Gal4; UAS-SP-IR2*) were used. The third SP knock-down line (*UAS-SP-IR3*) was not used because, although like the first two lines it produced knock-down levels of SP, the insert appeared to be unstable. We worked with two matched pairs of experimental and control lines: *+;UAS-SP-IR1* and *+;UAS-SP-IR2* males acted as controls for *Acp26Aa-P-Gal4;UAS-SP-IR1* and *Acp26Aa-P-Gal4; UAS-SP-IR2* males respectively. Five virgin male and five virgin female parents of each cross were placed into vials for 3 days and transferred into fresh vials for a further 3 days. Virgin male offspring were collected from these vials over 4 days. For all experimental and control crosses, 1-3 day-old virgin male offspring were mated *en masse* to females from their own vials and stored in single sex groups of 10 for 1-2 days before use in the experiments.

Receptivity Assay. To determine the effect of the SP on female receptivity, wild-type females were mated once to SP knock-down or control males, then exposed to wild-type males in a receptivity assay 24 and 48 h later. Virgin wild-type females were collected at eclosion and stored 10 per vial for 2 days. Pairs consisting of a single 4-5 day-old knock-down or control male, and a single 4 day-old wild-type virgin female, were then put into individual vials. Approximately 130 pairs of each cross and control

were set up. Pairs were observed for about 8 h and 120-150 pairs from each line mated. Immediately after mating, males were removed. After 24 h, a 5 day-old wild-type male was introduced into each vial and the number of females remating within 1 h recorded. This test was repeated 48 h after the initial matings, with 6-day-old wild-type males.

Oviposition Assay. Two day-old wild-type virgin females were mated in single pairs to 4-5 day-old SP knock-down or control males. 60 pairs of each cross and control were set up. Females were then transferred to fresh vials every 24 h for 5 days and the number of eggs laid every day counted.

Ovulation Assay. Three day-old virgin wild-type females were mated in single pairs to 5-6 day-old SP knock-down or control males. 70 pairs of each cross and control were set up. At 6, 24 and 48 h after the first mating, subsets of approximately 25 females from each group were dissected in Phosphate Buffered Saline (PBS) to determine the % of females in each group with an egg in the uterus (5).

Egg Fertility Assay. Vials from days 1, 3 and 5 after mating in the oviposition assay described above were retained to count progeny in order to determine % egg fertility (number of pupae / number of eggs laid x 100).

Results and Discussion

Recovery of SP RNAi and Gal4 transgenic stocks. Three independent SP-RNAi transgenic lines were recovered, *UAS-SR-IR1*, *UAS-SP-IR2* and *UAS-SP-IR3*, with insertions on chromosomes III, II and II respectively. The *UAS-SP-IR3* insertion appeared to be unstable, as w^1 revertants were sometimes observed in the stock. For this reason, experiments were conducted with two of the transgenic stocks only (i.e. *UAS-SP-IR1*, *UAS-SP-IR2*). For the *Acp26Aa-P-Gal4* driver line, one insert was recovered and mapped to the X chromosome. To confirm that the transgene drives accessory-gland-specific expression, virgin transgenic females were crossed to males homozygous for *UAS-lacZ*, and the accessory glands of the adult male progeny were stained for lacZ expression (Fig. 1). LacZ expression was seen in the accessory glands of *Acp26Aa-P-Gal4; UAS-lacZ* males (Fig. 1a) but not homozygous *UAS-lacZ* control males (Fig. 1b).

SP is undetectable following RNA interference. Western blotting (Fig. 2) showed that males carrying the *Acp26Aa-P-Gal4* driver and any of the three *UAS-SP-IR* transgenes produced no detectable SP. All

other control males produced detectable SP. RNAi activated specifically in the male accessory glands knocked down the SP to levels that were not detectable by Western analysis, in both 3 day-old SP knock-down virgin males (Fig. 2a) and 3 day-old SP knock-down males mated when 2 days old (Fig 2b). SP RNA levels in SP knockdown males were strongly reduced (10.5% and 1.2% of control levels for *Acp26Aa-P-Gal4;UAS-SP-IR1* and *Acp26Aa-P-Gal4;UAS-SP-IR2* males respectively). In contrast, the level of Dup99B RNA in SP knockdown males was high (94.9% and 74.1% of control levels for *Acp26Aa-P-Gal4;UAS-SP-IR1* and *Acp26Aa-P-Gal4;UAS-SP-IR2* males respectively). Results were averaged across two RNA extractions.

