The roles of reproductive proteins in determining male and female fitness in *Drosophila melanogaster*

Thesis submitted for PhD

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Abstract

In this thesis I use *Drosophila melanogaster* as a model organism to study the roles of reproductive proteins in determining male and female fitness. Many of these proteins are likely involved in mediating sexual conflict between the sexes, therefore I focus on how males and females interact at the molecular level, in order to gain insight into the mechanisms underlying sexually antagonistic coevolution.

I provide the context for the work (Chapter 1) and describe the general methods and stocks used throughout (Chapter 2). I show that the sex peptide receptor (SPR) found in females, dramatically changes the fitness benefits to males after early rematings (Chapter 3). I also describe my investigation into the structure of the mating plug formed within females mated to males lacking the mating plug protein, PEBII (Chapter 4). I then test two candidate genes, *Acp26Aa* and *Spn2*, for roles in sperm competition and compare the results obtained from functional tests and correlative studies (Chapter 5). Next, I focus on the requirement of sex peptide (SP) for SPR and vice versa for inducing feeding responses in mated females and early changes in post mating egg laying and receptivity (Chapter 6). Carrying on from this, I investigate the role of SP and its related protein, Dup99B, in eliciting post mating responses in females (Chapter 7). Finally, I summarise the findings from my thesis and discuss ideas for future work to increase our understanding of the consequences of sexual conflict and sexually antagonistic coevolution in *Drosophila melanogaster* (Chapter 8).

My research shows that reproductive proteins play important roles in determining male and female fitness and provides further data supporting how sperm competition and molecular interactions between the sexes can generate and maintain genetic variation for sexual traits.

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Chapter 1. General introduction

In the following chapter I discuss sexual selection theory and how the realization of the existence of widespread polyandry within the animal kingdom has contributed to the understanding of male-female coevolution. Sexual conflict and sperm competition, and the behaviours associated with it, are of central importance to fitness. I discuss some adaptations to sperm competition and molecular interactions between the sexes which determine fitness outcomes. I discuss evidence for sexual conflict and sexually antagonist coevolution and the approaches commonly used for detecting their presence in various species. I conclude with an outline of the model species used in this thesis, *Drosophila melanogaster*, and the ways in which males and females of this species interact at the molecular level, which have shed light on the mechanisms underlying sexual conflict and sexually antagonistic coevolution.

1.1 Sexual selection and sexual conflict

The term sexual selection was used by Darwin to explain the interactions between males of a given species to gain mating opportunities and also encompasses how particular male phenotypes form the basis upon which females choose a mating partner (Darwin, 1871). Sexual selection provides an explanation for why secondary sexual characteristics (such as antlers of male deer for male-male competition or ornate peacock feathers for female choice) are observed in nature. It is a logical extension of the Darwin and Wallace theory of natural selection which posits that individuals that have traits that are best suited for a particular environment will be the ones to survive and reproduce at a higher rate than individuals that lack such beneficial phenotypes (Darwin, 1859). The net result of both natural and sexual selection determines an individual's fitness, which is commonly measured as the number or quality of offspring across one or several generations. Developments of sexual selection theory after Darwin (e.g. Fisher, 1930, Lande, 1981, Kirkpatrick, 1982, Kokko *et al.*, 2002) describe how variation within secondary sexual characters determine mating and reproductive success and how such traits may be selected or become exaggerated. Traditionally this focus has been limited to traits which either gave a male an advantage over other males for access to females (intrasexual selection) or which increased the attractiveness of the male to females (intersexual selection).

Traditionally, models of sexual selection view males as competitive and promiscuous and females as the sex that expresses choice. The models are based upon on the principle of anisogamy, which states that male gametes (sperm) are cheap and easily produced, while female gametes (eggs) require more investment and are a limiting resource. In some species females may be further constrained by the requirements of parental care (Trivers, 1972). The differences in per gamete investment and parental care lead to promiscuous males (generally) having higher variance in potential reproductive rate than females (Edward & Chapman, in press).

The focus of Darwin, and subsequent evolutionary biologists, on sexual selection was initially limited to 'precopulatory' processes. This was based on the assumption that females were monogamous and therefore selection would act on a male's pursuit of mating opportunities and corresponding female preferences for indicators of male quality. Although Darwin observed instances of female multiple mating in his barnacle studies (Darwin, 1887), societal norms at the times are thought to have shaped Darwin's conclusion that multiple mating was neither normal nor desirable for females (for review see Simmons, 2001, Arnqvist & Rowe, 2005). Indeed, little benefit could be ascribed to females mating multiply. By the 1970's this view was increasingly challenged when it was recognized that females of many species do mate multiply within a reproductive cycle and that sexual selection would, therefore, continue to affect the reproductive success of males beyond the act of mating itself ('postcopulatory sexual selection') (Parker, 1970). The currency of sexual selection therefore changed from a sole focus on selection acting on males to mate with as many females as possible, to selection on males to fertilize as many eggs as possible. Because the average reproductive success of males and females within a given species is equal (Fisher, 1930), it was considered that mating should be a cooperative effort between the sexes (Krebs & Davies, 1993). However, with the rise of the study of behavioural ecology and the recognition that selection ultimately acts at the level of genes by favouring particular phenotypes (Hamilton, 1964, Dawkins, 1976), it became increasingly clear that traits involved in the interaction of the sexes could be shaped more by conflicts of interest as opposed to cooperation (Trivers, 1972, Dawkins, 1976). This gave rise to the theory of sexual conflict (Parker, 1979).

Sexual conflict is broadly defined as 'differences in the evolutionary interests between males and females' (Parker, 1979). It can occur over traits such as mating frequency, fertilization, relative parental effort, female remating behaviour, female reproductive rate

and clutch size. It is now recognised that males and females may evolve sexually antagonistic traits owing to fundamental differences in the costs and benefits of reproductive decisions. Ongoing differences in cost / benefit fitness ratios of interactions between the sexes can result in cycles of sexually antagonistic coevolution. In this, the optimal value of traits shared across the sexes differs for males and females, resulting in antagonistic selection for opposing values of reproductive traits that influence shared phenotypes (Rice, 1998).

Sexual conflict is classified as either intralocus or interlocus depending on whether the genes that encode traits subject to antagonistic selection are the same or different in males and females (Arnqvist & Rowe, 2005, Chapman *et al.*, 2003b). An example of intralocus sexual conflict is found in the evolution of the human hip. Selection is likely to have favoured an increase in hip width in females to accommodate giving birth (Lavelle, 1995), however this may have come at a cost to locomotion (Rice & Chippindale, 2001). Since males should not benefit from increasing hip width and face instead only the cost of decreased locomotion, hip width should be subject to opposing selection pressures in males and females. Therefore hip width may be at neither the male nor the female optimum. A tug-of-war between the sexes is therefore expected to occur, and possible outcomes are the evolution of genes that modify expression, or the evolution of sex limited control of hip width, which could allow each sex to express its own sex-specific optimum (Rice, 1984, Rhen, 2000, Gibson *et al.*, 2002). Intralocus conflict may be important in generating sexual dimorphism, however, evidence also suggests that it may be an important constraint in the evolution of many traits because, until sex limitation can be achieved, traits subject to sexually antagonistic selection are not at their sex specific optima (Arnqvist & Rowe, 2005).

Interlocus conflict involves evolutionary conflicts of interest between different genes in males and females. It is predicted to occur over a range of traits such as courtship, mating frequency, time or place of mating, mate choice, fertilization, female reproductive output or parental care (reviewed in Arnqvist & Rowe, 2005, Chapman *et al.*, 2003b). One of the earliest examples of sexual conflict was described by Geoff Parker based on his studies of the yellow dung fly, *Scatophaga stercoraria* (Parker, 1979). In this species females are sometimes damaged or drowned in dung as males compete for matings. Parker's models showed that male yellow dung flies would benefit from traits that increased their competitive ability and that selection would favour these traits even if they sometimes led

to female damage or death (Parker, 1979). Selection should then favour females with traits that allow them to avoid or resist male-induced harm which would then reduce the benefit of the male traits. This produces a cycle of 'unresolvable evolutionary chases' (Parker, 1979) as each sex evolves traits that increase their own fitness either by increasing the level of manipulation or by decreasing the harm caused by manipulative traits in the opposite sex.

1.2 Contrasting sexual selection and sexual conflict models of evolutionary change

Traditional models of sexual selection propose that genes that confer a reproductive advantage to males will also confer fitness gains for females mating with males carrying these genes (Andersson, 1994). This co-operative view states that coevolution of male sexual traits and female preference traits increase the average fitness of both sexes until checked by other process, such as natural selection. Mutually beneficial coevolution is thus promoted because the evolutionary interests of the two sexes are aligned (Pizzari & Snook, 2003). Males gain fitness benefits by gaining paternity while females gain by mating with males that provide direct benefits such as nuptial gifts (Vahed, 1998), parental care (Clutton-Brock, 1991) or access to good territories (Emlen & Oring, 1977). Females may also gain indirect benefits by mating with attractive males because those males should pass on genes for attractiveness to sons, resulting in an increase in the likelihood of sons being successful at gaining matings, as has been shown in field crickets, *Gryllus bimaculatus* (Wedell & Tregenza, 1999). This was first described by Fisher and is known as the 'runaway process' (Fisher, 1930). It predicts that the strength of female preferences for a male display trait will increase with increased levels of the male display trait (Fisher, 1930). Another alternative route through which there may be indirect benefits is when females prefer males that provide 'good genes' that confer increased fitness in offspring of both sexes in traits such as offspring growth, survival or parasite resistance (reviewed in Kirkpatrick & Ryan, 1991) or prefer to mate with males that are genetically compatible (Zeh & Zeh, 1996, 1997, Tregenza & Wedell, 2000).

In contrast to traditional models of sexual selection that predict co-operative (or cost neutral) fitness payoffs from interactions between the sexes, under sexual conflict models, the interactions are antagonistic. Sexual conflict theory recognizes that some genes that confer a reproductive advantage to males may in fact result in the expression of phenotypes that cause harm to females, resulting in fitness losses for females compared to the level

obtained by females that mate with males that do not carry antagonistic genes (Parker, 1979, Lande, 1981). Sexual conflict has been suggested to result in 'chase-away' dynamics (Holland & Rice, 1998) whereby selection acting on females to evolve counter-adaptations to reduce the harm caused by male adaptations leads to a cyclic arms race scenario (in the case of interlocus sexual conflict) or a 'tug of war' over the outcome of a particular trait (in the case of intralocus sexual conflict). The chase-away hypothesis (Holland & Rice, 1998) predicts that a particular genotype that makes a male more harmful to his mate can be selected for if it also increases his fitness relative to the fitness of other genotypes. Thus the chase-away hypothesis provides functional explanations for maladaptive reproductive behaviours like super- and suboptimal remating frequency (reviewed in Arnqvist & Nilsson, 2000) and potentially maladaptive partner choice (Holland & Rice, 1998) which are unaccounted for by traditional models.

Sexual conflict models are firmly rooted within the central tenets of natural and sexual selection such that any trait which increases the likelihood of gaining matings or fertilizations will be favoured. The main difference between models of sexual conflict and traditional models of selection is that the former predicts a negative intersexual fitness relationship while the latter predicts a positive relationship. Thus sex-specific net fitness payoffs must be measured to determine which models are likely to be influencing the outcome of sexual interactions in any particular species (Pizzari & Snook, 2003 see section 1.5).

1.3 Evolution of polyandry

In nature, females of many animal species mate with more than one male during their lifetime or within a reproductive cycle (Jennions & Petrie, 2000, Hosken & Stockley, 2003, Simmons, 2003). Polyandry is predicted to have evolved either because females gain direct or indirect benefits from mating with multiple males or because remating rates are driven by sexual conflict, i.e. because of selection on males to mate multiply. Numerous studies have shown that females can gain fitness benefits from polyandry (reviewed by Arnqvist & Nilsson, 2000, Simmons, 2005). Possible direct benefits include guaranteeing an adequate sperm supply (Turner & Anderson, 1983), benefiting from nuptial gifts or access to other resources (Markow & Ankney, 1984), or avoidance of harm caused by male harassment (Gowaty & Buschhaus, 1998). However, some of these direct benefits are predicted to be similar in polyandrous females and females that mate with the same male

repeatedly. In contrast, indirect benefits have been hypothesized to be greater in polyandrous females than in monogamous females remating with the same male. Mating with multiple males may serve as a mechanism of inbreeding avoidance (Tregenza & Wedell, 2000) or to increase genetic diversity of the offspring in order to increase disease resistance. Polyandry may also serve to reduce parental incompatibilities in offspring resulting from endosymbionts such as *Wolbachia* (Zeh & Zeh, 1997, Charlat *et al.*, 2007). Lastly, females may gain indirect fitness benefits by 'trading-up' and mating with males with 'good-genes' or 'good-sperm' (Jennions & Petrie, 2000).

1.4 Polyandry and sperm competition – male and female adaptations

Interestingly, although sexually antagonistic selection arising from sexual conflict may increase the likelihood that females will remate, the effect of this on individual males creates further intrasexual conflict between them to gain fertilization of eggs, leading to sperm competition between males. Parker (1970) was the first to document the existence of sperm competition and describe its evolutionary significance in his studies on the yellow dung fly, *S. stercoraria*. The process of sperm competition is defined as 'the competition between the sperm of two or more males for the fertilisation of a given set of ova' (Parker, 1984). It has since been shown to exist across the plant and animal kingdoms and has been reported in birds, mammals, fish, amphibians, reptiles and invertebrates. It is particularly common and much studied in insects (see Birkhead & Møller, 1998). Insects have proven to be extremely good model organisms for the study of sperm competition because they often store viable sperm for long periods of time and are amenable to controlled, laboratory studies. However, it can still be difficult to determine the precise mechanisms of sperm competition that allow males to outcompete other males, even when techniques allow for the visualization of competing sperm *in vivo* (Civetta, 1999, Price *et al.*, 1999, Manier *et al.*, 2010). Across various taxa, several factors are known to affect the outcome of sperm competition. They include the number of sperm transferred during mating, the frequency of male mating (leading to differences in ejaculate depletion amongst males), the age of the competing males (leading to differences in sperm quality measured as the number of live sperm and sperm velocity), mating duration (leading to possible increases in sperm and / or ejaculate amounts transferred and likely stored), copulation frequency and remating rate of the female (leading to differences in sperm usage / dumping patterns at time of remating) (reviewed in Birkhead & Møller, 1998). In Chapter 3, I examine the roles of female

receptivity state and remating interval as determinants of sperm competition outcomes and male fitness. Specific adaptations that are thought to have evolved in order to increase a male's success in sperm competition include increases in sperm size or number (Parker, 1990), investment in different types of sperm (Cook & Gage, 1995), and the transfer of ejaculatory proteins that reduce female remating, increase sperm storage, increase egg laying rate, or that provide a nuptial gift (Engqvist & Sauer, 2001, Gillott, 2003). Additionally, it has been shown that the genotype of the current male, the genotypes of previous and subsequent males and the genotype of the female interact to determine sperm competition outcomes (Clark & Begun, 1998, Clark *et al.*, 1999, Bjork *et al.*, 2007). In the following sections I describe the dynamics of the adaptations that influence the outcome of sperm competition.

1.4.1 Adaptations in sperm and seminal fluids in response to sperm competition

The relative number of sperm transferred by competing males and the relative fertilization efficiency of an ejaculate are two broadly defined traits that affect the outcome of sperm competition (Pizzari & Parker, 2009). The evolution of ejaculate expenditure in terms of sperm number has been the focus of extensive theoretical (Parker, 1998) and empirical work (Wedell *et al.*, 2002). Comparative studies have tested the theoretical prediction that male investment in ejaculates (measured as testes size for example) should increase with increasing levels of sperm competition. Consistent with this prediction, males in polyandrous mating systems have relatively larger testes (primates, Harcourt *et al.*, 1981, amphibians, Jennions & Passmore, 1993, birds, Møller & Ninni, 1998, butterflies, Gage, 1994, Karlsson, 1995, bats, Hosken, 1997, fish, Stockley *et al.*, 1997). Positive associations have been found between sperm competition intensity and relative gonad weight and sperm numbers in closely related fish species (Stockley *et al.*, 1997) and testis mass positively associates with social group size (an indicator of levels of polyandry) in bats (Hosken, 1997). Numerous associations between female mating frequency and testis size have been found in insects (Simmons & Siva-Jothy, 1998, Simmons, 2001) and have been most clearly demonstrated in butterflies. Gage (1994) showed that relative testis size was significantly positively correlated with increasing numbers of spermatophores per female in 74 species from five families. Likewise, Karlsson (1995) found a positive correlation between residual ejaculate weight and degree of polyandry across 21 species of butterflies. Intra-specific ejaculate allocation has also been studied in response to artificial selection.

Hosken and Ward (2001) created monogamous and polyandrous replicate populations of the yellow dung fly, *S. stercoraria*, and showed a rapid increase in testis size (after 10 generations) in response to the level of sperm competition.

Increases in gonad investment may not always lead to changes in sperm number. Another important factor in determining the competitive ability of sperm can be size, especially if changes in size increase the probability of fertilization (Parker, 1970). An important component of sperm size is total length. Longer sperm may be more competitive if they are more motile or fill the female's sperm storage organs more effectively and hence better resist displacement (Simmons & Siva-Jothy, 1998, Snook, 2005). Comparative studies across a variety of taxa, and most thoroughly in Drosophila (Pitnick & Markow, 1994), have concluded that relatively long sperm provide an advantage in sperm competition (Briskie & Montgomerie, 1992, Briskie *et al.*, 1997, Gage, 1994, Gomendio & Roldan, 1991, LaMunyon & Ward, 1999, Ward, 1998). An additional explanation for the evolution of longer sperm may be that longer sperm serve as a more efficient delivery device for seminal fluid peptides delivered by an ejaculate (the roles of which are discussed in sections 1.4.2, 1.5.3 and 1.6.2) (Pizzari & Parker, 2009).

Males may also adapt to sperm competition by evolving more than one morphological type of sperm. Polymorphic sperm are found in some species of Diptera and Lepidoptera. Typically, one type of sperm is capable of fertilising eggs and the other is non-fertilising (Silberglied *et al.*, 1984, Swallow & Wilkinson, 2002). Non-fertilising sperm (parasperm) may be energetically less costly to produce and may function as a 'cheap filler' of the female sperm storage organ (Wedell, 2001).

The role of seminal fluid peptides in sperm competition has been extensively reviewed (Chapman, 2001, Simmons, 2001, Poiani, 2006, Ravi Ram & Wolfner, 2007). Most work in this area has been carried out in *Drosophila melanogaster* where specific accessory gland proteins have been demonstrated to increase male success in sperm competition (Clark *et al.*, 1995, Chapman *et al.*, 2000, Fiumera *et al.*, 2005, 2007). I examine two *D. melanogaster* seminal fluid proteins for roles in sperm competition in Chapter 5. Seminal fluid may also serve to increase ejaculate volume which would be predicted to increase the displacement ability of a competing male's ejaculate (Gilchrist & Partridge, 1995, Harshman & Prout, 1994). In *D. melanogaster* prolonged copulation allows for the transfer of a wide range of seminal fluid proteins that increase the inhibition of female receptivity

and act to promote sperm displacement (Gilchrist & Partridge, 2000, Chapman & Davies, 2004). In the following section, I further describe the ways that male seminal fluids in Drosophila act as chemical mate guards.

There is also ample evidence that males respond to the degree of sperm competition and accordingly adjust their investment in a given ejaculate (snails, Oppliger *et al.*, 1998, insects, Gage & Baker, 1991, Simmons *et al.*, 1993, Cook & Wedell, 1996, Simmons & Kvarnemo, 1997, Wedell & Cook, 1999, Martin & Hosken, 2002, birds, Pizzari *et al.*, 2003, fish, Pilastro *et al.*, 2002). For example, male butterflies, *Pieris rapae*, increase the number of fertilising sperm as a proportion of total sperm when they encounter previously mated females in comparison to virgin females (Wedell & Cook, 1999). In field collected bush crickets, *Requena verticalis*, males transferred greater numbers of sperm to older females, as predicted by theory, since older females are more likely to have previously mated (Simmons *et al.*, 1993). In *D. melanogaster*, males have been shown to adjust the components of their seminal fluid depending on the mating history of the female they encounter (Sirot *et al.*, 2011).

1.4.2 Behavioural adaptations in response to sperm competition

A taxonomically widespread male adaptation to sperm competition is mate guarding behaviour, either during or after copulation (Parker, 1974, Grafen & Ridley, 1983, Mathews, 2002, Plaistow *et al.*, 2003). By extending the period of exclusivity of his sperm in the female reproductive tract, a male can increase the opportunity for females to utilise his sperm before remating, thus reducing the opportunity for sperm competition.

Male water striders maintain their grasp of females with which they have just mated in order to defend them against aggressive courtship and mating attempts from rival males, ensuring that more of their sperm will be used for fertilization (Clark, 1988, Rowe, 1994, Jablonski & Vepsalainen, 1995). Mate guarding can also take the form of prolonged copulation as a way of excluding rival males until the female is unreceptive (Wedell, 1993) or reaches peak fecundity. Increased mating duration may allow for a greater number of sperm being transferred and / or stored (Alcock, 1994, Dickinson, 1986, Sakaluk, 1986, Siva-Jothy & Tsubaki, 1989) or may provide time for the transfer of greater quantities of seminal fluid (Sakaluk, 1986, Svensson *et al.*, 1990, Wedell, 1993).

Mate guarding does not always require the physical presence of the male. Some seminal fluid proteins have been demonstrated to modulate female physiology and behaviour in ways that effectively restrict females from mating with other males (reviewed in Simmons, 2001, Gillott, 2003). The most obvious of these effects is the formation of a mating plug formed by coagulation of the ejaculate in the female reproductive tract (I examine the dynamics of mating plug formation and its consequences on female remating in Chapter 4). Mating plugs may increase the efficiency of sperm storage (Polak *et al.*, 1998, Polak *et al.*, 2001, Lung & Wolfner, 2001) or decrease the chance that females will remate (Contreras-Garduno *et al.*, 2006, Orr & Rutowski, 1991, Polak *et al.*, 1998, Polak *et al.*, 2001). Large ejaculates have been shown to increase the refractory period of females in bush crickets and Lepidoptera (Wedell, 2001) and one key seminal fluid protein, the sex peptide, has been identified in *D. melanogaster* that directly reduces female remating frequency for several days after mating (Chen *et al.*, 1988, Liu & Kubli, 2003, Chapman *et al.*, 2003c, Ravi Ram & Wolfner, 2009).

1.4.3 Female adaptations in response to sperm competition

Females are expected to evolve mechanisms of choice that operate during and / or after mating. Lloyd (1979) hypothesized that females, especially in species where males prevent pre-mating choice, may manipulate ejaculates by selectively storing, using, or digesting sperm depending on the characteristics of the male. Thus, multiple mating may provide the opportunity for females to select the best mate to father their offspring or to increase offspring genetic diversity (Lloyd, 1979, Walker, 1980, Thornhill & Alcock, 1983, Eberhard, 1996). Thornhill and Alcock (1983) coined the term 'cryptic female choice' to describe female responses that select among competing males and their sperm. Cryptic female choice can involve overt behavioural biases, such as decisions to oviposit or remate, or be less obvious, involving changes in hormone titres, or differential transport, storage, usage or killing of sperm in storage. William Eberhard expanded on the idea of cryptic female choice, arguing that even slight changes in female reproductive morphology, behaviour or physiology can “tilt the playing field” and “change the rules” for sperm competition (Eberhard, 1996, 1998), giving females a large degree of control in biasing paternity. However determining the degree to which female choice and sperm competition interact to determine reproductive success has proven difficult.

Females of some species have been shown to be capable of manipulating ejaculates according to various mechanisms. For example, in feral chickens, females consistently bias sperm retention in favour of the preferred, dominant male phenotype. If they are sexually coerced by subordinate males, they will eject the subordinate's ejaculate, and preferentially retain the sperm of dominant males (Pizzari & Birkhead, 2000). In the damselfly, *Calopteryx splendens xanthostoma*, females also have the capacity to alter the proportions of sperm stored in the spermathecae (often from several previous males) and in the bursa (predominantly from her last mate) and thus control which sperm are used to fertilize her eggs (Siva-Jothy & Hooper, 1996).

Evidence that females create the conditions favouring particular sperm is observed in sperm competition studies demonstrating conspecific sperm precedence between related species that can nonetheless hybridize. In studies of *Allonemobius fasciatus* and *A. socius* ground crickets, only sperm from conspecific males fertilize the majority of eggs, regardless of the order in which males from the two species mate with a female (Gregory & Howard, 1993). This was shown to not be a consequence of a lack of sperm transfer by heterospecific males nor a result of differential sperm storage. Sperm precedence was instead attributed to differences in sperm motility between males, with conspecifics maintaining motile sperm within the female spermathecae (Gregory & Howard, 1994). Thus the female reproductive tract provides conditions that are favourable to sperm from one species of male over the other.

Another female adaptation to sperm competition is less obvious, though equally important from the point of view that females should have traits that reduce the harm caused by male adaptations for sperm competition, specifically harassment caused by courting males. In some cases females have evolved mechanisms, such as larger spines in water striders (*Gerris incognitus*) which allow them to resist unwanted mating attempts (Arnqvist & Rowe, 1995). In some species females succumb and remate in order to avoid costly male harassment, a behaviour which is referred to as convenience polyandry (Thornhill & Alcock, 1983).

1.4.4 Male x female interactions and the maintenance of polymorphisms

The 'lek paradox' states that choice by females for the highest quality males will deplete, through directional selection, genetic variance in male traits to the point where there would no longer be benefits to female choice (Arnqvist & Rowe, 2005). However, empirical

evidence shows that genetic variance for fitness (Houle, 1992, Burt, 1995, 2000) and sexually selected traits (Pomiankowski & Møller, 1995) is often quite high. In species that mate multiply, the fitness of the individual interacting parties at each mating is determined by an interaction between the female and all the males with which she has mated and whose sperm she retains (Rice, 1998, Ward, 1993). These interactions can serve to slow the erosion of genetic variation.

Within *Drosophila* fruit flies, it has been shown that the genes encoding seminal fluid proteins are often highly polymorphic (Begun *et al.*, 2000, Tsaur *et al.*, 2001) and a male's genotype correlates with sperm competition success (Clark *et al.*, 1995). Females also show high levels of genetic variation in their ability to allow some males to displace sperm, suggesting that the genotype of females determines how particular morphs of seminal fluid proteins are received and processed (Clark & Begun, 1998). It has also been demonstrated that particular male and female genotypes interact with one another during bouts of sperm competition (Clark *et al.*, 1999, Long *et al.*, 2006, McGraw *et al.*, 2009, also see sections 1.6.3 and 1.6.5).

1.5 Investigating sexual conflict and sexually antagonistic coevolution

Sexual conflict can occur whenever the evolutionary interests of the sexes differ, such that males and females have different fitness optima for a given action or trait, leading to cycles of sexually antagonistic coevolution (Rice, 1996). Sexual conflict is often demonstrated through 'economic studies' that measure the cost and benefits of particular traits to males and females (Arnqvist & Rowe, 2005). Although sexual conflict is likely to form a part of all reproductive interactions between the sexes, demonstrating that sexually antagonistic coevolution (compared to e.g. good genes models) can be a challenge (Arnqvist & Rowe, 2005). In sexually antagonistic evolution, the trait that gives an advantage to males in mating is disadvantageous to females. This contrasts with models based on direct benefits where the trait that gives a mating advantage to males is also advantageous to females. Both kinds of models predict co-evolutionary change, with the former model predicting that the beneficial male trait reduces female fitness creating selection on females to reduce the cost of the male trait, while the latter model predicts runaway coevolution of male signal and female preference (Pizzari & Snook, 2003, Arnqvist & Rowe, 2005). Thus the key evidence required for demonstrating sexually antagonistic evolution is the proper assessment of costs and benefits of particular traits to males and females along with

empirical data on the forces of selection acting on each sex presently and in the past (Rowe, 1994, Chapman *et al.*, 2003a, 2003b, Cameron *et al.*, 2003, Cordero & Eberhard, 2003). This is a difficult task because of the difficulties in measuring all possible costs and benefits and because sexual conflict may be hidden due to past adaptation or because of a lack of understanding of the relevant biological mechanisms. Techniques that have been employed to detect sexually antagonistic coevolution include experimental evolution (e.g. Holland & Rice, 1999, Rice, 1996, Martin & Hosken, 2003a), cross-species comparative studies (e.g. Civetta & Singh, 1995, Arnqvist & Rowe, 2002, Swanson & Vacquier, 2002) and intra-specific population crosses (e.g. Andrés & Arnqvist, 2001, Brown & Eady, 2001). The works described in this thesis rely on genetic experiments which make use of mutants in order to demonstrate the role of specific proteins in sexual conflict (following e.g. Chapman *et al.*, 1995, 2000, Lung *et al*., 2002, Liu & Kubli, 2003).

1.5.1 Evidence for sexually antagonistic coevolution from experimental evolution studies

Some of the best lines of evidence for sexual conflict leading to sexually antagonistic coevolution come from experimental evolution studies with *D. melanogaster*. Sexual conflict is predicted to occur over mating in fruit flies because females suffer costs that result from the actions of male seminal fluid proteins (sfps). Sexually antagonistic coevolution is thus predicted to occur as females evolve resistance to the harmful effects of sfps. Rice (1996) used a stock of fruit flies that allowed males to evolve against a static female genotype. After 30 generations, males had adapted to the static female line and increased their fitness relative to control males. The adapted males had evolved higher mating rates and sired more offspring than control males. Females suffered a decline in lifespan when mating with the adapted males, as would be expected if no resistance mechanism was allowed to evolve. These findings are consistent with the idea of sexually antagonistic coevolution.

In a separate experimental evolution study, Holland and Rice (1999) enforced monogamy on a line of fruit flies, thus also removing the potential for sperm competition, sexual conflict, and cryptic female choice. Control populations were also created in which polyandry was allowed to occur. After 32-47 generations, males from the monogamous lines evolved to be less harmful to females than males from the polyandrous lines. Females mated to monogamous line males lived longer and produced more eggs then females mated to control males. The results were consistent with the theory that monogamy males should

evolve to be less harmful to females because the potential fitness benefits accrued by mating should be equal for both sexes (Fisher, 1930). Similar results were found by Hosken *et al.* (2001) in yellow dung flies, *S. stercoraria*, and in a follow up study on the Holland and Rice (1999) *D. melanogaster* lines described above, by Pitnick *et al.* (2001a,b).

1.5.2 Evidence for sexual conflict arising from energetic costs associated with mating

Mating is expected to be costly for males and females. Females in many insect species show reluctance to remate and males have evolved adaptations to gain matings with both receptive and unreceptive females. Male adaptations such as mating guarding, courtship, and attempted copulation can also result in energetic costs of reproduction in females. These costs are significant in water striders (Gerridae) where the mating system is characterised by intense premating contests, and where longer struggles tend to result in copulation and shorter struggles do not (Arnqvist, 1992). There are energetic costs to females engaged in these struggles (Arnqvist, 1992, Rowe, 1994, Watson *et al.*, 1998) which are higher during premating struggles compared to during mating itself. This suggests that females may accept matings to reduce the costs of the premating struggle and may represent 'convenience polyandry' for female water striders (Rowe, 1992).

In the dung fly, *Sepsis cynipsea*, females shake vigorously in an attempt to dislodge males that attempt to copulate (Blanckenhorn *et al.*, 2002). In contrast to water striders, the costs of shaking are likely to be minor compared to the costs incurred from mating (Muhlhauser & Blanckenhorn, 2002). In *D. melanogaster* courtship alone is costly to females (Partridge & Fowler, 1990, Friberg & Arnqvist, 2003). Pre-copulatory mate guarding causes energetic costs to female isopods, *Idotea baltica* (Jormalainen *et al.*, 2001). Females struggle against guarding males and suffer a cost from being guarded in the form of producing smaller eggs than unguarded females (Jormalainen *et al.*, 2001).

1.5.3 Sexual conflict arising from harm to females caused by male seminal fluid products

In *D. melanogaster*, female mating costs are caused by the actions of male seminal fluid proteins. The female cost of mating is independent of other male-related and reproductive costs, such as courtship and egg production, and arises from mating *per se* (Fowler & Partridge, 1989). Experimental manipulations to reduce the female mating frequency showed that 'low-mating' females lived longer than 'high-mating' females but that both

groups produced eggs at the same rate (Fowler & Partridge, 1989). Further investigation revealed that the female cost of mating results from the actions of seminal fluid proteins produced in the main cells of the male accessory glands that are transferred to females during mating, and that the more of these accessory gland products that were received the shorter the female's lifespan (Chapman *et al.*, 1995). Seminal fluid proteins have a variety of male-benefiting functions (see next section). One particular seminal fluid protein, sex peptide, induces mating costs in females (Wigby & Chapman, 2005). Therefore the interaction between sex peptide, and it's recently discovered receptor SPR, is likely to mediate sexual conflict because the effects of SP are beneficial to males and can be harmful to females (Chow *et al.*, 2010 also see Chapter 6).

1.5.4 Sexual conflict arising from female costs resulting from male paternity assurance

Despite the cost of mating, females in many species benefit from mating more than once (see section 1.3). However, it is always in the interests of males to delay or prevent females from remating to avoid sperm competition, thus providing the potential for conflict over female remating rate. In the bumble bee, *Bombus terrestris*, females benefit from mating multiply because colonies with high genetic diversity benefit from reduced parasite load (Baer & Schmid-Hempel, 1999, 2001). However, in the wild *B. terrestris* females can only mate once because males prevent females remating by transferring seminal proteins that produce a mating plug (Schmid-Hempel & Schmid-Hempel, 2000). In the cockroach, *Nauphoeta cinerea*, males insert their spermatophore directly in the bursa copulatrix of their mates which inhibits the sexual receptivity centre in the female's brain (Montrose *et al.*, 2004). Recently mated males may also transfer a spermatophore which is sperm depleted which results in sperm competition avoidance for the male, but decreased fitness for the female because they receive insufficient sperm to fertilize all their eggs (Montrose *et al.*, 2004). Apart from physical mating plugs, the seminal fluid of many male insects often contains substances that delay remating (Gillott, 2003). Civetta and Clark (2000) found that *D. melanogaster* males that performed better in sperm competition and delayed female remating for longer also caused early female mortality, providing evidence of the costs of sexual conflict to females.

1.6 The model species used in this thesis, *Drosophila melanogaster*

The fruit fly, *D. melanogaster*, that is used in this thesis has long been established as a powerful model for testing questions in evolutionary biology and is well suited for studies of sexual conflict and sexually antagonistic coevolution (e.g. Chippindale *et al.*, 2001, Holland & Rice, 1999, Rice, 1996, Wigby & Chapman, 2005). Much is known of *D. melanogaster* genetics, physiology and behaviour and there are ample tools available that make it amenable to genetic manipulations. The ability to identify and manipulate the proteins of the male accessory glands has been particularly useful and helped to uncover their roles in mediating sexual conflict in this species (e.g. Chapman *et al.*, 1995, Wigby & Chapman, 2005). Females are also polyandrous both in the lab and in the field (Harshman & Clark, 1998) which makes them well suited to studies of sperm competition. *D. melanogaster* are cheap and easy to maintain and have a relatively short life cycle (~10 days from egg to adult) which makes them ideal model organisms for generating experiments to test theories and mechanisms of sexual conflict quickly and efficiently. In the following sections I discuss aspects of the behaviour, physiology and genetics of *D. melanogaster* that are relevant to the studies conducted in this thesis.

1.6.1 Courtship and copulation in *Drosophila melanogaster*

The mating behaviour and reproductive process in *D. melanogaster* is well studied and provides a useful background to begin to understand the nature of sexual conflict. Male courtship precedes mating and is required to stimulate receptivity in females. Courtship includes orientation towards the female, foreleg tapping upon the female's abdomen, following females, wing vibration to a produce a 'courtship song', genital licking and attempted copulation (Spieth, 1974, Hall, 1994). Depending on a female's receptivity state she will respond in one of two ways. To accept a mating a female stops moving and assumes a mating posture while manipulating her external genitalia (Spieth, 1974, Hall, 1994). Rejection behaviours include abdomen bending, ovipositor extension and kicking (Spieth, 1974, Connolly & Cook, 1973). Once courtship leads to a successful mounting, copulation duration appears to be primarily under male control (MacBean & Parsons, 1967) and typically lasts around 20 minutes. Sperm transfer appears to be complete in most matings by 8 minutes with the remaining time likely required for full transfer of seminal fluid. Only matings of a full duration induce a decrease in receptivity (Gilchrist & Partridge, 2000). Hence, continuation of copulation after sperm transfer may be

detrimental to females because they are no longer gaining benefits (such as more sperm) and may represent a source of conflict between the sexes if females are being manipulated into an unreceptive state. All of the studies conducted in this thesis involved the careful observation of mating behaviour in order to determine characteristics such mating latency, mating duration, and remating intervals.

1.6.2 The seminal fluid proteins of male *Drosophila melanogaster*

The transfer of seminal fluid proteins by males is a common feature found across many insect species (Gillott, 2003), although they have been particularly well studied in fruit flies. Seminal fluid proteins are a major determinant of post mating fitness for both males and females and range in size from small peptides to large glycoproteins (Wolfner *et al.*, 1997, Chapman & Davies, 2004). Recent estimates suggest there are over 130 unique molecules being transferred to females in the seminal fluid (Findlay *et al.*, 2008, 2009). Some sfps remain in the genital tract while a few target specific structures such as the ovaries or sperm storage organs (Heifetz *et al.*, 2000, Ravi Ram *et al.*, 2005, Wolfner, 1997). Other sfps are capable of crossing the posterior vaginal wall and entering the circulatory system (Lung & Wolfner, 1999) where they may target structures in the nervous system and brain (Ding *et al.*, 2003, Ottiger *et al.*, 2000).

Some sfps affect female behaviour and physiology, for example they stimulate ovulation (Heifetz *et al.*, 2000, 2001, Herndon & Wolfmer, 1995) oogenesis (Aigaki *et al.*, 1991, Chen *et al.*, 1988, Soller *et al.*, 1999), increase sperm storage (Neubaum & Wolfner, 1999a), decrease receptivity to mating (Aigaki *et al.*, 1991, Chen *et al.*, 1988, Manning, 1967), and are essential for mating plug formation (Lung & Wolfner, 2001). Sfps also have important roles in sperm competition (Chapman *et al*., 2000, Fiumera *et al*., 2005, 2007). In addition, the seminal fluid contains antibacterial peptides, proteases, protease inhibitors, lectins, lipases and CRISPs (Chapman *et al.*, 2001, Lung *et al.*, 2001, 2002, Mueller *et al.*, 2004). Sfps in *D. melanogaster* are rich in predicted regulators of proteolysis (Swanson *et al.*, 2001, Mueller *et al.*, 2004). Well over a quarter of all known sfps are predicted to be either proteases or protease inhibitors and likely control other seminal fluid proteins either by releasing biologically active peptides through cleavage or, alternatively, restricting the time that a specific sfp is available to act (Wolfner, 2002, Heifetz *et al.*, 2005, Ravi Ram & Wolfner, 2007). Proteolysis regulators may regulate the exposure of proteins on the sperm surface and / or protect sperm from degradation (Lung *et al.*, 2002). Some sfps are

predicted to be degenerate in function (i.e. they overlap in their function to some degree). In Chapter 7 I studied the roles sex peptide and Dup99B, two proteins which show substantial amino acid homology due to a likely duplication of the SP gene (Saudan *et al.*, 2002, Kim *et al.*, 2010), in producing short term egg laying and receptivity responses in mated females.

