Sexual conflict in *Drosophila melanogaster*

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Abstract

Conflicts between the sexes over mating and reproduction are common in many taxa. In *Drosophila melanogaster* sexual conflict over mating frequency occurs because male fitness is maximised by a higher mating frequency than that which maximises female fitness. In addition, females incur survival and reproductive costs from mating frequently. The female cost of mating is mediated by male accessory gland proteins (Acps), which are transferred to females in the seminal fluid of males during mating. Theory predicts that sexual conflict can lead to antagonistic coevolution between the sexes in which males evolve traits that benefit males but harm females and females evolve traits to minimise the extent of male-induced harm. This coevolution may be rapid and has the potential to promote speciation.

In this thesis I used *Drosophila melanogaster* as a model organism to investigate the evolution and genetics of sexual conflict. In Chapters 3 and 4 I used selection lines in which the level of sexual conflict had been experimentally manipulated. Chapter 3 describes how females in the selection lines adapted to the level of sexual conflict by evolving different levels of resistance to male-induced harm. Chapter 4 shows that the selected females did not differ in pre-mating resistance to males, suggesting instead that the resistance to male harm had evolved via post-mating changes. Chapter 5 describes how a single Acp, the sex peptide (SP), which reduces female receptivity and increases egg production after first matings, also causes female mating costs. Chapter 6 confirms that SP benefits males by increasing their post-mating reproductive success. Thus, SP benefits males but harms females suggesting that the *SP* gene underlies sexual conflict in *D. melanogaster*. Finally, Chapter 7 summarises the thesis and discusses future directions for investigating sexual conflict in *Drosophila.*

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Declaration

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

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Stuart Wigby

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Chapter 1. General Introduction

1.1 Sexual selection and sexual conflict

Darwin (1871) introduced the concept of sexual selection after considering why in many species there are physical and behavioural differences between the sexes and why some of these sex-specific traits do not appear to increase the ability of individuals to survive. For example, the males of some insects possess appendages on their abdomens that help to prevent females from escaping during copula, and in many bird species males have large plumages that serve to attract females but that seem unlikely to increase the survival of the bearers (Darwin, 1871). Darwin realised that the fitness of individuals was not only dependent on their ability to survive but was also dependent on the ability of individuals to gain mates and produce fit offspring. Darwin concentrated on the way in which variation in secondary sexual characteristics between individuals determined the mating success and reproductive success of those individuals relative to others of the same sex and species (Darwin, 1871). For over a century following Darwin's discussion of sexual selection, the main focus of research in this area was on competition and conflict between individuals of the same sex (usually males) or on the preference of individuals (usually females) for the 'best' quality mates. For example, the results of a study on elephant seals suggested that males fight for social status to gain access to females (Le Boeuf & Peterson, 1969) and the results of a study on long-tailed widowbirds suggested that female long-tailed widowbirds preferred males with experimentally extended tails (Andersson, 1982). Interactions between the sexes for the purposes of reproduction were often considered to be relatively cooperative and harmonious (Krebs & Davies, 1993). However, over the last three decades traditional thought has been overturned by the idea that traits involved in mating and reproduction can instead be shaped by conflicts of interest between males and females (e.g. Trivers, 1972; Dawkins, 1976; Parker, 1979). Because there are physical and behavioural differences between the sexes and because males and females have different roles in reproduction, there can be differences in the evolutionary interests of the sexes over reproductive decisions (sections 1.2 and 1.3, below). As a result, traits expressed in both sexes (section 1.2) or sex specific traits (section 1.3) that promote the genetic interests of one sex to the detriment of the other sex may evolve antagonistically between the sexes. The last thirty years has seen increasing numbers of studies on a wide range of sexual organisms that support

the idea that such evolutionary conflicts of interest between the sexes (sexual conflicts) are widespread.