Effect of SP knock-down on female receptivity. The receptivity of females mated to SP knock-down males (*Acp26Aa-P-Gal4;UAS-SP-IR1* and *Acp26Aa-P-Gal4;UAS-SP-IR2*) was compared to females mated with the respective controls (+;*UAS-SP-IR1* and +;*UAS-SP-IR2*) at 24 and 48 h after their first matings (Fig. 3). Females that mated with males deficient in the SP were significantly more receptive than were females mated to control males. At 24 h the receptivity of females mated to SP knock-down males was intermediate between that of females mated to control males and that of virgin females. By 48 h the receptivity of females mated to SP knock-down males was similar to that of virgins. Females mated to SP knock-down males therefore did not behave like virgin females in terms of receptivity until 48 h after mating; there was some residual reduction in receptivity caused by matings to SP knock-down males.

Effect of SP knock-down on oviposition and ovulation. On each of the 5 successive days after mating, females mated to SP knock-down males laid significantly (with one exception) fewer eggs than did females mated to control males (Fig. 4a). On days 1-2, females mated to SP knock down males laid eggs at a level intermediate between that of females mated to control males and that of virgin females. Thereafter, the number of eggs deposited by mates of the SP knock-down males was similar to that of virgins (Fig. 4a). At 6, 24 and 48 h after mating, females mated to SP knock-down males from both lines showed significantly (with one exception) lower ovulation than mates of control males, and significantly (with one exception) higher ovulation than virgin females (Fig. 4b). Thus mates of SP knock-down males did not show a full mated response and their egg laying dropped down again to virgin levels 2-3 days after mating.

The egg-production and ovulation tests showed that females mated to males deficient in the SP produced significantly fewer eggs than females mated to control males for the 5 days after mating. However, in the first 1-2 days after matings to SP knock-down males, females did not behave like

virgins, although their egg-production did become comparable to that of virgins after 2-3 days. Thus some residual stimulation of egg-production is achieved following matings to SP knock-down males. This stimulation of egg-production and ovulation is presumably caused by the transfer of other ejaculate proteins, such as Acp26Aa and Dup99B.

Effect of SP knock-down on egg fertility. There were no significant differences in egg fertility in mates of SP knock-down and control males on days 1 and 3 after mating (Fig. 5). On day 5 the egg fertility of mates of control males was significantly lower than of mates of SP knock down males (Fig. 5). Egg-production was significantly higher in the control females, which would have led them to run out of sperm more quickly (44) than females mated to SP knock-down males. On day 5, all females laid equal numbers of fertile eggs, suggesting that there were no significant differences in the numbers of sperm stored across treatments. The results show that SP knock-down males transferred sperm and that these sperm were stored and used in comparable numbers to those of control males.

Conclusions.

The results confirm that SP is necessary for some post-mating responses of females. We used two matched pairs of experimental and control lines, and the consistent findings with them indicate that the effects on the post-mating responses were attributable to the absence of the SP, rather than to some other effect of genetic background. Females mated to SP knock-down males produced by RNA interference were significantly more receptive and laid and ovulated significantly fewer eggs, than did mates of control males. RNAi has therefore proved to be a powerful technique for the *in vivo* characterisation of SP protein function. There was some residual reduction in receptivity and stimulation of egg-production in the mates of SP knock-down males. We conclude that these residual effects in mates of SP knock-down are due to ejaculate components and not to pheromone transfer or mating itself, because mates of DTA-E males (which mate and transfer pheromones but no Acps or sperm), show virgin levels of egg-production and receptivity (26). The residual effects in mates of SP knock-down males were presumably due at least in part to as-yet unidentified ejaculate component(s), because the other molecules so far shown to mediate these effects have smaller and/or shorter-lived effects (Dup99B), or affect only egg production but not receptivity (Acp26Aa).

Our results are quantitatively similar to those of Liu and Kubli (45) who analysed the responses of females mated to *SP* gene knockout males produced by homologous recombination. Mates of SP knockout males also showed some residual reduction in receptivity and stimulation of egg-production.