1.6.3 Sperm competition in *Drosophila melanogaster*

Females can retain viable sperm for at least two weeks (Neubaum & Wolfner, 1999a) and are polyandrous, thus providing the necessary conditions for the ejaculates of different males to compete. As previously discussed in section 1.4, the determinants of sperm competition are complex and involve many different physiological factors. Of particular interest in understanding the maintenance of genetic variation is the finding that male genotypes show extensive variation in their sperm competitive success (Civetta & Clark, 2000, Clark *et al.*, 1995, Hughes, 1997) and that females also influence sperm competitiveness depending on their particular genotype (Clark & Begun, 1998). This leads to complex outcomes that depend on the female and rival male traits to determine the outcome of sperm competition (Clark *et al.*, 2000, Bjork *et al.*, 2007).

1.6.4 Reproductive proteins in female *Drosophila melanogaster*

Proteins produced in the female reproductive tract carry out important functions such as egg activation, lubrication, or defence against pathogens (Wolfner *et al.*, 2005). Some are also likely to interact with male proteins to determine sperm storage, control of oogenesis and ovulation, and control over remating rate (Swanson *et al.*, 2004). An evolutionary EST (expressed sequence tag) screen of the female reproductive tract in *D. simulans* and *D. melanogaster* identified 526 independent genes predicted to mediate a diverse range of biological functions. These include a number of candidate proteins likely to interact with sfps (Swanson *et al.*, 2004).

D. melanogaster females have two types of organs dedicated to sperm storage. The seminal receptacle is the primary site of sperm storage and contains the majority (65–80%) of the sperm (Lefevre & Jonsson, 1962, Neubaum & Wolfner, 1999b), whereas the paired spermathecae are the site of long-term storage (Bloch Qazi *et al.*, 2003). Sperm are stored in the spermathecal lumen, which receives proteins of unknown function from surrounding secretory epithelial cells (Filosi & Perotti, 1975). Important female reproductive proteins

are likely to be found in organs dedicated to the storage of sperm, as has been found in some social insects (Wheeler & Krutzsch, 1994, Collins *et al.*, 2004, 2006). Prokupek *et al.* (2008) undertook an examination of the proteins of the spermathecae and documented categories of genes that could play a role in spermathecal functions such as storing, maintaining, and utilizing sperm. Prokupek *et al.* (2008) found that many of the most rapidly evolving genes were proteases with secretion signals, which are likely to interact with ejaculate proteins and coevolve with them. A further study found that the spermathecae were enriched for proteases and metabolic enzymes while the seminal receptacle was enriched for genes involved in protein localization, signalling and ion transport (Prokupek *et al.*, 2009).

To date, only a single female reproductive protein, sex peptide receptor, as been identified which is known to directly interact with a male sfp (sex peptide) (Hasemeyer *et al.*, 2009, Yapici *et al.*, 2008, Ribeiro & Dickson, 2010, Chow *et al.*, 2010). SPR is expressed within the reproductive tract and in parts of the nervous system and brain. SPR modulates female post mating responses such as increases in egg laying and feeding and decreases receptivity (Yapici *et al.*, 2008).

1.6.5 Interactions of male and female reproductive proteins in sperm competition and inducing female post mating responses in *Drosophila melanogaster*

As mentioned in the previous section, to date the only known pair of interacting male and female proteins are sex peptide and sex peptide receptor. Recent research has revealed intriguing allelic interactions for sperm competition outcomes between the sex peptide (SP) gene and its receptor, SPR, in females (Chow *et al.*, 2010). The proportion of first male offspring (P1) fathered by males with different SP alleles depended on a female's SPR genotype, with the interaction resulting in up to 8 fold differences in P1 scores. Further study and identification of other interacting male and female reproductive proteins should provide important molecular insight into sperm competition, sexual conflict, and / or cryptic female choice.

Two male sfps, Acp26Aa and Acp36DE are synthesized in the male's accessory gland although cleaved forms of the proteins are found after their transfer to the female (Monsma *et al.*, 1990, Bertram *et al.*, 1996). This suggests that processing of these sfps requires a contribution from the female, although the specific female contribution is unknown. Interestingly, processing in the female also requires another sfp, CG11864 (Ravi Ram *et*

al., 2006). Taken together, this shows how sfp proteolysis is a step-wise process and is often regulated by both males and females. The female might contribute enzymatic co-factors, or members of a proteolytic cascade that facilitates or mediates the action of CG11864. Alternatively, the female reproductive tract could provide an ionic environment or a pH environment that is optimal for the activity of CG11864 (Ravi Ram & Wolfner, 2007). Possible reasons for this interplay between proteolysis regulators could be to regulate the processing of reproductive molecules with essential physiological functions in ways which serve particular male or female interests in reproduction. Male and female proteolysis regulators may face sexually antagonistic selection in order to control the time that an intact signal peptide is available to act within the female (Wolfner, 2002, Heifetz *et al.*, 2005).

Spermathecal proteases identified by Prokupek *et al.* (2008, 2009) may be involved in interactions with male reproductive proteins, or play roles functionally analogous to male reproductive proteins. Female proteases might act to control the viscosity of the lumen of the spermathecae analogous to the semen coagulation role played by a serine protease produced in the prostate of male primates (PSA) (Malm *et al.*, 2000). An exciting possibility is that male-derived protease inhibitors might inhibit female proteases secreted into the reproductive tract in a specific male–female molecular interaction (Prokupek *et al.*, 2008). Such interactions are of considerable interest in the context of sexual conflict, because sperm competition between males can fuel antagonistic selection within males, and between males and females (Rice, 1998, Arnqvist & Rowe, 2005). Understanding the intricacies of the molecular interplay between males and females can thus provide crucial information to answer interesting evolutionary questions related to sexual cooperation and conflict at the molecular level.

1.7 Outline of Thesis

The work in this thesis was funded by the Natural Environment Research Council and was performed under the supervision of Tracey Chapman (Principal Supervisor) and Matt Gage (Second Supervisor). All experiments were performed by the author unless otherwise indicated. The aim of the thesis was to investigate the underlying genetics of sexual conflict in *Drosophila melanogaster* by identifying the roles of male and female reproductive proteins, using targeted genetic manipulations.

Chapter 2 describes the general materials and methods used in the thesis.

Chapter 3 investigates the role of a female reproductive protein, the sex peptide receptor (SPR), in determining the fitness of first and second males to mate across a range of remating intervals. This work was carried out with Claudia Fricke who helped with experiments and contributed data on the remating frequency of wild type females. The results show that rematings at early *vs.* late intervals, and the presence *vs.* absence of SPR, dramatically change the fitness benefits to males and contributes to our understanding of potentially antagonistic male and female interactions. The work is currently in revision following a first round of review at *Proceedings of the Royal Society B-Biological Sciences* with co-authors Claudia Fricke, Victoria Ng and Tracey Chapman.

Chapter 4 describes my contribution to a paper published in the *Journal of Insect Physiology* (2010, Vol. 56, pages 107-113) with co-authors Amanda Bretman, Mara Lawniczak and Tracey Chapman. I investigated the structure of the mating plug formed within females mated to males lacking the mating plug protein, PEBII, and demonstrated that these plugs showed significant differences in structure at 5-10min after the start of mating (ASM). The work demonstrates the importance of the mating plug in determining male post-copulatory success.

In Chapter 5 I investigated whether two male accessory gland proteins, Acp26Aa and Spn2, which show associations with sperm competition outcomes (Clark *et al.*, 1995, Fiumera *et al.*, 2005), have direct effects on sperm competition dynamics by conducting direct functional tests with RNAi mutants. There was only partial concordance between the results of the association tests the results of my genetic tests. Therefore, I discuss the possible benefits and drawbacks of both types of approaches for determining the functions of specific seminal fluid protein genes.

Chapter 6 describes experiments that tested for interactions between sex peptide (SP) and its receptor (SPR) and tests for roles for other seminal fluid proteins in modulating short and long term changes in egg laying and receptivity state. Additionally, this chapter demonstrates that SP and SPR are required for elevating feeding rate in mated females. This work adds to our understanding of possible degeneracy within male-female molecular interactions and expands on the known phenotypes under the control of SPR.

Chapter 7 investigates the role of SP and its related protein, Dup99B, which is produced from a sex peptide-like gene duplicate, in eliciting post mating responses 1 and 2 days after mating. I used single and double mutants for SP and Dup99B, in the first study to explore the effects of Dup99B in the absence of SP. This work makes a significant contribution to establishing which sfps have strong post mating effects and to examining potential degeneracy between SP and Dup99B.

Chapter 8 is a general discussion of the findings of this thesis and their wider implications. I discuss the roles of male x female genetic interactions for the maintenance of polymorphisms and the role of fluctuating selection for driving the increasing diversity of male and female reproductive proteins. The chapter discusses the evolution of degeneracy of protein function and concludes with ideas for future work to increase our understanding of the consequences of sexual conflict and sexually antagonistic coevolution in *Drosophila melanogaster*.

Appendix I outlines the experiment undertaken to develop a new dominant marker line for studies of SPR and remating interval in sperm competition under taken in Chapter 3

Appendix II is a copy of the submitted manuscript showing the full context of Chapter 4: A mating plug protein reduces early female remating in *Drosophila melanogaster*. Journal of Insect Physiology 56:107-113.

Chapter 2. General materials and methods

2.1 Fly culturing

2.1.1 Cages, bottles and vials

Stocks were maintained in glass bottles (189ml) containing 70ml food medium. The *Dahomey* wild type stock (see below) was maintained in plastic cages (45 x 25 x 25cm) containing bottles of SYA food medium (described below). Experimental flies and some stocks were maintained in glass vials (23x73mm) containing 7ml of SYA food. Flies were kept on a 12:12h light:dark cycle in humidified rooms at 25°C, or at 18°C for stocks not in current use.

2.1.2 Sugar-yeast-agar medium

Sugar-yeast-agar (SYA) food consists of 100g autolysed yeast powder, 50g sugar, 15g agar, 30ml Nipagin (10% w/v solution) and 3ml propionic acid per litre of distilled water. The yeast, sugar, agar, and water were brought to the boil and then left to simmer for several minutes before removing from the heat. After the mixture cooled to 60°C the Nipagin and propionic acid were added and then the medium was immediately dispensed into bottles or vials.

2.1.3 Maize-yeast medium

Maize-yeast medium (ASG) consists of 20g autolysed yeast powder, 60g maize meal, 85g sugar, 10g agar and 25ml Nipagin (10% w/v solution) per litre of distilled water. The medium was prepared and dispensed as described for SYA medium as described above.

2.1.4 Grape juice medium

In order to collect larvae at a standard density for experiments, a grape juice medium was used to collect larvae. The medium consisted of 1.1L water, 50g agar, 600ml red grape juice concentrate and 42.5ml Nipagin (10% w/v solution). The agar was added to 1L of water and brought to the boil. The red grape juice concentrate was then added and the mixture returned to the boil and allowed to simmer for a few minutes. The remaining water was added and the mixture left to cool to 60°C before the Nipagin was added. The medium was then dispensed into plastic Petri dishes and allowed to set at room temperature.

2.1.5 Standard density collections

All flies used in experiments were collected at standard density, to minimise environmentally determined variation in adult body size. Mated females were allowed to lay eggs on Petri dishes containing grape juice medium for 18-24h. 3-8h old larvae were collected from the grape medium with the use of a dissecting pin and placed into vials of SYA medium at a constant density of 100 per vial.

2.2 Stocks

The functions of genes can be inferred by comparing the mutant phenotype against the wild type, a process known as reverse genetics. I used two general types of mutants which I refer to as null mutants and RNA interference (RNAi) lines. Null mutants are flies that lack the gene product of the gene under study, either because the gene is altered or absent. Such knock out / loss of function mutations can be produced through untargeted mutagenesis with radiation or chemicals (with desired mutants collected in a later screening study) or can be created in a targeted fashion using processes such as homologous recombination (Rong & Golic, 2000, Rong *et al.*, 2002). Most knock out stocks are maintained as heterozygotes, with the mutant chromosome paired with a balancer chromosome. Balancer chromosomes rescue wild type gene function and prevent recombination, which could otherwise cause the mutation to become unstable (Thompson, 1977).

RNA interference (RNAi) is a naturally occurring process that influences gene regulation and intracellular immune defence (Hannon, 2002). It works through the actions of micro and short interfering RNAs, which, in concert with naturally occurring cellular RNAi proteins, can target specific messenger RNAs (mRNA), thus providing an early, post-transcriptional, block in protein synthesis (Qiu *et al.*, 2005). An RNAi response is triggered by the presence of double stranded RNA (dsRNA) which serves as a template for RNAi proteins that break down the double stranded RNA and continue to degrade any endogenous mRNA with a complementary sequence (Hannon, 2002). dsRNA can be produced in RNAi knock down mutants through the insertion and activation of a cDNA transgene encoding a short sequence of the target gene. The nucleotide sequence of the transgene is designed in a sense-antisense orientation so that when transcribed into RNA it forms a double stranded, hairpin loop, which then catalyses the destruction of the native

mRNAs with the target sequence (see below). I refer to the sense-antisense transgene as an inverted repeat (IR).

In order to drive expression of the IR at the correct time and in the desired tissue, the *Gal4/UAS* system was used (Brand & Perrimon, 1993). Gal4 is a transcription activator protein found in yeast. The *Upstream Activation Sequence* (*UAS*) is a short section of a yeast promoter region to which Gal4 binds in order to activate gene transcription. Transgenes are thus designed so that expression of the IR construct is under the control of the *UAS* promoter. The general procedure is as follows: a driver line of flies which expresses Gal4 in a particular tissue (e.g. accessory glands) is crossed to another line carrying the *UAS* promoter immediately upstream of the IR construct (**Figure 2.1**). In the progeny of this cross, Gal4 in the tissue of interest will bind to *UAS* and activate transcription of the IR sequence which leads to the production of dsRNA, thereby triggering the RNAi response against endogenous expression of the target gene specifically in that tissue. An important difference between null mutants and RNAi lines is that the latter still contain a fully functional copy of the gene of interest. RNAi is rarely 100% efficient, thus the technique may not totally eliminate translation of a protein. Therefore RNAi flies are referred to as "knockdown" mutants.

2.2.1 Wild type stock (*Dahomey*)

The laboratory wild type stock was collected in Dahomey (now Benin) in 1970 and has been maintained since then in large population cages with overlapping generations. Each cage contained 12 bottles of SYA food. Every week the oldest 3 bottles are replaced with new bottles containing fresh SYA medium.

2.2.2 *WhiteDahomey*

A white eyed, *whiteDahomey* (*wDah*) stock was generated by repeatedly backcrossing the w^{1118} strain into the *Dahomey* genetic background (Broughton *et al.*, 2005). *wDah* was used in the formation of controls when a mutant stock originates from a w^{1118} genetic background.

2.2.3 *UAS-sex peptide-stop*

The w^{1}*;P[*w^{+mC}*,UAS-SP-STOP.L61] (UAS-SP-stop)* line contains a transgenic construct in which the coding sequence of sex peptide (SP) has an early stop codon. This transgene

carries the *mini-white* eye colour marker (which produces a red eye) in the same w^1 background as used to construct the PEBII, Acp26Aa, and Spn-2 inverted repeat lines, and was therefore used as a control for genetic background. More importantly this line also allowed us to control for eye colour differences which can affect male mating success (Amanda Bretman and Tracey Chapman, unpublished data). The *UAS-SP-stop* construct has no obvious detectable effects on mating traits tested so far.

2.2.4 *Sex peptide receptor* null and controls

Sex peptide receptor lacking females (*SPR* null) were from the *Df(1)Exel6234* stock (Bloomington Drosophila Stock Center #7708) which was backcrossed into the *Dahomey* genetic background for six generations to produce *wDah,Df(1)Exel6234*. The X-chromosome of *Df(1)Exel6234* contains a deletion of the entire *SPR* gene and homozygous females from this stock do not produce SPR (Yapici *et al.*, 2008). *SPR* control females, *wDah,Df(1)Exel6234/SPR*$^+$, were produced by crossing *SPR* null females with *Dahomey* males to provide a functional copy of the *SPR* gene. Thus, *SPR* control females were from the same genetic background as *SPR* null females.

Following backcrossing, the *SPR* null line was verified in a bioassay by first mating the females to *Dahomey* males and then remating them after 48h. *SPR* null females remated significantly more than controls (*SPR/wDah* heterozygotes) (proportion remating, *SPR* null: 0.86; control: 0.12, Fishers Exact Test $\chi^2 = 59.9$, $p < 0.001$) indicating that the deletion was present in the new background.

2.2.5 *Stubble* competitor line

Stubble, *Sb*, is a dominant, homozygous lethal marker characterised by short thick bristles. *Birmingham;Sb*^{1}*/TM6* (Bloomington Drosophila Stock Center #2539) was backcrossed into the *Dahomey* strain for six generations to produce *Dah;Sb*^{1} which was used in the sperm competition experiment described in Chapter 3 to distinguish offspring fathered by competitor males. This line was selected based upon extensive preliminary tests that established the similarity of *Dah;Sb*^{1} to *Dahomey* in mating duration, mating latency and sperm competitiveness (Appendix I).

2.2.6 *Sparkling poliert* stock

Dah;;;sv$^{spa\text{-}pol}$ flies contain the *sparkling poliert* (*spa*) allele of the *shaven* gene (described in Partridge *et al.*, 1994). *Spa* was backcrossed into the *Dahomey* wild type background for 5 generations and was used to generate females and competitor males for sperm competition experiments described in Chapter 5. *Spa* flies are homozygous for a recessive mutation that produces small, smooth, glassy eyes that are easily distinguishable from wild type flies when viewed under a dissecting microscope. The stock used was maintained in SYA bottle cultures prior to experiments.

2.2.7 *PEBII* knockdown and controls

A *w*^{1}*;P[w*$^{+mC}$*,UAS-PEBII-IR.13-7]* inverted repeat (IR) line (*PEBII 13-7*) generated by Dr Mara Lawniczak was used to generate males lacking PEBII using the *Gal4/UAS* system. In brief, PEBII knockdown males were derived by crossing *PEBII 13-7* males to virgin females carrying a ubiquitous Gal4 driver (*w*^{1118}*;P[w*$^{+mC}$*,Act*^{5C}*-Gal4]25FO1/CyO*, Bloomington Drosophila Stock Center #4414, referred to as *Actin Gal4/CyO*) to produce *w*^{1118}*/w*^{1}*;P[w*$^{+mC}$*,Act*^{5C}*-Gal4]25FO1/P[w*$^{+mC}$*,UAS-PEBII-IR.13-7]*. Separate control lines for the Gal4 driver and the PEBII IR construct were generated from crosses with the *UAS-SP-stop* line. The Gal4 driver control males were generated from crosses of virgin *Actin Gal4/CyO* females with *UAS-SP-stop* males to produce *w*^{1118}*/w*^{1}*;P[w*$^{+mC}$*,Act*^{5C}*-Gal4]25FO1/P[w*$^{+mC}$*,UAS-SP-STOP.L61]*. The knockdown control males were generated from crosses of virgin *UAS-SP-stop* females with *w*^{1}*;UAS-PEBII-IR* males to produce *w*^{1}*;P[w*$^{+mC}$*,UAS-PEBII-IR.13-7]/P[w*$^{+mC}$*,UAS-SP-STOP.L61]*.

Quantitative real-time PCR showed that the PEBII 13-7 RNAi knockdown line significantly reduced the expression of PEBII. Transcript levels of PEBII were found to be reduced 16-fold in the knockdown line compared to the controls (Bretman *et al.*, 2010).

2.2.8 *Acp26Aa* knockdown

Acp26Aa knockdown males were derived by crossing males from the *UAS-Acp26Aa-IR* line, *w*^{1118}*;;P[GD5250]* (Vienna Drosophila RNAi Center stock #41193) with virgin females from the *Acp26Aa-Gal4* driver line, *w*^{1118}*;P[Acp26Aa-GAL4.C]* (described in Chapman *et al.*, 2003c) to produce *w*^{1118}*;P[Acp26Aa-GAL4.C];P[GD5250]*. This directs the RNAi silencing effect to the accessory glands only, where Acp26Aa is produced.

Knockdown control males were created by crossing *UAS-Acp26Aa-IR* line males to virgin females from the *UAS-SP-stop* line to produce w^{1118};*P[*w^{+mC}*,UAS-SP-STOP.L61];P[GD5250]*. The Gal4 driver control males were created by crossing *UAS-SP-stop* males with *Acp26Aa Gal4* driver virgin females to produce w^{1118};*P[Acp26Aa-GAL4.C]/P[*w^{+mC}*,UAS-SP-STOP.L61]*.

Quantitative real-time PCR (see below) showed that the *Acp26Aa-IR* RNAi knockdown line significantly reduced the expression Acp26Aa. Transcript levels of Acp26Aa were found to be reduced 55.8 fold in the knockdown line compared to the knockdown control and 18.3 fold compared to the Gal4 driver control.

2.2.9 *Spn2* knockdown

Spn2 knockdown males were derived by crossing males from the *UAS-Spn2-IR* line, w^{1118}*; P[KK106352]VIE-260B* (Vienna Drosophila RNAi Center stock #100958) with virgin females from the *Acp26Aa Gal4* driver line, w^{1118};*P[Acp26Aa-GAL4.C]* (described in Chapman *et al.*, 2003c) to produce w^{1118};*P[Acp26Aa-GAL4.C]/P[KK106352]VIE-260B*. This directs the RNAi silencing effect to the accessory glands only, where Spn2 is produced. Knockdown control males were created by crossing *UAS-Spn-2-IR* line males to virgin females from the *UAS-SP-stop* line to produce w^{1118};*P[*w^{+mC}*,UAS-SP-STOP.L61]/P[KK106352]VIE-260B*. The Gal4 driver control males were created by crossing *UAS-SP-stop* males with *Acp26Aa Gal4* driver virgin females to produce w^{1118};*P[Acp26Aa-GAL4.C]/P[*w^{+mC}*,UAS-SP-STOP.L61]*.

Quantitative real-time PCR (see below) showed that the Spn2-IR knockdown line significantly reduced the expression of Spn2. Transcript levels of Spn2 were found to be reduced 55.5 fold in the knockdown line compared to the knockdown control and 36.8 fold compared to the Gal4 driver control.

2.2.10 *Sex peptide* mutants

Sex peptide (SP) null males were generated by crossing the w^{1118};;*0325/TM3,Sb,ry* (*0325*) with the w^{1118};;*Δ130/TM3,Sb,ry* (*Δ130*) to produce w^{1118};;*0325/Δ130*. *0325* is a SP null mutant construct created through targeted homologous recombination (as described in Liu and Kubli 2003). *TM3,Sb,ry* is a balancer chromosome that restores wild type function. Some initial studies with *SP* null males used w^{1118}*;;0325/0325* homozygotes as SP nulls

and w^{1118}*;;0325/TM3,Sb,ry* as controls (Chapter 6). However, *0325/0325* homozygous males are slow to mate and so were crossed with *Δ130* in a subsequent experiment (Chapter 7). *Δ130* contains a deletion that includes the *SP* gene (as described in Liu and Kubli 2003). Controls for the *0325/Δ130* SP null males were created by crossing *Δ130* with w^{1118}*;;0416/TM3,Sb,ry* (*0416*) to produce w^{1118}*;;0416/Δ130*. *0416* was produced during the process of making the *0325 SP* null line and contains a copy of the mutant *SP* gene and a wild type copy (as described in Liu and Kubli 2003). Thus *0325/Δ130* SP null and the *0416/Δ130* control share the same genetic background. All sex peptide stocks were kindly donated by Professor Eric Kubli.

SP lines were verified using egg laying and receptivity bioassays. Wild type females that did not receive SP laid significantly fewer eggs than those that did (mean eggs laid ± se, SP null: 41.9 ± 2.6, SP control: 59.9 ± 3.2, *Dahomey* control = 61.6 ± 1.7; ANOVA $F_{2,134}$ = 18.073, $p < 0.001$). They were also significantly more likely to remate (proportion remated, SP null: 0.93, SP control: 0.07, *Dahomey* control = 0.04; G-test G=111.49, *d.f.* = 2, $p < 0.001$).

2.2.11 *Ductus ejaculatoris peptide 99b* (*Dup99B*) mutants

w^{1118}*;;071E/071E* homozygous males were collected from the w^{1118}*;;071E/TM3,Sb,Ser* line (donated by the Kubli lab). *071E* is a Dup99B null mutant construct donated by Professor Eric Kubli. w^{1118}*;;071E/TM3,Sb,Ser* males were used as controls. Lack of Dup99B protein expression in null lines was confirmed by Western blot (**Figure 2.2**, see below).

2.2.12 *SP/Dup99B* double null mutants

w^{1118}*;;0325,072/0325,072* homozygous males were collected from the w^{1118}*;;0325,072/TM3,Sb,Ser* double mutant line. This line was donated by Professor Eric Kubli and contains the *0325* SP null construct described above and *072*, a Dup99B null mutant construct. w^{1118}*;;0325,072/TM3,Sb,Ser* males were used as controls. Lack of Dup99B protein expression in null lines was confirmed by Western blot (**Figure 2.2**, see below).

2.3 Molecular biology

2.3.1 Quantitative PCR

Quantitative PCR (qPCR) is a technique based on the real time monitoring of a PCR reaction and was used to calculate the knockdown efficiency of the Acp26Aa and Spn2 knockdown males compared to their controls (obtained as described above). RNA was extracted from sample of males using the mirVana miRNA isolation kit (Ambion, USA) according to the manufacturer's protocol. The *Precision* reverse transcription kit (PrimerDesign, UK) was used for cDNA synthesis according to the manufacturer's protocol. Real-time PCR was performed using an Applied Biosystems Prism 7500 Sequence Detection System, and low-ROX MasterMix (PrimerDesign, UK). The ratio of selected gene transcripts (measured as change in real-time fluorescence during the qPCR) was compared to a ubiquitously expressed control gene to determine the relative expression level of the gene of interest. *αTubulin84b* was used as the control gene to correct for slight differences in the amount of cDNA template added to each well. PCR primers for *Acp26Aa*, *Spn-2* and αTubulin84b were custom ordered from PrimerDesign. PrimerDesign is a highly sensitive qPCR assay that utilizes a fluorescent probe (tagged with FAM) designed to target the gene of interest.

Acp26Aa primers
Sense primer: TGTGAACCGCAGACTTTTGG
Anti-sense primer: TGGCTTCCTGAAACTGATTGG
Spn2 primers
Sense primer: AGTTCCAGCTAGTACCGAGTTA
Anti-sense primer: GCGTCTGATTATCTACCCAATTATT
αTubulin84B primers
Sense primer: CCGTGTGCATGTTGTCCAA
Anti-sense primer: GAACTCTCCCTCCTCCATACC

Quantification of transcript levels in knock-down males relative to their controls (all standardized to *αTubulin84b*) was performed using a standard curve method following Applied Biosystems protocols. Ct values were averaged across two independent sample replicates and knockdown efficiency was calculated using the $2^{-\Delta\Delta Ct}$ method (Schmittgen

& Livak, 2008). This work was carried out at the Biomedical Research Center at UEA with the aid of Dr Caroline Pennington.

2.3.2 Western blot

The level of Dup99B in Dup99B null and Dup99B/SP double null males compared to controls (obtained as described above) was determined by Western blotting. 5 day old mated males were collected from stock vials and assigned to null or control treatments based on the presence or absence of the balancer chromosome. Groups of 20 males from each treatment were placed into Eppendorf tubes and snap frozen in liquid N_2. 100 µl of homogenization buffer (Hepes lysis buffer,15 mM EDTA, 0.1% IGEPAL, 10% protease inhibitor) was added to each tube followed by homogenization with a plastic pestle. Vials were then left on ice for 30min followed by centrifugation at 5000rpm for 5min.

Protein levels for each extraction were quantified with a BCA protein assay kit (Thermo Scientific, USA) according to manufacturer's protocols. 1µg of protein was added to new Eppendorfs and mixed with 5µl homogenization buffer and 2µl 1M DTT before boiling at 110°C for 3min. An equal amount of protein extract for each line was loaded on an SDS polyacrylamide gel (15% acrylamide/bisacrylamide) and subjected to electrophoresis at 200V for 30min. The gel was then blotted with Towbin buffer on Hybond ECL nitrocellulose membrane (Amersham Pharmacia).

The membrane was washed in blocking solution [5% low-fat dry milk in PBS0.1% Tween 20 (PBS-T)] for 1h and incubated overnight at 4°C with the primary antibody (anti-Dup99B rabbit antibodies, Genscript Ltd (USA). After washing with PBS-T solution, the membrane was incubated with peroxidase-labeled anti-rabbit secondary antibody (Amersham Pharmacia) for 1h, and then treated with the ECL Western blotting detection system (Amersham Pharmacia), according to the manufacturer's instructions. The result of the Western blot showed that the Dup99B null and the Dup99B/SP double null do not express Dup99B protein at detectable levels (**Figure 2.2**).

Figure 2.1. Illustration of *Gal4-UAS* system. A fly containing the *Gal4* driver is mated to another fly that contains the *UAS-target gene-Inverted Repeat (IR)* (*top*). In the progeny of this mating, *Gal4* is expressed in a tissue specific manner and activates the *UAS-Gene X-IR* (*bottom*) (adapted from Brand and Perrimon, 1993).

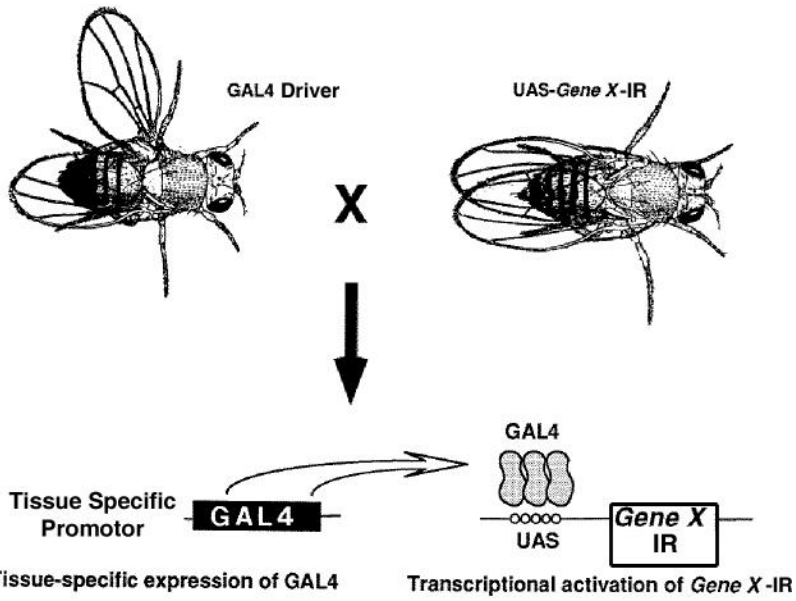
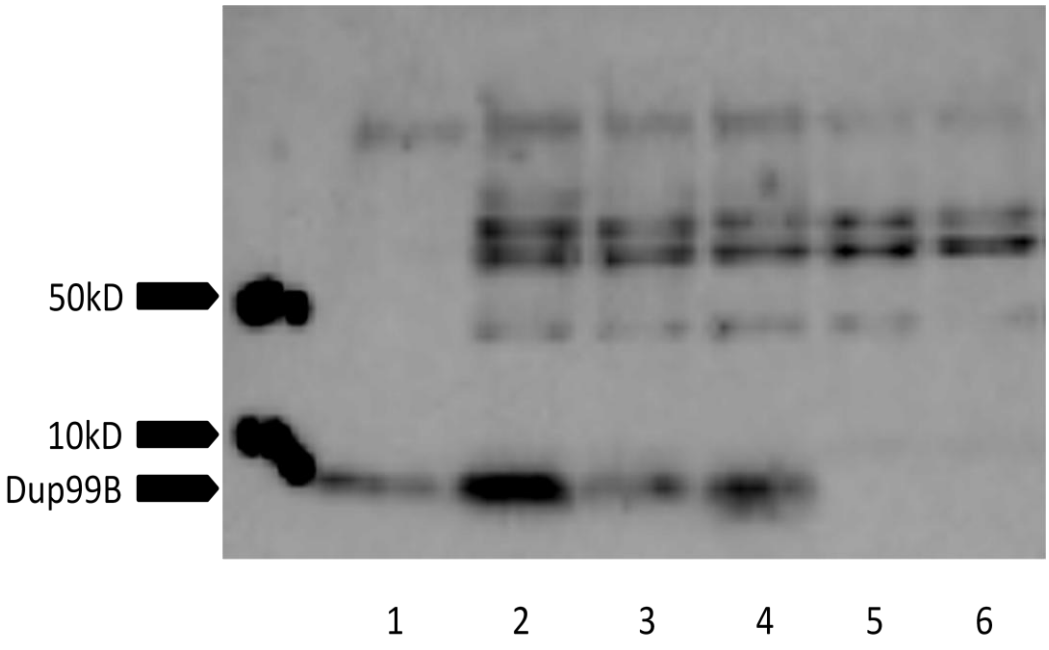


Figure 2.2. Dup99B Western blot results. Lanes (from left to right): **(1)** *wDahomey*, **(2)** *0416/Δ130* (*SP* control), **(3)** *0325/Δ130* (*SP* null), **(4)** *0325/0325* (*SP* null), **(5)** *SP,Dup99B* null, **(6)** *Dup99B* null.



Chapter 3. Sex peptide receptor and remating interval alter sperm competition dynamics and the benefits of remating in *D. melanogaster*.

3.1 Summary

In multiply-mating species fitness gains for individuals at each mating are determined by a multi-way interaction between the female, her current and previous mates. Recent research in *D. melanogaster* is now revealing some of the interacting genes involved. These include *sex peptide* (*SP*) and its receptor (*SPR*). These genes are predicted to be subject to sexually antagonistic selection because of the opposing effects of SP on male and female fitness. Here we measured the fitness of second mating males in rematings from 3h to 48h, with SPR-lacking and control females. Early rematings with *SPR* null females resulted in significantly increased last male, and decreased first male, fitness. Therefore the absence of SPR significantly altered the fitness gains to males of gaining, and preventing, early rematings. This is consistent with the idea that SPR is subject to sexually antagonistic selection due to its effects on the fitness of competing males. In contrast, early remating with control females produced relatively equitable paternity share, and later rematings exhibited typical second male precedence. The frequency of early remating in wild type females was surprisingly high, with 25-40% of wild type females remated within 4h under intermittent and continual male exposure, respectively. Early rematings are therefore biologically important and the potentially antagonistic interactions between a female and her mates will be strongly selected.

3.2 Introduction

Sperm competition is defined as competition between the ejaculates from two or more males for the fertilization of a given set of eggs (Parker, 1970). Females of many species in the animal kingdom remate before using all their stores of viable sperm (Simmons, 2001), creating the conditions for strong post-copulatory sexual selection between the sperm/ ejaculates of different males within the female reproductive tract (Parker, 1970). In *Drosophila melanogaster* the majority of wild caught females carry sperm from more than one male (Imhof *et al.*, 1998) showing that multiple mating with different males is frequent in the natural context. Females also remate before exhausting the sperm from previous matings (Gromko & Markow, 1993). *D. melanogaster* females store sperm in two distinct organs within the reproductive system: the tubular seminal receptacle and the paired, mushroom-shaped spermathecae (Lefevre & Jonsson, 1962). The storage of sperm within these sperm storage organs provides an arena in which sperm competition can occur. Sperm competition has been studied widely in *Drosophila* and represents a significant opportunity for selection to shape both male and female reproductive traits (Pitnick *et al.*, 2001a, Clark, 2002).

In a laboratory setting, the proportion of offspring in *D. melanogaster* that are fathered by the second male to mate (P2) is typically >0.8 (Simmons, 2001). However, the temporal effects of remating and of sperm storage on sperm competition have been little explored (van Vianen & Bijlsma, 1993, Bloch Qazi *et al.*, 2003). In *Drosophila*, sperm storage is the process whereby sperm are directed to the seminal receptacle and spermathecae for current and future use (Bloch Qazi *et al.*, 2003). Sperm storage, from initial fertilization to complete storage within the seminal receptacle and spermathecae, can take up to 6h (Lefevre & Jonsson, 1962, Gilbert, 1981, Neubaum & Wolfner, 1999a, Tram & Wolfner, 1999). The outcome of sperm competition is therefore likely to depend upon the timing of female remating, as this will determine not only the relative levels of sperm present, but also whether the storage of sperm from the previous male is complete. Early rematings (<6h for *Drosophila*) provide the opportunity for sperm mixing to occur before sperm storage is complete. This could lead to sperm competition outcomes that are more dependent on 'fair raffle' mechanisms of sperm use (Parker, 1990) instead of the usual observed pattern of sperm displacement leading to high last male sperm precedence (Harshman & Prout, 1994). A prediction of the fair raffle mechanism is that P2 should be

in direct proportion to the number of sperm transferred (Parker, 1990). Hence, if *Drosophila* males transfer equal numbers of sperm we predict that early rematings, before transport of the first male's sperm to the storage organs is finalised, should result in P2 values of 0.5. During later rematings, when sperm storage is complete (≥6h), the second male must displace the first male's sperm from storage if he is to gain a significant share of paternity. The process of remating itself is reported to cause females to dump a portion of resident sperm from storage back into the uterus (Snook & Hosken, 2004). It is unknown whether this requires seminal fluid or is a wholly female mediated response. In the subsequent period during which first and second male sperm from the uterus are restored, the second male sperm can physically displace any remaining first male sperm in storage (Price *et al.*, 1999, Manier *et al.*, 2010). Movement of rival sperm within the female reproductive tract is a dynamic process, with high sperm motility leading to low levels of stratification of sperm within the sperm storage organs (Manier *et al.*, 2010).

In many studies of sperm competition rematings are, however, conducted after 1-3 days (Avila *et al.*, 2010, Chow *et al.*, 2010, Manier *et al.*, 2010), which far exceeds the period needed for sperm storage to occur. This protocol has been used because it is often assumed that female *D. melanogaster* will not remate for at least a day following a mating. However, it is becoming clear that this view is incorrect and that there is more opportunity for early rematings than has yet been realised. For example, Bretman *et al.* (2010) report remating rates of 70-75% within 4h in laboratory experiments while remating rates of 30-50% within 6h have also been reported previously (Vanvianen and Bijlsma, 1993). It is clear therefore that systematic investigations of the frequency of early rematings, and their effects on sperm competition dynamics, are required.

The outcome of sperm competition can depend on the genotypes of the first and second males and of the female with which they mate (Prout & Bundgaard, 1977, Prout & Clark, 1996, Wilson *et al.*, 1997, Clark *et al.*, 2000, Bjork *et al.*, 2007, Chow *et al.*, 2010). Therefore, it is the interplay between molecules and behaviours in males and females that ultimately determines reproductive success. For example, the initiation of post mating responses in females involves interactions between seminal fluid molecules transferred by males during mating, and specific target molecules in the female (Ravi Ram *et al.*, 2005, Ravi Ram & Wolfner, 2007). The striking changes in female behavior and physiology following mating (Chapman, 2001) and the associated significant fitness effects of such

responses can also lead to strong antagonistic selection between genes responsible for male and female fitness (Rice, 1996, Rice & Holland, 1997, Mueller *et al.*, 2005).

Several of the >130 seminal fluid proteins transferred from males to females during mating (Findlay *et al.*, 2008) have significant effects on sperm competition (e.g. Fiumera *et al.*, 2005, 2007). Both sequence variation in, and the expression of, genes coding for seminal fluid proteins are associated with male success in sperm competition (Clark *et al.*, 1995, Fiumera *et al.*, 2005, 2007). One key protein, sex peptide (SP), increases egg laying rate while decreasing female remating for 7-10 days after mating (Chapman *et al.*, 2003c, Liu & Kubli, 2003). SP should therefore have a significant impact upon a male's reproductive success through sperm competition. The data are consistent with this finding. In two separate studies, SP affects the proportion of offspring fathered by the first (P1) but not second (P2) male to mate, when matings occur 1 day after the first mating (Fricke *et al.*, 2009, Avila *et al.*, 2010). Males that lack SP do not stimulate female egg laying and sperm use to control male levels, and more of their sperm are present in storage when remating occurs resulting in higher P1 values. Hence SP alters the outcome of sperm competition by changing patterns of sperm use (Avila *et al.*, 2010). SP also alters a male's reproductive success through its effects on female receptivity. Males that transfer SP have a higher 'per mating' reproductive success because they father more offspring before the female remates (Fricke *et al.*, 2009). Together these findings indicate that egg laying, receptivity and the amount of sperm in storage can (i) have significant effects on the outcome of sperm competition and (ii) be influenced by SP. Manipulation of the SP transduction pathway therefore offers an exciting opportunity to test the effects on sperm competition of different remating intervals and of the importance of post mating responses that occur in females. It also gives an unparalleled opportunity to investigate female influences, given that the sex peptide receptor (SPR) has now been identified (Yapici *et al.*, 2008). Consistent with the interest of this pathway in sperm competition, variation in SPR and SP has been linked with phenotypic differences in female fecundity, receptivity and sperm competition outcomes (Chow *et al.*, 2010).