1.2 Types of sexual conflict and sexually antagonistic evolution

1.2.1 Intralocus sexual conflict

There are two manifestations of sexual conflict: intralocus and interlocus conflict. I give here a brief description of intralocus conflict, though the primary focus of this thesis is intersexual conflict (described in section 1.2.2). Traits that are expressed in both sexes but for which the fitness optima differ between males and females, create intralocus conflict (conflict between the sexes over the same gene). A hypothetical example comes from the evolution of human hip width (Rice & Chippindale, 2001). During the evolution of modern humans the hip width of females appears to have increased to accommodate the increasing head width of foetuses at birth (Lavelle, 1995), despite the potential cost to the efficiency of locomotion (Rice & Chippindale, 2001). However, genes that increase hip width are likely to have been selected against when expressed in males because males would not have gained the benefits at child birth from increased hip width but would have still suffered the potential locomotion cost (Rice & Chippindale, 2001). Thus, adaptations that benefit individuals of one sex may result in reduced fitness when the same adaptation is expressed in individuals of the opposite sex (Parker & Partridge, 1998). This may lead to each sex impeding the evolution of the other (Rice, 1984; Slatkin, 1984; Rice, 1992). Antagonistic evolution between the sexes may ensue, whereby selection acts in opposing directions when the trait is expressed in different sexes. This is predicted to lead to selection for sex limitation of the antagonistic genes (Rice, 1984; Rhen, 2000; Gibson et al., 2002) potentially resulting in sexual dimorphism or possibly genomic imprinting (Day & Bonduriansky, 2004).

Experimental evidence for intralocus sexual conflict has thus far been largely limited to studies on *Drosophila melanogaster* (e.g. Rice, 1992; Vieira et al., 2000; Chippindale et al., 2001) because of the ability of researchers to use genetic manipulations which are currently unavailable in many other organisms. For example, Chippindale et al. (2001) cloned *D. melanogaster* haploid genomes, placed them in random wild-type genetic backgrounds and measured the fitness of males and females containing the cloned haplotypes. The study showed that the fitness of haplotypes was negatively correlated between males and females as adults,

suggesting that genomes which produce high fitness adult individuals of one sex produce low fitness adults in the other sex (Chippindale et al., 2001). Importantly, there was no negative correlation in the fitness of male and female haplotypes at the larval stage (Chippindale et al., 2001) during which the optima for most traits are likely to be highly concordant across the sexes. The results of Chippendale et al. (2001) are therefore consistent with intralocus conflict theory because conflict is only predicted to occur when there are differences in the optima for males and females. Intralocus conflict could potentially be common among species (Rice & Chippindale, 2001). However, the evolutionary importance of intralocus conflict might depend on the rate at which sex limitation evolves. If sex limitation is rapid then intralocus conflict will be resolved quickly and periods when each sex constrains the evolution of the other will be of relatively short duration. For example, from studies on mammals and birds there is evidence of rapid adaptive change in sexual size dimorphism (the difference in body size between males and females) suggesting that there are genetic mechanisms that may rapidly minimize intralocus conflict (reviewed in Badyaev, 2002).

1.2.2 Interlocus sexual conflict

Genes involved in interlocus conflict are sex-limited and code for behavioural, physical or physiological traits in one sex whose effects result in a reduction in fitness for individuals of the opposite sex with which they interact. Traits can evolve that harm individuals of the opposite sex if those traits elevate the spread of the genes encoding the harming traits (e.g. if they increase the fitness of the harming individual, despite the costs they cause to mates). Conflict can occur over interactions such as courtship, mating frequency, time or place of mating, mate choice, fertilization, rate of female reproductive output, total female reproductive output, clutch size or parental care (reviewed in Chapman et al., 2003b). For example, in the yellow dung fly, *Scatophaga stercoraria*, sexual conflict occurs at mating because females are sometimes damaged or drowned as males compete for access to females (Parker, 1979). Thus, male *S. stercoraria* appear to be subject to selection for traits that increase their competitive ability in the struggle for access to females even if this sometimes leads to female damage or death (Parker, 1979). Individuals of the sex whose fitness is most reduced by the interaction are predicted to evolve traits to minimise the extent harm sustained (resistance). In the example of the yellow dung fly, females are predicted to be selected to avoid or resist the male-