The responses were smaller in magnitude than those observed in the present study, possibly attributable to differences in the fly strains used, or differences in the fly food or culturing techniques. For instance, the rate of egg-laying by virgin females differed in the two studies. This trait shows substantial natural genetic variation between strains as well as clinal, geographic variation (46).

Sperm transfer and use appeared normal in matings with SP knock-down males, because egg fertility was unimpaired. Despite the presence of sperm, females mated to SP knock-down males showed post-mating responses similar to those of mates of *son-of-tudor* males, which transfer Acps and other ejaculate proteins but no sperm (26, 27). These findings show that the 'sperm effect' is in fact an effect of SP, but one that is manifest only in the presence of sperm. Sperm may act as carriers of SP, with slow release prolonging the SP response.

Our results suggest SP is unlikely to cause the increased mortality in females that is attributable to as yet unidentified Acps (33). There is no reduction in the cost of mating in mates of *son-of-tudor* males, which do not transfer sperm, compared to mates of wild-type males (47). Therefore, because sperm are necessary for full SP transfer, SP is unlikely to be responsible for the Acp-mediated cost of mating in females (33). Further work with these SP knock-down males is necessary to confirm this hypothesis, and to determine the net effect of SP on male and female reproductive success.

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Fig. 1. Specificity of expression driven by the *Acp26Aa-P-Gal4* transgene, demonstrated by lacZ expression in *Acp26Aa-P-Gal4;UAS-lacZ* males. (a) *Acp26Aa-P-Gal4* drives expression of *UAS-lacZ* in male accessory glands (AG) but not the ejaculatory duct (ED) or ejaculatory bulb (EB). (b) No lacZ expression in is detected in control males, homozygous for *UAS-lacZ* but lacking the driver.

Fig. 2. Western blot showing levels of SP in SP knock-down and control males (a) in 3 day old virgin males and (b) in 3-day old males mated when 2 days old. From left, in lanes 1-4 homozygous *UAS-SP-IR1*, *UAS-SP-IR2*, *UAS-SP-IR3* and *Acp26Aa-P-Gal4* control males produced SP (molecular weight 4428Da (22)). In lanes 5, 7 and 9, control males carrying the inverted repeat insert without the Gal4 driver (*+;UAS-SP-IR1*, *+;UAS-SP-IR2* and*+;UAS-SP-IR3*) produced SP. In lanes 6, 8 and 10, males with the inverted repeat and the Gal4 driver (*Acp26Aa-P-Gal4;UAS-SP-IR1*, *Acp26Aa-P-Gal4;UAS-SP-IR2* and *Acp26Aa-P-Gal4;UAS-SP-IR3*) produced no detectable SP.

Fig. 3. Effect of SP on female receptivity. % females remating within 1 h in a receptivity test with wild-type males, 24 and 48 h after initial matings to *Acp26Aa-P-Gal4;UAS-SP-IR1* SP knock-down males, *+;UAS-SP-IR1* control males, *Acp26Aa-P-Gal4;UAS-SP-IR2* SP knock-down males or *+;UAS-SP-IR2* control males. The receptivity of virgin females is also shown. The numbers of females that did and did not mate following each type of initial mating were analysed in 2 x 2 contingency tables using Fisher exact tests. Females mated to SP knock-down males (*Acp26Aa-P-Gal4;UAS-SP-IR1* and *Acp26Aa-P-Gal4;UAS-SP-IR2*) were significantly more receptive than their respective controls (i.e. mates of *+;UAS-SP-IR1* and *+;UAS-SR-IR2* males), $P < 0.0001$ all tests. Females mated to both lines of SP knock-down males were significantly less receptive than virgin females at 24 h ($P < 0.0001$ both comparisons). At 48 h, females initially mated to *Acp26Aa-P-Gal4;UAS-SP-IR1* males were only marginally less receptive than virgins ($P = 0.03$) and females mated to *Acp26Aa-P-Gal4;UAS-SP-IR2* males did not differ significantly in receptivity compared to virgin females.