This chapter describes the systematic study of the dynamics of sperm competition, utilizing a design that allowed for the examination of outcomes across a range of remating intervals from 3h to 48h after initial matings. These times were based on measurements of the frequency of early rematings in our wild type population using both continual and

intermittent male exposure designs, to determine the frequency at which such early rematings might typically occur (contributed by Claudia Fricke and Victoria Ng). To test the influence of females on sperm competition patterns following early rematings comparisons were made between control females and those that could not respond to the delivery of SP. Using a *sex peptide receptor* (SPR) deletion mutant, the experiments tested whether females that were unresponsive to SP would show different sperm competition outcomes *vs.* SP responsive control females. Females that lack SPR do not increase their egg laying rate and are more likely to remate following copulation (Yapici *et al.*, 2008). This study therefore examined female remating effects on sperm competition, and elucidated the critical importance of egg laying and remating rate in understanding how sperm competition shapes the evolution of reproductive traits in this species.

3.3 Materials and methods

3.3.1 Fly stocks

Wild type male flies were from the *Dahomey* laboratory population (see Chapter 2 for stock details). *Dahomey* (*Dah*) containing the dominant marker, *Stubble, Dah;;Sb*^{1}, was used as the competitor strain. Sex peptide receptor-lacking (*SPR* null) females were homozygotes of *wDah,Df(1)Exel6234* (Bloomington Drosophila Stock Center #7708). *SPR* control females were *wDah,Df(1)Exel6234/SPR*$^{+}$.

3.3.2 Effect of SPR and remating interval on sperm competition

At four days post-eclosion 200 *SPR* null and 200 control females were transferred singly into experimental vials using ice anaesthesia. The following day *Dah;;Sb*^{1} males were aspirated singly into the vials containing single females. The time at which mating started and ended was then recorded. Flies that did not mate within 2h were discarded. After mating had ended the male was removed and the female was assigned to one of four treatments. 59-60 females from each genotype were assigned to each of the 3h and 5h remating interval treatments. 33-37 females were assigned to each of the 24h and 48h remating interval treatments. At the designated remating interval, females were aspirated singly into new mating vials containing a single *Dahomey* male. Time to mating (latency), and the start and end of mating were again recorded. Flies that did not mate within 2h were discarded. Females that remated were allowed to lay eggs for 24h and were then discarded. Total numbers of progeny produced during the remating interval were counted. Progeny

produced after the second mating were sorted according to genotype based on the presence or absence of the *Sb* marker phenotype. The paternity of the marker male was calculated as twice the number of marker offspring observed (since the *Dah;;Sb*^{1} males were heterozygous for *Sb*^{1}). The proportion of offspring fathered by the second male (P2) was calculated as *Dahomey* offspring/total number of offspring.

3.3.3 Frequency of early rematings in wild type females continually exposed to wild type males for 8h following first matings (contributed by Claudia Fricke and Victoria Ng)

100 virgin wild type *Dahomey* females were each mated once to a wild type *Dahomey* male. The time of introduction, start and end of matings were recorded. Immediately after the end of the first mating, males were removed via aspiration and a second male introduced to each female. Pairs were then observed continuously for the occurrence of a second mating over a period of 8h. The start and end of all second matings observed was recorded. The time between the start of the first and second matings was the remating interval.

3.3.4 Frequency of early rematings in wild type females intermittently exposed to wild type males for 48h following first matings (contributed by Claudia Fricke and Victoria Ng)

200 virgin wild type *Dahomey* females were each mated to a wild type male as above, and the time of introduction, start and end of matings recorded. Starting at 2, 4, 6, 8, 24 and 48h after the first mating, groups of 30 randomly chosen females were given the opportunity to remate with a fresh wild type male. Pairs were observed for 30mins and the number of females remating was recorded. Females were assayed at only one of these time points, hence each remating interval was an independent test. These assays were performed in two blocks with 22 or 30 females per time point per block, respectively.

3.3.5 Statistical analysis

P2 was analyzed using a generalized linear model in R 2.12.0 (R Development Core Team, 2010, Crawley, 2007). Quasibinomial errors were used to increase goodness of fit and to correct for overdispersion in the model. Female genotype (control *vs. SPR* null) and remating interval (time) were the fixed factors. Progeny produced before mating

(progeny1), first mating duration (duration1), second mating duration (duration2), and second mating latency (latency2) were covariates. The significance of all factors was tested with an Analysis of Deviance through subtraction from the full model. Total wild type and marker offspring produced were analyzed using a similar procedure, except that quasipoisson errors were used. The rate of progeny production after the first mating was square root transformed to improve normality and then analyzed using *t* tests in SPSS 16 (SPSS, Inc., Chicago IL). Correlations between factors were tested using the Spearman's rank correlation coefficient, in SPSS 16. Means ± standard errors (s.e.) are presented throughout. Mean P2 was calculated as the overall proportion of the sum of all wild type and marker offspring produced within each treatment. Standard errors for P2 were calculated using the formula: $s.e. = \sqrt{P2(1-P2)/n}$ (Sokal & Rohlf, 1995). To test for the effects of time on the frequency of remating in wild type females we used a generalized linear model with binomial errors, with length of remating interval and block as fixed factors.

3.4 Results

3.4.1 Effect of SPR and remating interval on male fitness: second male paternity share (P2)

Males remating with *SPR* null females gained significantly higher P2 at 3h than did males mating with control females (*SPR* null: 0.76 ± 0.07, control: 0.48 ± 0.09; **Figure 3.1a**). However, P2 was similar for rematings in both groups at 5h and beyond (5h *SPR* null: 0.60 ± 0.08, control: 0.64 ± 0.10; 24h *SPR* null: 0.61 ± 0.09, control: 0.68 ± 0.13; 48h *SPR* null: 0.66 ± 0.09, control: 0.72 ± 0.10). The difference between *SPR* null and control females over time was evident through the significant interaction between female genotype and remating interval (**Table 3.1**). P2 was significantly affected by the number of progeny produced in the intermating interval with P2 being generally higher in females that produced fewer progeny before remating. There was also a positive effect of the second mating duration on P2 (**Table 3.1**; Spearman's *rho* = 0.258, *d.f.* = 216, $p < 0.001$, data not shown).

3.4.2 Effect of SPR and remating interval on male fitness: absolute number of first and second male offspring produced.

Consistent with the results for P2 paternity share above, 3h rematings with *SPR* null females produced significantly more second male (and fewer first male) offspring than with controls (**Figure 3.1b,c, Table 3.2**). At >3h remating intervals control females produced significantly more second male offspring than *SPR* null females, while the number of first male offspring produced by control and *SPR* null females were similar. The significant difference over time in second male progeny production by *SPR* null and control females was evident in the significant interaction between female genotype and remating interval (**Table 3.2**). Consistent with the P2 results above, there was a significant positive effect of second male mating duration on second male progeny produced (**Table 3.2** and Spearman's *rho* = 0.197, *d.f.* = 216, $p = 0.003$, data not shown).

3.4.3 Effect of SPR on remating rate and number of progeny produced before remating.

As expected, significantly more *SPR* null females remated than controls across all remating intervals (% remated, *SPR* null: 88.2, control: 49.2; Fisher's exact test, $p < 0.0001$, **Table 3.3**). Overall, *SPR* null females also produced offspring at a significantly lower rate than controls after the first mating (offspring/h, *SPR* null: 3.26 ± 0.24, control: 4.37 ± 0.35; $t_{216} = 2.441$, $p = 0.015$; data not shown), though this difference was not consistent at all remating intervals (**Figure 3.2**). There was no difference, for example, in the number of offspring produced by *SPR* null females and controls before the remating test at 3h (*SPR* null: 14.49 ± 1.54, control: 16.47 ± 1.89; $t_{69} = 0.817$, $p = 0.42$).

3.4.4 Frequency of early rematings in wild type females continually exposed to wild type males for 8h following first matings

A total of 60 out of 99 females (60.60%) remated within the 8h observation period. As shown in **Figure 3.3a**, females started to remate as early as 1h after their initial mating, with a roughly linear increase in number of rematings until approximately 320min (~5.5h) after their initial mating, when rematings ceased except for 1 additional mating at 371min.

3.4.5 Frequency of early rematings in wild type females intermittently exposed to wild type males for 48h following first matings

In the intermittent remating tests, there was up to 10% remating even after only 2h, and by 4-24h there was ~25% remating, with a consistent dip across both replicate blocks to ~15% at 8h. By 48h remating was up to ~50%. Hence, the magnitude of female remating was significantly dependent on remating interval (Analysis of Deviance, $G^2 = 29.59$, $d.f. = 5$, $p < 0.0001$, **Figure 3.3b**), with evidence of some replicable periodicity in remating frequency over time. There was no significant effect of block ($G^2 = 0.23$, $d.f. = 1$, $p = 0.635$).

3.5 Discussion

This study showed significant effects of SPR and remating interval on male fitness through sperm competition. Early rematings at 3h with females lacking SPR resulted in significantly higher P2, and higher absolute second male progeny, than in the control females. Hence the removal of SPR increased the benefit of gaining early rematings, consistent with the prediction that SP and SPR are subject to sexually antagonistic selection. As expected, in control females there was evidence for relatively equitable paternity among the two males when remating occurred after 3h and sperm mixing was likely. Later rematings resulted in the expected second male precedence. The frequency of early rematings in unmanipulated wild type females was surprisingly high. 50% remating was observed after about 300mins (5h) of continual exposure to males and > 25% remating after only 4h in tests of intermittent male exposure. These findings show that the fitness benefits and costs of early rematings are highly biologically relevant.

The results reveal a novel role for SPR in determining the magnitude of fitness benefits to remating males. The benefit of gaining early rematings was increased in mates of females lacking SPR. Likewise, there was a greater cost to the first mating male in *SPR* null females that remated early. Hence the removal of SPR, and the loss of the ability to regulate sexual receptivity, significantly altered the balance of fitness benefits and costs to successive mates of the female. The optimal remating interval for the parties involved therefore shifted significantly in the absence of SPR. Given that second male paternity is tightly linked to indices of male lifetime reproductive success (Fricke *et al.*, 2010), the benefits of gaining rematings are particularly important for a male's overall fitness. In

control but not SPR-lacking females, the benefits for remating males will potentially be higher if they can avoid remating with recently mated females and instead focus their efforts on females with whom they could achieve high P2. Data suggest that males can detect female mating status (Friberg, 2006) and may be able to adjust their ejaculate accordingly (Wedell *et al.*, 2002, Bretman *et al.*, 2011). However, there has been little work so far to dissect at a finer scale the degree to which males can choose between females based upon optimal remating interval.

The results are consistent with the idea that the three-way interaction between competing males and the female can have significant fitness effects on the parties involved. This system is therefore likely to be subject to sexually antagonistic selection both between males and between males and the female (Rice, 1998). However, we do not yet know the fitness effects of SPR removal, and hence the loss of responses to SP, for females. Presumably there is variation in the expression of SPR across females (as there is variation in SP expression across males (Smith *et al.*, 2009) and it will be interesting in future work to investigate the fitness consequences of this for females and for successive competing males.

The pattern of paternity was in contrast to what would be predicted based on the likely numbers of sperm in storage. There were no differences in the number of offspring produced by SPR-lacking or control females before remating at 3h **(Figure 3.2)**, suggesting that the number of sperm in storage may have been similar. P2 value should therefore not differ across females, and there should be relatively equitable paternity for first and second males due to higher sperm mixing at the early remating interval. However, only the control females showed P2 of approximately 0.5, while P2 in SPR-lacking females was 0.76. At later remating intervals control females produced significantly more progeny then the *SPR* null females before remating, suggesting that control sperm stores were depleted to a greater extent. This effect should be exacerbated given that, in the absence of SP and presumably also in SPR-lacking females unable to respond to SP, sperm show slower depletion from storage (Avila *et al.*, 2010). Hence there should have been higher P2 in controls than SPR-lacking females, but this was not observed. Control females did, however, show the expected gradual increase in P2 with longer remating intervals. The findings suggest that the predicted number of sperm present in storage at the time of

remating (Letsinger & Gromko, 1985) are consistent with paternity patterns in control but not SPR-lacking females.

SPR could interact with ejaculate components and influence sperm motility, storage or retention. If sperm in SPR null females took longer to be stored, then earlier remating could result in more first male sperm being dumped (Snook & Hosken, 2004, Manier *et al.*, 2010), giving second male sperm a higher numerical advantage in sperm competition. A reduction in sperm motility could result in less mixing and more stratification of rival ejaculates within storage, which could favour the second mating male (Simmons & Siva-Jothy, 1998). These possibilities could explain why there was no difference in P2 after 3h rematings, with greater remating intervals compensating for slower sperm storage. SPR is expressed in only one of the two sperm storage sites (the spermathecae and not the seminal receptacle (Yapici *et al.*, 2008), whereas SP is delivered to both storage sites bounds to sperm. This could explain why patterns of paternity predicted by sperm numbers in storage in the presence and absence of SP (Avila *et al.*, 2010) contrast with what was seen here in SPR-lacking females. The significance of delivering SP to sites in the female, e.g. the seminal receptacle, where its receptor is not expressed is not yet known.

The duration of the second mating was positively associated with relative and absolute second male paternity across both female genotypes. Manier *et al.* (2010) found a significant correlation between the size of the second male's ejaculate and the amount of resident sperm displaced from storage and suggest that the increased sperm numbers in larger ejaculates may represent an adaptation to facilitate physical displacement of resident sperm. Our results are consistent with this idea, assuming that longer matings result in the transfer of a larger ejaculate. Fricke *et al.* (2010) show that P2 is positively associated with a male's relative and absolute lifetime reproductive success. These results confirm that mating duration is a potentially important fitness trait.

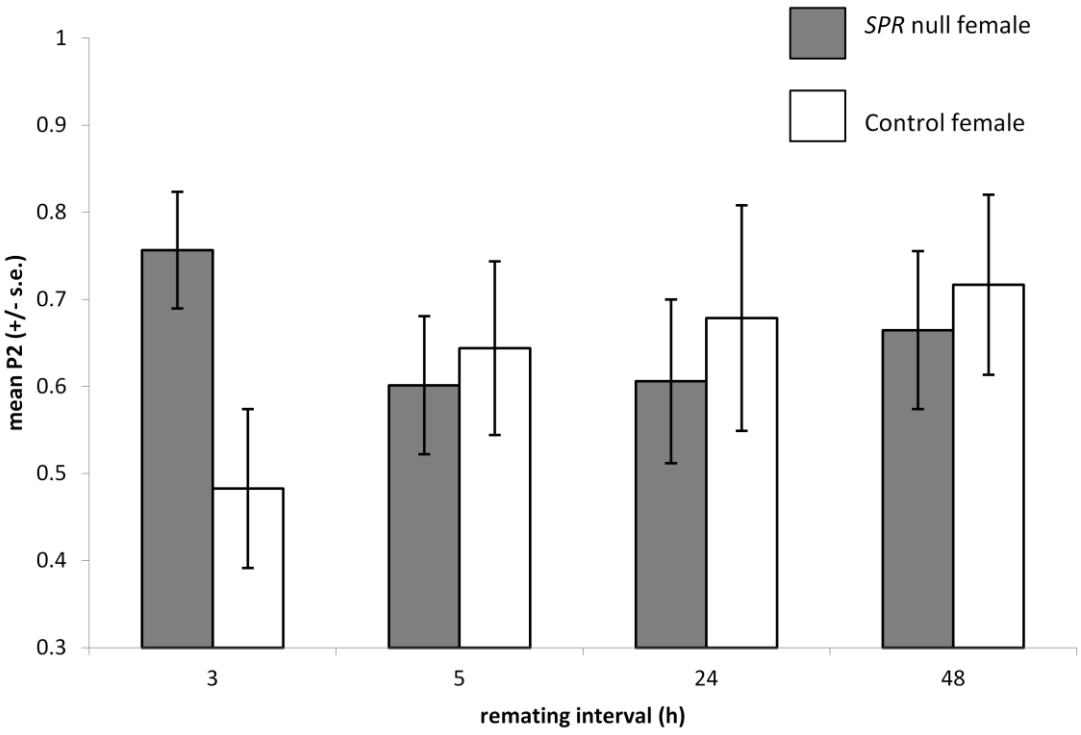
The high frequency of early rematings observed in wild type females is important because it shows that the study is set in a relevant biological context. It is often assumed that remating in *D. melanogaster* only occurs after several days. However, the results of this study and previous reports (van Vianen & Bijlsma, 1993, Bretman *et al.*, 2010) reveal that a substantial fraction of rematings can occur within just a few hours. This was evident in this study under both continual and intermittent male exposure regimes. The early remating profiles also hint at some consistent rhythmicity, with the intermittent exposure tests

showing higher frequencies of remating at 4h and 6h than at 8h. Further study of the fine scale temporal variation in female receptivity and its impact on sperm competition dynamics would be very useful. These findings are important in the context of previous work that has tended to examine sperm competition outcomes at a longer remating interval, e.g. after 3 days (Simmons & Siva-Jothy, 1998, Simmons, 2001, Avila *et al.*, 2010, Manier *et al.*, 2010). For example, Long *et al.* (2010) examined P2 after 1-3 day remating intervals in *D. melanogaster* and found that second males fathered significantly more offspring as the remating interval increased. This is consistent with a reduction in the number of first male sperm in storage over 1-3 days.

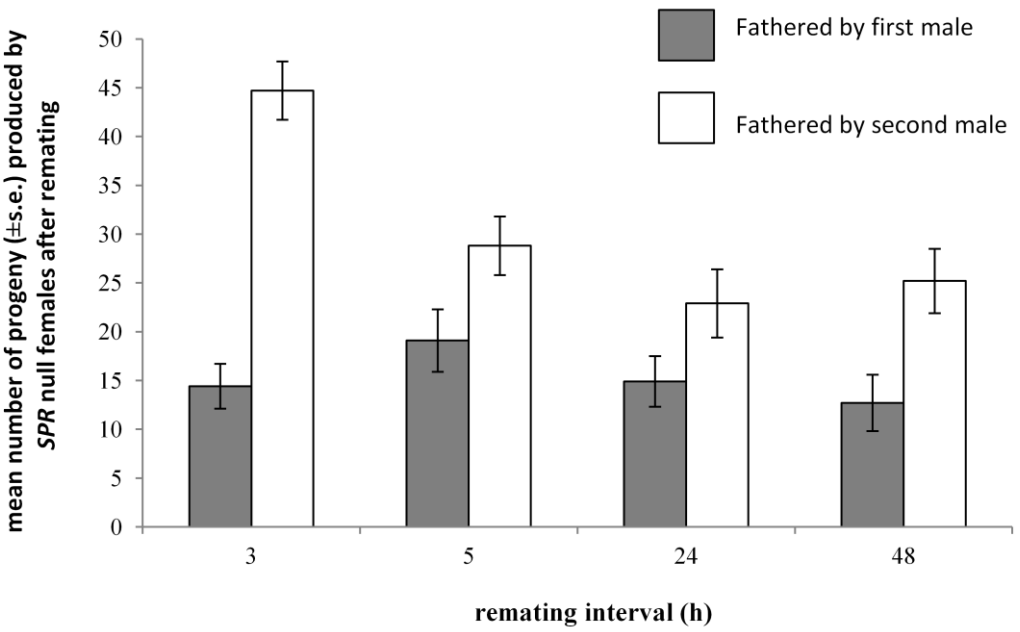
Overall this study shows that the removal of SPR can significantly alter the costs and benefits of early rematings for successive mates of the female. The substantial level of rapid remating in wild type females suggests that early rematings are biologically important. Hence variation in fitness over short remating intervals offers a significant opportunity for selection. The possibility that the SP-SPR pathway is subject to sexually antagonistic selection, suggested by the finding that the absence of SPR (and any female resistance to SP) was beneficial to early remating males, deserves further study. Removal of SPR also altered fitness gains for competing males and shifted the optimal remating interval for males in the first *vs.* second mating roles. Hence the results also show a role for SPR in mediating conflicts between different competing males. This highlights the multi-way interaction between successive male mates and the female (Rice, 1998). The continued study of the dynamics of sperm competition and time-dependent changes in paternity should also help to illuminate the complex mechanisms contributing to the fitness of males in competition (Bussiere *et al.*, 2010).

Figure 3.1. Effect of SPR on male fitness. (a) Second male paternity share (P2). Mean P2 (± s.e.) for males remating with either SPR null or control females after 3, 5, 24 or 48h. Filled bars: SPR null females; open bars: SPR control females. (b) Absolute number (mean ± s.e.) of offspring fathered by the first or second males following rematings at 3, 5, 24 or 48h with SPR null females. Filled bars: first male progeny, open bars: second male progeny. (c) Absolute number (mean ± s.e.) of offspring fathered by the first or second males following rematings at 3, 5, 24 or 48h with control females. Filled bars: first male progeny, open bars: second male progeny.

(**a**)



(b)


Fathered by first male
Fathered by second male
mean number of progeny (±s.e.) produced by SPR null females after remating
50
45
40
35
30
25
20
15
10
5
0
3
5
24
48
remating interval (h)

(c)

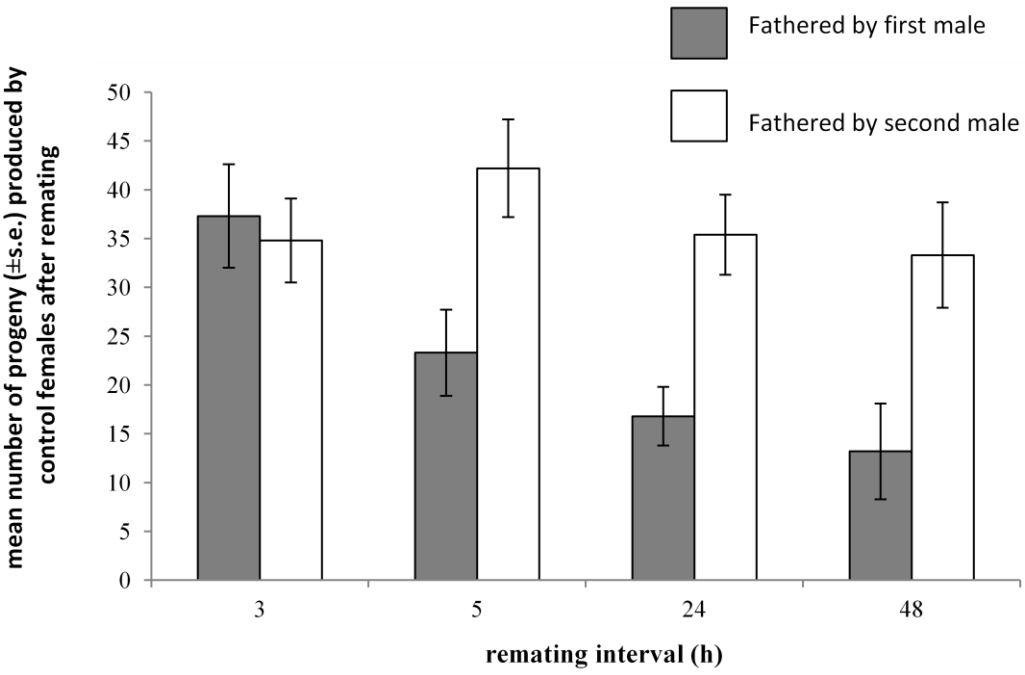

Fathered by first male
Fathered by second male
mean number of progeny (±s.e.) produced by control females after remating
50
45
40
35
30
25
20
15
10
5
0
3
5
24
48
remating interval (h)

Figure 3.2. Effect of SPR on the number of offspring produced before remating. Mean (± s.e.) number of offspring produced before rematings at 3, 5, 24 or 48h, by *SPR* null or control females. Filled bars: *SPR* null females, open bars: *SPR* control females.

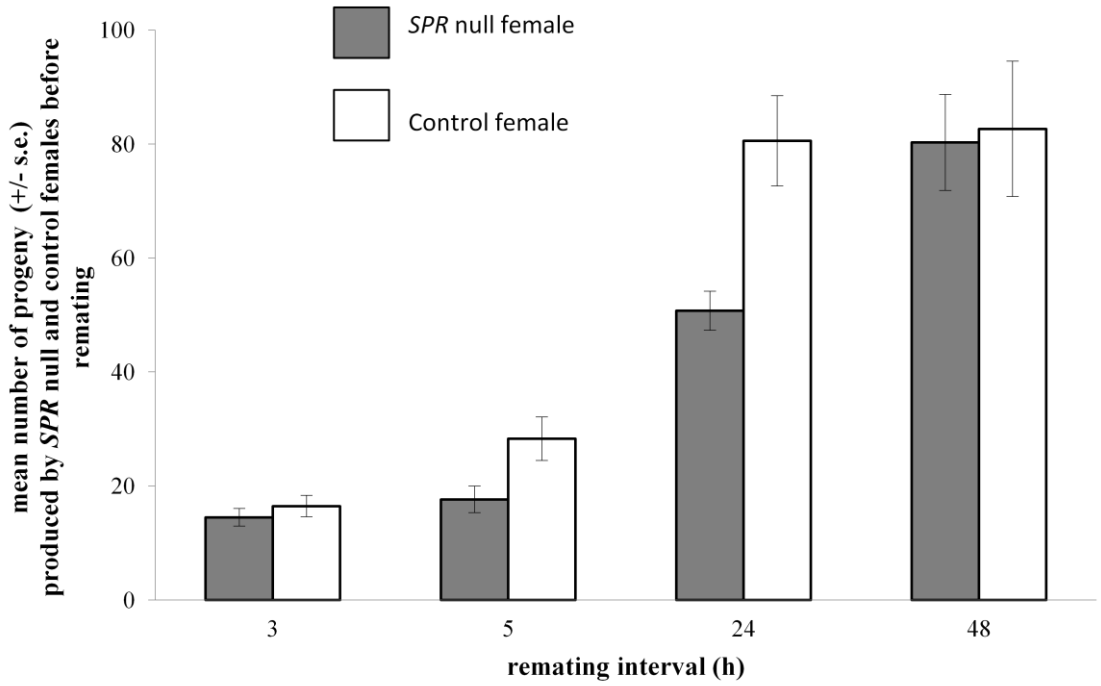
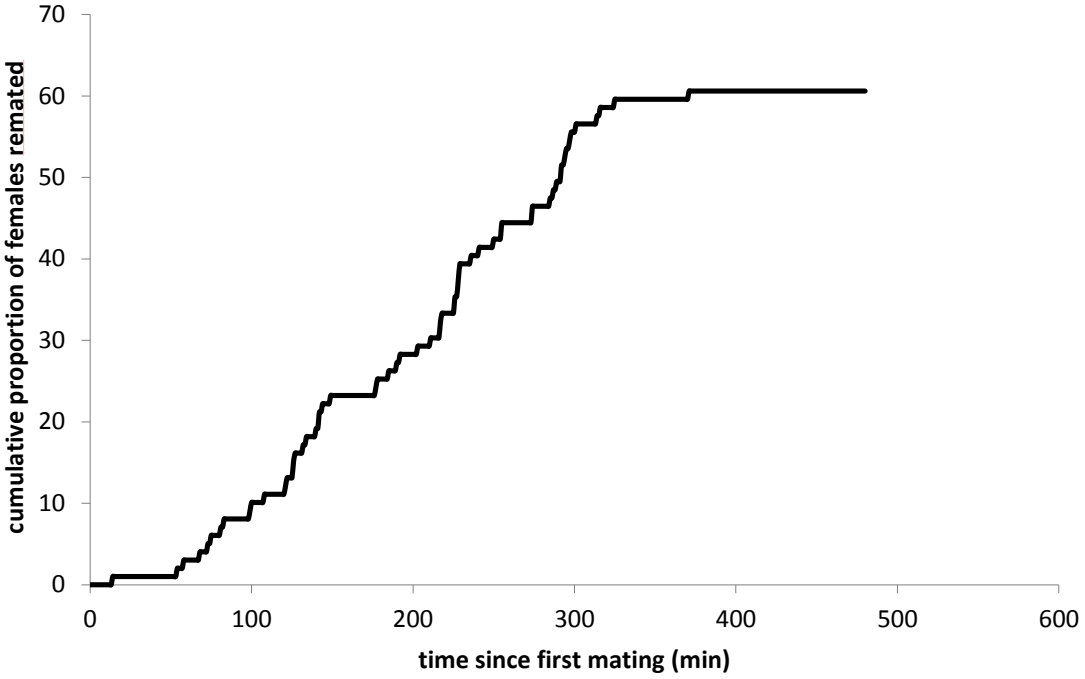


Figure 3.3. Frequency of early remating in wild type females. (a) Rematings following continuous exposure to males after first matings. The graph shows the cumulative proportion of wild type females remating over an 8h observation period (in minutes). 60/99 females remated within this time interval. (b) Rematings following intermittent exposure to males. Proportion of females remating in block 1 (dark grey) and block 2 (light grey). Females were given the opportunity to remate with males at 2, 4, 6, 8, 24 or 48h. Each time point is an independent test done on a different sample of the same female cohort for blocks 1 and 2, respectively.

(a)



(b)

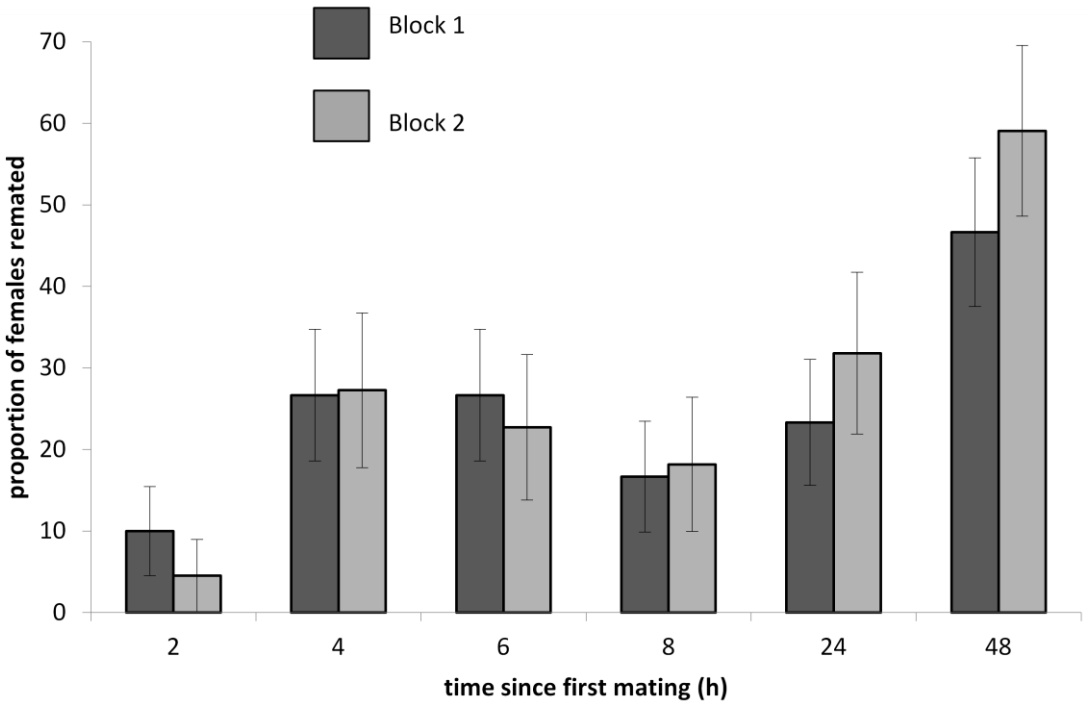

Block 1
Block 2
proportion of females remated
70
60
50
40
30
20
10
0
2
4
6
8
24
48
time since first mating (h)

Table 3.1. Effect of female genotype (SPR null *vs.* control) on second male paternity share (P2). The results of a generalized linear model to test for differences in P2 in males mated to either SPR null or control females. Female genotype and length of remating interval (time) were fixed factors. Progeny after first mating (progeny1) and second mating duration (duration2) were included as covariates. The significance of all factors was tested in an Analysis of Deviance through subtraction from the full model. The quasibinomial dispersion parameter was 20.87. Significant terms are highlighted in bold.

factor	*d.f.*	deviance	*F*	*p*
female genotype	1	36.22	1.662	0.1987
time	3	132.77	2.031	0.1106
progeny1	1	106.37	4.883	**0.0282**
duration2	1	355.62	16.321	**<0.0001**
female genotype:time	3	361.73	5.777	**0.0008**
time:duration2	3	171.03	2.731	**0.0449**
progeny1:duration2	1	121.70	5.831	**0.0166**

Table 3.2. Effect of female genotype (SPR null *vs.* control) on the absolute number progeny produced following remating. The results of generalized linear models to test for differences in the absolute number of (**a**) first and (**b**) second male offspring produced following rematings at 3, 5, 24 and 48h by SPR null or control females. Female genotype and length of remating interval (time) were fixed factors. Progeny produced before remating (progeny1) and second mating duration (duration2) were included as covariates. The significance of all factors was tested in an Analysis of Deviance through subtraction from the full model. The quasipoisson dispersion parameters were 17.34 and 11.62 for the first and second male progeny analyses respectively. Significant terms are highlighted in bold.

(**a**) Number of first male progeny produced after remating

factor	*d.f.*	deviance	*p*
female genotype	1	132.72	**0.0067**
time	3	284.79	**0.0012**
progeny1	1	92.05	**0.0238**
duration2	1	178.25	**0.0017**
female genotype:progeny1	1	144.40	**0.0039**

(**b**) Number of second male progeny produced after remating

factor	*d.f.*	deviance	*p*
female genotype	1	49.50	**0.0438**
time	3	75.74	**0.1015**
progeny1	1	14.44	**0.2764**
duration2	1	181.31	**<0.0001**
female genotype:time	3	194.32	**0.0008**
female genotype:progeny1	3	60.66	**0.0223**

Table 3.3. Remating frequency of SPR null and control females at 3, 5, 24 and 48h. Numbers in parentheses indicate sample sizes used for statistical analyses after excluding females that were sterile.

remating interval	*SPR* null			*SPR* control		
(h)	mated	unmated	% mated	mated	unmated	% mated
3	53 (41)	7	88.3%	33 (30)	27	55.0%
5	44 (38)	15	74.6%	26 (23)	34	43.3%
24	34 (27)	0	100%	15 (13)	19	44.1%
48	33 (27)	0	100%	20 (19)	17	54.1%
overall	164	22	88.2%	94	97	49.2%

Chapter 4. The effect of PEB II on early remating in female *D. melanogaster*

4.1 Summary

Across a range of taxa seminal fluid is required for efficient fertilization and to stimulate maximum female fecundity. Seminal fluid proteins can also improve male fertilization success in sperm competition. In some species, components of the seminal fluid can form a mating plug within the female reproductive tract that can prevent remating or the loss of sperm from the female reproductive tract. In *D. melanogaster* the mating plug is comprised of distinct anterior and posterior parts. Products of the ejaculatory bulb form the major constituents of the posterior plug. Bretman *et al.* (2010) show (in a study to which I contributed the findings described in this chapter) that the PEBII ejaculatory bulb protein plays a role in determining the frequency of early female remating. As part of this study, I investigated whether the structure of the mating plug formed within females mated to PEBII knockdown males was altered in comparison to the mating plugs from control matings. By visualising the mating plugs that were formed in the presence or absence of the PEBII protein, I demonstrated that mating plugs lacking PEBII showed significant differences in structure at 5-10min after the start of mating (ASM). Such structural changes in mating plugs may affect the function of sfps involved in early remating responses since it has been proposed that the mating plug serves a role as a scaffold which directs sperm and sfps to specific locations within the female reproductive tract. This study also highlights that distinct seminal fluid proteins reduce female remating at early *vs.* later times following initial matings, and that remating conditions (continual *vs.* periodic confinement with males) can greatly affect female remating rate.

4.2 Introduction

Males of many animals transfer ejaculates that contain, in addition to sperm, seminal fluid substances that play important roles in postcopulatory sexual selection (Simmons, 2001, Eberhard, 1996, Arnqvist & Rowe, 2005). Across a range of taxa seminal fluid is required for efficient fertilization, to simulate maximum female fecundity and to increase male fertilization success in sperm competition (Birkhead *et al.*, 2009). In some species, components of the seminal fluid can form a mating plug, i.e. a mass within the female reproductive tract that can prevent remating or the loss of sperm from the female reproductive tract. In butterflies, the mating plug is referred to as a 'sphraga', which functions to prevent remating and potentially serves as a signal to other males to indicate that a female has been mated (Orr, 2002). Likewise the spermatophore of male locusts, *Locusta migratoria*, acts as a temporary plug by sealing up the female's genital tract until oviposition during which it is ejected (Simmons & Siva-Jothy, 1998). In some Hymenopteran species the mating plug has also been shown to stimulate oviposition in mated females (Melo *et al.*, 2001). The mechanisms involved in mating plug formation have not been well studied, but are likely to involve an interaction between seminal fluid molecules and conditions or molecules in the female (Birkhead *et al.*, 2009).

Previous studies concluded that the mating plug in *Drosophila* does not serve to prevent remating, since females remain unreceptive even after the plug is degraded (Eberhard, 1996). However other work suggested that the function of the plug in *Drosophila* is to facilitate sperm storage and to reduce female remating. For example, in *Drosophila hibisci*, previously mated males produce smaller mating plugs that are less effective at preventing sperm loss from the female sperm storage organs than are the plugs of virgin males (Polak *et al.*, 1998). In a further study with previously mated males, Polak *et al.* (2001) showed how the mating plug reduces female remating by both inhibiting any subsequent male courtship behaviour and by reducing female receptivity. However, the *D. hibisci* studies do not control for the effect of other ejaculatory proteins which can affect female remating rate. Previously mated males would be expected to transfer lower amounts of all accessory gland proteins, not just those responsible for the formation of the mating plug.

The mating plug in *D. melanogaster* is divided into two distinct anterior and posterior regions, which differ in their timing of formation as well as their constituent proteins (**Figures 4.1, 4.2**). The posterior portion is formed from male ejaculatory bulb (PEB)

proteins (Bairati, 1968) and begins to form 3-5min after the start of mating (ASM), before sperm transfer takes place (Gilchrist & Partridge, 2000, Lung & Wolfner, 2001). The anterior region is comprised of accessory gland proteins and is observed 20min ASM (Lung & Wolfner, 2001). The major constituent of the posterior plug is the large, 38 kD PEB-me protein (Lung & Wolfner, 2001). PEB-me shares amino acid motifs with spider flagelliform silk and mussel byssal thread proteins, and these motifs are associated with the ability to coagulate (Lung & Wolfner, 2001). The observation that the posterior mating plug undergoes coagulation has led to the theory that its role could be to direct and facilitate sperm movement into the uterus (Bairati, 1968). A second idea is that the posterior plug can facilitate the correct placement within the female reproductive tract of the sfps forming the anterior portion of the plug (Lung & Wolfner, 2001). This idea is supported by the finding that the anterior plug contains Acp36DE, which facilitates the movement of sperm into the sperm storage organs (Lung & Wolfner, 2001, Neubaum & Wolfner, 1999a). The formation of the mating plug may also control the timing of movement of sfps (such as Acp26Aa and Acp62F) from the reproductive tract into the hemolymph (Lung & Wolfner, 1999, Park & Wolfner, 1995).