induced harm. If resistance by the harmed sex reduces the beneficial effects of the harming trait to the harming sex then theory predicts that there will be selection on individuals of the harming sex to overcome this resistance by increasing the level of their harming trait. Thus, interlocus conflict may lead to sexually antagonistic coevolution in which members of each sex evolve traits that elevate their own fitness relative to other members of their same sex but at the expense of members of the opposite sex (Rice, 1996). It is predominately interlocus conflict that is investigated and discussed in this thesis. Therefore, except where stated otherwise, the term 'sexual conflict' is henceforth used to refer to interlocus conflict.

Sexual conflict can result in a reduction in fitness of individuals of one sex as a result of fitness-enhancing adaptations in individuals of the opposite sex. Thus, there is predicted to be variation in the costs and benefits of antagonistic sexual interactions for each sex as a result of natural variation in the traits involved in sexual conflict. However, it should be noted that at the population level, when there is an equal sex ratio, the average fitness of males and females are always equal (Fisher, 1930; Arnqvist, 2004). Thus, a reduction in the fitness of ~~fitness~~ one sex resulting from interlocus conflict leads to a reduction in the fitness of the population as a whole (Arnqvist, 2004), because male fitness is achieved through females (Fisher, 1930).

1.3 Contrasting sexual selection and sexual conflict models of evolutionary change

Traditional coevolutionary models of sexual selection focus on the way in which females gain from choosing the 'best' males. Females could gain from choosing 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 could also gain indirect genetic benefits from choosing males that are successful at gaining matings because the offspring of such males are also likely to be successful at gaining mates. For example, in field crickets, *Gryllus bimaculatus*, males that are successful at gaining matings sire sons that are also successful at gaining matings (Wedell & Tregenza, 1999). Such indirect genetic benefits have the potential to result in a genetic association between the female preference and male attractiveness (i.e. the Fisher 'runaway' process; Fisher, 1930; Lande, 1981; Kirkpatrick, 1982; Iwasa & Pomiankowski, 1991; Pomiankowski et al., 1991). Also, females may

prefer males that provide 'good genes' for traits such as offspring growth and survival (e.g. peacocks; Petrie, 1994), or parasite resistance (reviewed in Siva-Jothy & Skarstein, 1998). The Fisherian runaway process and 'good genes' are not mutually exclusive theories of sexual selection but represent opposite ends of the indirect genetic benefits model of sexual selection (Kokko et al., 2002).

Sexual conflict models of coevolution differ from models of coevolution via traditional sexual selection in that they focus on the way in which females are harmed or exploited by males and the way in which females evolve to minimise the resulting fitness loss (e.g. Parker, 1979; Arak & Enquist, 1993; Arak & Enquist, 1995; Holland & Rice, 1998; Wachtmeister & Enquist, 2000; Gavrilets et al., 2001). Traditional models of sexual selection do not consider harm to females or males resulting from interactions between the sexes. Sexual conflict models of evolutionary change show that males and females can coevolve antagonistically, with each new adaptation that benefits males decreasing the fitness of females with which they interact and *vice versa*. Consequently, sexual conflict models make different predictions about the evolution of female 'preferences' to those made by sexual selection models, and may be based on different assumptions. For example, Fisherian sexual selection models predict that the strength of female preferences for a male display trait will increase with increased levels of the male display trait, whereas the 'chase-away' model of Holland and Rice (Holland & Rice, 1998; see section 1.4.1) predicts that female preferences will decrease as the male display trait increases. Also, the mate-choice model of Gavrilets et al., 2001 is based solely on sexual conflict over mating patterns (see section 1.4.1) and is not constrained by the assumptions of traditional sexual selection models such as positive genetic correlations between male traits and female preferences, (Lande, 1981; Kirkpatrick, 1982; Pomiankowski et al., 1991; Iwasa & Pomiankowski, 1995) and the existence of condition dependent male traits, (Rowe & Houle, 1996; Iwasa & Pomiankowski, 1999). Sexually selected indirect benefits to females are predicted to occur even in a conflict scenario (e.g. Cordero & Eberhard, 2003), because the most harmful males could be the fittest and therefore sire the fittest male offspring. However, such indirect benefits to females may be small (Kirkpatrick & Barton, 1997; Cameron et al., 2003) when compared to the direct costs that females can incur from males (see section 1.6). The relative importance of indirect benefits in sexual conflict is,