Fig. 4. Effect of SP on oviposition and ovulation. (a) The median (± interquartile range) numbers of eggs laid per 24 h by females over 5 days, after mating to SP knock-down or control males. Data for unmated virgin females are also shown. The data were analysed using Wilcoxon tests. Females mated to *Acp26Aa-P-Gal4;UAS-SP-IR1* SP knock-down males did not differ at 24 h in egg-production from mates of their respective controls (mates of +; *UAS-SP-IR1* males), but laid significantly fewer eggs than their mated controls on days 2-5 ($P < 0.0001$, all comparisons). Females mated to *Acp26Aa-P-Gal4;UAS-SP-IR2* males laid significantly fewer eggs than their controls (mates of *+;UAS-SP-IR2*

males) on all days ($P < 0.0001$ all comparisons). Females mated to both SP knock-down line males produced significantly more eggs than virgin females on the first day after mating ($P < 0.001$, both tests). Females mated to *Acp26Aa-P-Gal4;UAS-SP-IR1* males also produced significantly more eggs than virgin females on day 2 after mating ($P < 0.0001$), but the egg-production of these females then became similar to that of virgin females on day 3 ($P = 0.3$), 4 ($P = 0.02$) and 5 ($P = 0.7$). Egg-production of females mated to *Acp26Aa-P-Gal4;UAS-SP-IR2* males was not significantly different from that of virgins on days 2-5 ($P > 0.1$ all tests). (b) The percentage of females with a egg in the uterus 6, 24 and 48 h after mating to SP knock-down or control males. Data for virgin females are also shown. The numbers of females that did and did not have an egg in the uterus were analysed in 2 x 2 contingency tables using Fisher exact tests. Females mated to SP knock-down males were significantly less likely to have an egg in the uterus than were their respective control females (females mated to *Acp26Aa-P-Gal4;UAS-SP-IR1* males *versus* their controls, at 6 h $P = 0.1$, at 24 h $P = 0.03$, at 48 h $P = 0.0003$; females mated to *Acp26Aa-P-Gal4;UAS-SP-IR2* males *versus* their controls, at 6 h $P = 0.02$, at 24 h $P = 0.003$, at 48 h $P < 0.0001$). Females mated to SP knock down males were also significantly more likely (in all comparisons except one) to have an egg in the uterus than were virgin females (females mated to *Acp26Aa-P-Gal4;UAS-SP-IR1* males *versus* virgins, at 6 h $P = 0.03$, at 24 h $P < 0.0001$ at 48 h $P = 0.002$; females mated to *Acp26Aa-P-Gal4;UAS-SP-IR2* males *versus* virgins, at 6 h $P = 0.002$, at 24 h $P < 0.0001$ at 48 h $P = 0.1$).

Fig. 5. Effect of SP on egg fertility. Egg fertility (number of pupae / number of eggs laid x 100, ± interquartile range) of females 1, 3 and 5 days after mating to SP knock-down or control males. The data were analysed using Wilcoxon tests. On days 1 and 3, the fertility of eggs produced by females mated to SP knock-down males was not significantly different from the fertility of eggs produced by females mated to control males (females mated to *Acp26Aa-P-Gal4;UAS-SP-IR1* males *versus* their controls, on day 1, $P = 0.2$, on day 3, $P = 0.05$; females mated to *Acp26Aa-P-Gal4;UAS-SP-IR2* males *versus* their controls, on day 1, $P = 0.5$, on day 3, $P = 0.2$). On day 5, the fertility of eggs laid by control females was significantly lower than that of females mated to SP knock-down males (females mated to *Acp26Aa-P-Gal4;UAS-SP-IR1* males *versus* their controls, $P < 0.0001$, females mated to *Acp26Aa-P-Gal4;UAS-SP-IR2* males *versus* their controls, $P = 0.0007$). On day 5, females of all lines produced non-significantly different numbers of fertile eggs (females mated to *Acp26Aa-P-Gal4;UAS-SP-IR1* males *versus* their controls, $P = 0.8$, females mated to *Acp26Aa-P-Gal4;UAS-SP-IR2* males *versus* their controls, $P = 0.6$).