Another component of the ejaculatory bulb proteins is PEBII, a smaller (7.2 kD) protein encoded by a gene located immediately upstream of PEB-me on the second chromosome (http://flybase.org/reports/FBgn0011694.html). Sequence variation near the PEBII locus has been associated with varying levels of male induced female mortality (Fiumera *et al.*, 2006) suggesting that PEBII could mediate sexual conflict by reducing costly female remating (Chapman *et al.*, 1995). In the study by Bretman *et al.* (2010), we investigated the role of PEBII in mediating female post mating responses and in the formation of the mating plug. RNAi lines were used to specifically target PEBII, thus avoiding the pitfalls of manipulating plug formation by controlling mating duration or history. We showed that PEBII plays a role in early female remating, using two independent lines of PEBII RNAi knockdown and control males. As part of this study, I investigated whether matings with PEBII knockdown males would lead to any gross changes in the structure of the mating plug, in order to investigate how the structure of mating plugs and the dynamics of their formation might impact on female remating.

4.3 Materials and methods

4.3.1 Fly stocks

PEBII knockdown males were *w^{1118}/w^{1};P[w^{+mC},Act^{5C}-Gal4]25FO1/P[w^{+mC},UAS-PEBII-IR.13-7]* (see Chapter 2 for stock details). Separate control lines for the *Gal4* driver and the construct were generated with crosses with the *UAS-SP-stop* line to control for eye colour differences. Knockdown control males were *w^{1118}; P[w^{+mC},UAS-SP-STOP.L61]/P[UAS-PEBII-IR-13-7]*, and the Gal4 driver controls were *w^{1118}/w^{1};P[w^{+mC},Act^{5C}-Gal4]25FO1/P[w^{+mC},UAS-SP-STOP.L61]*. Wild type *Dahomey* females were generated according to standard methods.

4.3.2 Visualisation of mating plug in PEBII lacking and control males

Wild type *Dahomey* females were mated to knockdown males lacking PEBII or to control males at 5 days post-eclosion. Mated females were frozen in liquid Nitrogen either 5-10min or 20min ASM and stored at -80˚C until dissection. We chose these time points based on previous work as they were known to span the peak period of mating plug formation (Lung & Wolfner, 2001). Females were dissected in Phosphate Buffered Saline (PBS) and their lower oviducts, extending from the ovipositor to just anterior of the spermathecae, were removed. Dissected reproductive tracts were mounted on slides in PBS with a cover slip gently placed over the top. Slides were viewed using a Ziess Plan Apochromat 10x/0.45 Ph1 objective and a DAPI reflector on a Ziess Axioplan 2 microscope (using the Henry Wellcome Biomedical Imaging Facility at UEA). Mating plugs were easily visualized because of the auto fluorescent properties of the mating plug (**Figure 4.1**; Lung and Wolfner, 2001). Images were recorded using an AxioCamHR CCD camera at identical exposure settings and viewed using AxioVision LE software. Unmodified images were printed on a HP Laserjet P1505n printer. Five volunteer respondents were then asked to score the images (blind with respect to identity) on a scale of 0 to 3 based on levels of fluorescence in the anterior and posterior area of the lower oviduct (with 0 = no fluorescence to 3 = highly fluorescent; **Figure 4.2**). The sample sizes were between 8 and 26 for each of the 6 time point / treatment combinations.

4.3.3 Statistical analysis

Fluorescence scores were transformed into a percent and analyzed using a general linear mixed effects model (GLMM) in R 2.8.0 (R Development Core Team, 2008, nlme library). Treatment, responder, and their interaction were treated as fixed effect variables. Responder nested in Picture ID was treated as a random effect variable in order to correctly assess variation across responders in a repeat measures design. Helmert contrasts (Crawley, 2007) were used to compare the experimental line to the controls and the controls against each other. Repeatability, *r*, of responders' fluorescence scores is given by $r = S^2A/(S^2 + S^2A)$, where S^2A is the among-groups variance component and S^2 is the within-group variance component calculated from the mean squares of an ANOVA with Picture ID as the factor (Lessells & Boag, 1987) conducted with SPSS 16 (SPSS, Inc., Chicago IL). All presented results show means ± standard error, unless otherwise indicated.

4.4 Results

4.4.1 Visualisation of mating plugs

At 5-10min after the start of mating (ASM), there was a consistent pattern of females mated to males lacking PEBII having lower mating plug fluorescence scores than females mated to control males (**Table 4.1, Figure 4.3**). At 5-10min ASM, females mated to males lacking PEBII showed significantly lower posterior mating plug fluorescence scores compared to controls (**Table 4.1, Figure 4.3a**; knockdown: 2.04 ± 0.08, driver control: 2.42 ± 0.08, knockdown control: 2.56 ± 0.05; GLMM t_{58} = -2.761, p = 0.008). No difference was observed for the anterior mating plug (knockdown: 1.61 ± 0.09, driver control: 1.80 ± 0.09, knockdown control: 1.77 ± 0.07; GLMM t_{58} = -1.099, p = 0.276). At 20min ASM, there was no consistent pattern or significant difference in the degree of anterior or posterior fluorescence between the mating plugs of females across treatments (**Table 4.1, Figure 4.3b**; anterior, knockdown: 2.10 ± 0.12, driver control: 1.72 ± 0.12, knockdown control: 2.36 ± 0.13, GLMM t_{25} = 1.092, p = 0.285; posterior, knockdown: 2.23 ± 0.13, driver control: 2.64 ± 0.07, knockdown control 1.98 ± 0.08 GLMM t_{25} = -0.339, p = 0.737). In assessing the effectiveness of the responders to consistently score fluorescence, repeatability was high for both the posterior (r = 0.59) and anterior (r = 0.58) fluorescence scores. In addition, it is noted that amongst the 48 possible treatment by

responder interactions, only two cases of a significant effect were found (see attached manuscript, Appendix II). There is no evidence therefore that the results were affected by any bias from responder differences.

4.5 Discussion

The work of Bretman *et al.* (2010) showed that the transfer of PEBII to females during mating in the male ejaculate causes females to be significantly less likely to remate again in the 4h after mating. By visualising the mating plugs that were formed in the presence or absence of the PEBII protein, I contributed to this conclusion by demonstrating that PEBII was responsible for differences in the levels of fluorescence 5-10min ASM. This effect was only observed in the posterior portion of the plug and disappeared by 20min ASM. This suggests that the plugs of PEBII knockdown males contain less material or have different properties, for example, they may diffuse or consolidate at different rates (Lung & Wolfner, 2001). Physical deficiencies in the mating plug may hamper the movement or function of other sfps involved in the early remating response, since it has been proposed that the mating plug serves a role as a scaffold which directs sperm and sfps to specific locations within the female reproductive tract (Lung & Wolfner, 2001).

It is known that other seminal fluid proteins transferred to females during mating also affect female remating, but so far this has been observed at later remating times. For example, male sex peptide (SP) effectively reduces female remating for up to 7 days after mating (Peng *et al.*, 2005a, Liu & Kubli, 2003) and Dup99B increases female rejection behaviour for up to 24h when it is injected directly into females (Rexhepaj *et al.*, 2003). It should be noted however, that the effect of Dup99B has not yet been replicated using knock out mutant males (Rexhepaj *et al.*, 2003, I use a knockout mutant to test the role of Dup99B in Chapter 7). It is often overlooked that it can take several hours for females to fully develop a reduced receptivity phenotype. For example, 4h after mating with SP null males, females showed similar receptivity to controls (Liu & Kubli, 2003). However, by 12h, 60% of SP-null mated females had remated, which indicates that SP takes some time to cause reduced female receptivity, and therefore that other molecules must be responsible for the initial decrease in receptivity. PEBII appears to partially fill this gap by reducing the early female remating rate either directly or indirectly through the formation of the mating plug. Thus PEBII, and potentially Dup99B, alters early remating rate.

Most studies that have examined female receptivity or sperm competition have tended to remate females after 24 or 48h, and the first hours after mating have so far seldom been examined for these traits (e.g. Avila *et al.*, 2010). Likewise, most studies control remating interval by keeping males and females separate during the inter-mating interval and then allowing them a restricted time opportunity to remate. This leads to lower levels of remating at early time intervals (Liu & Kubli, 2003) as compared to conditions of continual confinement, where it has been reported that 50-75% of females will remate 4-6h after their first mating (Bretman *et al.*, 2010, van Vianen & Bijlsma, 1993). Within the laboratory, males and females are normally kept in continual confinement within large population cages so it is likely that cage females face similar continual courtship exposure as in this experiment; however density and sex ratio differences are likely to cause fluctuations in levels of male harassment (Friberg & Arnqvist, 2003). It is uncertain which experimental protocol (continual *vs.* periodic confinement with the opposite sex) best reflects the natural environment, although it is likely that females would face either scenario depending on population density and availability of oviposition sites (Gromko & Markow, 1993).

Males who do not form a mating plug properly are likely to lose out on gaining paternity because their mates will be more likely to remate. We would expect that plug formation should then be under strong selection for high expression of mating plug proteins, yet there is a high level of variation in expression of PEBII across naturally derived lines (Fiumera *et al.*, 2005). These differences in expression levels do not correlate with differences in sperm competition outcomes, female remating rate after 3 days, or fecundity (Fiumera *et al.*, 2005; Amanda Bretman, data not shown). We show here that variation in expression levels (through the use of RNAi) causes significant differences in early female remating rate possibly because of defects in the formation of the mating plug. Males who transfer reduced levels of PEBII are unable to reduce early female remating. Females who remate sooner will, over the course of their lifetime, have more matings and experience a higher cost of mating (Chapman *et al.*, 1995). Thus in nature, PEBII could be mediating sexual conflict by reducing costly female remating. Interestingly, one particular polymorphism within a 1500bp region upstream of the *PEBII* gene is associated with a three-fold increase in mating-induced female mortality (Fiumera *et al.*, 2006). It is unknown whether this polymorphism is correlated with differences in expression levels of PEBII. The findings of large variation in expression levels and alleles with large differences in male induced

female mortality suggest that PEBII may be involved in sexually antagonistic interactions which could constrain selection for an optimal male phenotype.

Figure 4.1. Example of a mating plug from a normal mating. *Left*, brightfield view of female lower reproductive tract (top of image is anterior, ovaries have been removed). SP=spermathecae; OV=oviduct; Vu=vulva. *Right*, UV fluorescence view of the same reproductive tract showing shape and location of the auto fluorescent mating plug. 5X magnification, scale bar, 100µm.

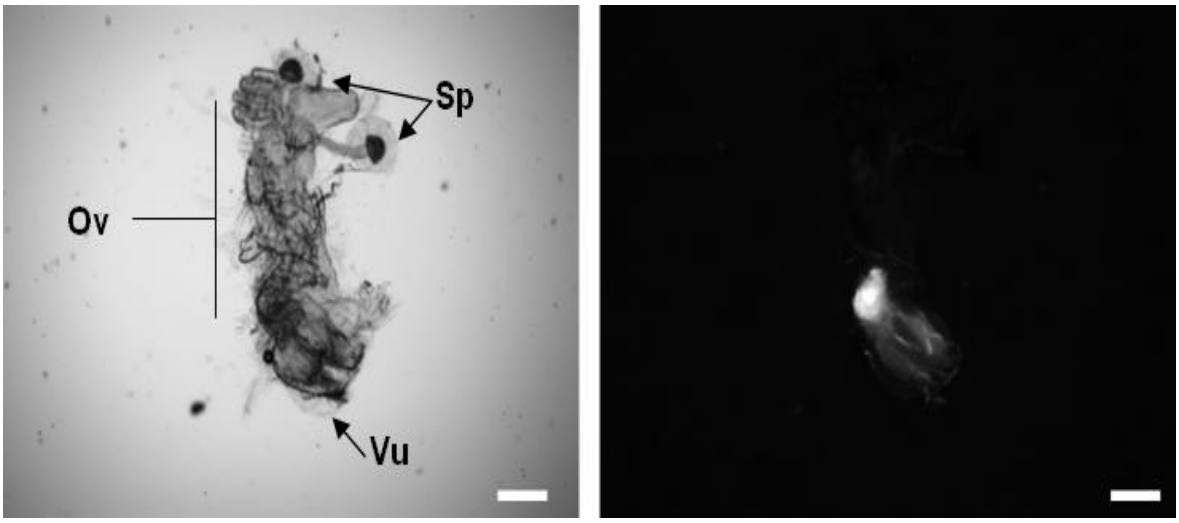


Figure 4.2. Mating plugs showing representative auto-fluorescence scores of 1-3. Female reproductive tracts were dissected as described in the methods. Images of the lower oviduct were divided into a posterior region consisting of the cuticle surrounding the vulva and ovipositor and anterior region extending to below the spermathecae. Images were scored on a scale of 0-3 based on levels of fluorescence in the anterior and posterior area of the lower oviduct (with 0 = no fluorescence to 3 = highly fluorescent). *Abbreviations*: OV, ovaries; SP, spermathecae; VV, vulva. Scale bar = 75µm.

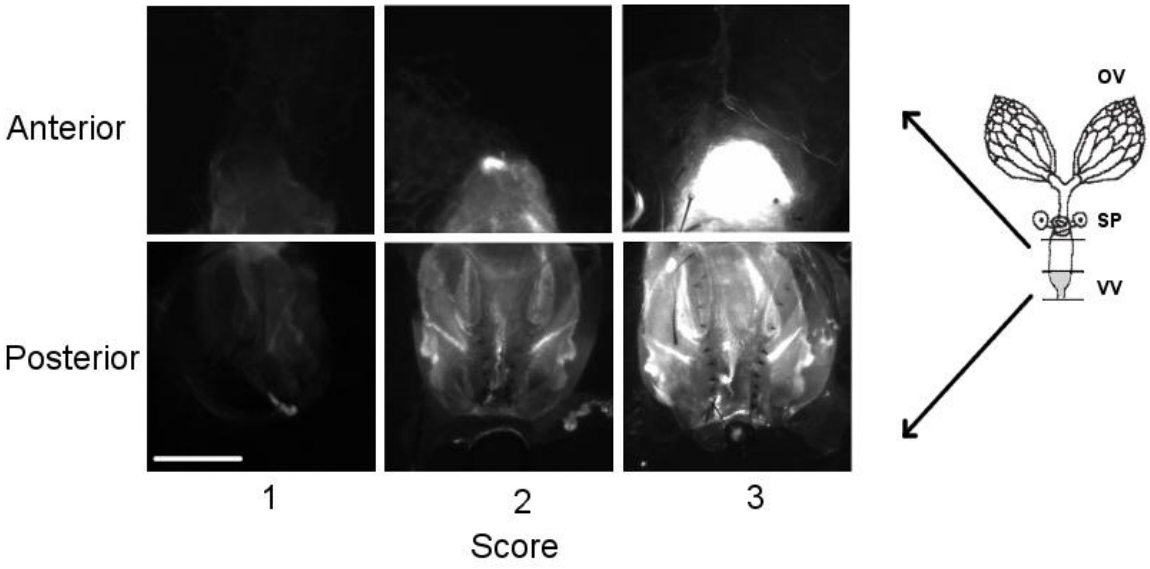
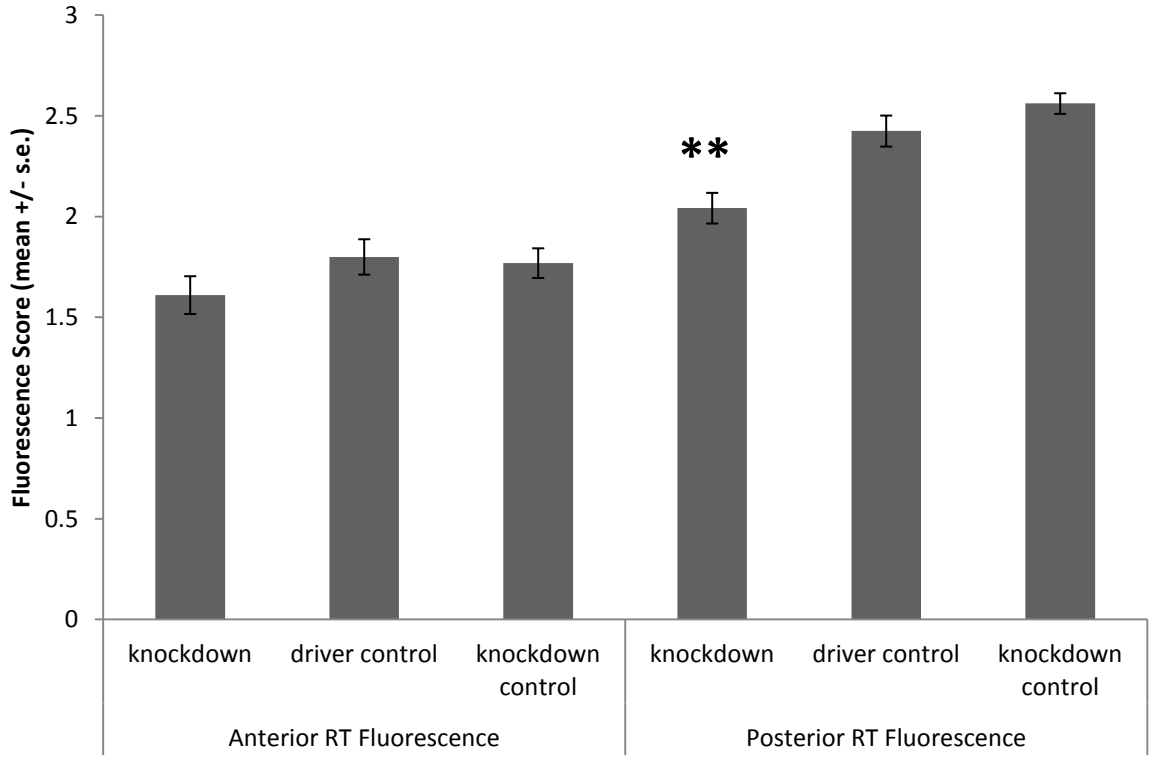


Figure 4.3. Mean fluorescence scores (0=no fluorescence, 3=highly fluorescent) of lower reproductive tract (RT) of wild type females 5-10min (a) and 20min (b) after the start of mating. Females were mated to PEBII knockdown (*w^{1118}/w^{1};P[w^{+mC},Act^{5C}-Gal4]25FO1/ P[w^{+mC},UAS-PEBII-IR.13-7]*), knockdown control (*w^{1};P[w^{+mC},UAS-PEBII-IR.13-7]/P[w^{+mC},UAS-SP-STOP.L61]*) or *Gal4* driver control males (*w^{1118}/w^{1};P[w^{+mC},Act^{5C}-Gal4]25FO1/P[w^{+mC},UAS-SP-STOP.L61])*. Fluorescence was scored separately for the anterior and posterior reproductive tract. Asterisks indicate a significant difference (p < 0.01).

(a) 5-10min ASM



(b) 20min ASM

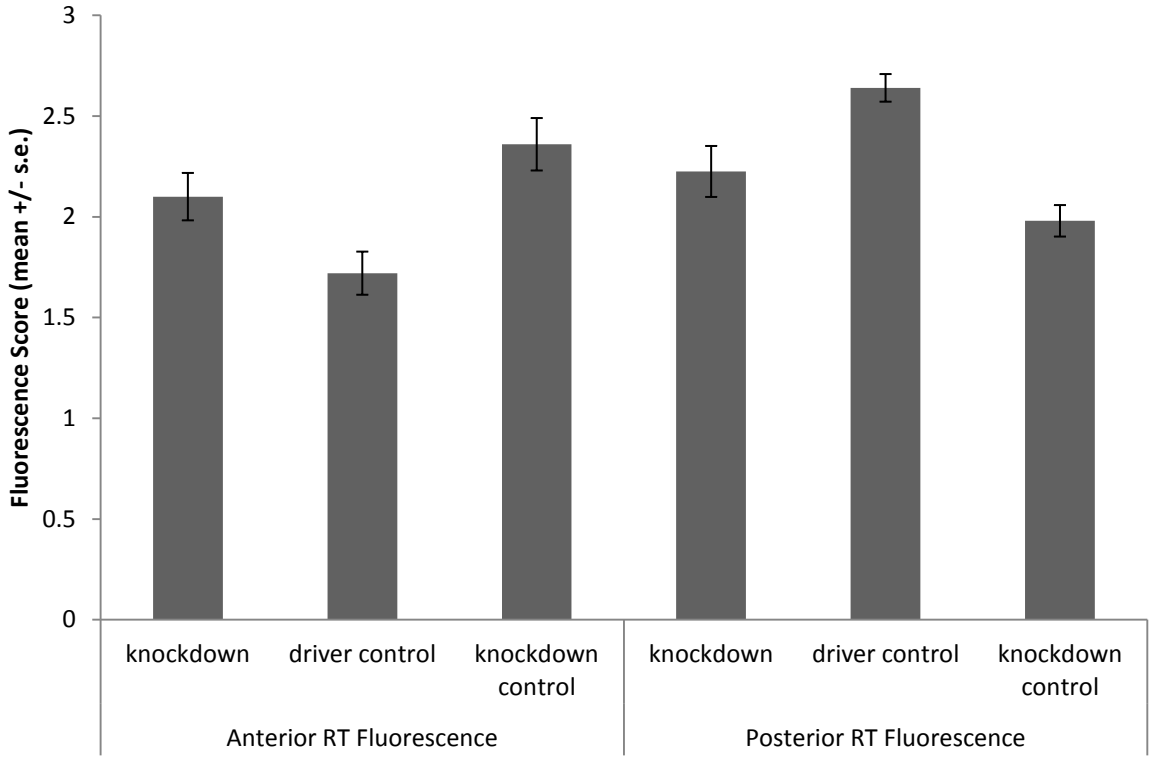

Fluorescence Score (mean +/- s.e.)
3
2.5
2
1.5
1
0.5
0
knockdown
driver control
knockdown control
knockdown
driver control
knockdown control
Anterior RT Fluorescence
Posterior RT Fluorescence

Table 4.1. Summary results of general linear mixed effects model to examine the effect of PEBII knockdown on anterior and posterior auto-fluorescence in mating plugs. Females were mated to PEBII knockdown (Kd) or control males (DC and KC, where DC = *Gal4* driver control and KC = *PEBII* inverted repeat control) either 5-10 or 20min after the start of mating (ASM). Treatment, responder, and their interaction were treated as fixed effect variables. Responder nested in Picture ID was treated as a random effect variable in order to correctly assess variation across responders in a repeat measures design. Helmert contrasts were used to examine specific comparisons between the knockdown line and controls and between the controls themselves. *P* values are shown for each Helmert contrast. Significant terms highlighted in bold.

5-10min ASM		*d.f.*	*t*	*p*
Anterior	*Kd vs. DC* and *KC*	58	-1.099	0.276
	DC vs. KC	58	-0.317	0.752
Posterior	*Kd vs. DC* and *KC*	58	-2.761	**0.008**
	DC vs. KC	58	0.941	0.351

20min ASM		*d.f.*	*t*	*p*
Anterior	*Kd vs. DC* and *KC*	25	1.092	0.285
	DC vs. KC	25	2.008	0.056
Posterior	*Kd vs. DC* and *KC*	25	-0.339	0.737
	DC vs. KC	25	-1.923	0.066

Chapter 5. The role of Acp26Aa and Spn2 in determining sperm competition outcomes

5.1 Summary

Within *Drosophila*, numerous studies report high levels of second male sperm precedence and have demonstrated the importance of seminal fluid proteins (sfps) in sexual selection and in determining male fitness. In previous work by other researchers, significant associations have been found between sperm competitive ability and allelic variation in or near to sfp genes. Two such genes with predicted effects on sperm competition are *Acp26Aa* and *Spn2*. Association tests provide some evidence for the potential functions of genes, but may not always be able to conclusively attribute a phenotype to a particular gene because of biological or statistical limitations. For example linkage disequilibrium with other key loci or a high false positive detection rate due to the large number of statistical tests needed could result in erroneous predictions. Direct functional assays involving targeted manipulation of genes may therefore be a better way to measure the causative role of a gene in sperm competition. In the experiments described in this chapter I took this direct approach to examine the roles of Acp26Aa and Spn2 in determining sperm competition outcomes. I used RNAi lines to significantly reduce the amount of either Acp26Aa or Spn2 produced by males and transferred to females during mating. Spn2 was found to have no effect on P2, which corresponded to the lack of a reported association between it and sperm displacement. However, in contrast to the expectation from the association studies, the results showed no effect of Acp26Aa on the proportion of offspring produced by a treatment male when he was the second male to mate (P2). I discuss the possible reasons for the discrepancy between the results obtained by association and functional studies in general.

5.2 Introduction

Postcopulatory sexual selection is a critical component of male reproductive fitness in multiply mating species and there is well documented selectable variation among males in sperm competitive ability (e.g. Clark *et al.*, 1995, Preston *et al.*, 2003, Konior *et al.*, 2005, Malo *et al.*, 2005). Sperm competitive ability is a multi-component trait that is influenced by a number of variables, including ejaculate volume (Harcourt *et al.*, 1981, Preston *et al.*, 2003, Dixson & Anderson, 2004) sperm motility (Gage *et al.*, 2004), sperm morphology (Oppliger *et al.*, 2003, Dixson & Anderson, 2004), and the actions of seminal fluid proteins (sfps) (reviewed in Poiani, 2006). Within *Drosophila*, numerous studies report high levels of second male sperm precedence and demonstrate the importance of sfps in sexual selection and in determining male fitness (Clark *et al.*, 1995, Wolfner, 2002, Chapman & Davies, 2004, Fiumera *et al.*, 2005, 2007). For example, males deficient in Acp36DE, a sfp with an important role in promoting sperm storage, lose out significantly in sperm competition (Chapman *et al.*, 2000).

Accessory gland protein 26Aa (Acp26Aa) and serine protease inhibitor 2 (Spn2) are two sfps identified in previous studies as candidate genes likely to have a role in determining sperm competition outcomes (Clark *et al.*, 1995, Fiumera *et al.*, 2005). Acp26Aa, also known as ovulin, is a well-studied sfp that increases post mating ovulation rate (Herndon & Wolfner, 1995). Spn2 is one of four sfps known to enter the ovaries and is detected on the surface of eggs laid by mated females (Ravi Ram *et al.*, 2005). Previous researchers have shown a role for protease inhibitors in preventing premature processing of sfps and possibly protecting sperm from proteolysis (Lung *et al.*, 2002, Ravi Ram & Wolfner, 2009, Ravi Ram *et al.*, 2006) along with involvement in the coagulation of the mating plug (Coleman *et al.*, 1995). However the actual effect (if any) of Spn2 on male and female fitness is unknown. Clark *et al.* (1995) and Fiumera *et al.* (2005) used permutation tests to search for associations between sperm competitive ability and polymorphic markers found within, or near to, male sfp genes. In the laboratory, sperm competition is often measured by mating a female to two males in succession and determining offspring paternity following the second mating. Clark *et al.* (1995) used a series of 152 lines, derived from natural populations and made homozygous for the second and / or third chromosomes while Fiumera *et al.* (2005) used 101 lines that were identical for the third, fourth, and sex chromosomes, with each line containing a unique second chromosome. The offspring produced from females mated to a chromosomal substitution male and a standard

competitor male were scored to determine first and second male paternity share (defined as P1 and P2, respectively (Boorman & Parker, 1976) for the given lines. Clark *et al.* (1995) found significant associations between particular sfp alleles at four different loci (Acp26Aa/Ab, Acp29B, Acp36DE and Acp53E) and the ability of males to resist displacement by subsequent sperm (P1). Fiumera *et al.* (2005) found a non-conservative amino acid change from serine to isoleucine at position 207 of Acp26Aa (Acp26s2201) that was associated with the proportion of offspring sired by the second male, P2. Additionally, a polymorphism within Spn2 (CG8137s1910) was associated with P1 (Fiumera *et al.*, 2005). However no association between Spn2 and P2 has been reported.

Association tests can provide suggestions for possible gene function, however these studies may not always conclusively attribute a phenotype to a particular gene, because the data are correlational and may also be subject to epistatic interactions with other loci. Likewise, any association found could be unique to the particular study population, a problem that may be exacerbated in the case of chromosomal substitution lines, because of the standardized, identical genetic background against which the effects of variation are measured. In contrast, direct functional assays involving targeted manipulation of genes are a relatively powerful way to measure the causative role of genes in sperm competition. Thus far, in the case of candidate genes for which there are functional data, there is low concordance of sperm competition phenotypes obtained between from gene association studies and direct tests (**Table 5.1**). Clark *et al.* (1995) provide evidence for an association between Acp26Aa and P1, but no effect has yet been reported in direct functional tests with a knockout mutant (Herndon & Wolfner, 1995). Likewise, Fiumera *et al.* (2005) report an association between Spn2 and P1, while no effect was observed in a direct functional test with a knockdown mutant (Amanda Bretman / Mara Lawniczak / Tracey Chapman, unpublished).

I used RNA interference (RNAi) to reduce the expression of Acp26Aa or Spn2, to examine their role in sperm competition. I used two lines carrying either an Acp26Aa or Spn2 sense-antisense transgene, and drove the expression of these constructs in their normal sites of expression, the male accessory glands. Sperm competition assays were conducted to determine whether direct functional tests for Acp26Aa and Spn2 would reveal a role for these proteins in determining P2 outcomes. Based on the findings of Fiumera *et al.* (2005), removal of Acp26Aa from the second male to mate is predicted to influence P2, while

removal of Spn2 should have no effect on P2, as no association between it and sperm displacement has been reported.

5.3 Materials and methods

5.3.1 Fly stocks

Females and competitor males homozygous for the recessive *spa* eye phenotype were collected (see Chapter 2 for stock details) and used to determine paternity (see below). Acp26Aa knockdown males were w^{1118};*P[Acp26Aa-GAL4.C];P[GD5250]*. Knockdown control males were w^{1118};*P[*w^{+mC}*,UAS-SP-STOP.L61];P[GD5250]*, and the Gal4 driver controls were w^{1118};*P[Acp26Aa-GAL4.C]/P[*w^{+mC}*,UAS-SP-STOP.L61]*. Spn2 knockdown males were w^{1118};*P[Acp26Aa-GAL4.C]/P[KK106352]VIE-260B*. Knockdown control males were w^{1118};*P[*w^{+mC}*,UAS-SP-STOP.L61]/P[KK106352]VIE-260B*, and the Gal4 driver controls were w^{1118};*P[Acp26Aa-GAL4.C]/P[*w^{+mC}*,UAS-SP-STOP.L61]*.

5.3.2 Sperm competition assay – measuring P2

Two independent assays were conducted to test the role of Acp26Aa and Spn2 in sperm competition. In order to test the effect of Acp26Aa and Spn2 on P2 outcomes, homozygous *spa* females were first mated to homozygous *spa* males. The next day, the females were remated to Acp26Aa or Spn2 treatment males exhibiting the wild type, red eye phenotype. Thus, offspring with the *spa* eye phenotype were fathered by the first male, while offspring with the red eye phenotype were fathered by a treatment male.

The full procedure was as follows: At 5 days post-eclosion virgin *spa* females were placed singly in yeasted SYA vials. Single *spa* males were then introduced into the vial and allowed 2h to mate. Time to mate (latency) and mating duration was noted for each vial. *Spa* males were discarded and the females allowed to lay eggs for 24h. The following day the females were randomly mated to males from one of 3 male treatment groups. For the Acp26Aa experiment samples sizes ranged from 22-29 doubly mated females. For the Spn2 experiment the samples sized ranged from 26-31. Males were 5 days post-eclosion at first mating and were kept in single sex vials containing 10 individuals with live yeast until the mating assay. Females were allowed 2h to mate with the treatment male or else were discarded. Latency and mating duration were again noted for each vial. Females were allowed to lay eggs for 2 days and were transferred to vials containing fresh yeasted food

after the first 24h. Total progeny produced over 2 days were counted and parentage was determined based on the eye colour of the offspring. Sterile females and females that had offspring of only one eye colour were omitted from analysis (Fiumera *et al.*, 2005, Clark & Begun, 1998).

5.3.3 Statistical analysis

Differences between second mating latency and second mating duration among treatment males were analysed by ANOVA in SPSS 16 (SPSS, Inc., Chicago IL). Second mating duration was transformed to improve normality by taking the natural logarithm. P2 was analysed using a general linear model for analysis of differences using R 2.10.0 (R Development Core Team, 2008, Crawley, 2007). Quasibinomial errors were used to increase goodness of fit and control for overdispersion of the model. For all models, male type was assigned as a fixed factor and first mating progeny, first mating duration, second mating latency, second mating duration and total progeny after remating were assigned as covariates. The significance of all factors was tested with an analysis of deviance through subtraction from the full model. In the case of Acp26Aa, first mating progeny and total progeny after remating remained as significant covariates. For Spn2, only second mating duration remained as a significant covariate. Total wild type offspring produced was analysed using ANOVA for Acp26Aa and the Kruskal-Wallis test for Spn2, conducted with SPSS 16. Correlations between factors were tested using the Spearman correlation coefficient, *rho*, in SPSS 16. Means ± standard errors (s.e.) are presented throughout.

5.4 Results

5.4.1 The effect of Acp26Aa on second male sperm precedence

There were no significant differences in the time it took treatment males to begin to mate (second mating latency (minutes), Acp26Aa knockdown: 73.5 ± 9.3, Gal4 driver control: 61.2 ± 8.0, Acp26Aa-IR control: 58.5 ± 8.9; ANOVA $F_{2,72} = 0.82$, $p = 0.446$). There were also no significant differences in the mating duration of treatment males (second mating duration (minutes), Acp26Aa knockdown: 18.1 ± 1.1, Gal4 driver control: 17.2 ± 0.6, Acp26Aa-IR control: 15.7 ± 0.9; ANOVA $F_{2,72} = 1.910$, $p = 0.156$). Thus the knockdown line did not show any obvious differences in mating behaviour compared to controls.

Analysis of P2 showed no significant differences between treatment males (P2, Acp26Aa knockdown: 0.686 ± 0.065, Gal4 driver control: 0.751 ± 0.054, Acp26Aa-IR control: 0.572 ± 0.070; GLM $F_{2,70}$ = 2.342, p = 0.104; **Figure 5.1a**, **Table 5.2**). The covariates, progeny from the first mating (before remating) and total number of progeny after remating, had significant impacts on P2 (1st progeny, $F_{1,70}$ = 10.71, p = 0.002; total progeny after remating, $F_{1,70}$ = 4.04, p = 0.048). Although they accounted for a significant amount of the variation in P2 in the GLM, the correlations between the covariates and P2 were not consistent in direction and were mostly non-significant when split by treatments and were thus not predictive of a particular P2 outcome (**Table 5.3)**. In accordance with the GLM, analysis of total counts of offspring fathered by the treatment male revealed a significant difference between the controls, but no difference between the knockdown line and the controls (total wild type offspring, Acp26Aa knockdown: 102.2 ± 14.2, Gal4 driver control: 134.0 ± 11.9, Acp26Aa-IR control: 73.6 ± 14.3; ANOVA, $F_{2,72}$ = 5.19, p = 0.008, Tukey B post hoc test p = 0.05; **Figure 5.2b**).

5.4.2 The effect of Spn2 on second male sperm precedence

There were no significant differences in the time it took treatment males to begin to mate (second mating latency (min), Spn2 knockdown: 69.5 ± 7.9, Gal4 driver control: 64.2 ± 8.7, Spn2-IR control: 71.4 ± 6.8; ANOVA $F_{2,85}$ = 0.234, p = 0.792). There was a significant difference in mating duration, with Gal4 driver control males mating on average 3min longer than the other treatments (second mating duration (min), Spn2 knockdown: 16.3 ± 0.6, Gal4 driver control: 19.5 ± 1.1, Spn2-IR control: 16.0 ± 0.6; ANOVA $F_{2,85}$ = 5.618, p = 0.005, Tukey B post hoc test p = 0.05). The Spn2 knockdown line did not show any obvious differences in mating behaviour compared to controls.

Analysis of P2 showed no significant differences between treatment males (P2, Spn2 knockdown: 0.612 ± 0.069, Gal4 driver control: 0.663 ± 0.059, Spn2-IR control: 0.546 ± 0.056; GLM $F_{2,84}$ = 1.311, p = 0.275 **Figure 5.2a**, **Table 5.4**). Second mating duration had a significant impact on the variation in P2 (2nd Mating Duration $F_{1,84}$ = 4.695, p = 0.033). The correlations between second male mating duration and treatment P2 were not always significant, but the positive slope suggests that increased second mating duration can lead to higher P2 (**Table 5.5**). In accordance with the GLM, analysis of total counts of offspring fathered by the treatment male revealed no significant difference between the treatments

(total wild type offspring, Spn2 knockdown: 88.0 ± 15.4, Gal4 driver control: 102.6 ± 14.0, Spn2 control: 71.6 ± 10.1; Kruskal Wallis, $\chi^2 = 1.259$, *d.f.* = 2, $p = 0.553$; **Figure 5.2b**).

5.5 Discussion

The experiments reported here used RNAi knockdown lines to significantly reduce the amount of either Acp26Aa or Spn2 produced by males and transferred to females during mating. The results show no effect of Acp26Aa or Spn2 on the proportion of offspring produced by a treatment male when he was the second male to mate (P2). Analysis of the total number of offspring produced by the second male further supported the conclusion that neither protein plays an important role in P2 outcomes. Although Gal4 control females produced significantly more offspring compared to the Acp26Aa-IR control, neither differed significantly from the Acp26Aa knockdown treatment. The number of offspring produced after the first and second mating had significant effects on the variation in P2 for the Acp26Aa experiment while only second mating duration had significant effects on P2 for the Spn2 experiment. The number of offspring produced and mating duration commonly appear as significant covariates in analyses of sperm competition (Fricke *et al.*, 2009, also see Chapter 3) and this highlights the roles that the number of previous male's sperm in storage and the amount of displacing ejaculate transferred have in determining P2. Fiumera *et al.* (2005) found an association between Acp26Aa and P2, but no association between Spn2 and P2. Therefore the findings of no effect on P2 for either protein are only partially consistent with the results of the association tests reported in the studies by Clark *et al.* (1995) and Fiumera *et al.* (2005) and further adds to the general discordance between association and functional studies reported in **Table 5.1**. In the following sections I discuss the statistical and biological explanations for why this discordance may occur.

In association studies, the large number of correlational tests that need to be performed for each marker across multiple lines and multiple phenotypes means that the probability of finding false positives may often be quite high. An important and difficult task within association studies is determining the threshold levels of significance for assigning associations. Most candidate gene studies prefer to use a liberal false discovery rate (FDR) threshold, which means that many false positive candidate genes are likely to be detected. Simultaneously, the chance of missing true associations may be increased (Hirschhorn & Daly, 2005). Among the 62 suggestive associations reported by Fiumera *et al.* (2005),

calculation of the FDR reveals that approximately 31 of them may be false positives. A 50% FDR may seem unacceptably high, but if the functional tests are not difficult to conduct then follow up experiments could reveal 31 associations reflecting real effects, which would represent an important step forward. Because of these strengths, association tests can be a powerful tool for surveying a large number of genes across large populations in order to observe the role of natural variation in determining the function of essential genes (Hirschhorn & Daly, 2005).

The second broad category of explanation for discordance between association and functional tests regards the relationships between sfp microsatellites and hidden sources of variability. In association studies, as in any correlative study, the observed association between measures of sperm competition and variation near or within sfp alleles is not a demonstration of causality. The actual causative loci may be physically linked or simply in linkage disequilibrium with the typed marker (Clark *et al.*, 1995, Fiumera *et al.*, 2005). It is also likely that the lines used would have contained variation for other traits involved in sperm competiveness such as ejaculate volume, sperm motility or morphology, which could have influenced the associations observed. Variation in genes coding for these other phenotypes would not have been detected since the genetic markers used only spanned sfp loci and not these other genes. An interesting possibility is that pleiotropy or physical linkage between particular sfp and ejaculate volume genes (perhaps transcription factors) could be an important factor in determining sperm competition outcomes.