however, a current topic of debate (e.g. Chapman et al., 2003a; Chapman et al., 2003b; Córdoba-Aguilar & Contreras-Garduño, 2003; Eberhard & Cordero, 2003).

1.4. Models of sexual conflict

1.4.1 Models of sexual conflict over mating

The first formal theoretical exploration of sexual conflict provided by Parker (1979) cited the example of yellow dung flies, *Scatophaga stercoraria*, in which males are selected for traits that increase their competitive ability relative to other males in the struggle for access to females, even if this harms females (described in section 1.2.2, above). Females are therefore predicted to be subject to selection to avoid or reduce the costs of being harmed by males. Parker's (1979) model considered a trait that yielded a mating advantage to males but caused a cost to females. Parker (1979) recognised that females would gain indirect benefits from mating with harmful males but realised that if the direct cost to females exceeded the indirect benefit, then sexual conflict would exist. The model showed that, under certain conditions, sexual conflict over mating could result in unpredictable and irresolvable evolutionary chases (Parker, 1979). This suggests that traits involved in sexual conflict in males and females can coevolve indefinitely. Clutton-Brock and Parker (1995) modelled ways in which males may coerce females into mating (forced copulation, harassment and intimidation) and found that such forms of coercion are predicted to evolve to levels 'where they have appreciable costs to both sexes' (Clutton-Brock & Parker, 1995). The multitude of ways in which females avoid coercion (reviewed in Clutton-Brock & Parker, 1995) or evolve traits that reduce the frequency or duration of matings (e.g. in water striders, dung flies and bean weevils; sections 1.6.1. and 1.6.3.) suggests that costs of mating in females are potentially high and can have important evolutionary implications for both sexes. Empirical evidence for sexual conflict over mating is discussed in section 1.6.

1.4.2 Models of sexual conflict over male displays and mate choice

The chase-away model of Holland & Rice (1998) is a verbal model based on the idea that male sexual display traits evolve to exploit pre-existing female sensory biases (sensory exploitation) and that females, in response, evolve reduced preference for the male traits (resistance). In the chase-away model, evolutionary chases between males and females over a male display trait can reach a resolution which is predicted to lead to selection on males to 'recurrently evolve new display

traits' (Holland & Rice, 1998). Mathematical models based on artificial neural networks suggest that males can evolve to exploit the hidden preferences of females through courtship or secondary sexual characters (Arak & Enquist, 1993). Such models contrast with the chase-away model in that they suggest that equilibria between male signals and female receptors might never occur and thus neither sex may 'win' the conflict (Arak & Enquist, 1993; Arak & Enquist, 1995; Wachtmeister & Enquist, 2000). A quantitative genetic model by Gavrilets et al. (2001) considered female mate choice resulting from the avoidance of mating costs. The outcome suggested that 'sexual conflict over mating patterns alone could drive rapid coevolution of male display traits and costly female resistance to these traits' (Gavrilets et al., 2001). Consistent with the chase-away model of Holland and Rice (1998) the model of Gavrilets et al. (2001) predicts that females will deviate little from their optimum mating pattern but that males may be left with costly display traits whose sole purpose is to 'achieve threshold levels of stimulation in females' (Holland & Rice, 1998). Thus, unlike the 'no winner' models of Arak and Enquist (1993, 1995) the model of Gavrilets et al. (2001) and the chase-away model of Holland and Rice (1998) suggest that females can end up 'winning' (or at least losing less than males do) antagonistic coevolution over male sexual displays.