Figure 1:

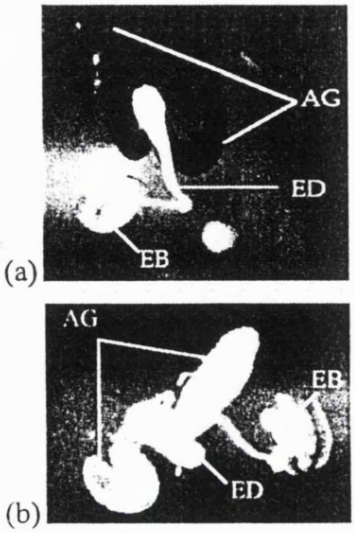


Figure 2:

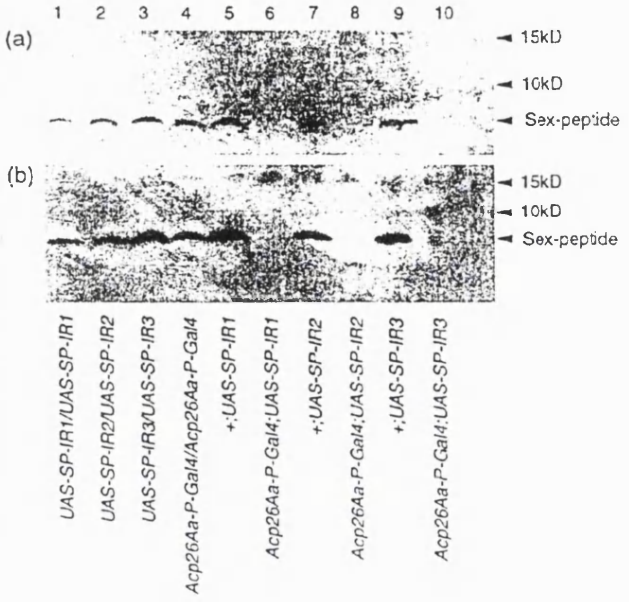

1 2 3 4 5 6 7 8 9 10
(a)
15kD
10kD
Sex-peptide
(b)
15kD
10kD
Sex-peptide
UAS-SP-IR1/UAS-SP-IR1
UAS-SP-IR2/UAS-SP-IR2
UAS-SP-IR3/UAS-SP-IR3
Acp26Aa-P-Gal4/Acp26Aa-P-Gal4
+;UAS-SP-IR1
Acp26Aa-P-Gal4;UAS-SP-IR1
+;UAS-SP-IR2
Acp26Aa-P-Gal4;UAS-SP-IR2
+;UAS-SP-IR3
Acp26Aa-P-Gal4;UAS-SP-IR3

Figure 3:

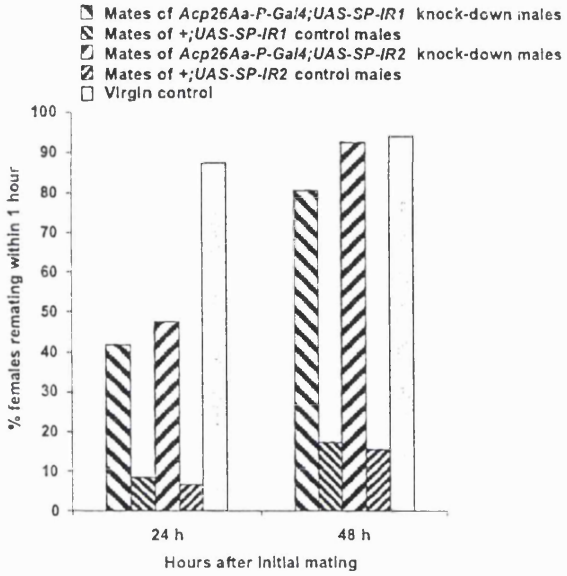
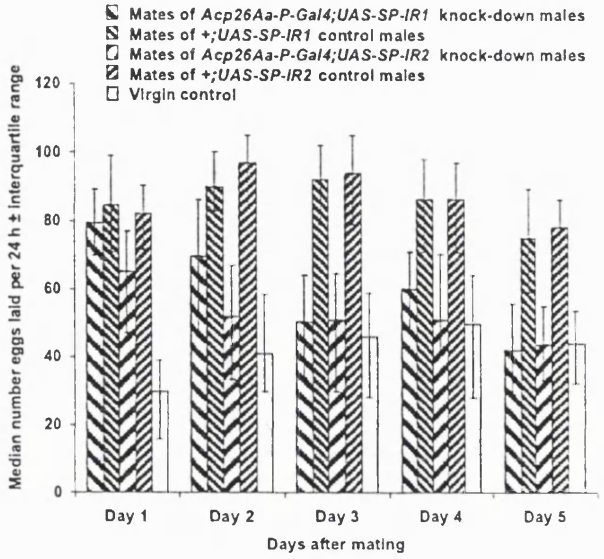
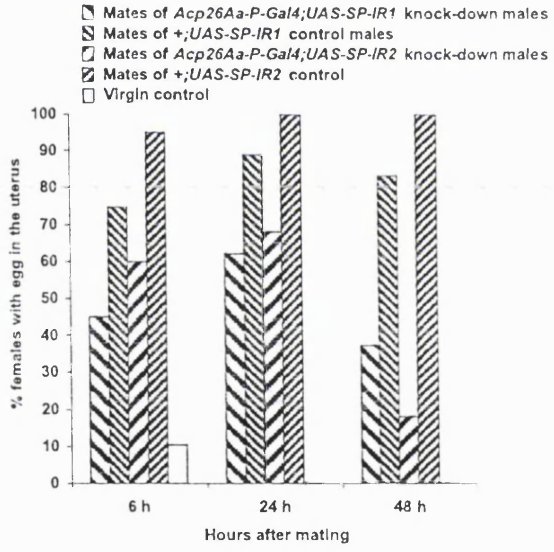