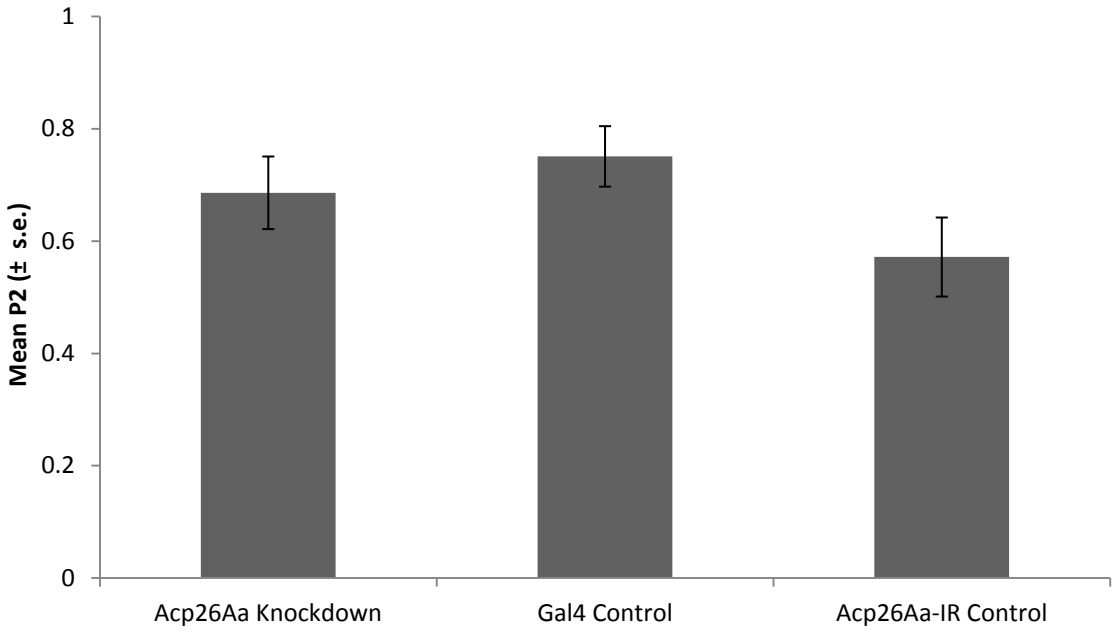
Fiumera *et al.* (2005) report 8 highly significant correlations between transcript abundance of Acp26Aa and 9 other sfps tested (mean ± s.e. r^2 =0.530±0.19) across the 101 lines used in their study. This suggests that lines that did poorly in sperm competition tests did so partly because of a reduction in overall sfp activity. Under this scenario, associations may arise because of the effects of lines that show reduced function of multiple sfps, which together have a non-additive effect on determining sperm competition outcomes (Hughes, 1997). With the present study, differences in P2 caused by the lack of just Acp26Aa may not have been detected because of the presence of other sfps that were able to compensate for the lack of Acp26Aa. These other sfps may not have been able to compensate for Acp26Aa in association studies, because males with lowered amounts of Acp26Aa would have also shown an overall reduction in sfp transcript levels according to the above correlation. Therefore, association studies may in fact be better at detecting the roles of

sfps with small effects, which would otherwise be missed in targeted knockout experiments such as this one.

Clark *et al.* (1995) used lines which were homozygous for either the second or third chromosome while Fiumera *et al.* (2005) used lines which were homozygous and identical for the third, fourth, and sex chromosomes, with each line containing a unique homozygous second chromosome. Uniformity in backgrounds is useful for increasing statistical power to find associations between polymorphisms on a focal chromosome with sperm competition. However, this population structure could also lead to spurious associations which are specific to the background used. The variable chromosomes represented by each substitution line could produce different pleiotropic and epistatic interactions within a standard background. For example Rice and Chippindale (2002) reported substantial epistatic interactions for male fitness between Y chromosomes and autosomes, while Jiang *et al.* (2010) reported significant Y-by-autosomal background effects on genome-wide gene expression in males. Specific male-by-female genotype interactions have been observed in *D. melanogaster* with both male and female fitness often dependent on the genotype of their mates (Clark & Begun, 1998, Clark *et al.*, 1999, Long *et al.*, 2006, McGraw *et al.*, 2009, Bjork *et al.*, 2007). In this regard, it would be interesting to make a comparison to the results obtained in genome-wide association studies (GWAS) performed in human populations, to determine markers for disease susceptibility. Two genetic markers for Crohn's disease identified in a previous study were not associated with the disease in a Japanese population (Georges, 2011). This may reflect gene-by-environment or gene-by-gene interactions, although it may also indicate that none of the corresponding genes are causative. The most reliable evidence of a true genetic association is replication of the association across multiple populations (Manolio, 2010, Georges, 2011). GWAS would be a very useful tool for dissecting the role of natural variation in male and female genotypes on reproductive phenotypes in *D. melanogaster*. The ability to produce large sample sizes and carry out simple assays in *D. melanogaster*, along with the increasing ease of next generation sequencing would make these experiments practical and extremely informative.

Figure 5.1. (**a**) Mean (± s.e.) male sperm offence ability (P2) for Acp26Aa knockdown males compared to driver control males and knockdown control males. No significant differences were found (see **Table 5.2**). (**b**) Mean (± s.e.) number of wild type offspring produced by females mated to either Acp26Aa knockdown males, driver control males or knockdown control males. Small letters denote homogenous subgroups determined by Tukey B post hoc comparisons ($p < 0.05$).

(a)



(b)

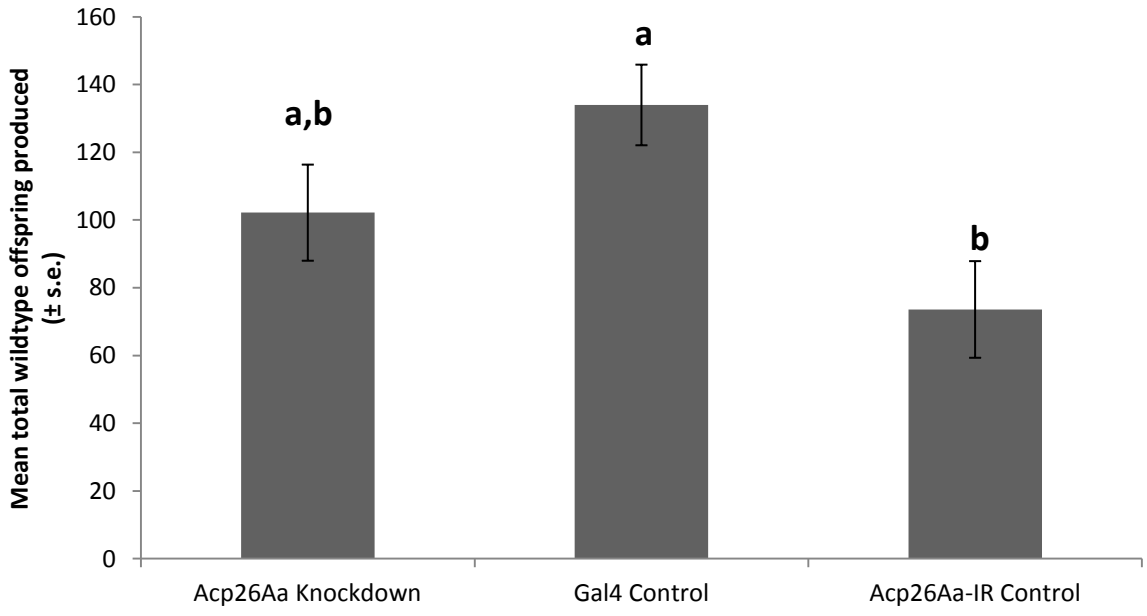
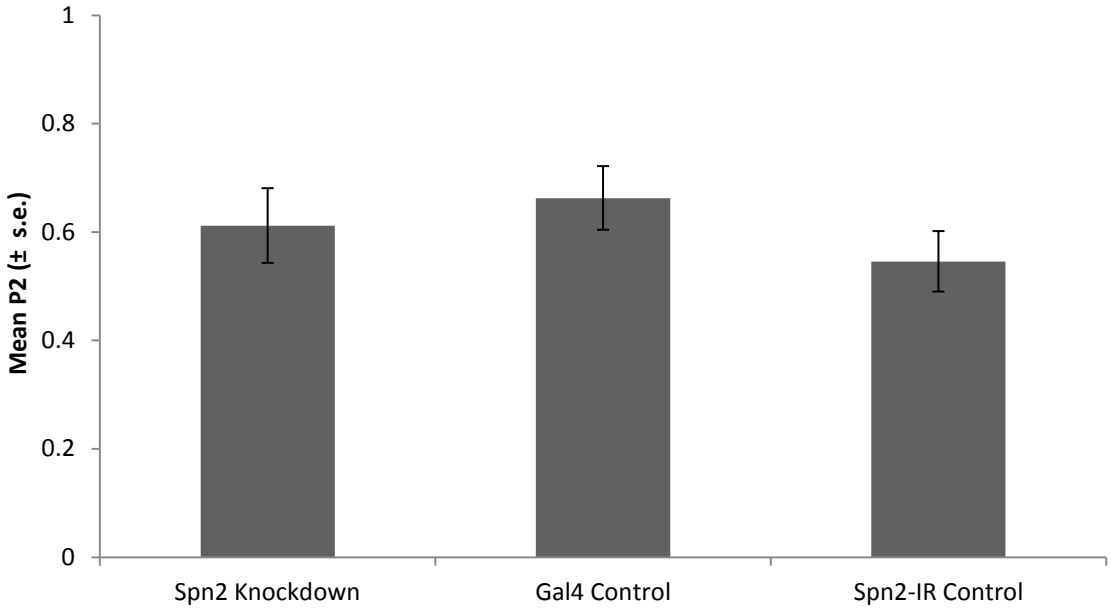


Figure 5.2. (**a**) Mean (± s.e.) male sperm offence ability (P2) for Spn2 knockdown males compared to driver control males and knockdown control males. No significant differences were found (**Table 5.4**). (**b**) Mean (± s.e.) number of wild type offspring produced by females mated to either Spn2 knockdown males, driver control males or knockdown control males. No significant differences were found.

(**a**)



(**b**)

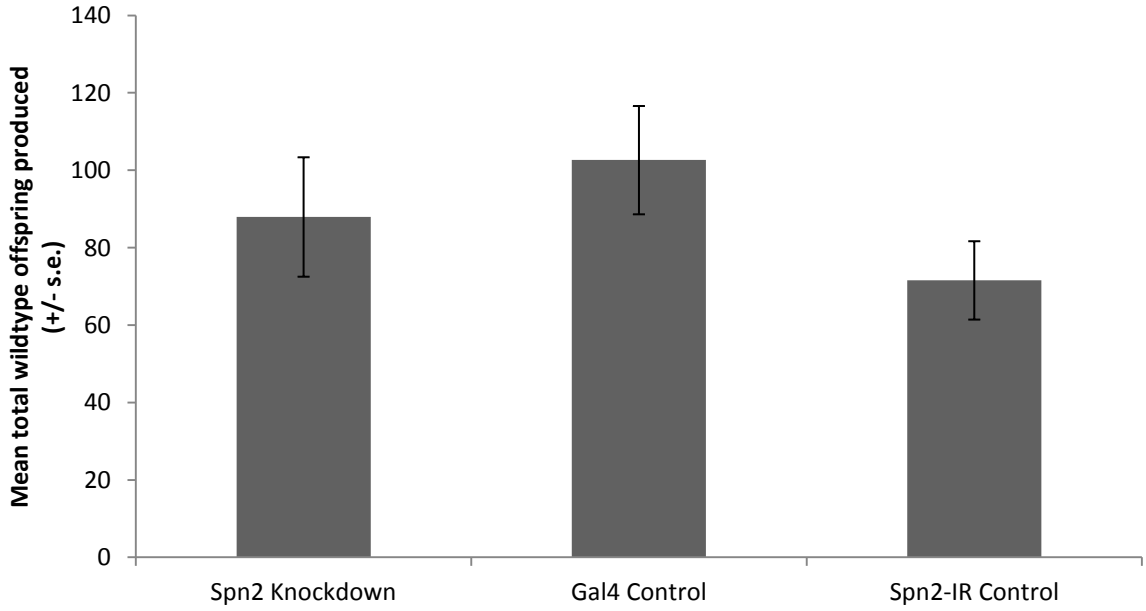


Table 5.1. Examples of concordance between association tests and functional tests. 'Yes' / 'No' indicate whether an effect was observed in P1 and / or P2.

Locus	Reported association with -		Effect in functional test with mutant		Concordance between association and functional tests?
	P1	P2	P1	P2	
Acp26Aa	Yes [1]	Yes [2]	No [4]	?	at least 50%
Acp33A	Yes [2]	Yes [2]	No [5]	No [5]	100% Different
Acp36DE	Yes [1]	No [1,2]	Yes [6]	Yes [6]	50%
Acp62F	No [1,3]	Yes [3]	Yes [7]	No [7]	100% Different
Sex Peptide	No [1,3]	No [1,3]	Yes [8]	No [8]	100% Same
Spn2	Yes [2]	No [1,2]	No [5]	?	at least 50%

(1) Clark *et al*., 1995, (2,3) Fiumera *et al*., 2005, 2007

(4) Herndon and Wolfner, 1995, (5) Bretman, Lawniczak, Chapman, unpublished

(6) Chapman *et al.*, 2000, (7) Mueller *et al.*, 2008, (8) Fricke *et al*., 2009

Table 5.2. The results of a generalized linear model to test for differences in P2 between Acp26Aa knockdown males and the Gal4 driver and Acp26Aa knockdown controls. Male type was assigned as a fixed factor and 1st mating progeny and total progeny after remating were assigned as covariates. The significance of all factors was tested in an Analysis of Deviance through subtraction from the full model. Quasibinomial errors were used with a dispersion parameter factor of 61.835 to increase goodness of fit and control for overdispersion of the model.

Factor	*d.f.*	deviance	*F*	*p*
male type	2	289.6	2.34	0.104
1st mating progeny	1	662.4	10.71	**0.002**
total progeny after remating	1	250.1	4.04	**0.048**

Table 5.3. Correlations between the mean of P2 and the covariates: 1st mating progeny and total progeny after remating for Acp26Aa knockdown and control treatments. Numbers indicate the Spearman's *rho* value with significance shown in brackets. KD=Knockdown.

Treatment P2	1st male progeny	total progeny after remating
Acp 26Aa KD	0.176 (0.411)	0.310 (0.141)
Gal4 control	**-0.416 (0.025)**	-0.078 (0.687)
Acp26-IR control	0.319 (0.148)	**0.491 (0.020)**

Table 5.4. The results of a generalized linear model to test for differences in P2 between Spn2 knockdown males and the Gal4 driver and Spn2 knockdown controls. Male genotype was assigned as a fixed factor and 2nd mating duration was assigned as a covariate. The significance of all factors was tested in an Analysis of Deviance through subtraction from the full model. Quasibinomial errors were used with a Dispersion parameter factor of 66.567 to increase goodness of fit and control for overdispersion of the model.

factor	*d.f.*	deviance	*F*	*p*
male genotype	2	174.5	1.31	0.275
2nd mating duration	1	312.6	4.70	**0.033**

Table 5.5. Correlations between the mean of P2 and 2nd mating duration for Spn2 knockdown and control treatments. Numbers indicate the Spearman's rho value with significances shown in brackets. KD=Knockdown.

Treatment P2	2^{nd} mating duration
Spn2 KD	0.090 (0.661)
Gal4 Control	**0.353** **(0.051)**
Spn2-IR Control	0.078 (0.678)

Chapter 6. Interactions between sex peptide and sex peptide receptor: feeding, fecundity and remating rate

6.1 Summary

Study of the mechanisms involved in male-female interactions at the molecular level will broaden our understanding of the costs and constraints imposed on the sexes by sexual selection and further elucidate the dynamics of coevolution between the sexes. The experiments described in this chapter tests for phenotypes under the control of a key set of male and female reproductive proteins, sex peptide (SP) and it's receptor in females, sex peptide receptor (SPR). SP triggers several post mating responses in mated females, such as an increase in egg laying, decrease in receptivity to further matings and stimulation of post mating feeding. Interestingly, females that are mated to SP-lacking males still show a decrease in receptivity and a slight increase in egg laying compared to virgin levels, for about 1 day after mating. This suggests the involvement of other seminal fluid proteins with partially degenerate roles to SP. The target of SP in females, sex peptide receptor (SPR) is known to modulate the egg laying and receptivity phenotype of SP. However, little is known about other post mating responses controlled by this receptor. To address this question I tested whether SPR is also required for the elevation of feeding observed in mated females. I compared feeding, receptivity and egg laying responses in SPR-lacking and control females that did and did not receive SP. The results show that SPR is required to elevate female feeding rate and that this elevation in feeding continues for at least 5 days after mating. SP and SPR were both required to increase feeding and egg laying and to reduce receptivity. I was unable to detect strong evidence for degeneracy within the network of male-female molecular interactions for feeding rate, egg laying and receptivity in mated females.

6.2 Introduction

Reproduction has been assumed to be a cooperative effort between males and females to produce offspring for the next generation. In multiply mating species, the sexes are expected to vary in reproductive effort, with males seeking to maximise per mating fitness returns and females seeking to maximise lifetime fitness (Bateman, 1948, Parker, 1970). The difference in reproductive optima between the sexes can lead to sexual conflict and adaptations that benefit one sex but that may cause costs in the other (Chapman *et al.*, 2003b, Arnqvist & Rowe, 2005). In insects such as *Drosophila melanogaster*, an important element in the interaction of the sexes is that females receive a signal to change from a virgin state (low rate of egg laying, high receptivity) to a mated state (increased egg laying, reduced receptivity) (Wolfner, 2002). Since *Drosophila* females can remate, males attempt to maximise the number of eggs fertilized by their sperm before the female remates. They do this by maximising the female's rate of egg laying and delaying rematings (Wigby & Chapman, 2004). The induced egg laying rate is apparently higher than the female optimum, leading to costs in females (Partridge *et al.*, 1987, Sgro & Partridge, 1999). This is an example of the conflict that exists between males and females in *D. melanogaster*. Further study of the mechanisms involved in male-female interactions at the molecular level will broaden our understanding of the costs and constraints imposed on the sexes by sexual selection and further elucidate the dynamics of coevolution between the sexes. The experiments described in this chapter tests for phenotypes under the control of a key set of male and female reproductive proteins, SP and it's receptor in females, SPR.

Most of the proteins transferred by *Drosophila* males in their ejaculate fall within conserved classes (e.g. proteases, lipases, lectins, protease inhibitors, odorant binding proteins, etc), and some post mating traits appear to be under the control of multiple sfps (Chapman & Davies, 2004, Ravi Ram *et al.*, 2005, Findlay *et al.*, 2008). SP on the other hand is capable of triggering several post mating responses in mated females, such as an increase in egg laying and decrease in receptivity to further matings (Chen *et al.*, 1988, Chapman *et al.*, 2003c, Liu & Kubli, 2003). Receipt of SP also causes the release of juvenile hormone (Moshitzky *et al.*, 1996), stimulates the release of antimicrobial peptides (Peng *et al.*, 2005b, Domanitskaya *et al.*, 2007); stimulates post mating feeding (Carvalho *et al.*, 2006, Barnes *et al.*, 2008), and reduces female longevity (Wigby & Chapman, 2005). Virgin females that are injected with SP increase egg laying and decrease receptivity as if

mated, though the effect only lasts for 1-2 days (Chen *et al.*, 1988), opposed to the 7-10 days observed in normal matings (Chapman *et al.*, 2003c, Liu & Kubli, 2003). This difference in post mating effect duration is because SP is normally bound to sperm and released from it over time by proteolytic enzymes, triggering the long term receptivity and egg laying effects following normal matings (Peng *et al.*, 2005a).

Interestingly, females that are mated to SP lacking males still show a decrease in receptivity and a slight increase in egg laying compared to virgin levels, although only for about 1 day after mating (known as the short term response) (Chapman *et al.*, 2003c, Liu & Kubli, 2003). This suggests the involvement of other seminal fluid proteins (sfps) with partially degenerate roles in this short term response. Candidate proteins causing this effect are Acp26Aa, which increases ovulation for 1 day after mating (Herndon & Wolfner, 1995), or possibly Dup99B (Saudan *et al.*, 2002, Rexhepaj *et al.*, 2003). When injected into virgin females, SP and Dup99B elicit the same remating and egg laying responses, but this effect only lasts for 24h (Saudan *et al.*, 2002), as noted above. Thus SP and Dup99B share some degeneracy in post mating effects, although it is unclear what the role of Dup99B is in normal matings. SP is believed to have arisen relatively recently because SP-like genes can only be detected within the Sophophora and Drosophila subgenuses (Kim *et al.*, 2010). Dup99B has high sequence similarity to SP and is only found within the melanogaster group suggesting that it has a more recent origin and may have arisen from a duplication of the SP gene (Saudan *et al.*, 2002, Kim *et al.*, 2010).

Although SP has been the focus of much research for nearly 25 years, its target in females, SPR, was only recently discovered (Yapici *et al.*, 2008). Females that lack SPR do not show an elevation in egg laying nor a decrease in receptivity 48h after mating with wild type males (Yapici *et al.*, 2008, Hasemeyer *et al.*, 2009). SPR is expressed in the female's central nervous system, predominantly in a subset of neurons that also express the *fruitless* gene and which are known to modulate sex-specific reproductive behaviour (Kvitsiani & Dickson, 2006, Yapici *et al.*, 2008). Within the reproductive tract, expression is observed in the lower oviduct and spermathecae, though not in the seminal receptacle which is the primary sperm storage organ (Yapici *et al.*, 2008). In cell culture assays, SP strongly binds to SPR, but the receptor also shows affinity for the related peptide, Dup99B. This suggests that SPR is the target of more than one biologically relevant seminal fluid protein and further supports the possibility that Dup99B may be responsible for the short term response

observed in wild type females mated to SP lacking males (I test for this in Chapter 7). Unlike its known ligands SP and Dup99B, SPR is highly conserved across several insect genomes (Yapici *et al.*, 2008). The ancestral ligands for SPR belong to another well conserved family of proteins and are known as myoinhibitory peptides (MIPs) (Kim *et al.*, 2010). The role of MIPs is unclear as they are not transferred by males and do not trigger post mating changes *in vivo* (Kim *et al.*, 2010). The lack of a clear, ancestral role of SPR in triggering post mating changes in females suggests that coevolution between the sexes has lead to the co-opting of the MIP signalling pathway for the benefit of males.

Apart from initial reports describing the egg laying and receptivity phenotype of SPR, little is known about other post mating responses controlled by this receptor. Since SP causes many post mating responses in females it is reasonable to assume that many (possibly all) are mediated by SPR. Previous work showed that receipt of SP in females increased feeding rate (Carvalho *et al.*, 2006), but that this was dependent on the female having a functioning ovary (Barnes *et al.*, 2008). Thus, SP indirectly increases feeding rate through its direct effect on rate of egg production, possibly mediated through an elevation in juvenile hormone triggered by SP in the corpora allata (Moshitzky *et al.*, 1996). SPR lacking females have normal, functioning ovaries yet do not show the elevation in egg production produced by the receipt of SP. Based on this reasoning, feeding should also be indirectly dependent on SPR, because SPR is required to increase egg laying rate (Yapici *et al.*, 2008). Alternatively, SP may bind to other receptors within the female that cause an increase in feeding rate. To address these questions I tested whether SPR is required for the elevation of feeding observed in mated females.

I made comparisons between the phenotypes observed in SPR-lacking and control females that did and did not receive SP. Differences in the phenotypes observed would be evidence for some level of degeneracy (either partial or full) within the network of male-female molecular interactions and could show whether SP binds to other targets within the female or whether SPR is the target for other seminal fluid proteins that affect female post mating responses. To test this interplay between the sexes, I measured SPR null and control females mated to either SP null or control males or left virgin for differences in levels of feeding and also for differences in egg laying and receptivity to remating.

6.3 Material and methods

6.3.1 Fly stocks

Sex peptide receptor-lacking (SPR null) females were homozygotes of *wDah,Df(1)Exel6234* (see Chapter 2 for stock details). SPR control females were *wDah,Df(1)Exel6234/SPR+*. Sex peptide null males were w^{1118};;*0325/Δ130*. Sex peptide controls were w^{1118};;*0416/Δ130*. Wild type *Dahomey* males were used as competitors in the remating assay. Throughout the text, SPR+ or SPR- indicates the presence or absence of sex peptide receptor in the female. SP+ or SP- indicates the presence or absence of sex peptide in the male with whom the female mated. Virgin means that the female was not mated.

6.3.2 Mating Assay

Four day old SPR null and control females were randomly assigned to 3 treatments, mated to SP+ or SP- males or left virgin (total of 6 treatments) and then placed in individual vials. The following morning single SP null or control males were aspirated into vials containing females assigned for the mating treatments. The time of entry, initiation, and completion of mating were recorded on the vial. Virgin treatments were treated as per mated females, except that they were not given a male. The first 60 females from each group to complete mating were aspirated into new vials in groups of 3 (virgin females were intermittently grouped in the same manner approximately 30-60min after the experiment began) and placed on the feeding assay viewing rack (6 treatments total; 10 vials and 30 females each). Males were removed and the remaining vials and the females were kept for remating assays (see below).

A replicate experiment was done 2 months later to include tests of early egg laying responses. Flies were reared and mated using identical procedures to those described above, except that 120 females from each group were aspirated into new vials in groups of 3 (6 treatments total; 20 vials and 60 females each). Females were transferred into new vials after 3, 6, 12 and 24h and only egg counts were performed.

6.3.3 Effect of SPR on the feeding response to SP

How best to measure feeding behaviour has been subject to debate. Carvalho *et al.* (2006) measured feeding rate by labelling food with a radioactive isotope and measuring

subsequent uptake in females. However the accuracy of this approach has been questioned (Wong *et al.*, 2008). Direct observation of proboscis extension onto the food was used here because it does not require exposure to radioactivity and is tightly correlated with food consumption (Wong *et al.*, 2009). Direct observation also allowed the same flies to be examined over multiple days and for multiple purposes.

The feeding assay began 2-4h after initial matings occurred. Vials were scanned continuously for female feeding behavior (proboscis extension on food) and scored until all vials had been observed 20 times (Barnes *et al.*, 2008). A fly was determined to be feeding if its proboscis was extended and touching the food. In cases where the view of the proboscis was blocked, characteristic head bobbing while remaining motionless was scored as feeding. The feeding assay was repeated at the same time for 5 consecutive days. Females were transferred to a newly yeasted vial after each assay.

6.3.4 Effect of SPR on the egg laying response to SP

The vials from the feeding assay were used to collect egg laying data 6h after mating and 1-5 days after mating. For the replicate experiment, eggs were counted 3, 6, 12 and 24h after mating. Total number of eggs laid was counted from all vials using a Leica MZ75 dissecting microscope. Eggs were counted immediately after flies were transferred or were frozen to prevent eggs hatching and counted at a later date.

6.3.5 Effect of SPR on the remating response to SP

Two sets of females were kept from the initial mating assay. One group was tested 24h after mating and the other 48h after mating. Individual females were given a single 5 day-old *Dahomey* male and observed for 1h or until remating occurred. Males of the same age were used on each day.

6.3.6 Statistical analysis

The feeding data are proportional, and as such the sampling variance is greatest for values near 0.5. The dependence of the variance on the mean therefore necessitated the use of the arcsine square root transformation for all feeding data. Normality tests were conducted with Kolmogorov-Smirnoff tests and homogeneity of variance with Levene tests, using SPSS 18 (SPSS, Inc., Chicago IL). The egg count data were square root transformed to improve normality for the repeated measures test.

The results of the feeding and egg laying assays from the first experiment were analysed using a repeated measures ANOVA. Mauchly's test of sphericity was used to check the assumption that the variation between treatments was equal. For the egg laying assay, Mauchly's test indicated that the assumption of sphericity had been violated ($\chi^2 = 43.6$, $p < 0.05$). Therefore the degrees of freedom were corrected using Huynh-Feldt estimates of sphericity (epsilon = 0.86). One way ANOVAs were also performed to look for significant differences in feeding and egg laying across specific time intervals. For all tests, when the assumption of equal variance was met, Tukey B post hoc tests were performed to distinguish significantly different treatments. Dunnets C post hoc tests were used when the assumption of equal variance was not met.

Virgin SPR+ and SPR- females differed slightly in feeding and egg laying rates. I corrected for this by applying a correction factor based on virgin rates to equalise the non-significant difference between SPR+ and SPR- genotypes, therefore producing a more conservative test. Correction factors were calculated independently for each test performed based on the raw numbers of feeds observed or eggs counted between the virgin SPR+ and virgin SPR- treatments. Correction factors for each test, where applicable, are given in the results.

The proportion of females that remated was analysed using a G-test (Sokal & Rohlf, 1995) with χ^2 statistics and critical values calculated using R 2.12.0 (R Development Core Team, 2010). Pairwise comparisons were conducted using Fisher's exact tests and Bonferroni corrections to control for multiple testing.

6.4 Results

6.4.1 Effect of SPR on the feeding response to SP

Total feeding counts over 5 days: raw and standardised data

The results show that the proportion of observed females feeding between the six treatments differed significantly (Repeated measures ANOVA, $F_{5,54} = 13.8$, $p < 0.001$; **Figure 6.1**). Post hoc tests revealed that SPR+ females mated to SP+ males fed at the highest rate over the course of the experiment (Tukey B $p = 0.05$). Interestingly, the results also suggested that SPR+ females mated to SP- males showed an intermediate level of feeding compared to mated SPR- females (Tukey B $p = 0.05$), suggesting that SPR may be

a target for sfps, other than SP, that also increase levels of feeding. However, the difference between the female genotypes in virgin levels of egg laying mean caution is needed in interpreting this result. There was a significant effect of day (Repeated measures ANOVA, $F_{5,270} = 9.72$, $p < 0.001$) generally observed as an increase in feeding after early compared to later time points. There was a near-significant interaction between treatment and day (Repeated measures ANOVA, $F_{25,270} = 1.52$, $p = 0.059$) suggesting that all treatments showed slightly different patterns of increased or decreased rate of feeding over time.

When feeding data were combined for all days, SPR+ females mated to SP+ males fed at the highest rate and SPR+ females mated to SP- males showed an intermediate level of feeding compared to mated SPR- and virgin females (ANOVA, $F_{5,54} = 12.64$, $p < 0.001$, Tukey B $p = 0.05$, **Figure 6.2a**), as observed in the repeated measures analysis above. Consistent with the raw data, analysis of the standardized feeding rates (1 SPR+ = 1.43 SPR-) showed that SPR+ females mated to SP+ males significantly fed at the highest rate over the course of the experiment and SPR+ females mated to SP- males still showed an intermediate level of feeding compared to the remaining treatments ($F_{5,54} = 7.09$, $p < 0.001$, Tukey B $p = 0.05$, **Figure 6.2b**). However, in contrast to the raw data, the SPR+/SP- treatment was not significantly different from mated SPR- and virgin females. The results show that SPR and SP are both required for inducing the feeding response and that the response can be observed for at least 5 days after mating.

Total feeding 24h after mating: raw and standardised data

An analysis combining early feeding rates from 6h to one day after mating showed that SPR+ females mated to either SP+ or SP- males fed at the highest rate ($F_{5,54} = 10.49$, $p < 0.001$, Tukey B $p = 0.05$, **Figure 6.3a**). Analysis of the results after standardizing both female types by virgin female feeding rates (1 SPR+ = 1.95 SPR-) showed that SPR+/SP+ females still fed at the highest rate, but at the same level as SPR+/SP- and SPR-/SP+ females ($F_{5,54} = 5.72$, $p < 0.001$, Tukey B $p = 0.05$, **Figure 6.3b**). Likewise, SPR+/SP- females fed at a similar level as all other treatments, with the exception of SPR-/SP- females. The results show that SPR and SP are both required for inducing early feeding rate increases.

6.4.2 Effect of SPR on the egg laying response to SP

Total egg laying over 5 days

The number of eggs laid differed significantly between treatments (Repeated measures ANOVA, $F_{5,54}$ = 18.469, p < 0.001; **Figure 6.4**). Post hoc tests revealed that SPR+ females mated to SP+ males laid significantly more eggs than other treatments over the course of the experiment (Dunnet C p = 0.05). There was a significant effect of day ($F_{4.3,232.0}$ = 351.17, p < 0.001) which was most apparent in the difference between number of eggs laid after 6h as compared to later time points. There was a significant interaction between treatment and day ($F_{21.5,232.0}$ = 5.624, p < 0.001) indicating that some of the treatments showed different dynamics of increased or decreased rate of egg laying over time. Following the same pattern as observed with the feeding assay, SPR+ females mated to SP- males showed an intermediate level of egg laying compared to mated SPR- females, suggesting that SPR may be a target for sfps other than SP that also increase egg laying rate. However the difference between the female genotypes in virgin levels of egg laying mean that this interpretation should be treated with caution.

Replicate 1 - total egg laying 24h after mating: raw and standardised data

Analysis of the total number of eggs counted one day after mating showed that SPR+ females that mated with either SP+ or SP- males laid significantly more eggs than the other treatments ($F_{5,54}$ = 17.54, p < 0.001, Tukey B p = 0.05; **Figure 6.5a**). Standardizing for virgin levels of egg laying (1 SPR+ = 1.18 SPR-) did not affect the main finding that SPR+/SP+ females laid significantly more eggs ($F_{5,54}$ = 5.80, p < 0.001, Tukey B p = 0.05; **Figure 6.5b**). However, the standardisation removed the significant difference between SPR+/SP- females and the remaining treatments. The lack of resolution to determine significantly different subsets of treatments may have been the result of low sample size.

Replicate 2 - total egg laying 24h after mating: raw and standardised data

One day after mating, SPR+/SP+ laid significantly more eggs than the other groups ($F_{5,112}$ = 32.30, p < 0.001, Tukey B p = 0.05; **Figure 6.6a**). SPR+/SP- females again showed intermediate rates of egg laying but this effect was no longer significant after standardization by virgin levels of egg laying (1 SPR+ = 1.43 SPR-) ($F_{5,112}$ = 8.29, p <

0.001, Tukey B $p = 0.05$; **Figure 6.6b**). The results show that SPR and SP have equal effects on egg laying in both the short and long term.

6.4.3 Effect of SPR on the remating response to SP

In the first day after mating, SPR+/SP+ females remated significantly less frequently than all the other treatments (G-test, $G = 297.3$, $d.f. = 5$, $p < 0.001$; **Figure 6.7a**). There were no significant differences in remating rate between any of the remaining treatments (Fisher's exact test $p < 0.05$). The same result was obtained two days after mating (G-test, $G = 282.8$, $d.f. = 5$, $p < 0.001$; Fisher's exact test $p < 0.05$; **Figure 6.7b**). SPR+/SP+ females remated at a slightly higher rate after two days compared to after the first day (40 *vs.* 29%, respectively), however this difference was not significant (Fisher's exact test, $p = 0.265$). There was also no difference in remating rate between SPR- females that received SP, when given the opportunity to remate after one day compared to two days (80 *vs.* 86%, respectively; Fisher's exact test, $p = 0.411$). These results indicate that SPR and SP together are required for the decrease in remating rate observed after mating both in the short and long term.

6.5 Discussion

In this experiment I tested whether SPR was required for the partial degeneracy of short term responses observed in mated females that do not receive SP, which suggested that other seminal fluid proteins are triggering changes in fecundity and receptivity. Because SPR has been shown to bind Dup99B, and because Dup99B can elicit the same responses as SP, I tested whether SPR is required for the short term receptivity and egg laying response. I also tested whether feeding rate was dependent on the presence of SPR.

The results show that SPR is required for the elevation in female feeding rate observed after mating, and that this elevation in feeding continues for at least 5 days after mating. There was also a suggestion that SPR had effects on feeding in addition to those caused by SP, indicated by the elevated feeding observed in mated control females that did not receive SP. The same pattern was reflected in the egg laying data: control females that received SP laid significantly more eggs. However, significant effects of SPR in the absence of SP were only detected in the raw data and not after standardizing the treatments for underlying fecundity differences, based on virgin levels of egg laying. Although the SPR+/SP- treatment was not significantly higher in terms of feeding and egg laying than

SPR- treatments in all tests performed, it was the only treatment that consistently formed an homogenous subset with SPR+/SP+ females in post hoc tests. The likely conclusion of this result is that SPR is activated by another early acting peptide. Dup99B remains as a potential candidate for this activation based on its sequence similarity to SP and its binding affinity for SPR (Yapici *et al.*, 2008, I test for this in the following chapter). Likewise, this work also highlighted the role of SPR in initiating early post mating responses. Previous work measured the effect of SPR only after 48h (Yapici *et al.*, 2008, Hasemeyer *et al.*, 2009) while this experiment showed that feeding, egg laying, and delayed remating responses were dependent on the presence of both SPR and SP within the first 24h. The inclusion of increased feeding rate adds to the list of post mating phenotypes regulated by SPR. Further candidate phenotypes involved in the SPR/SP signalling cascade include 'siesta sleep inhibition'(Isaac *et al.*, 2010), increases in innate immune responses (Peng *et al.*, 2005b), and reduction in female longevity (Wigby & Chapman, 2005).

Recent work has shown that the SPR/SP signalling system is involved in modulating a change in diet in females from a mainly carbohydrate diet when virgin to a protein diet when mated, to support increased egg production (Ribeiro & Dickson, 2010). Similar to the results presented here, Ribeiro and Dickson (2010) show that other SPR ligands are likely to contribute to female nutritional decision making. From a mechanistic point of view, it is interesting to study the connections between the SPR/SP pathway and other important signalling cascades (such as TOR-S6K involved in metabolism) in determining post mating switches, and to further understand how the brain processes these signals to produce the desired outputs. Although the elevation in feeding rate is dependent on the presence of a functioning ovary, the switch in diet from carbohydrates to protein is an independent and direct effect of SPR signalling in the nervous system and does not depend on a feedback mechanism involving egg production (Ribeiro & Dickson, 2010). Likewise, the role of SP in delayed rematings is independent of the ability to produce and lay eggs (Barnes *et al.*, 2007) suggesting a direct neurological pathway with no feedback between receptivity and fecundity state.

Knowledge of how SPR and SP have evolved across invertebrate species is important for understanding the history of coevolution between the sexes. SPR is evolutionarily conserved across a large range of invertebrates including gastropods, Lepidoptera, and Diptera species suggesting that it is a receptor with important, ancestral functions (Yapici

et al., 2008, Kim *et al.*, 2010). Likewise, conservation of SPR across invertebrates matches the evolutionary conservation of MIPs, which are hypothesised to be the ancestral ligands of SPR (Kim *et al.*, 2010). This pattern of conservation is in contrast to SP, which can only be detected within a limited number of *Drosophila* species. Because MIPs are not detected in the male reproductive system, and because they are not capable of triggering post mating responses, the current role of SP as a ligand for SPR capable of inducing post mating effects is both unique and novel (Kim *et al.*, 2010). This is consistent with the idea that proteins that control important processes such as egg laying, receptivity, and feeding can evolve fairly rapidly. The recent duplication of SP to create the Dup99B gene is further evidence of this phenomenon.

SP and SPR are the only male-female interacting proteins known to date and further research of this system, including the identification of the existence and role of further players, is important for understanding male-female coevolution. Polymorphisms in SPR and SP genes give rise to strong male × female interactions that modulate remating rate and P1 (Chow *et al.*, 2010). Multiple lines of evidence suggest that SP can induce some post mating responses without binding to SPR. Firstly, SP binds to sites where SPR is not expressed (Ottiger *et al.*, 2000, Ding *et al.*, 2003, Yapici *et al.*, 2008) so it is likely that there are other receptors for SP. Secondly, examination of the amino acid sequence of SP reveals 3 separate regions capable of inducing egg laying and receptivity responses, immune responses, and stimulation of juvenile hormone synthesis, respectively, suggesting that different regions of the protein could bind to separate targets within the female (Saudan *et al.*, 2002, Ding *et al.*, 2003). These interactions are important to study because they can highlight the constraints to evolution in the SP/SPR system.