1.4.3 Models of male-induced harm: 'deliberate' harm or a side effect?

Male-induced harm to females may be an unselected side-effect of male-benefiting adaptations (Parker, 1979; Chapman et al., 1995; Morrow et al., 2003a). Alternatively, males could gain directly from harming females if harmed females were more likely to delay remating and consequently delay the onset of sperm competition and/or sperm displacement (Johnstone & Keller, 2000). In addition, males could gain from harming their mates if females responded to harm by reallocating resources from somatic maintenance to current investment in reproduction. This might occur because the future reproductive value of harmed females would be decreased (Lessells, 1999). This would benefit males by increasing the rate of fertilizations in their mates.

The few empirical studies that test the 'deliberate' harm hypotheses do not support the idea that males gain directly from harming their mates. One prediction from Johnstone and Keller's (2000) model is that the more harm females encounter (e.g. the more males and matings they encounter) the less frequently females should

remate. A study in the dung fly, *Sepsis cynipsea*, a species in which females attempt to dislodge courting males by shaking (Hosken et al., 2003), provided results that contrast with this prediction. Hosken et al. (2003) found that female reluctance to mate (shaking duration) decreased with increasing numbers of previous copulations (Hosken et al., 2003). Thus, it appeared that the more harm females had received, the more willing they were to mate - a finding that opposes the prediction from the model of Johnstone and Keller (2000). This suggests that in *S. cynipsea* male-induced harm is not directly selected in males, but is a pleiotropic effect of other, unknown, male-benefiting adaptations (Hosken et al., 2003; see also Pitnick & García-González, 2002). The available data therefore suggest that, instead of benefiting males, the harming of females appears to be associated with increases in female remating frequency. This is predicted to reduce male fitness because if females remate quickly then males encounter sperm competition and sperm displacement sooner, leading to reduced paternity. A study involving three insect species (*Callosobruchus maculatus, Drosophila melanogaster* and *Tribolium castaneum*) found that females did not responsd to generalised harm by delaying remating or increasing current investment in reproduction (Morrow et al., 2003a). The female insects were experimentally harmed by either ablation of a leg, an antenna or a wing, or by a puncture to the thorax or the abdomen. However, these methods of harm infliction and the magnitude of harm were unlikely to have realistically simulated natural male-induced harm. Therefore, for future studies it will be crucial to understand the mechanism by which male-induced harm occurs and to test the deliberate harm theory by experimental manipulations of natural harming mechanisms.

1.4.4 Models of sexual conflict over parental care

Sexual conflict is predicted to occur between the sexes over parental care in species where parental care is provided by both sexes. Each parent would prefer greater investment by the other parent in the care of their offspring (Trivers, 1972; Clutton-Brock, 1991). Thus, there is the potential for sexually antagonistic coevolution of behavioural adaptations that influence the investment in offspring by each parent (Chapman et al., 2003b). For example, males can gain fitness by mating with multiple females. However, in pair forming species, in which both parents contribute parental care (e.g. in many bird species and in insects such as burying beetles), it is in the interests of females to inhibit their mates from copulating with other females.