Mates of Acp26Aa-P-Gal4;UAS-SP-IR1 knock-down males
Mates of +;UAS-SP-IR1 control males
Mates of Acp26Aa-P-Gal4;UAS-SP-IR2 knock-down males
Mates of +;UAS-SP-IR2 control males
Virgin control
% females remating within 1 hour
100
90
80
70
60
50
40
30
20
10
0
24 h
48 h
Hours after initial mating

Figure 4:

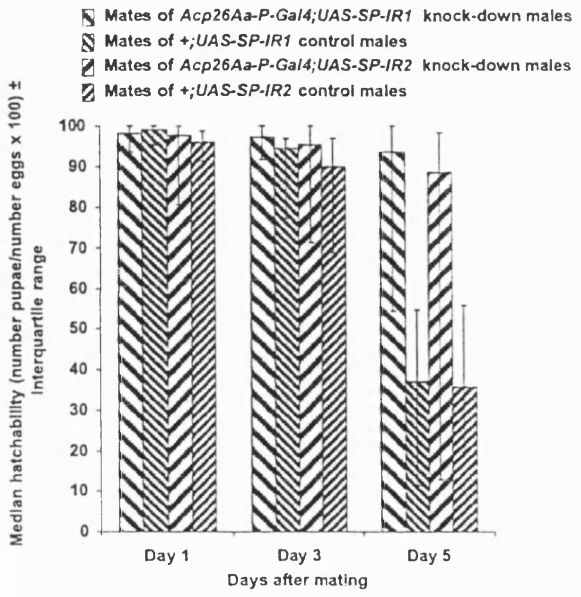

Mates of Acp26Aa-P-Gal4;UAS-SP-IR1 knock-down males
Mates of +;UAS-SP-IR1 control males
Mates of Acp26Aa-P-Gal4;UAS-SP-IR2 knock-down males
Mates of +;UAS-SP-IR2 control males
Virgin control
Median number eggs laid per 24 h ± interquartile range
120
100
80
60
40
20
0
Day 1
Day 2
Day 3
Day 4
Day 5
Days after mating

(a)


Mates of Acp26Aa-P-Gal4;UAS-SP-IR1 knock-down males
Mates of +;UAS-SP-IR1 control males
Mates of Acp26Aa-P-Gal4;UAS-SP-IR2 knock-down males
Mates of +;UAS-SP-IR2 control
Virgin control
% females with egg in the uterus
100
90
80
70
60
50
40
30
20
10
0
6 h
24 h
48 h
Hours after mating

(b)

Figure 5:


Mates of Acp26Aa-P-Gal4;UAS-SP-IR1 knock-down males
Mates of +;UAS-SP-IR1 control males
Mates of Acp26Aa-P-Gal4;UAS-SP-IR2 knock-down males
Mates of +;UAS-SP-IR2 control males
Median hatchability (number pupae/number eggs x 100) ± Interquartile range
100
90
80
70
60
50
40
30
20
10
0
Day 1
Day 3
Day 5
Days after mating