The presence or absence of links between post mating effects caused by the same reproductive protein provide an opportunity to study how such responses evolve. Evolution should be less constrained if different responses are not strongly linked in a mechanistic sense (Barnes *et al.*, 2008). A system of one to one relationships and tight, exclusive interactions between molecules would be expected to be very resistant to evolutionary change, as for example enzymes involved in DNA copy and repair that are conserved across kingdoms. However, systems where a molecule interacts with many receptors or where multiple cofactors are required, could allow for more fine tuning and fault tolerance. Ravi Ram and Wolfner (2009) demonstrate the existence of a regulatory long term

response (LTR) network of seminal fluid proteins that controls the fate of SP, regulating its binding to sperm and its eventual cleavage within a complex multistep network. Seminal proteins that function interdependently are likely to be constrained in their ability to evolve rapidly. Within the LTR network, CG17575 is highly conserved, whereas CG9997 shows signals of rapid evolution, showing that within a network, some proteins are capable of rapid change. Whether this is because of a coevolutionary arms race, or the result of genetic drift on a weakly selected trait, is unknown and deserves future study.

Figure 6.1. Mean (± s.e.) proportion of feeding events observed per vial over 5 days for SPR null (dashed lines) or control females (solid lines) either mated to SP+ control males (triangles), SP- null males (circles), or left virgin (squares).

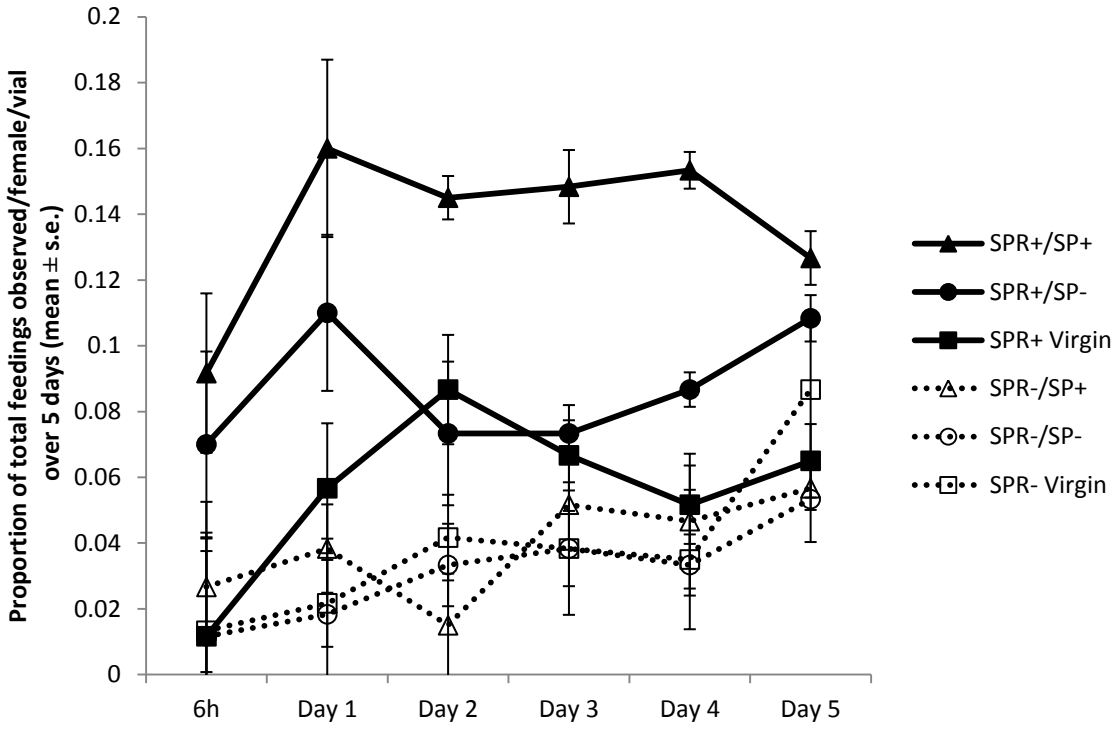
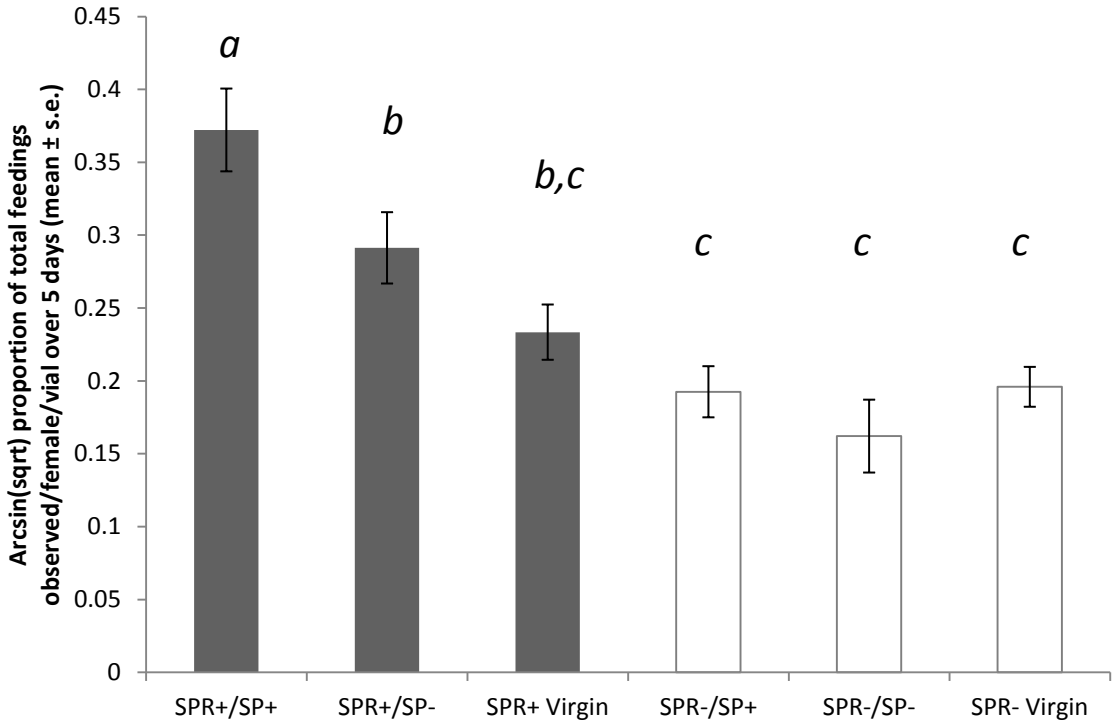


Figure 6.2. (**a**) Mean (± s.e.) arcsine square root proportion of total feeding observed per vial summed across 5 days after mating for SPR null or control females mated to SP null or control males or left virgin. (**b**) as above, but standardized so that SPR null and control virgin females have the same rate of feeding. Shaded bars (SPR control females); open bars (SPR null females). Small letters denote homogenous subgroups determined by Tukey B post hoc comparisons (p = 0.05).

Feeding – 5 day counts

(**a**)



(**b**)

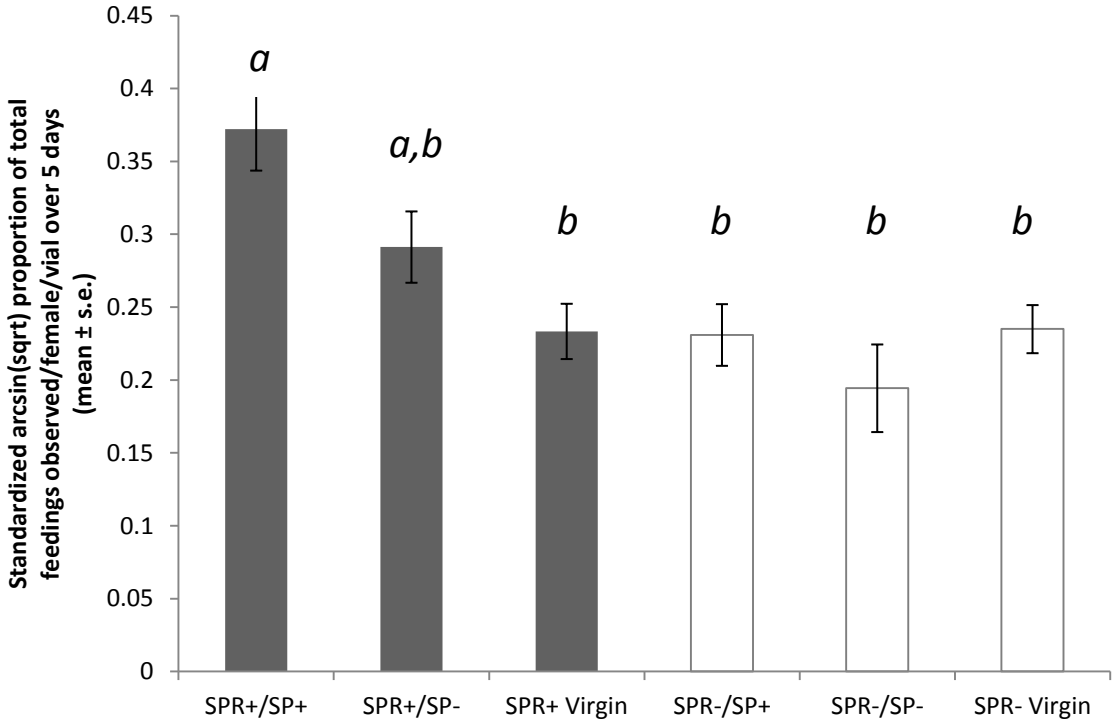
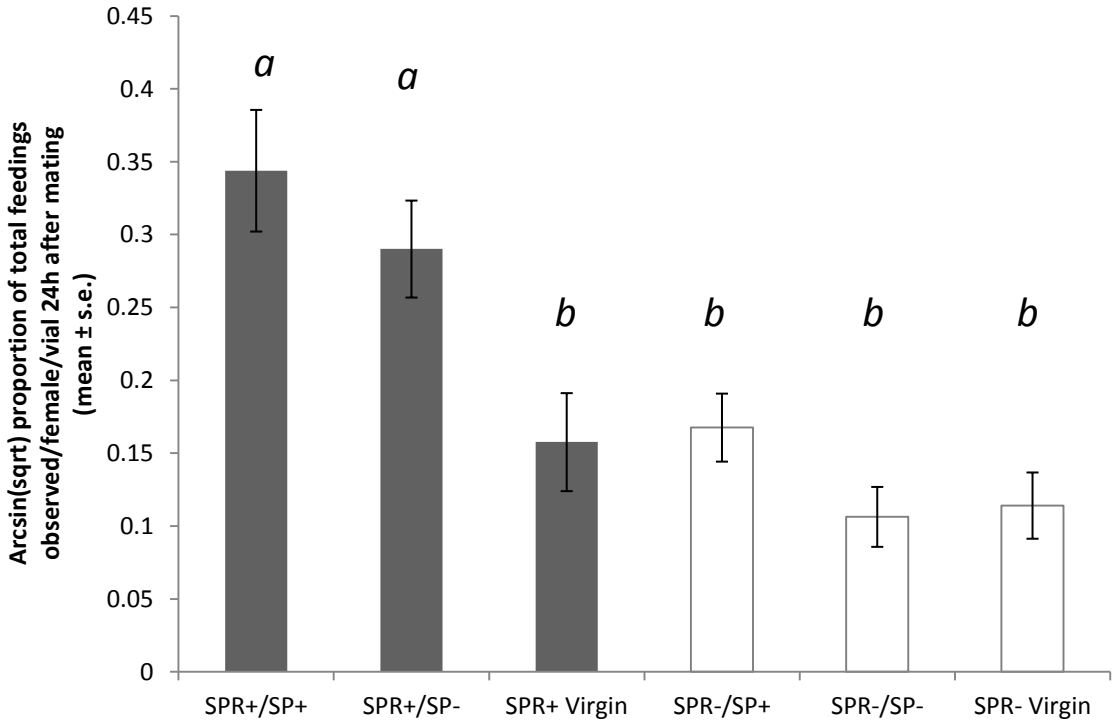

Standardized arcsin(sqrt) proportion of total feedings observed/female/vial over 5 days (mean ± s.e.)
0.45
0.4
0.35
0.3
0.25
0.2
0.15
0.1
0.05
0
a
a,b
b
b
b
b
SPR+/SP+
SPR+/SP-
SPR+ Virgin
SPR-/SP+
SPR-/SP-
SPR- Virgin

Figure 6.3. (**a**) Mean (± s.e.) arcsine square root proportion of feeding observed per vial summed across the first 24h after mating for SPR null or control females mated to SP null or control males or left virgin. (**b**) as above, but standardized so that SPR null and control virgin females have the same rate of feeding. Shaded bars (SPR control females); open bars (SPR null females). Small letters denote homogenous subgroups determined by Tukey B post hoc comparisons ($p < 0.05$).

Feeding – 24h counts

(**a**)



(**b**)

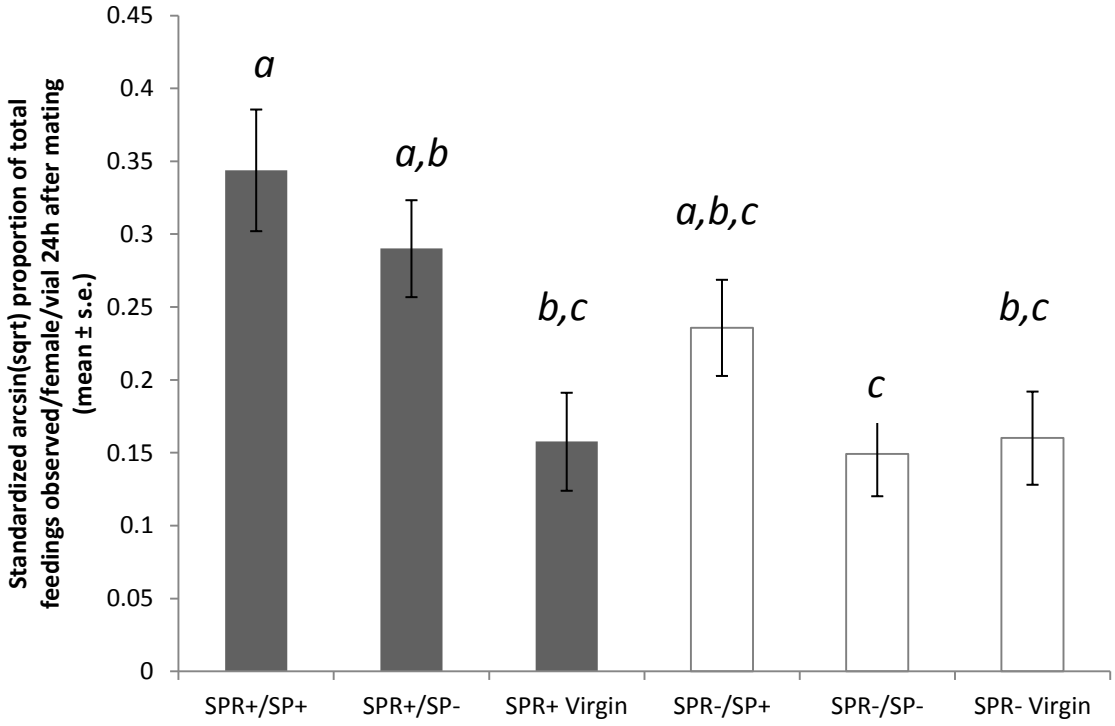


Figure 6.4. Mean (± s.e.) number of eggs laid per vial over 5 days for SPR null (dashed lines) or control females (solid lines) either mated to SP+ control males (triangles), SP- null males (circles), or left virgin (squares).

Egg laying over 5 days

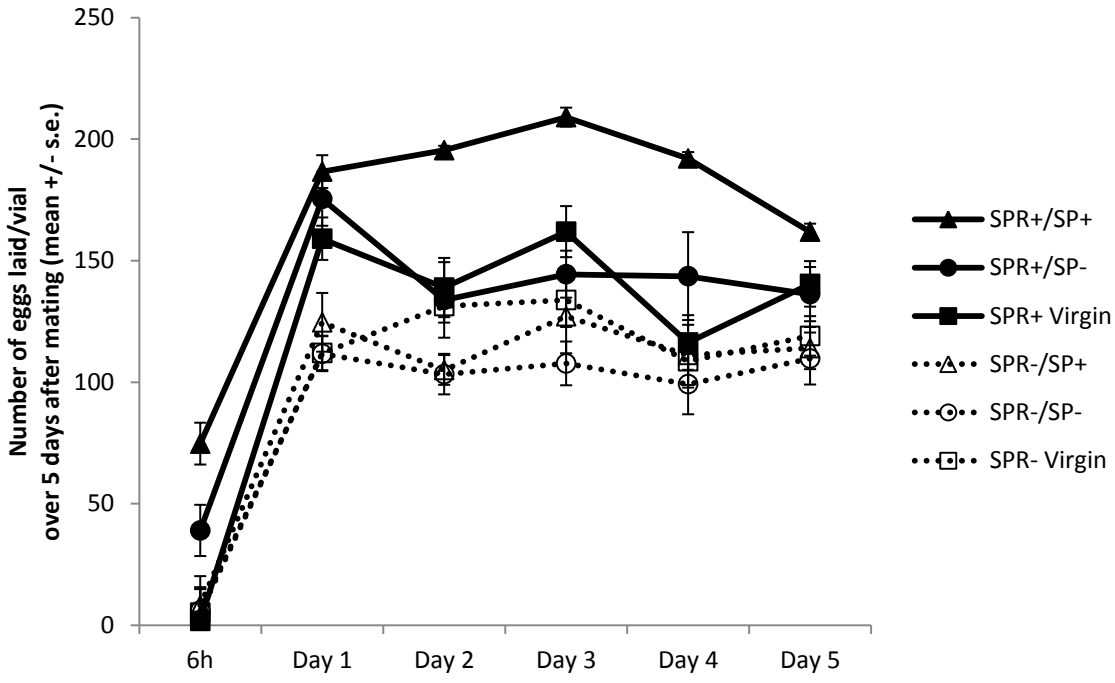
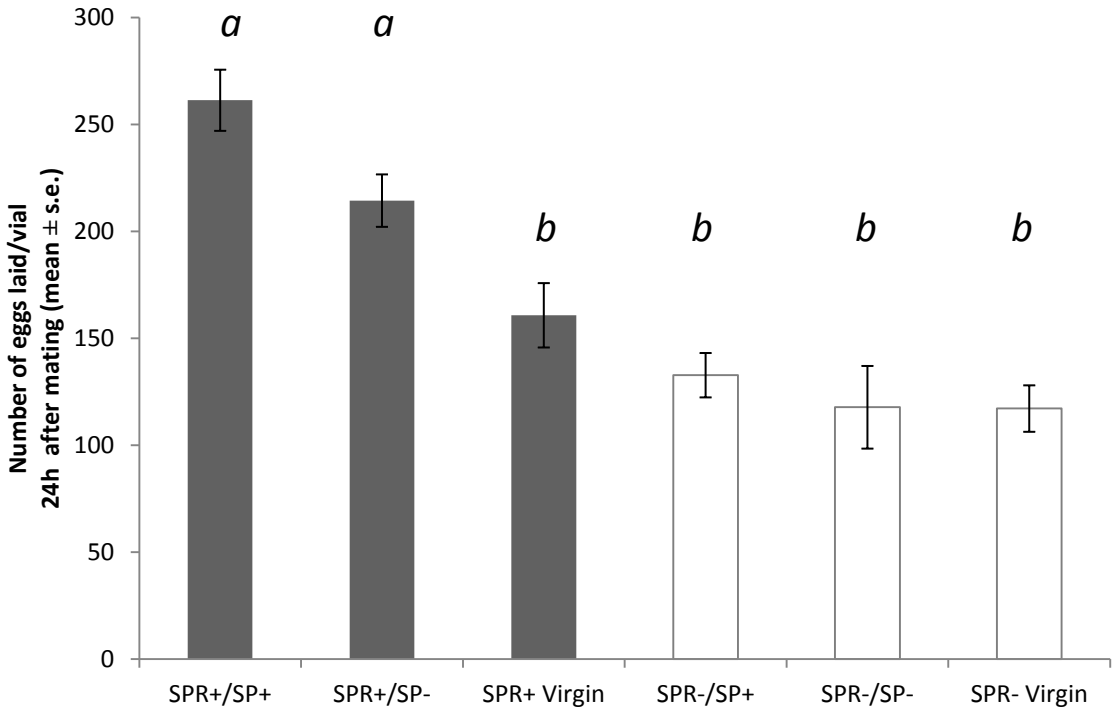


Figure 6.5. Replicate 1 (**a**) Mean (± s.e.) number of eggs laid per vial summed across the first 24h after mating for SPR null or control females mated to SP null or control males or left virgin in experiment 1. (**b**) as above but standardized so that SPR null and control virgin females have the same rate of egg laying. Shaded bars (SPR control females); open bars (SPR null females). Small letters denote homogenous subgroups determined by Tukey B post hoc comparisons ($p < 0.05$).

Egg laying – 24h counts – Replicate 1

(**a**)



(**b**)

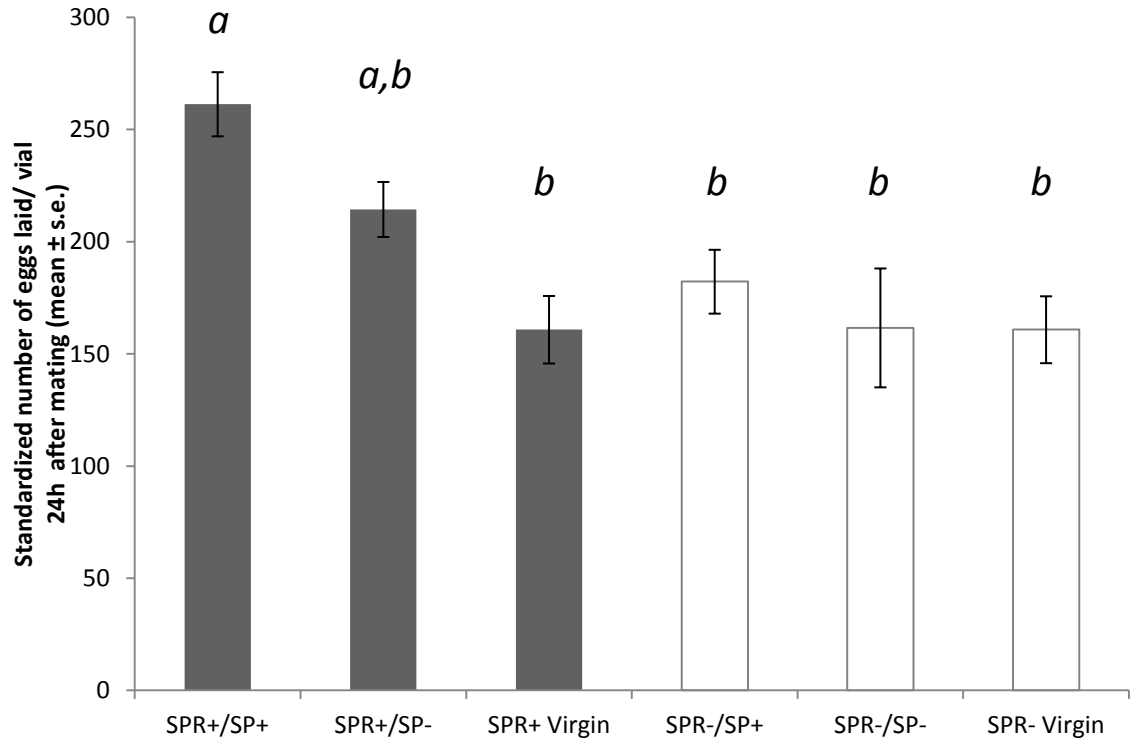
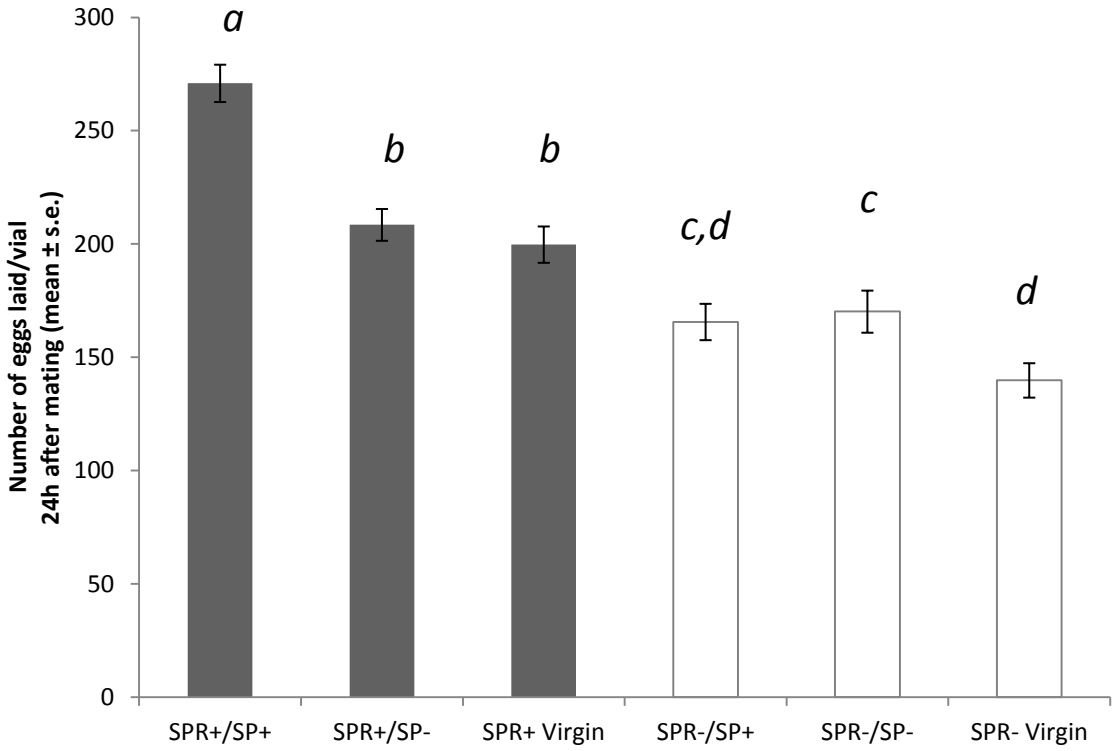

Standardized number of eggs laid/ vial 24h after mating (mean ± s.e.)
300
250
200
150
100
50
0
a
a,b
b
b
b
b
SPR+/SP+
SPR+/SP-
SPR+ Virgin
SPR-/SP+
SPR-/SP-
SPR- Virgin

Figure 6.6. Replicate 2 (**a**) Mean (± s.e.) number of eggs laid per vial summed across the first 24h after mating for SPR null or control females mated to SP null or control males or left virgin in experiment 2. (**b**) as above but standardized so that SPR null and control virgin females have the same rate of egg laying. Shaded bars (SPR control females); open bars (SPR null females). Small letters denote homogenous subgroups determined by Tukey B post hoc comparisons ($p < 0.05$).

Egg laying – 24h counts – Replicate 2

(**a**)



(**b**)

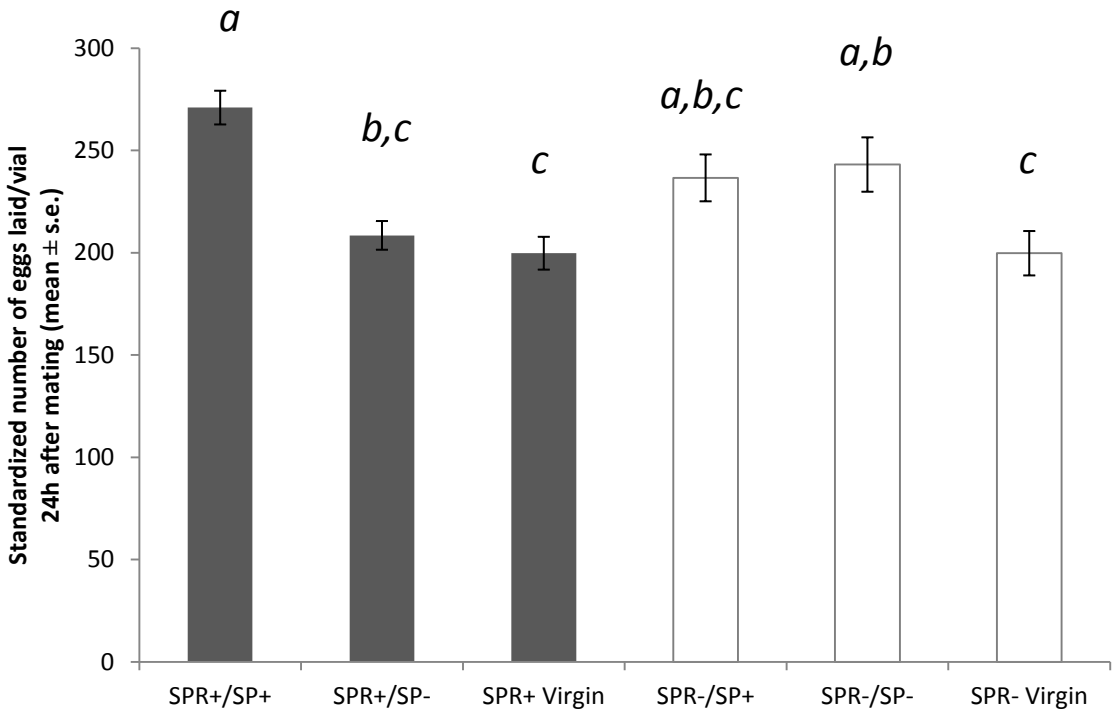
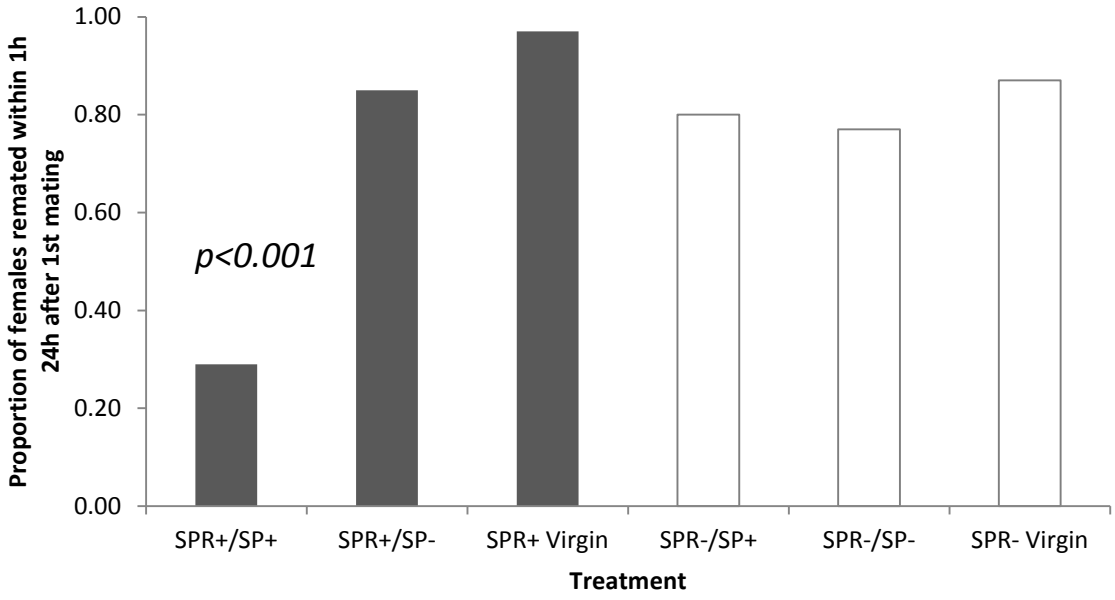

a
b,c
c
a,b,c
a,b
c
300
250
200
150
100
50
0
Standardized number of eggs laid/vial
24h after mating (mean ± s.e.)
SPR+/SP+
SPR+/SP-
SPR+ Virgin
SPR-/SP+
SPR-/SP-
SPR- Virgin

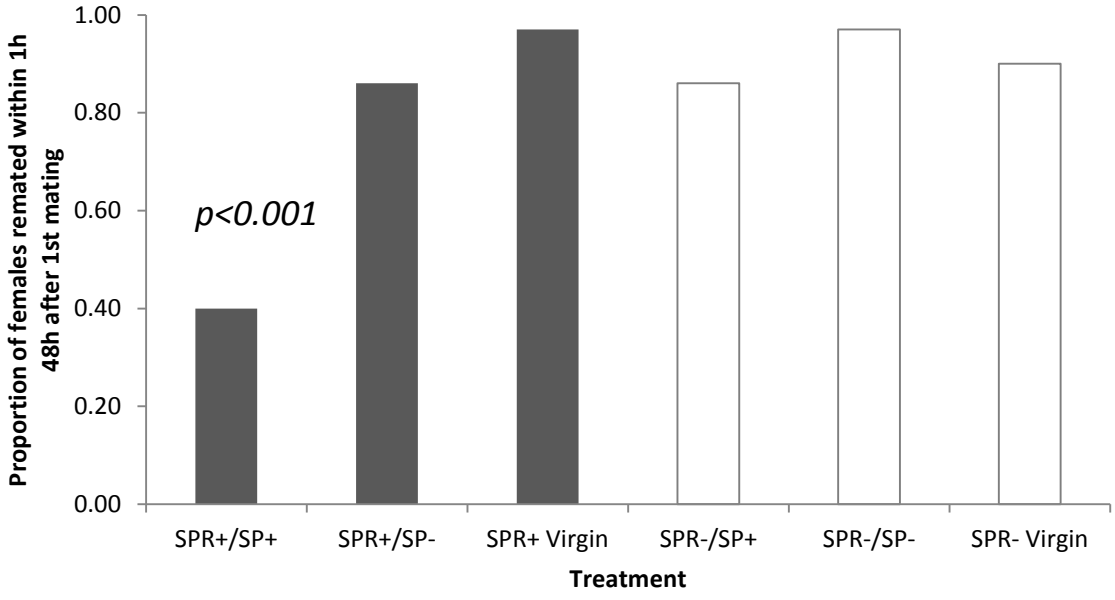
Figure 6.7. Proportion of SPR null or control females that remated to a *Dahomey* male within 1h, (**a**) 24h or (**b**) 48h after mating to either SP null males, control males or left virgin.

Remating – 24h and 48h

(**a**)



(**b**)



Chapter 7. Seminal fluid protein interactions – sex peptide and Dup99B

7.1 Summary

Seminal fluid proteins (sfps) and female reproductive proteins form a complex web of interactions and multiple sfps may sometimes be required to induce specific post mating responses, e.g. increased post mating fecundity. Sex peptide (SP) and Acp26Aa increase egg laying in mated females but act at two different stages of this process. SP increases the rate of oogenesis after mating while Acp26Aa stimulates ovulation. There is also evidence to suggest the possible involvement of a third sfp, *ductus ejaculatoris* protein 99B (Dup99B). Likewise, the observation that females mated with SP null males still show short term changes in egg laying and receptivity suggests the action of multiple sfps in these processes. A possible key candidate responsible for triggering these short term responses is Dup99B based on previous studies using injection experiments. Dup99B shares partial sequence homology to SP and also shows affinity for sex peptide receptor (SPR). In this chapter I tested the hypothesis that the presence of Dup99B is responsible for the short-term mating responses observed in females mated to SP null males. This is the first study to examine the effect of Dup99B in the absence of SP. The results showed no evidence that Dup99B influenced female post mating responses after 24 and 48h. This suggests that Dup99B does not fulfil a degenerate role, despite its sequence similarity to SP and affinity for the sex peptide receptor.

7.2 Introduction

Seminal fluid proteins (sfps) and female reproductive proteins form a complex web of interactions and multiple sfps may sometimes be required to induce specific post mating responses (Ravi Ram & Wolfner, 2007, Chapman & Davies, 2004, Swanson *et al.*, 2004, Prokupek *et al.*, 2008, 2009). The increased fecundity in females post mating is an example, as multiple processes influenced by different sfps act in concert to increase overall egg laying rate. The process begins with the progression of developing eggs from germ cells in the ovary (oogenesis). Mature eggs are released from the ovary (ovulation) and pass through the lateral, then common oviducts to reach the uterus where they are fertilized and finally laid (Bloch Qazi *et al.*, 2003). Sex peptide (SP) and Acp26Aa increase egg laying in mated females but act at two different stages of this process. SP increases the rate of oogenesis after mating while Acp26Aa stimulates ovulation (Chapman *et al.*, 2003c, Liu & Kubli, 2003, Herndon & Wolfner, 1995, Heifetz *et al.*, 2000). There is also evidence to suggest the possible involvement of a third sfp, *ductus ejaculatoris* protein 99B (Dup99B), which has been reported to increase egg laying for 1h after mating through an unknown mechanism (Saudan *et al.*, 2002, Rexhepaj *et al.*, 2003). Although much is known about the individual effects of these sfps (Chapman & Davies, 2004, Ravi Ram *et al.*, 2005), how they could also interact with each other to induce post mating phenotypes has been less well studied empirically. Heifetz *et al.* (2000) suggest a model whereby Acp26Aa stimulates the release of mature eggs which allows for the increase in the rate of oogenesis triggered by other sfps such as SP.

Another post mating phenotype that is likely affected by the actions of multiple sfps is the reduced receptivity observed in mated females for at least 2 days after mating. Females mated to SP null males remate more readily than mates of normal males (Liu & Kubli, 2003, Chapman *et al.*, 2003c) indicating SP's involvement in reducing female receptivity. Dup99B can also reduce receptivity when injected in virgin females (Saudan *et al.*, 2002). Dup99B has high sequence similarity to SP and shows high amounts of amino acid homology at the C-terminal end of the peptide, which is known to be required for inducing egg laying and receptivity responses (Saudan *et al.*, 2002). However, when females are mated to males that do not produce Dup99B, the effect of Dup99B appears to be very weak and transient (Rexhepaj *et al.*, 2003).

SP transferred during a normal mating can illicit post mating responses for 7-10 days (Chapman *et al.*, 2003c, Liu & Kubli, 2003). SP binds to sperm by its N-terminus and is gradually released from the sperm tails through proteolytic cleavage (Peng *et al.*, 2005a). This slow release is critical for the maintenance of post mating changes in females over the long term (Peng *et al.*, 2005a). Interestingly, in the absence of SP females still show an increase in egg laying and reduction in receptivity, but this response only lasts 24h (Chapman *et al.*, 2003c, Liu & Kubli, 2003) which indicates that other sfps are also capable of influencing these phenotypes. A possible key candidate responsible for triggering these short term responses is Dup99B. SP and Dup99B elicit similar increases in egg laying rate and reduced remating when injected into virgin females, but this response only lasts 24h (Saudan *et al.*, 2002). Dup99B lacks homology to SP within the N terminus of the protein (Saudan *et al.*, 2002) and, unlike SP, only remains bound to sperm for a few hours (Peng *et al.*, 2005a). This could provide the explanation for why Dup99B is only capable of producing a short term response.

The aim here was to test the hypothesis that the presence of Dup99B is responsible for the short-term mating responses observed in females mated to SP null males. In mating experiments comparing Dup99B mutant males with controls, Dup99B appeared to play a negligible part in the short term response (Rexhepaj *et al.*, 2003). However, because of the strong, overriding effects of SP, it is unknown whether Dup99B is actually capable of eliciting a stronger post mating response in females during regular matings. Using a Dup99B mutant and a Dup99B/SP double mutant, I tested whether the absence of Dup99B would cause a reduction in female post mating responses after 24 and 48h.