This is because the effort invested by a male in extra-pair copulations could instead be invested in the offspring of the primary pair, which would increase the inclusive fitness of the female parent (Westneat & Sargent, 1996; Wachtmeister & Enquist, 2000; Royle et al., 2002). Consequently, behavioural adaptations in females are predicted to evolve to enforce monogamy in their mates (e.g. in starlings, *Sturnus vulgaris,* Eens & Pinxten, 1996; in burying beetles, *Nicrophorus defodiens*, Eggert & Sakaluk, 1995). In males, adaptations are predicted to evolve to resist female control (e.g. 'peace-keeping' in the cichlid fish, *Lamprologus ocellatus*, Walter & Trillmich, 1994). Sexually antagonistic coevolution in behavioural traits associated with parental care is theoretically similar to other types of sexually antagonistic coevolution (Chapman et al., 2003b) and may be a potent evolutionary force in species in which parents provide care for their offspring.

1.4.5 Models of sexually antagonistic coevolution and speciation

Sexually antagonistic coevolution is predicted to drive rapid, non-directional evolutionary change in traits associated with mating and reproduction. Thus, sexually antagonistic coevolution could promote divergence in allopatric (geographically separated) populations, potentially facilitating reproductive isolation and speciation (Rice & Holland, 1997; Rice, 1998). In a model by Gavrilets (2000), sexual conflict over mating rate was predicted to lead to rapid coevolutionary change in reproductive traits in both sexes, promoting the formation of reproductive barriers in allopatric populations. Interestingly, the formation of reproductive barriers was predicted to occur faster in large populations (Gavrilets, 2000), which is the opposite to the prediction made by earlier models of allopatric speciation via sexual selection in which smaller populations were predicted to diverge faster (e.g. Lande, 1981). However, the model of Gavrilets (2000) has been questioned because of concerns about the generality of the assumptions of the model (Tregenza et al., 2000). For example, the model of Gavrilets (2000) assumes that sexual conflict will occur over matings between morphologically, genetically, or physiologically incompatible males and females. However, Tregenza et al. (2000) argue that matings between incompatible male and female phenotypes will be rapidly selected against and thus the potential for conflict will be limited. Another model suggested that reproductive isolation could also occur in sympatry (without geographic separation) via conflict over mating rate (Gavrilets & Waxman, 2002). In this model females can diversify genetically into separate groups without geographic isolation and, if

the proportion of males that are compatible with a given female is sufficiently small, males can also diversify (Gavrilets & Waxman, 2002). This diversification in sympatry can theoretically lead to the formation of reproductively isolated groups (Gavrilets & Waxman, 2002).

Game theory models suggest that both intralocus and interlocus sexual conflict could play major roles in speciation (Parker & Partridge, 1998). However, when incipient speciation occurs in sympatric populations or in populations mixing after a period of allopatry 'females will act as a force favouring pre-mating isolation and males as a force against it' (Parker & Partridge, 1998). Thus, the probability of speciation may depend upon whether the strength of selection is greater in males to force mating or in females to avoid mating and also upon which sex has the greater 'power' to enforce it's evolutionary interests (Parker & Partridge, 1998).

1.5 Empirical methods for investigating sexual conflict and sexually antagonistic coevolution

Demonstrating the occurrence of sexual conflict is theoretically straightforward. If an action or trait of one sex reduces the inclusive fitness of the members of the opposite sex with which they interact (i.e. if the sexes have different fitness optima for the action or trait) then sexual conflict may be operating. Measurements of life history traits such as survival, fecundity and fertility combined with various experimental manipulations have been used to detect sexual conflict. Examples of experimental manipulations used to detect sexual conflict include altering the ability of females to resist matings (e.g. Arnqvist & Rowe, 1995), altering the ability of females to control copulation duration (e.g. Crudgington & Siva-Jothy, 2000), altering mating rate (e.g. Fowler & Partridge, 1989; Baer & Schmid-Hempel, 1999; Baer & Schmid-Hempel, 2001; Morrow & Arnqvist, 2003), removing mate choice (e.g. Pitnick & García-González, 2002) and altering the number of parental carers (e.g. Royle et al., 2002). Evidence for sexual conflict is discussed in the next section (1.6).

Sexually antagonistic coevolution (Rice, 1996) is the predicted adaptation and counter-adaptation of sexes in response to sexual conflict (see section 1.2.2). Sexual conflict is a pre-requisite for sexually antagonistic coevolution but the occurrence of sexual conflict does not necessarily mean that sexually antagonistic coevolution will

occur. For example, there may be genetic or environmental constraints that prevent adaptation in response to sexual conflict. Demonstrating sexually antagonistic coevolution is less straightforward than demonstrating sexual conflict and studies so far have, at best, only provided indirect evidence. To provide direct evidence would require firstly demonstrating the occurrence of male adaptations that harm females and female adaptations for resistance, changing over time. Secondly, the change in adaptations over time would have to alter the costs and benefits of interactions between the sexes. Techniques that have been used to investigate sexually antagonistic coevolution include experimental evolution (artificial selection) (e.g. Rice, 1996; Holland & Rice, 1999; Martin & Hosken, 2003; see section 1.6.8), cross-species comparative studies (e.g. Civetta & Singh, 1995; Bergsten et al., 2001; Arnqvist & Rowe, 2002a; Swanson & Vacquier, 2002; see section 1.6.9) and intra-specific population crosses (e.g. Andrés & Arnqvist, 2001; Brown & Eady, 2001; see section 1.6.10). However, the lack of both current theory and knowledge of the mechanism of conflict in many study species, in addition to fact that genes underlying sexual conflict have not been identified in any species, means that not all the data from these studies can be said to provide clear evidence for sexually antagonistic coevolution. For example, a recent model (Rowe et al., 2003) questioned the reliability of predictions about the outcome of intra-specific population crosses that have been used to infer sexually antagonistic coevolution in a number of studies (see section 1.6.10). Also, some experimental evolution studies had potentially confounding differences in inbreeding and body size between treatments that weaken the strength of evidence for sexually antagonistic coevolution (reviewed in Chapman et al., 2003b; see section 1.6.8).

1.6 Empirical evidence for sexual conflict and sexually antagonistic coevolution over mating, in the insects and other invertebrates

The largest body of empirical evidence for sexual conflict and sexually antagonistic coevolution comes from studies on species in which there are potential conflicts over mating frequency, mate choice and paternity assurance. It is sexual conflict over mating frequency in the model species *Drosophila melanogaster* upon which this thesis focuses. There is the potential for sexual conflict over mating in polygamous species such as *D. melanogaster* because the mating frequency that maximises male fitness tends to exceed that which maximises female fitness (Bateman, 1948). This stems from anisogamy, the difference in size of the gametes

of males and females. It is often assumed that the small gametes of males are energetically 'cheaper' to produce than the larger gametes of females (Parker et al., 1972). A consequence of anisogamy is that females generally invest more resources into offspring production (e.g. via the production of nutritious eggs) than do males (Trivers, 1972). Thus, the potential lifetime reproductive success of females tends largely to be determined by the resources available to invest in processes such as egg production and parental care, whereas the lifetime reproductive success of males tends largely to be determined by the number of females available for insemination (Clutton-Brock & Vincent, 1991). This leads to selection on males to seduce or coerce females into mating with them (even when it is not in the female's interest to do so) and selection on females to resist superfluous matings (examples reviewed in Clutton-Brock & Parker, 1995).