7.3 Materials and methods

7.3.1 Fly stocks

Dup99B single mutant males were w^{1118}*;;071E/071E* homozygotes (see Chapter 2 for stock details). Males with a balancer chromosome, w^{1118}*;;071E/TM3,Sb,Ser*, were used as controls. *SP* null males were w^{1118}*;;0325/0325* homozygotes and males with a balancer chromosome w^{1118}*;;0325/TM3,Sb,ry* were used as controls. *SP/Dup99B* double null mutant males were w^{1118}*;;0325,072/0325,072* homozygotes. Males with a balancer chromosome, w^{1118}*;;0325,072/TM3,Sb,Ser*, were used as controls. *Dahomey* wild type males and females were also collected.

7.3.2 Effect of sex peptide and Dup99B on receptivity to remating

In this experiment I mated wild type females to either SP null, Dup99B null, SP/Dup99B double nulls, or control males and tested female receptivity after 24 or 48h. 5 day-old treatment males were placed into individually labelled vials of SYA medium supplemented with granules of live yeast and 5 day old virgin *Dahomey* females were aspirated singly into the mating vials. 80 mating pairs were set up in this fashion for each male treatment. Introduction time, copulation start and end time were noted. After mating the males were removed to ensure no remating could occur. Flies that did not mate within 2h were discarded. An unmated virgin female treatment was set up by adding females to individual vials that contained no males. Females from each treatment where then randomly assigned to either the 24h or 48h remating assay. At the designated remating interval, single wild type males were aspirated into the mating vials. The number of females to remate within 1h of introduction was then recorded.

7.3.3 Effect of sex peptide and Dup99B on egg production

In this experiment I mated females to either SP null, Dup99B null or SP/Dup99B double mutant males and tested their effect on egg production. *Dahomey* females were mated to treatment males as described above. Females were allowed 24h to lay eggs before being transferred to a new set of SYA vials supplemented with live yeast for a further 24h before being discarded. Vials were frozen and eggs counted.

7.3.4 Statistical analysis

The proportions of females that remated in each treatment were compared using G-tests. Expected values and the critical *G* value were calculated using R 2.8.0 (R Development Core Team, 2008). Fisher's Exact Tests were also performed using R 2.8.0. The mean numbers of eggs laid across treatments were analysed using ANOVA and Tukey B posthoc tests in SPSS 16 (SPSS, Inc., Chicago IL).

7.4 Results

7.4.1 Effect of sex peptide and Dup99B on receptivity to remating

24h remating interval

The number of females in each treatment that mated once ranged from 37-38, except for the SP null treatment, which had 22 successful matings. After 24h, females initially mated to SP null males remated at a significantly higher frequency than females that initially received SP (**Figure 7.1a,** proportions, SP null: 0.86, SP control: 0.08, Dup99B null: 0.16, Dup99B control: 0.14, Dup99B/SP control: 0.32, *Dahomey*: 0.03; G-test, $G = 206.74$, $d.f. = 7$, $p < 0.001$). The remating rate of females initially mated to Dup99B/SP null males (proportion = 0.95) was not significantly different from females initially mated to SP null males (proportion = 0.86) (Fisher's Exact Test, $p = 0.179$). Mates of Dup99B null males (proportion = 0.16) had remating rates that were not significantly different to Dup99B controls (proportion = 0.14) (Fisher's Exact Test, $p = 0.533$).

48h remating interval

The number of females in each treatment that mated once ranged from 35-38, except for the SP null treatment, which had 21 successful matings. After 48h, females initially mated to SP null males remated at a significantly higher frequency than females that initially received SP (**Figure 7.1b**, proportions, SP null: 1, SP control: 0.13, Dup99B null: 0.24, Dup99B control: 0.19, Dup99B/SP control: 0.27, *Dahomey:* 0.11; G-test, $G = 183.53$, $d.f. = 7$, $p < 0.001$). The remating rate of females initially mated to Dup99B/SP null males (proportion = 0.97) did not differ significantly from that of females initially mated to SP null males (proportion = 1) (Fisher's Exact Test, $p = 0.606$). Mates of Dup99B null males (proportion = 0.24) had remating rates that were not significantly different to Dup99B controls (proportion = 0.19) (Fisher's Exact Test, $p = 0.430$).

Overall the experiments showed no effect of Dup99B on receptivity, even in the absence of SP. These results were consistent after both 24 and 48h.

7.4.2 Effect of sex peptide and Dup99B on egg production

0-24h after mating

The number of females in each treatment that mated once ranged from 38-40, except for the SP null treatment, which had 31 successful matings. After 24h, females mated to SP null males laid significantly fewer eggs than females who received SP (**Figure 7.2a**; mean eggs laid ± se, SP null: 35.74 ± 3.4; SP Control: 51.86 ± 2.9; Dup99B null: 51.62 ± 2.9, Dup99B control: 52.45 ± 3.4, Dup99B/SP control: 48.92 ± 3.3, *Dahomey*: 54.79 ± 2.9; ANOVA, $F_{7,294}$ = 6.463, $p < 0.001$, Tukey B $p = 0.05$). Females mated to Dup99B null males did not differ significantly in the number of eggs laid in comparison to females mated to Dup99B controls (mean eggs laid ± se, Dup99B null: 51.62 ± 2.9; Dup99B control: 52.45 ± 3.4; Tukey B NS). Females mated to SP null males (but which received Dup99B) did not differ significantly in egg production from females mated to SP/Dup99B null males (mean eggs laid ± se, SP null: 35.74 ± 3.4, SP/Dup99B null: 37.13 ± 3.0, Tukey B NS).

24-48h after mating

After 48h, females mated to SP null males laid significantly fewer eggs than females that received SP (**Figure 7.2b**; mean eggs laid ± se, SP null: 40.65 ± 3.5; SP Control: 60.50 ± 2.5; Dup99B null: 57.35 ± 2.7, Dup99B control: 64.00 ± 3.3, Dup99B/SP control: 55.15 ± 3.5, *Dahomey*: 55.82 ± 2.6; ANOVA, $F_{7,294}$ = 10.163, p = 0.001, Tukey B p = 0.05). Females mated to Dup99B null males did not differ significantly in the number of eggs laid in comparison to females mated to Dup99B controls (mean eggs laid ± se, Dup99B null: 57.35 ± 2.7; Dup99B control: 64.00 ± 3.3; Tukey B NS). Females mated to SP null males (but which received Dup99B) did not differ significantly in egg production from females mated to SP/Dup99B null males (mean eggs laid ± se, SP null: 40.65 ± 3.5, SP/Dup99B null: 36.3 ± 2.2, Tukey B NS).

Thus these experiments showed no significant effect of Dup99B on egg laying, even in the absence of SP. These results were consistent after both 24 and 48h.

7.5 Discussion

Sex peptide and Dup99B are an interesting case study in the evolution and function of male seminal fluid proteins. Although much is known about their individual effects, how

they could also interact with each other to induce post mating phenotypes was unknown. The experiment reported here is the first study to examine the effect of Dup99B in the absence of SP, and showed no effect of Dup99B on female post mating responses after 24 and 48h. The typical short term response in reduced receptivity and increased egg laying elicited by SP null males was also observed in females who did not receive SP and Dup99B. Therefore Dup99B, even in the absence of SP, is not capable of influencing the receptivity or egg laying phenotypes in females despite its sequence similarity to SP and affinity for the sex peptide receptor (Saudan *et al.*, 2002, Yapici *et al.*, 2008). Effects of Dup99B have been reported to last for only 1h after mating (Rexhepaj *et al.*, 2003), so it will be important to repeat this study and measure responses at earlier time points post mating.

Expanding this work further, future experiments should include the use of double and triple mutants for SP, Dup99B and Acp26Aa. Experiments with those lines would allow the direct comparison of the effects of these sfps within the same background strain of fly. Combination experiments with Acp26Aa and SP will be elucidating in this regard because so much is already known about their mechanisms and timing effects. However, important questions remain unanswered about how these sfps may interact with each other to stimulate egg production. For example, whether Acp26Aa plays a larger role in the short term response in the absence of SP or whether ovulation (triggered by Acp26Aa) can proceed without SP initiating oogenesis is unknown. Likewise, how the responses caused by other sfps, such as an elevation in feeding rate or changes in hormonal levels, regulate egg laying and receptivity is an important area for future study. Further attempts to characterise the *Drosophila melanogaster* mating system and identify the key molecular players is crucially important for understanding male-female coevolution.

In sum, I was unable to attribute a phenotype to Dup99B in egg laying or receptivity responses in mated females. The possibility that Dup99B could be responsible for the short term post mating responses in females mated to SP null males (as discussed in Chapter 6) thus appears unlikely. This study is relevant to the study of the evolution of degeneracy of protein action which is hypothesised to form a major component of biological system (Edelman & Gally, 2001). Further discussion of the topic of degeneracy in sfp evolution is contained within the general discussion (Chapter 8).

Figure 7.1. Proportion of females that remated (**a**) 24 or (**b**) 48h after exposure to Dup99B null, SP null, Dup99B/SP null or control males. Females that did not receive sex peptide (SP null, Dup99B/SP null, virgins) were significantly more likely to remate than controls 24h after mating. There was no difference in the result after 48h. Small letters denotes a significant subset of treatments (based on G-test and Fisher's Exact Tests).

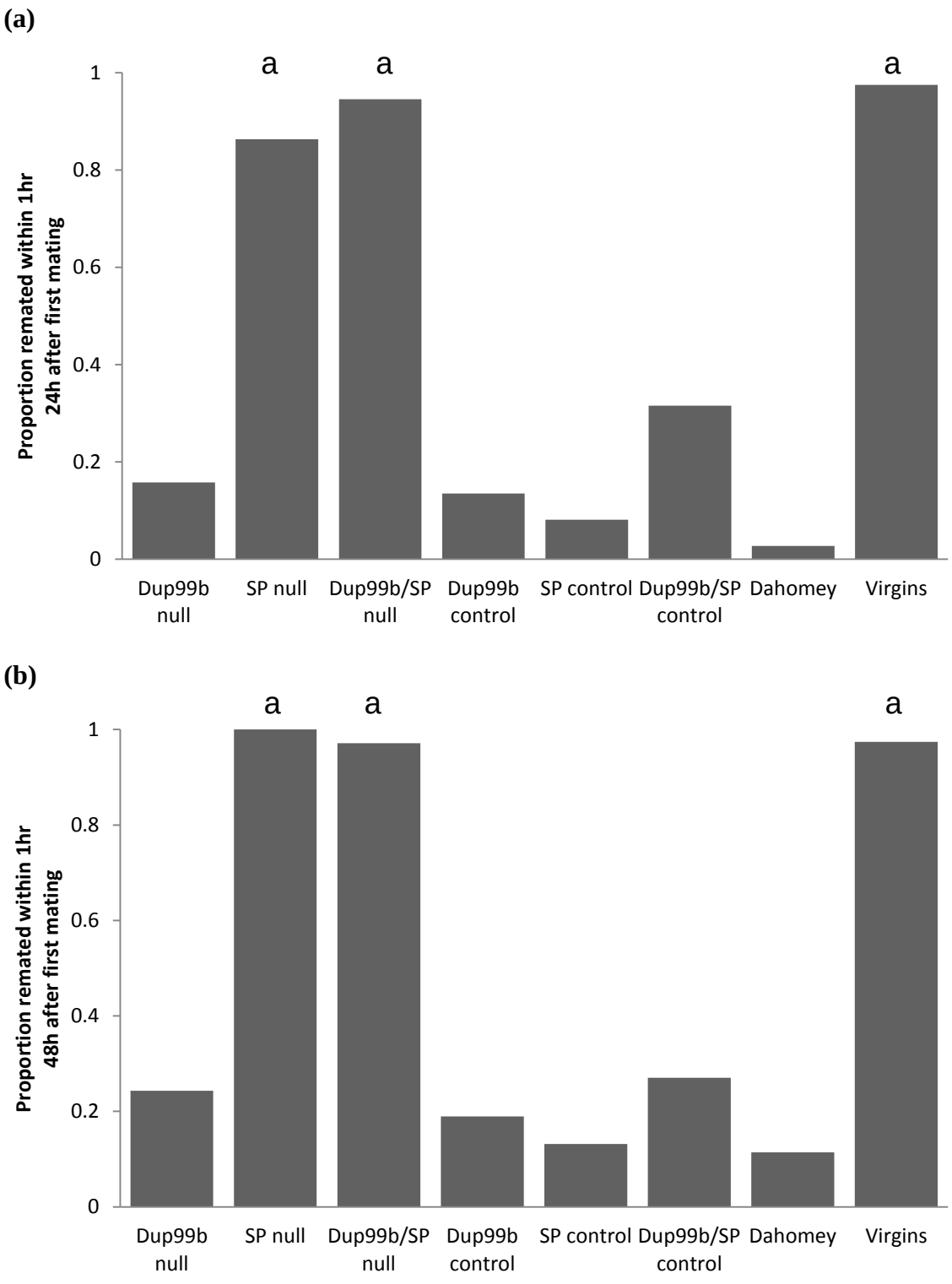
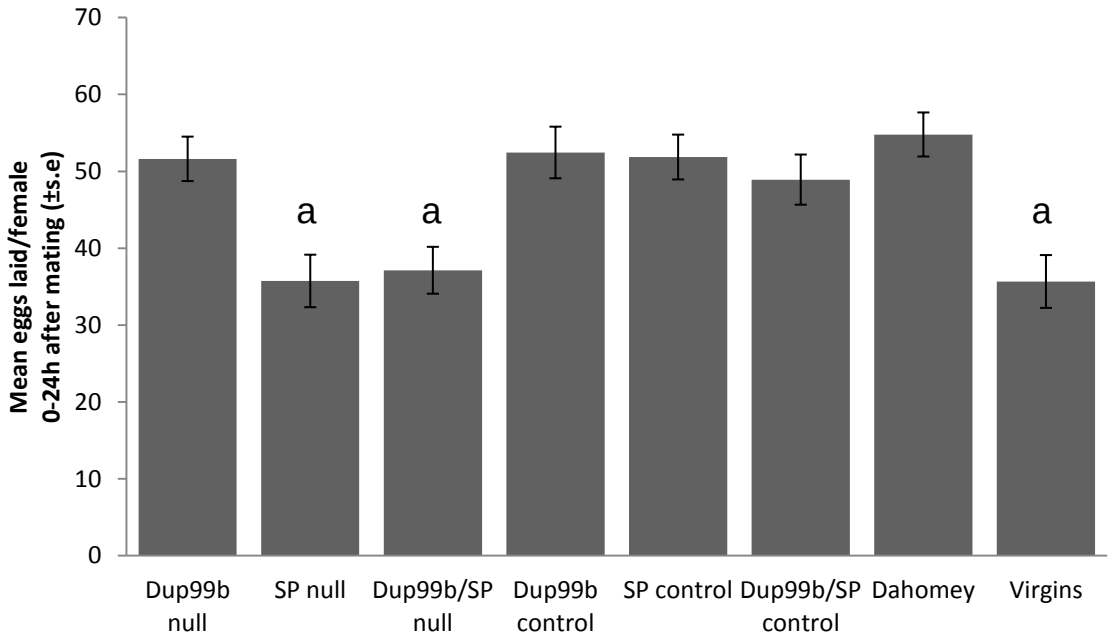
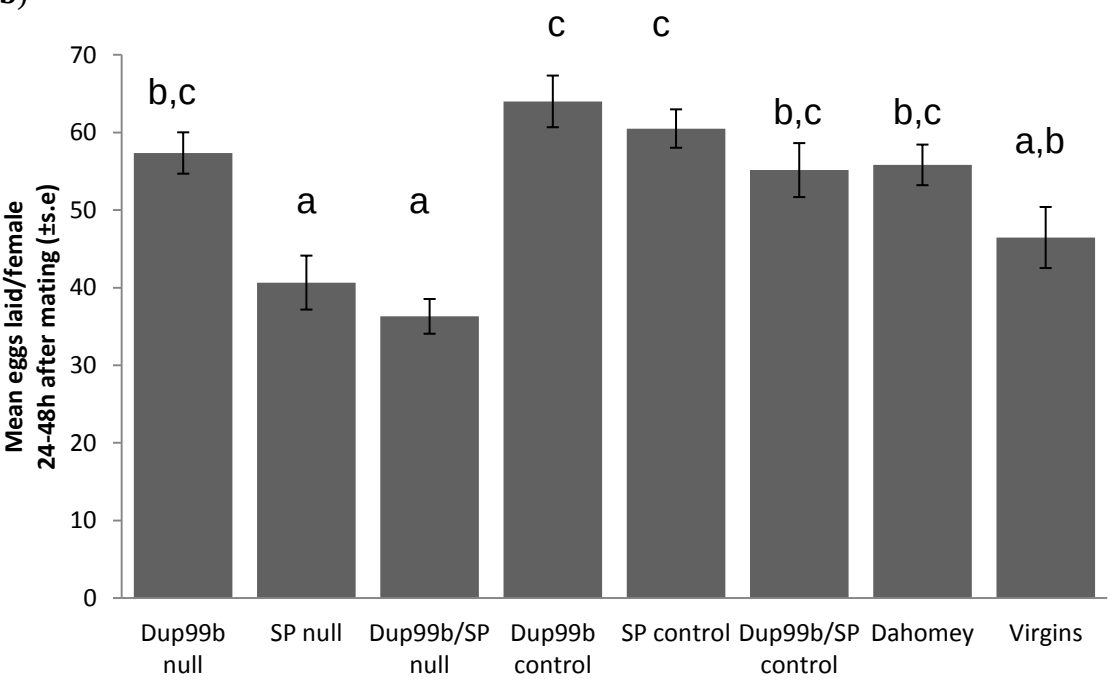


Figure 7.2. Mean (± s.e.) number of eggs laid per female during (**a**) 0-24h and (**b**) 24-48h after mating with males of indicated genotype. Females that did not receive sex peptide (SP null, Dup99B/SP null, virgins) laid significantly more eggs than controls 0-24h after mating. There was no difference in the results after 24-48h after mating. Females mated to either SP nulls or Dup99B/SP nulls laid significantly fewer eggs than all other groups. Small letters indicate homogenous subsets (Tukey B $p = 0.05$).

(**a**)



(**b**)



Chapter 8. Discussion

This chapter includes an overview of the main findings of this thesis (8.1) followed by their wider implications (8.2). I discuss in the latter the roles of male x female genetic interactions and fluctuating selection in the maintenance of polymorphisms and in driving the apparent increasing diversity of male and female reproductive proteins. I explore the evolution of degeneracy of protein function and conclude with ideas for future work that could be undertaken to increase our understanding of, and ability to detect, sexual conflict in nature.

8.1 Main Findings

8.1.1 Sex peptide receptor and remating interval alter sperm competition dynamics and the benefits of remating in *D. melanogaster* (Chapter 3)

I investigated the role of the sex peptide receptor (SPR) in determining the fitness of first and second males to mate across a range of remating intervals. This study showed significant effects of SPR and remating interval on male fitness through sperm competition. Early rematings with females lacking SPR resulted in significantly higher P2, and higher absolute second male progeny, while early rematings with control females produced relatively equal paternities for competing males. Later rematings resulted in the expected second male precedence across both treatments. The results revealed a novel role for SPR in determining the magnitude of fitness benefits to remating males. The benefits for remating males will therefore be potentially higher if they can avoid remating with recently mated females and instead focus their efforts on mating with females with which they would gain high P2 paternity share. Data suggest that males can detect female mating status (Friberg, 2006) and may be able to adjust their ejaculate accordingly (Wedell *et al.*, 2002, Bretman *et al.*, 2011). However, there has been little work so far to dissect at a finer scale the degree to which males can choose between females based upon optimal remating interval. Further experiments that focus on remating intervals between 0-5h after mating would be very useful to explore the complex mechanisms contributing to the fitness of males in competition. These could include sperm competition experiments with males from lines in which the sperm are labelled, to permit direct measurements of the outcome of sperm in competition in SPR females. Likewise, studies of whether remating decisions

taken by females within this early remating window reflect a lack of sfp efficacy or female 'trading up' for indirect benefits would be illuminating.

8.1.2 The effect of PEB II on early remating in female *D. melanogaster* (Chapter 4)

I investigated the structure of the mating plug formed within females mated to males lacking the mating plug protein, PEBII, and demonstrated that these plugs showed significant differences in structure at 5-10min after the start of mating (ASM). This work was included in the study by Bretman *et al.* (2010). The work linked physical deficiencies in the mating plug with differences in male post-copulatory success. Structural changes in mating plugs may affect the function of sfps involved in early remating responses, since it has been proposed that the mating plug serves a role as a scaffold which directs sperm and sfps to specific locations within the female reproductive tract (Lung & Wolfner, 2001). This idea is supported by the finding that the anterior plug contains Acp36DE, which facilitates the movement of sperm into the sperm storage organs (Lung & Wolfner, 2001, Neubaum & Wolfner, 1999a). Males who transfer reduced levels of PEBII are unable to reduce early female remating. Females who remate earlier will, over the course of their lifetime, have more matings and experience a higher cost of mating (Chapman *et al.*, 1995). Thus in nature, PEBII could be mediating sexual conflict by reducing costly female remating. Further experiments should explore the role of the mating plug in more detail, through targeted manipulation of the major mating plug protein, PEB-me, or through the identification of males carrying polymorphisms for PEB proteins to determine its precise role in determining male and female post mating fitness.

8.1.3 The role of Acp26Aa and Spn2 in determining sperm competition outcomes (Chapter 5)

I examined the roles of two sfps, Acp26Aa and Spn2, in determining sperm competitive ability. Based on the associations between allelic variation in seminal fluid protein loci and sperm competition outcomes reported by Fiumera *et al.* (2005), lack of Acp26Aa in the second male to mate with females was predicted to influence P2 scores. In contrast, data from the same association study suggest that removal of Spn2 should not affect P2. These associations were derived from measures of variation in polymorphic markers found within, or near, the genes coding for these proteins and sperm competition outcomes. I used RNAi to directly test the role of these proteins and found no effect of Acp26Aa or

Spn2 on P2. Therefore the findings of no effect on P2 for either protein only partially conformed to the results of the association tests, and add further to the general discordance between the results of association and functional studies. Association tests are a powerful tool for surveying a large number of genes across large populations in order to observe the role of natural variation in determining gene function (Hirschhorn & Daly, 2005). However, as my experiment shows, caution must be exercised when interpreting the results of such studies because of the inherent limitations due to statistical power, the likelihood of false positives, and the effects of genetic linkage, epistasis and protein degeneracy. In the future, it would be useful to engineer lines bearing different polymorphisms of the same gene (in the same genetic background), in order to measure the direct effects of different alleles on sperm competition outcomes.

8.1.4 Interactions between sex peptide and sex peptide receptor: feeding, fecundity and remating rate (Chapter 6)

I tested for phenotypes under the control of key male and female reproductive proteins, namely sex peptide and its target in females, sex peptide receptor. I measured SPR null and control females left virgin or mated to either SP null or control males, for differences in levels of feeding, egg laying and receptivity. I focussed on whether SPR was required for the partial degeneracy of short term receptivity and egg laying responses observed in mated females that did not receive SP. As predicted, post mating feeding was dependent on the presence of SPR and required the receipt of SP. I did not find significant evidence for degeneracy (either partial or full) within the network of male-female molecular interactions. However, females with SPR that did not receive SP, showed a trend towards intermediate levels of feeding and egg laying. An intriguing conclusion from this result is that SPR is activated by another early acting peptide. Dup99B is as a potential candidate for this activation based on its sequence similarity to SP and its binding affinity for SPR The inclusion of increased feeding rate adds to the list of post mating phenotypes regulated by SPR. Future work should study other candidate phenotypes to determine whether they are involved in the SPR/SP signalling cascade such as ‘siesta sleep inhibition’(Isaac *et al.*, 2010), increases in innate immune responses (Peng *et al.*, 2005b), and reduction in female longevity (Wigby & Chapman, 2005) in order to broaden our understanding of the costs and constraints imposed on the sexes by these interacting proteins and further elucidate the dynamics of coevolution between the sexes.

8.1.5 Seminal fluid protein interactions – sex peptide and Dup99B (Chapter 7)

In the final data chapter, I describe how I tested the hypothesis that Dup99B is responsible for the short-term changes in egg laying and receptivity observed in females mated to SP null males. Using Dup99B and SP single mutants and a Dup99B/SP double mutant, I tested whether the absence of Dup99B would cause a reduction in female post mating responses after 24 and 48h. This was the first study to examine the effect of Dup99B in the absence of SP and it showed no effect of Dup99B on female post mating responses after 24 and 48h. Therefore Dup99B, even in the absence of SP, is not capable of influencing the receptivity or egg laying phenotypes in females despite its sequence similarity to SP and affinity for SPR (Saudan *et al.*, 2002, Yapici *et al.*, 2008). Effects of Dup99B have been reported to last for only 1h after mating (Rexhepaj *et al.*, 2003), so it is possible that these experiments did not have sufficient temporal resolution to detect differences in post mating responses. It would therefore be informative to repeat this study and measure responses at earlier time points post mating. In expanding this work further, future experiments should include the use of double and triple mutants for SP, Dup99B and Acp26Aa in order to include all major sfps believed to increase egg laying. Experiments with these lines would allow the direct comparison of the effects of these sfps within the same background strain of fly.

8.2 Implications and conclusions - why so many reproductive proteins?

This thesis has shown how male and female reproductive proteins impact on the fitness of the individuals involved. Within *Drosophila melanogaster*, over 130 sfps interact with several classes of molecules, including other sfps (either from the same or other males), female reproductive tract proteins, and pathogens (Findlay *et al.*, 2008, 2009, Prokupek *et al.*, 2008, 2009). Selection acting on male and female reproductive success is predicted to be very strong and should lead to low levels of additive genetic variance for these proteins within populations (e.g. Civetta & Singh, 1995, Hughes, 1997). However, molecular studies in a diverse array of animal taxa show that high levels of variation are present and that genes involved in reproduction evolve at an accelerated rate relative to other genes (reviewed in Swanson & Vacquier, 2002). Such variation in reproductive proteins has been detected within the *Drosophila* genome in the form of high levels of polymorphisms, gene duplications, and accelerated rates of loss and gain of reproductive genes (Chapman, 2008). In the following sections I briefly discuss how sexual selection in the form of sperm

competition between rival males, and sexual conflict between males and females, could drive the rapid evolution of sfps, possibly leading to reproductive isolation and speciation. I also explore the idea of degeneracy as a general principle of complexity within biological systems and how it could promote the maintenance of genetic diversity. I then conclude with ideas for why more integrated approaches using networks may be necessary for determining the costs and benefits to each sex of particular reproductive strategies.

8.2.1 Response to sperm competition

Numerous studies have demonstrated significant variation in sperm competition success amongst males. Sexual selection could select for allelic variation as a mechanism for males to compete against the seminal fluid of other males to gain greater paternity share (Chapman, 2008). Duplication of an sfp locus followed by positive selection, which has been widely detected in *Drosophila* (Findlay *et al.*, 2008), could allow sfps to coevolve with other male sfps. Despite very strong selection on genes encoding seminal fluid, polymorphisms are maintained most likely by balancing selection brought about by negative frequency dependence (Prout & Clark, 1996). The outcome of sperm competition is dependent on chemical signalling between females and males and possibly between the ejaculates of rival males. Therefore it is not surprising that the presence of interactions between genotypes of females and males (Clark *et al.*, 1999) and of non-transitivity among male genotypes (Clark *et al.*, 2000) can determine the outcome of sperm competition. These outcomes are dependent on genetic variation in reproductive traits in both sexes (Clark *et al.*, 1995, Fiumera *et al.*, 2005, 2007, Chow *et al.*, 2010) which should then be maintained by these same interactions through fluctuating selection (Clark, 2002, Hall *et al.*, 2008).

8.2.2 Response to sexual conflict

It has recently been shown in *D. melanogaster* that the majority of genes associated with sex-specific fitness are also sexually antagonistic (Innocenti & Morrow, 2010), corroborating the hypothesis that genetic variation for fitness is maintained by sexually antagonistic selection. Previous findings in red deer have shown that optimal genotypes differ between males and females, because a genotype that produces a male phenotype with relatively high fitness will, on average, produce a phenotype with lower fitness when expressed in a female (Foerster *et al.*, 2007). Thus antagonistic selection is unable to

produce an optimal male and female genotype which would otherwise erode levels of genetic variation. Interlocus sexual conflict is predicted to promote the evolution of novel seminal fluid protein genes when the cost of investing in a particular seminal fluid protein become too high, or females become insensitive to it (Chapman, 2008). Duplication of an sfp locus followed by positive selection, could allow sfps to coevolve with receptors in the female reproductive tract (Findlay *et al.*, 2008).

8.2.3 Speciation

Sfps may be involved in the formation of new species (reviewed in Swanson & Vacquier, 2002, Martin & Hosken, 2003b, Clark *et al.*, 2006). Injections of conspecific *Drosophila* accessory gland extracts can rescue an infertile hybrid cross (Fuyama, 1983) which supports the theory that divergence of accessory gland proteins is partly responsible for reproductive isolation. In some *Drosophila* species, it has been shown that sfps are twice as diverse between species as are the non-reproductive proteins in terms of polymorphisms and instances of gene gains and loss (Civetta & Singh, 1995, Swanson *et al.*, 2001). In particular, *Acp26Aa* is one of the fastest evolving genes in the *Drosophila* genome, with a *dN=dS* ratio (non-synonymous *vs.* synonymous amino acid substitution ratio) of 1.6 between *D. melanogaster* and *D. yakuba* indicating that its evolution is strongly driven by positive selection (Tsaur & Wu, 1997, Tsaur *et al.*, 1998). Other accessory gland proteins that show signs of positive selection include *Acp36DE* (Begun *et al.*, 2000) and *Acp29AB* (Aguade, 1999). Sexual conflict may explain increased speciation rates through the rapid evolution of reproductive barriers after colonization of new habitats (Gavrilets, 2000, Gavrilets & Hayashi, 2005). Of particular interest is the possibility that sexual conflict could promote sympatric speciation. Gavrilets and Waxman (2002) show that under some conditions sexual conflict can cause females to diversify genetically into separate groups. In some cases, this can actually mediate conflict by "trapping" the males in the middle at a sub-optimal state. However males may respond to female diversification by diversifying themselves resulting in the formation of new species.

8.2.4 Degeneracy within reproductive proteins

Biological degeneracy is "the ability of elements that are structurally different to perform the same function or yield the same output" (Edelman & Gally, 2001). It differs from the term 'redundant' or 'partially redundant' which refers to identical copies of genes

performing identical roles. Degeneracy is present in all levels of biological organization (Edelman & Gally, 2001). Numerous authors have argued that degeneracy is an emergent property of biological complexity and common to gene networks, neural networks, and evolution itself (Thomas, 1993, Nowak *et al.*, 1997, Brookfield, 1997, Edelman & Gally, 2001, Greenspan, 2001, Frank, 2003).

Degeneracy may arise in order to achieve sufficient expression levels (e.g. recent sfp gene duplications), fidelity in responses across different environments (e.g. genotypes of females / rival males), because of partial overlap in function (e.g. role of sex peptide and Acp26Aa in fecundity), or as fail safe mechanisms (e.g. possibly sex peptide and Dup99B) (Thomas, 1993, Brookfield, 1997). Thus the presence of multiple sfps within redundant functional classes of proteins (such as protease, protease inhibitor, immunity, and lipid metabolism categories) may be relics of the ongoing arms race between male and female reproductive proteins or may reflect degenerate protein functions (Chapman, 2008). Putative degeneracy of sfps provides a tantalizing explanation for the lack of sperm competition phenotypes observed after targeted genetic manipulations of Acp26Aa or Spn2 (Chapter 5) and for the partial compensation of SP-SPR phenotypes observed in the absence of SP (Chapter 6).

8.2.5 Implications for studying sexual conflict and sexually antagonistic coevolution

The ubiquity of male x female interactions and degeneracy of protein function implies that multi-dimensional approaches are required for understanding the impacts of male and female reproductive genes on reproductive success and how these could be shaped by sexual selection / conflict. Quantitative genetic models based on targeted manipulations of genes have provided essential clues for how sexual conflict might operate and evolve. Likewise, statistical approaches such as the association studies reported by Clark *et al.* (1995) and Fiumera *et al.* (Fiumera *et al.*, 2005) (Chapter 5) have been essential for revealing how levels of allelic variation can produce significant differences in male and female reproductive success. What is ultimately required to demonstrate whether particular genes are mediating sexual conflict are more experiments which seek to combine an understanding of the allelic effects of polymorphic and / or degenerate genes with economic models that take into account the full costs and benefits to males and females across multiple generations.

For example, models by Alonzo and Pizzari (2010) demonstrate that a cost to females of mating and the existence of sperm competition alone are insufficient to demonstrate conflict between the sexes and among males over female remating. This is because under some scenarios of large, yet temporary, increases in post mating fecundity, female remating may actually benefit a first male if the second male increases female fecundity such that more of the first male sperm will be used then if she had otherwise not remated (even though remating could still lead to high P2). However, relevant mechanisms, such as sperm displacement / dumping, sperm death, and costs of remating to females would change the costs and benefits for all parties involved, further highlighting the importance of understanding and disentangling all aspects of reproductive behaviour in order to identify the ways in which sexual conflict shapes the evolution of species in nature. Only by looking at the wider picture, can we begin to understand how evolution is shaping the genome of life around us.

Appendix I. Experiment to determine marker lines

Introduction

I planned to test the effect of female remating interval on sperm competition outcomes by manipulating the sex peptide receptor in females (Chapter 3). This required the establishment of a reliable dominant male marker for measuring sperm competition outcomes. 12 dominant marker lines were ordered and backcrossed into the *Dahomey* wild type background and this protocol outlines the experiments used to test them.

I conducted experiments to produce P1 and P2 scores for each line along with matching P1 and P2 scores for the *Dahomey* males they were competed against. The marker line that showed the highest similarity with the P1 scores of their corresponding *Dahomey* males was selected for the experiment testing the effect of remating interval.

We were also interested in comparing this new dominant marker setup against our old sperm competition setup which used the recessive marker: *Sparkling* (*spa*). This allowed for a comparison of *spa* P1 and P2 scores against *Dahomey* and the selected dominant marker line.

Material and methods

Fly stocks

12 individual marker lines were ordered from the Kyoto Drosophila Genetic Resource Center and Bloomington Drosophila Stock Center (**Table AI.1**). They include 11 lines with the *Stubbly* (*Sb*) marker and 1 with the *Brown* (*BwD*) eye colour marker. These lines were backcrossed into the *Dahomey* (*Dah*) background (see Chapter 2 for stock details) for 6 generations. Flies with the dominant marker were selected from the stock bottles and placed on grape juice medium for larva collection at standard density. Heterozygous male offspring with the dominant marker were collected as virgins and used for experiments. Males were kept in single sex vials containing 10 individuals with live yeast until the mating assay at 5 days post-eclosion. Homozygous females and males with the recessive *sparkling* (*spa*) eye phenotype were used for comparisons with the marker lines. Adults were raised and collected as per above. *Dah* virgin female offspring and males were also raised and collected as per above.

Sperm competition assay 1 – measuring P1

At 5 days post-eclosion virgin treatment females were placed singly in yeasted vials (13 treatments containing *Dah* females, 1 treatment with *spa* females). The appropriate male was then introduced into each vial and allowed 2h to mate. Sample sizes ranged from 33-40 double matings per treatment.

11x - *Sb* male mated to *Dah* female
1x - *BwD* male mated to *Dah* female
1x - *spa* male mated to *spa* female
All 13 treatments remated to *Dah* males

1x - *Dah* male mated to *Dah* female
Not remated

Time to mate (latency) and mating duration were noted for each vial. The males were then discarded and the females allowed to lay eggs for 24h. The following day all the females (with the exception of the females mated to *Dah* males) were mated to *Dah* males. Females were given 2h to mate with the competitor male or else were discarded. Latency and mating duration was again noted for each vial. Females were given 24h to lay eggs. Total numbers of progeny produced over 24h were counted and parentage determined based on the presence of *Sb* or the eye colour of the offspring (for the *BwD* and *spa* lines). In the case of *BwD* and *Sb* paternity was deemed to be twice the number of offspring observed with the marker, since the fathers were all heterozygous. Sterile females and females that had offspring of only one marker type were omitted from the analysis.

Assay 1 analysis

The dominant marker males were screened to eliminate any lines which behaved in an obviously poor fashion. This was based on extreme P1 values (i.e. P1=0) and / or poor mating performance or poor fertility compared with the *Dah*/*Dah* treatment.

Sperm competition assay 2 – measuring P2

The exact set up as above was repeated, except that females were first mated to *Dahomey*. Also, no *Dah*/*Dah* treatment was set up. P2 was determined as above.

Statistical analysis to determine the best marker line

Mean P1 of each marker line was compared to the mean P1 of its corresponding *Dah* competitor with independent, pairwise generalized linear model tests in R 2.12.0 with quasibinomial errors (R Development Core Team, 2010, Crawley, 2007). Mean first mating latency of the marker lines were compared to *Dah* males using multiple Mann-Whitney tests in SPSS 16 (SPSS, Inc., Chicago IL). Mean first mating durations of the marker lines were compared to *Dah* males using ANOVA and Dunnet-t post hoc tests with *Dah* set as the control.

The line which showed the most non-significant differences in sperm competition outcomes and mating characteristics to *Dah* males was selected for use in further experiments.

Results

Stubbly line B was selected for further tests since it was the only line to show no significant differences in P1, mating latency, or mating duration in comparison to *Dahomey* males **(Table AI.2, Figures AI.1, AI.2)**. Although multiple lines showed no significant difference in P1 and mating latency, only line B passed all three tests. *Spa* males performed well in P1 tests compared to *Dah* males and they showed similar mating durations. This verified there use as good competitors in sperm competition experiments conducted in our lab (for example, Chapter 5)

Figure AI.1. P1 comparisons between multiple marker lines and *Dahomey* after performing reciprocal crosses over 2 experiments. *Stubbly* lines B and K, along with the recessive marker line *spa*, produced a similar P1 result as did *Dahomey* males in reciprocal crosses. Refer to **Table AI.1** for the identity of the specific lines

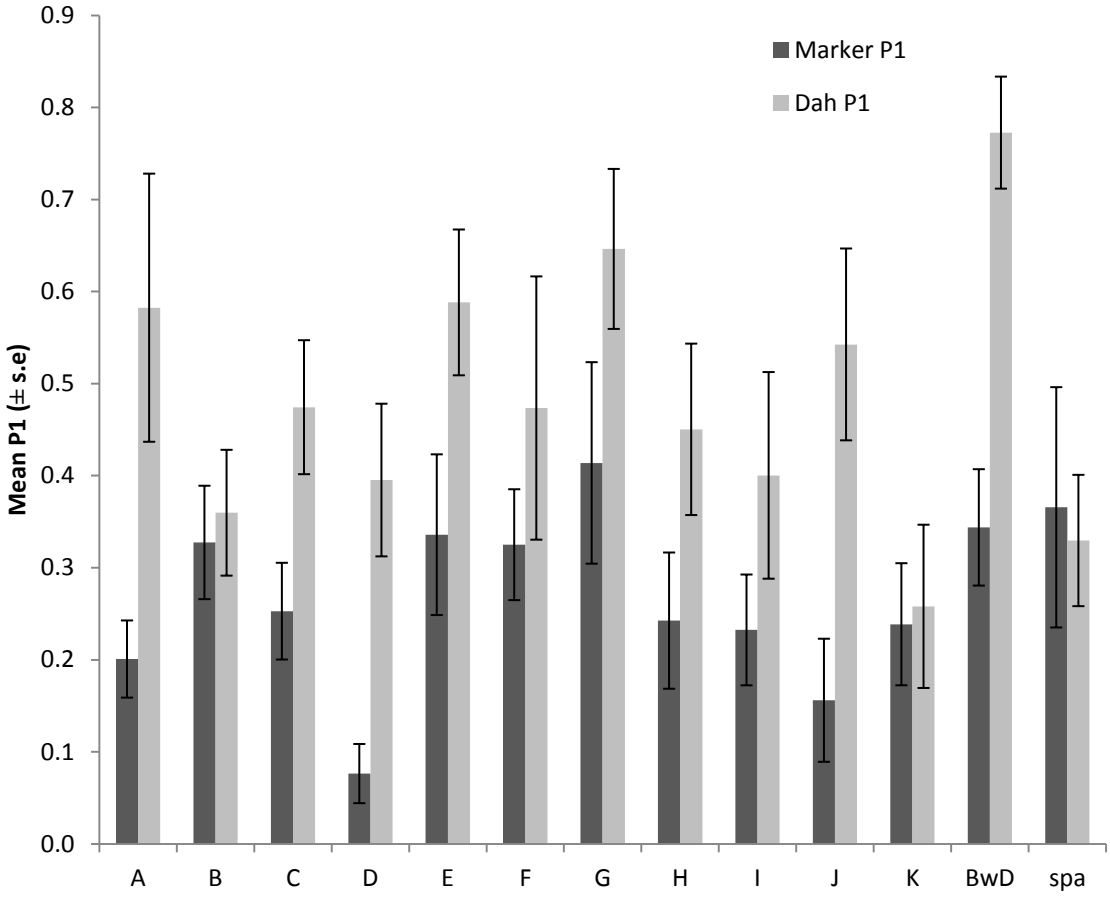
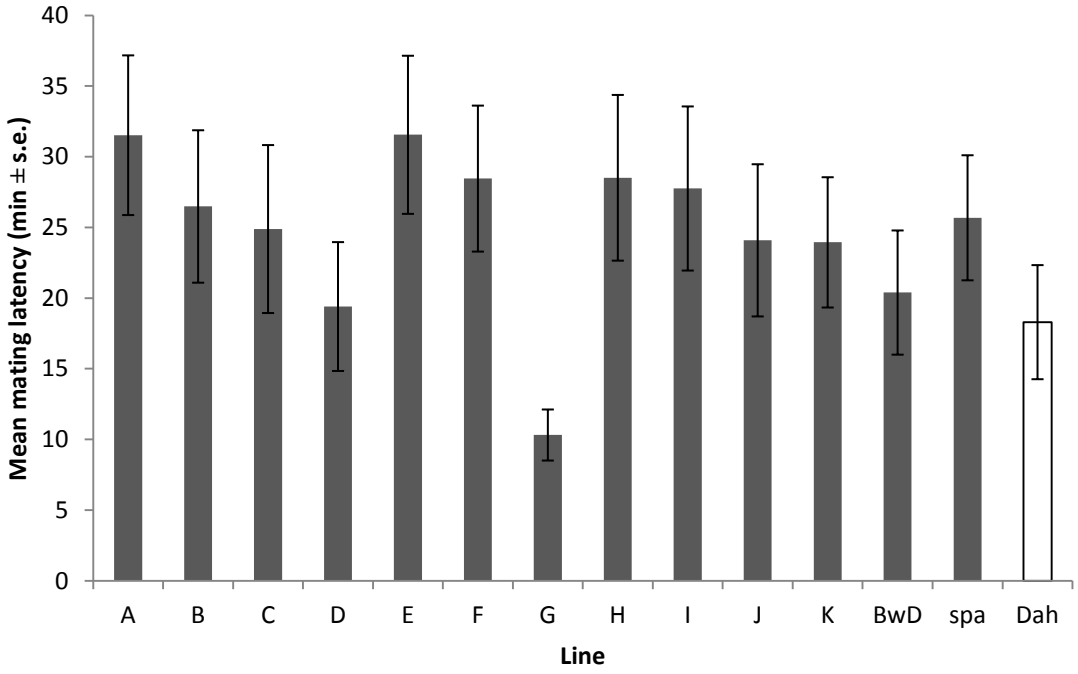


Figure AI.2.(**a**) Mean time to mate (latency) for each marker line and for wild type *Dahomey* males mated to *Dahomey* females. (**b**) Mean mating duration for each marker line and for wild type *Dahomey* males mated to *Dahomey* females. White bar indicates the *Dahomey* line to which all means were compared. Refer to **Table AI.1** for the identity of the specific lines

(**a**)



(**b**)

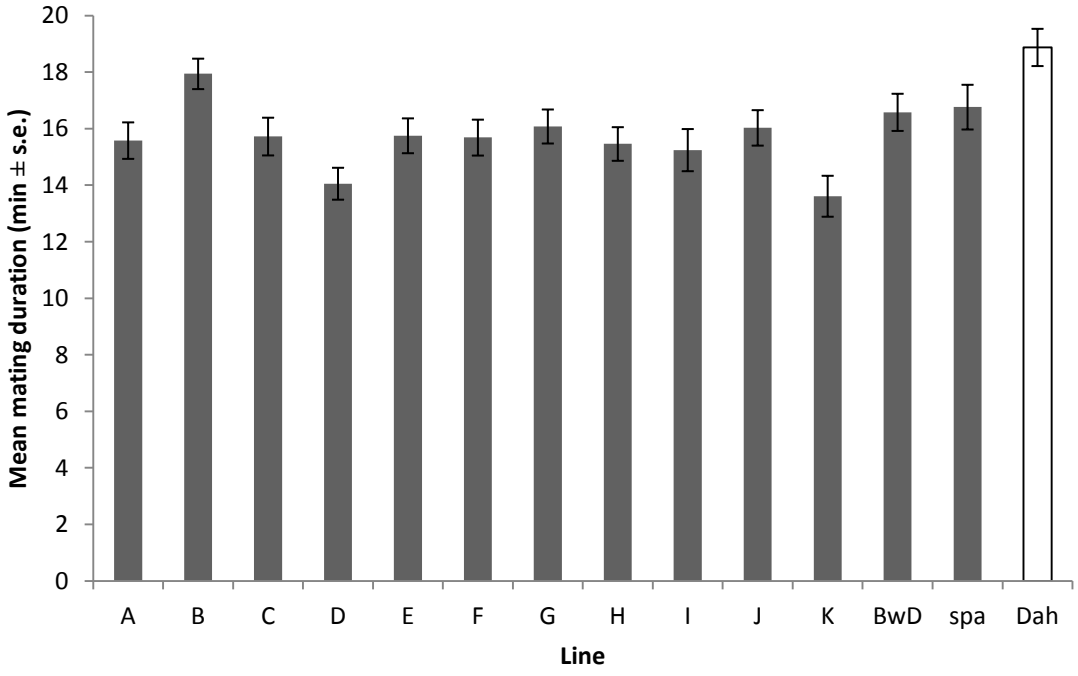


Table AI.1. Sources of the dominant marker lines used in the marker test sperm competition assay. Included is the collection from where they were ordered, a legend which shows the code used in **Figure AI1** and **Figure AI2**, the original genotype and the originating stock number.

collection	legend	original genotype	stock #
Bloomington Drosophila Stock Center	A	*Sb[1]/In(3LR)Ubx[101], Ubx[101]*	585
	B	*Birmingham; Sb[1]/TM6*	2539
	C	*w[*]; Sb[1]/TM3, P[w[+mC]=ActGFP]JMR2, Ser[1]*	4534
	D	*w[1]; Sb[1]/TM3, P[w[+mC]=GAL4-Hsp70.PB]TR2, P[w[+mC]=UAS-GFP.Y]TR2, y[+] Ser[1]*	5704
	E	*S[1]/SM1; Sb[1]/TM6*	8616
	F	*w[*]; spir[I83]/CyO; Sb[1]/TM3, Ser[1]*	8722
Drosophila Genetic Resource Center (Kyoto)	G	*Sb[1]/In(3LR)Ubx[101], Ubx[101]*	105994
	H	*W[1] Sb[1]/In(3LR)Cx*	106019
	I	*R[1] D[1] red[1] Sb[1]/TM6*	106454
	J	*D[1] red[1] Sb[1]/TM2*	106498
	K	*w[1]; Sb[1]/TM3, P[w[+mC]=GAL4-Hsp70.PB]TR2, P[w[+mC]=UAS-GFP.Y]TR2, y[+] Ser[1]*	107496
		bw[D]	101339

Table AI.2 Significance values for the comparisons conducted between marker lines and wild type *Dahomey* males. The mean values (± s.e.) are presented in **Figures AI.1-2**. Mean P1 of each marker line was compared to the mean P1 of its corresponding *Dah* competitor using GLM tests with quasibinomial errors. Mean first mating latency of the marker lines were compared to *Dah* males using multiple Mann-Whitney tests. Mean first mating durations of the marker lines were compared to *Dah* males using ANOVA and Dunnet t post hoc tests with *Dah* set as the control. Refer to **Table AI.1** for the identity of the specific lines. Dark shading highlights the selected *Stubbly* marker line, B, which was used for further tests. Lighter shading highlights the *spa* and *BwD* lines which have been used in previous tests.

Line	P1	latency	duration
A	<0.01	0.186	<0.01
B	0.640	0.224	0.963
C	<0.05	0.929	<0.01
D	<0.01	0.782	<0.001
E	0.083	0.033	<0.01
F	0.384	0.244	<0.01
G	0.283	0.530	<0.05
H	0.141	0.286	<0.01
I	0.333	0.159	<0.001
J	<0.05	0.219	<0.05
K	0.329	<0.05	<0.001
BwD	<0.001	0.822	0.096
spa	0.927	<0.05	0.156

Appendix II. A mating plug protein reduces early female remating in *Drosophila melanogaster*. Journal of Insect Physiology 56:107-113.

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A mating plug protein reduces early female remating in *Drosophila melanogaster*

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ABSTRACT

Mating plugs are formed within the female reproductive tract during mating from male ejaculate constituents or even from male genitalia themselves. Across species, mating plugs have roles in sperm storage and the prevention of female remating. In the fruitfly *Drosophila melanogaster*, accessory gland proteins such as the sex peptide are known to reduce female remating, however this effect can take some time to establish, hence other ejaculate components must also be involved. We hypothesised a role for the PEBII mating plug protein in the prevention of early female remating. Using RNA interference we produced PEBII knockdown males. We found that these males were significantly less able to prevent female remating in the 4 h following mating. The mating plugs produced by PEBII knockdown males also showed lower levels of autofluorescence in the first 10 min after the start of mating, suggesting they differed in composition to those of control males. Reduced levels of PEBII had no effect, however, on fecundity, progeny production or egg–adult viability in the first 24 after mating, suggesting there were no short-term effects of PEB II on sperm transfer, storage or use. Our results show that PEBII has a subtle but significant role in the prevention of early female remating.

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1. Introduction

In many species, a mating plug is formed within the reproductive tract during and/or after mating. Such plugs have been shown to have a range of functions related to sperm competition (e.g. Mann, 1984; Eberhard, 1996; Birkhead and Møller, 1998; Simmons, 2001). Most obviously, plugs form a physical barrier to prevent remating or to prevent loss of sperm female reproductive tract. In butterflies, plugs (referred to as sphraga) physically prevent remating and are also large and elaborate, perhaps to act as visual deterrents to other males (Orr and Rutowski, 1991). Plugs can reduce female receptivity in the short term (especially if they must be expelled to allow oviposition), or in extreme cases such as the bumblebee *Bombus terrestris*, the plug permanently switches off female receptivity (Baer et al., 2001). The constituents of mating plugs vary, and can comprise proteins or lipids (Mann, 1984), but plugs can also be formed from part of the male's genitalia, the formation of which irreversibly damages the male intromittent organ (e.g. in spiders Fromhage and Schneider, 2006) or even results in male death (e.g. stingless bees Colonello and Hartfelder, 2005). The mating plug can also signal the dominance of the previous male to further prospective mates, as for example in the Iberian rock lizard *Lacerta monticola* (Moreira et al., 2006). In some cases the mating plug is also essential for females to stimulate oviposition (Melo et al., 2001).

It has been suggested that the mating plug in *Drosophila* may not prevent remating, as females remain unreceptive until after plug is degraded (Eberhard, 1996). However, this overlooks the possibility that females are unreceptive because of chemical constituents of the plug, and this is the topic we investigate in this study. Recent evidence suggests that the mating plug in *Drosophila* reduces female remating and facilitates sperm storage. Experimental reduction of the mating plug size through manipulating mating duration in *Drosophila hibisci*, for example, demonstrates how the plug reduces female remating by inhibiting courtship by other males and by reducing female receptivity (Polak et al., 2001). Polak et al. (1998) also showed that the plug ensures sperm storage; smaller plugs from previously mated males could not prevent back-flow of sperm away from the sperm storage organs or out of the female.

In *Drosophila melanogaster*, the mating plug comprises two major portions; the posterior portion contains proteins synthesised in the male ejaculatory bulb (PEB-me, PEBII and PEBIII), and proteins from the male accessory gland (including Acp36DE) are contained within the anterior plug (Lung and Wolfner, 2001). The posterior plug is formed within 3 min after the start of mating (ASM) (Lung and Wolfner, 2001) but before sperm transfer

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(Gilchrist and Partridge, 2000), hence the sperm may permeate or move around the plug before the anterior plug is formed at 20 min ASM. Males lacking Acp36DE (the anterior plug) transfer normal amounts of sperm, but fewer of their sperm are stored and females mated to these males consequently have fewer offspring (Neubaum and Wolfner, 1999).

We investigated here the effect of one of the constituent proteins of the *D. melanogaster* mating plug, PEBII, on female receptivity immediately after the first mating. PEBII was singled out for testing because of its potential involvement in sexual conflict between the evolutionary interests of males and females. Association studies have linked variation at the PEBII locus to increased female death rate following single matings (Fiumera et al., 2006). Hence PEBII is predicted to be subject to selection arising from sexual conflict because its transfer and recruitment to the mating plug presumably increases male fitness, but seems to have a negative side effect on the fitness of the females with which the male mates. Given the reported effect of mating plugs on female receptivity in other *Drosophila* species (Polak et al., 2001) and the potential involvement of PEBII sequence variation in determining mating costs (Fiumera et al., 2006), we predicted that loss of PEBII would cause females to remate more quickly. We focused on the early remating phenotype for two reasons. First, because there were no reported associations between PEBII variation and female refractoriness when tested at longer time points such as 48 h after mating (Fiumera et al., 2005), suggesting that any receptivity effect would be seen at earlier time points. Second, we had noticed anecdotally from observations of mixed sex groups containing PEBII knockdown males, that females often remated again almost immediately (MKNL, unpublished observations).

We tested the prediction that PEBII plays a role in early female remating by developing lines of flies in which PEBII was knocked down using RNA interference, so the effect of PEBII could be examined directly. The speed with which females remated immediately after having mated to control (producing full mating plugs) or knock down (producing plugs with reduced levels of PEBII) males was then measured in two independent lines of PEBII knockdown and control males. We also characterised the degree of autofluorescence of the mating plugs formed in females mated to one of the PEBII knockdown lines at 5–10 and 20 min ASM to determine whether there were gross changes in the structure of mating plugs with reduced PEBII. To examine whether any effects on early remating were associated with fecundity or fertility differences, we then conducted a third experiment in which females were mated singly to PEBII reduced or control males.

2. Materials and methods

2.1. Fly rearing and food media

Fly rearing and all experiments were conducted in a 25 °C humidified room, with a 12:12 h light:dark cycle, on standard sugar-brewer's yeast-agar (SY) medium (Bass et al., 2007) supplemented with live yeast granules. Wild-type flies were from a large, outbred laboratory population originally collected in Dahomey (Benin) (Bass et al., 2007).

2.2. Generation of PEBII knockdown lines

PEBII knockdown males were generated by RNAi using the UAS/Gal4 system, as described in Chapman et al. (2003). Briefly PEBII knockdown males were produced by cloning tail-to-tail inverted repeats of a 763 bp sequence including the entire PEBII gene into the pWIZ vector (Lee and Carthew, 2003). The structure of this vector, named pPEBII-IR, was verified by multiple restriction digestions. Transgenic flies were then constructed using standard methods as described in Chapman et al. (2003) by injection of pPEBII-IR into a w^1 genetic background. Homozygous viable and fertile stocks were obtained by backcrossing *white*$^+$ individuals to the w^1 injection stock and then crossing *white*$^+$ individuals *inter se*. Thus the genetic background of the lines remained that of the injection stock.

Two independent and stable homozygous lines were obtained, PEBII 13-7 and PEBII 8-1. PEBII knockdown males were derived by crossing males from PEBII 13-7 and PEBII 8-1 stocks to virgin females from a ubiquitous driver line (*Actin Gal4/CyO* in the w^{1118} background, stock 4414 from the Bloomington Stock Centre). The efficiency of knockdown was confirmed by using quantitative PCR with SYBR green probes and standard procedures (as in Chapman et al., 2003). qPCR confirmed that significant knockdown was achieved through RNAi, however, there was a difference between the two lines in the efficiency of the knockdown; there was a 16-fold and 2-fold reduction of PEBII in lines 13-7 and 8-1, respectively.

2.3. Effect of PEBII transfer on immediate remating

The effect of PEBII on female remating was tested by first mating wild-type females to experimental males (knockdown or control) and then challenging them immediately with wild-type males. We used both of the PEBII knockdown lines obtained to test for repeatability, but also for dosage effects, as we knew that line 13-7 had a much more effective knockdown than line 8-1. Males from each knockdown line were mated with virgin females from the *Actin Gal4/CyO* driver line. Hence two types of control were necessary, to control for the chromosomal location of the *Gal4* driver and for the location of each of the 2 PEBII inverted repeat constructs. We also equalised as far as possible the degree of red eye-colour in the controls in comparison to the experimental lines. This was necessary because the degree of orange pigment in the eyes (as determined by the copy number of transgenic inserts marked with w^+) affects male mating success (AB and TC unpublished data) presumably because *white*-eyed males are visually impaired. Therefore, we controlled for this by creating control and experimental lines all with 2 transgene copies. To do this we crossed the *Actin Gal4/CyO* driver and PEBII inverted repeat lines separately to a transgenic line that contained a non-functional *UAS-Sex Peptide* construct (*UAS-SP-stop*, marked with w^+) in the same w^1 background as used for the PEBII inverted repeat lines. Specifically, knockdown controls were created by crossing PEBII 13-7 and 8-1 knockdown line males to virgin females from the *UAS-SP-stop* line, and the driver controls from crossing *UAS-SP-stop* males with *Actin Gal4* driver virgin females. The non-functional *UAS-SP-stop* construct has no detectable effects on any other mating traits tested so far and is simply used here to control for eye-colour.

Larvae from these crosses and from the Dahomey population were maintained at a standard density of 100 per vial. At eclosion offspring were collected using ice anaesthesia and separated into single sex vials at 10 per vial. At 4 days post-eclosion males were transferred singly into experimental vials using ice anaesthesia. The following day females were aspirated singly into the mating vials. Introduction time, copulation start and end time were noted, with great care taken not to disturb the vials (as this is known to significantly shorten mating duration, AB and TC unpublished data). Flies that did not mate within 1 h were discarded. After mating had ended the female was aspirated into a vial containing a wild-type male and again the introduction, start and end of mating times were noted. The transfer time between the end of the first

mating and the introduction of the female into the second vial was 15.1 ± 0.44 min (mean ± S.E.) and transfer time was not significantly different for treatment, line or the interaction (ANOVA line: $F_{1,453} = 0.009$, $P = 0.93$; treatment: $F_{2,452} = 0.41$, $P = 0.66$; line × treatment: $F_{2,452} = 0.005$, $P = 0.99$). Females were allowed 4 h to remate (final n per treatment 70–84).

2.4. Visualisation of mating plug in PEBII knockdown and control males

Wild-type Dahomey females were raised at standard densities (100 larvae per vial) and then stored 10 per vial until use in experiments. Females were mated to PEBII 13-7 knockdown or to control males (with separate controls for the PEBII 13-7 inverted repeat and for the *Gal4* driver, as above). Mated females were frozen in liquid nitrogen either 5–10 or 20 min ASM and stored at −80 °C until dissection. We chose these time points based on previous work that identified peak times for mating plug formation (Lung and Wolfner, 2001). Females were dissected in PBS and their lower oviducts, extending from the ovipositor to just anterior of the spermathecae, were removed. Dissected reproductive tracts were mounted on slides in PBS with a cover slip gently placed over the top. Slides were viewed using a Ziess Plan Apochromat 10×/0.45 Ph1 objective and a DAPI reflector on a Ziess Axioplan 2 microscope (using the Henry Wellcome Biomedical Imaging Facility at UEA). Mating plugs were easily visualized because of their autofluoresce (Lung and Wolfner, 2001). Images were recorded using an AxioCamHR CCD camera at identical exposure settings and viewed using AxioVision LE software. Unmodified images were printed on a HP Laserjet P1505n printer. Five respondents were then asked to score the images (blind with respect to identity) on a scale of 0–3 based on levels of fluorescence in the anterior and posterior area of the lower oviduct (with 0 = no fluorescence to 3 = highly fluorescent; Fig. 1). We chose this method, rather than digital quantification of fluorescence in the mating plugs, to reduce the effect of subjective decisions about what defined the edge of these diffuse and variable structures. The sample sizes were between 8 and 26 for each of the six time point/treatment combinations.

2.5. Effect of PEBII transfer on mating duration, fecundity, egg–adult survival and progeny production

To examine whether any effect of PEBII on early remating was mediated through effects on fecundity and egg fertility (i.e. possible short-term effects on sperm transfer or use), we conducted a third experiment. We examined fecundity and egg–adult viability of females in the 24 h following matings to PEBII knockdown and control males. We used the same PEBII 8-1 and 13-7 lines as above, but this time drove PEBII knockdown using a *Tubulin Gal4/TM3 Sb* driver line that had been backcrossed to the Dahomey wild-type background. Experimental males were the *PEBII/Tubulin Gal4* male offspring of PEBII 8-1 and 13-7 inverted repeat lines crossed to the *Tubulin Gal4* driver line, and controls were the male *PEBII/TM3Sb* offspring from the same cross. We also had an additional common control, the male offspring from the cross between *Tubulin Gal4/TM3 Sb* and *white*^{1} (the injection stock). Females were raised at standardised densities and mated singly with PEBII knockdown or control males. Matings were observed and mating durations recorded as above. Following matings, females were placed in oviposition vials. Eggs from the first 24 h period were counted and the vials then retained in order to count the emerging progeny 12–14 days later (final n per treatment 95–99).

2.6. Statistical analysis

Analysis of mating latency was done using SPSS v14.0 (SPSS Inc., Chicago, IL, USA). Data were tested for normality using Kolomogrov–Smirnov tests and for homogeneity of variance using Bartlett tests and transformed to normality where possible. Normally distributed data with homogeneous variances were analysed using ANOVA, non-normal data using non-parametric tests. Egg–adult survival data were analysed using analysis of deviance of general linear models (GLM) with quasi-binomial errors. Fluorescence scores were transformed into a percent and analysed using a general linear mixed effects model (GLMM) (R2.8.0, nlme library). Treatment, responder, and their interaction were treated as fixed effect variables. Responder nested in picture identity was treated as a random effect variable in order to correctly assess variation

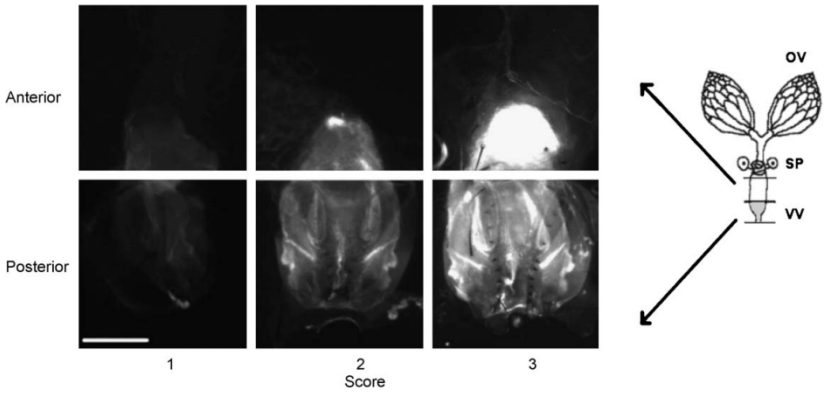

Fig. 1. Mating plugs showing representative autofluorescence scores of 1–3. Female reproductive tracts were dissected as described in Section 2. Images of the lower oviduct were divided into a posterior region consisting of the cuticle surrounding the vulva and ovipositor and an anterior region extending to below the spermathecae. Images were scored on a scale of 0–3 based on levels of fluorescence in the anterior and posterior area of the lower oviduct (with 0 = no fluorescence to 3 = highly fluorescent). *Abbreviations*: OV, ovaries; SP, spermathecae; VV, vulva. Scale bar = 75 μm.

across responders. Helmert contrasts (Crawley, 2007) were used to compare the experimental line to the controls and the controls against each other.

3. Results

3.1. Effect of PEBII transfer on immediate remating

We found that females mated to PEBII knockdown males subsequently mated significantly more quickly to wild-type males than did females that had previously mated to control males (Fig. 2). This was highly significant for line 13-7 males, with females in the experimental group remating 30 min faster than those in the control groups (Fig. 2A; ANOVA: $F_{2,236} = 9.95$, $P < 0.001$). Line 8-1 showed a consistent but non-significant trend in the same direction (Fig. 2B; ANOVA: $F_{2,217} = 0.56$, $P = 0.57$), in line with the fact that PEBII knockdown was less efficient in this line. Combined probabilities (Sokal and Rohlf, 1981) across both lines revealed that mating latency was significantly faster in females mated to PEBII knockdown males ($\chi^2_4 = 19.54$, $P = 0.0006$).

There was a significant difference in the proportion of females remating between treatments in line 13-7. (Experimental = 86%, driver control = 69%, knockdown control = 76% remating. All treatments $\chi^2_2 = 8.05$, $P = 0.018$; experimental versus knockdown control only $\chi^2_1 = 5.6$, $P = 0.018$.) Although this was also significant for line 8-1 ($\chi^2_2 = 5.9$, $P = 0.05$) this was caused by the difference between the driver control and the other two treatments. In line 13-7 there was no difference between treatments in the time taken to initiate the first mating (ANOVA: $F_{2,277} = 0.76$, $P = 0.93$). However, treatments did differ in duration of the first mating, but this was caused by the knockdown control group which mated for a shorter duration than the other groups and so this cannot explain the pattern in latency to remate (all treatments Kruskal–Wallis $\chi^2_2 = 20.3$, $P < 0.0001$, driver control versus knockdown Mann–Whitney $U_1 = -1.24$, $P = 0.22$).

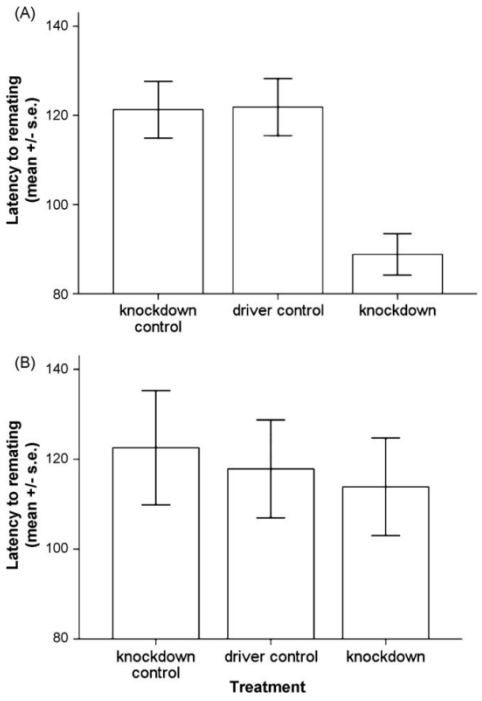

Fig. 2. Latency to remating (mean ± S.E.) for females previously mated to PEBII knockdown inverted repeat control (knockdown control) or *Gal4* driver control males. In line 13-7 (A) females mated to knockdown males remated significantly quicker than those mated to control males, when subsequently presented with a wild-type male. For line 8-1 (B) this trend was not significant, although a combined analysis across both lines revealed a significant decreased in latency in females receiving reduced PEBII.

Table 1
Summary results of general linear mixed effects model on anterior and posterior autofluorescence scores of mating plugs in females mated to PEBII knockdown (Kd) vs. control males (DC and KC, where DC = *Gal4* control and KC = PEBII inverted repeat control).

	df	*t*	*P*
5–10 min ASM			
Anterior fluorescence score			
Kd vs DC and KC	58	−1.099	0.276
DC vs KC	58	−0.317	0.752
Posterior fluorescence score			
Kd vs DC and KC	58	−2.761	0.008
DC vs KC	58	0.941	0.351
20 min ASM			
Anterior fluorescence score			
Kd vs DC and KC	25	1.092	0.285
DC vs KC	25	2.008	0.056
Posterior fluorescence score			
Kd vs DC and KC	25	−0.339	0.737
DC vs KC	25	−1.923	0.066

3.2. Visualisation of mating plug in PEBII lacking and control males

At 5–10 min after the start of mating (ASM), there was a consistent pattern of females mated to PEBII knockdown males having lower mating plug fluorescence scores than females mated to control males (Table 1, Fig. 3). This effect was significant for posterior fluorescence 5–10 min ASM ($t_{58} = -2.761$, $P < 0.01$) but not for the anterior fluorescence scores ($t_{58} = -1.099$, $P = 0.276$). At 20 min ASM, there was no consistent pattern or significant difference in the degree of anterior or posterior fluorescence between the mating plugs of females mated to PEBII knockdown or control males. Importantly, although there were significant differences in the way in which responders scored the different images (supplemental table), there were no significant interaction effects, hence no suggestion of any systematic bias due to responder.

3.3. Effect of PEBII transfer on mating duration, fecundity, egg–adult survival and progeny production

There were no significant differences between females mated to PEBII producing or lacking males (Table 2) in fecundity, egg–adult survival or progeny production in the first 24 h after mating. In this experiment there were also no differences in mating duration across the different male genotypes (Table 2).

4. Discussion

The most important result from our study was that PEBII, one of the ejaculatory bulb proteins that form the *D. melanogaster* mating plug (Lung and Wolfner, 2001) caused females to be significantly less likely to remate again in the 4 h after mating. Hence our hypothesis that PEBII plays a role in early remating was confirmed. In one line, knockdown of PEBII increased the number of females remating within 4 h by 10% and reduced the time females took to

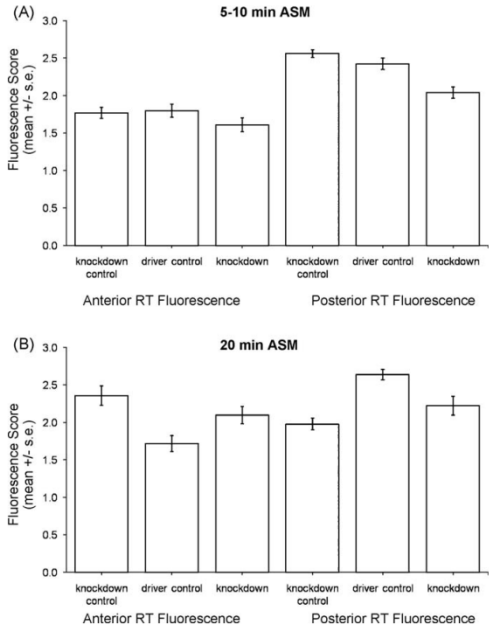

Fig. 3. Mean fluorescence scores (0 = no fluorescence, 3 = highly fluorescent, ±S.E.) of lower reproductive tract (RT) of wild-type females (A) 5–10 min and (B) 20 min after the start of mating. Females were mated to PEBII knockdown (PEB13-7), inverted repeat control (knockdown control) or *Gal4* driver control males. Fluorescence was scored separately for the anterior and posterior RT.

remate by 30 min. The effect was consistent but weaker in the other line, where the level of PEBII knockdown was much less, but the overall effect across both lines was significant. We also detected consistent differences between PEBII knockdown and control males in the form of the mating plug that was produced. There was less autofluorescence in both the anterior and posterior mating plug at 5–10 min ASM, although the effect was only significant in the posterior portion, and this effect disappeared by 20 min ASM. There was no effect of PEBII on fecundity or progeny production following single matings. Hence there is no evidence consistent with the idea that the remating effect is mediated through differences in fecundity or fertility. The evidence overall shows that the mating plug, specifically PEBII, has a subtle but significant effect on reducing early remating by females.

The effect of components of the ejaculate on remating has long been established (Manning, 1962, 1967; Scott, 1987). However, it is often overlooked that females can require several hours to develop the full refractory response, and most receptivity tests are usually conducted only after 24 or even 48 h (e.g. Chapman et al., 2003). In the experiments described in this study it was typical for 70–75% of females in control lines to remate within 4 h, and 30–50% remating by wild-type females within 6 h has been reported previously (Vanvianen and Bijlsma, 1993). Thus the effect of PEBII can still be evident even above high rates of remating. To date the seminal fluid protein most characterised in terms of female receptivity is the male accessory gland sex peptide (SP). The effects of SP on female remating have been assessed both by injection of purified or synthetic SP (Chen et al., 1988), and by using knockout (Liu and Kubli, 2003) and knockdown males (Chapman et al., 2003). Receptivity of females mated to SP-null males was very low after 4 h (<5% remating within 1 h) and comparable to that of control matings, but after 12 h, 60% of SP-null mated females were willing to remate (Liu and Kubli, 2003). This indicates that SP is not responsible for the reduction in receptivity immediately after mating and that other molecules such as PEBII play a separate role. It is possible that PEBII and SP could affect female remating by different routes. For example, SP increases female rejection behaviours such as ovipositor extrusion (Chen et al., 1988; Liu and Kubli, 2003). PEBII might also exert its effects via this route, or

Table 2
Effect of PEBII transfer on mating duration, fecundity, egg–adult survival and progeny production. Females were mated singly to males lacking PEBII (knockdown lines 13-7 or 8-1), or PEBII producing control males (knockdown control for each line or driver control common to both lines, see text for details). Fecundity (egg) and progeny counts were from the first 24 h after mating. (A) Mean (±S.E.) for mating duration (min), fecundity and progeny numbers, and median (interquartile range) egg to adult survival (i.e. progeny produced/eggs laid). (B) Results from ANOVA of the effect of male genotype on mating duration, fecundity and progeny number. (C) The effect of male genotype on egg to adult survival data was analysed using a GLM analysis of deviance with quasi-binomial errors. Egg–adult survival and mating duration were used as factors, the dispersion parameter for line 13-7 was 34.72 and for line 8-1 was 33.46.

	PEBII 13-7 inverted repeat control	PEBII 13-7 knockdown	PEBII 8-1 inverted repeat control	PEBII 8-1 knockdown	Common *Gal4* driver control
(A)					
Mating duration	18.67 (0.79)	18.72 (0.99)	18.56 (0.85)	19.61 (0.67)	19.57 (0.56)
Fecundity (eggs per 24 h)	83.59 (3.51)	83.28 (3.88)	74.84 (4.01)	73.57 (4.08)	82.19 (3.77)
Progeny number	74.67 (4.35)	72.53 (4.96)	66.13 (5.48)	65.39 (4.18)	71.14 (5.07)
Egg–adult survival	0.97 (0.84–1.00)	0.91 (0.82–1.00)	0.96 (0.83–1.00)	0.90 (0.85–0.97)	0.94 (0.88–1.00)

	PEBII 13-7 knockdown *vs* controls			PEBII 8-1 knockdown *vs* controls		
	F	*df*	*P*	*F*	*df*	*P*
(B)						
Mating duration	0.45	2, 95	0.64	0.72	2, 96	0.49
Fecundity (eggs per 24 h)	0.07	2, 99	0.93	0.35	2, 99	0.70
Progeny number	0.07	2, 99	0.93	0.18	2, 99	0.31

	PEBII 13-7 knockdown *vs* controls				PEBII 8-1 knockdown *vs* controls			
	df	Deviance	*F*	*P*	*df*	Deviance	*F*	*P*
(C)								
Egg–adult survival	1	55.92	0.80	0.45	1	55.82	0.83	0.43
Mating duration	1	57.61	0.83	0.44	1	133.89	2.00	0.14
Egg–adult survival × mating duration	1	50.46	1.45	0.23	1	55.44	1.66	0.20

could modulate female attractiveness via changes to pheromones. The receptivity pathways affected by PEBII will be interesting to investigate in future experiments. Our study emphasises an interesting, and perhaps overlooked, observation, that remating rates can be higher under continuous confinement with males in comparison to the situation when females are separated from males and then re-exposed. There could be two separate phenomena at work. First, the development of non-receptivity may take time, and during this period females may be susceptible to remating if males are around. Second, the development of non-receptivity may itself interact with presence or absence of males.

Polak et al. (2001) suggested that the *Drosophila* plug prevents remating. Manipulations of mating plug size in that study were achieved through varying copulation duration, hence those experiments vary many different components of the ejaculate and also curtail and alter the balance of transfer of particular types of ejaculate components, which is not uniform, at least in *D. melanogaster*, where different seminal compounds are transferred before and after sperm (Lung and Wolfner, 2001). The use of knockdown lines produced by RNAi was an advantage because it allowed us to specifically target one component of the mating plug whilst leaving all others unaffected. Using this technique we showed here that the mating plug structure differed when levels of PEBII were reduced. At 5–10 min ASM, the plugs of PEBII knockdown males were less fluorescent, which suggests that they contain less material in total, or that the plug diffuses more quickly (Lung and Wolfner, 2001). However, this difference was not evident at 20 min ASM, which could mean that at that time all plugs had become either more diffuse or more consolidated. Mating duration, though it was not associated with PEBII mediated remating latency, could have been associated with differences in plug fluorescence, e.g. between the control lines at 20 min, possibly through the increased transfer of other fluorescent plug components. However, this was not supported as a general explanation by the finding that there were no consistent differences between male genotypes across the two experiments in terms of mating duration.

Aside from the ability to prevent recently mated females from mating again, no other role has yet been detected for PEBII in determining male reproductive fitness, e.g. in sperm competition or in female remating at later times ASM (e.g. Fiumera et al., 2005). Our data are consistent with these earlier findings, as we also found no evidence that PEBII affects egg production, progeny number or egg to adult survival in the first 24 h after mating. Hence there is no evidence that the effect of PEBII on early female remating was mediated through differences in egg production or through differences in the transfer, storage or use of sperm shortly after mating. Our results do not, however, rule out longer-term effects on these traits.

Significant variation has been detected in PEBII transcript abundance among males leading to large fold differences among males in PEBII expression (Fiumera et al., 2005). Together with our results here this suggests that there may be considerable variation in the degree to which the PEBII phenotype on early remating is expressed. There are also correlations across PEBII and transcript abundance of 7/9 Acps tested (Fiumera et al., 2005) despite the fact that PEBII and the Acps are expressed in different parts of the male reproductive system. Furthermore, there are significant associations between sequence variants of PEBII and Acp53 (Fiumera et al., 2005). These associations may mean that males may be constrained in their ability to transfer optimal levels of PEBII. Conflicts between the optimal level of transfer of specific ejaculate proteins is an interesting possibility for further study.

The fact that sequence variation at or near the PEBII locus was associated with increased death rate in females following single matings (Fiumera et al., 2006) suggests that PEBII could play a role in mediating sexual conflict. Our findings suggest a plausible mechanism, females receiving lower levels of PEBII transfer will remate at higher frequency and hence incur higher costs of mating (e.g. Chapman et al., 1995). Why males might transfer lower levels of PEBII, which presumably decreases their reproductive success by increasing the frequency of early female remating, is not clear. The reasons for the striking variability among males in the amount of PEBII transcript made are therefore currently unknown. In terms of the mechanisms by which females remate so very quickly, we do not yet know whether females accept a lower level of courtship, or whether those females are more attractive than females mated to control males, which would be interesting to test in the future.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.jinsphys.2009.09.010.

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