Females should resist mating when the costs of mating outweigh the benefits of mating, or the costs of not mating (e.g. 'convenience polyandry' in water striders, Rowe, 1992; sections 1.6.1 and 1.6.2). If there is variation in the harmfulness of male courtship, harassment, intimidation, coercion or copulation itself, then females may bias copulation or fertilization towards certain male phenotypes (creating mate or sperm choice) to minimise harm and maximises their own fitness. Thus mate or sperm choice could potentially occur as a side effect of the avoidance or minimization of male-imposed mating costs (Arnqvist, 1992; Crean & Gilburn, 1998; Friberg & Arnqvist, 2003). Examples of the types of mating costs that females can incur (Daly, 1978) include morphological damage received during mating (e.g. in *Callosobruchus maculatus*, Crudgington & Siva-Jothy, 2000, and *Sepsis cynipsea* Blanckenhorn et al., 2002; section 1.6.3); post-mating harm incurred from male seminal fluid proteins (e.g. in *Drosophila melanogaster*, Chapman et al., 1995; section 1.6.4); energetic costs of mating caused by events occurring before, during and after mating (e.g. in water striders, Watson et al., 1998 and *Idotea baltica*, Jormalainen et al., 2001; section 1.6.1); increased predation risk (in water striders Rowe, 1994, and *Photinus collustrans*, Wing, 1988; section 1.6.2); risk of parasite infection (e.g. in *Adalia bipunctata*, Hurst et al., 1995; section 1.6.5) and lowered immunity (e.g. in *Tenebrio molitor*, Rolff & Siva-Jothy, 2002; section 1.6.7).

Whilst the optimum mating frequency of females tends to be lower than that of males, females also often derive benefits from multiple mating (see section 1.6.6).

However, it is in the interests of males to delay or even completely prevent their mates from remating, to avoid sperm competition or displacement. In many species, including *Drosophila melanogaster*, the last male to mate has an advantage in sperm competition ('last male precedence', Kaufman & Demerec, 1942; Prout & Bundgaard, 1977; Scott & Richmond, 1990; Scott & Williams, 1993; but see also Mack et al., 2003) and therefore the reproductive output of a male can be greatly reduced when their mate subsequently remates with a different male. Thus, sexual conflict over mating includes post-mating conflict over the length of time before females remate (section 1.6.6). Section 1.6 discusses empirical evidence for sexual conflict over mating. There is considerable evidence for sexual conflict over mating in vertebrate species, such as conflict over male-induced forced copulation and male harassment and punishment for female that refuse to mate (reviewed in Clutton-Brock & Parker, 1995). However, I focus here primarily on examples of sexual conflict in invertebrates.

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

Mating is expected to have energetic costs for both sexes. When it is not in the interest of females to mate, superfluous matings are predicted to cause some energetic cost, which is expected to result in reduced fitness. Females in many insect species often show considerable reluctance to mate and males have evolved a number of adaptations to persuade, or force, females to mate. Male adaptations such as mate guarding, courtship and attempted copulation can also cause energetic costs in females and these costs can be large in some species. For example, in the water striders (Heteroptera; Gerridae) the mating system is characterised by intense pre-mating contests in which longer struggles tend to result in copulation and shorter struggles do not (Arnqvist, 1992). Female energy expenditure increases considerably during pre-mating struggles and during mating itself (Arnqvist, 1992; Rowe, 1994; Watson et al., 1998; Ortigosa & Rowe, 2002). Furthermore, female energy expenditure in pre-mating struggles is higher than energy expenditure during mating which suggests that when females accept matings it may be, at least partly, to reduce the energetic costs (and to reduce the predation risk, see section 1.6.2) of the pre-mating struggle (Watson et al., 1998). Thus, superfluous matings may represent the 'best of a bad job', or 'convenience polyandry' for female water striders (Rowe, 1992; Watson et al., 1998).

There is evidence for energetic costs associated with pre-mating activity in Dipterans. In the dung fly, *Sepsis cynipsea*, females try to dislodge males that attempt to copulate with them by shaking vigorously (Blanckenhorn et al., 2000). This behaviour is consistent with sexual conflict over mating, and shaking females presumably incur energetic costs. However, such costs may be relatively minor when compared with the costs incurred from mating itself (Muhlhauser & Blanckenhorn, 2002; see section 1.6.3). In *Drosophila melanogaster* there is evidence that non-mating exposure to males is costly to females. Such costs could be a result of receiving male courtship (Partridge & Fowler, 1990). Female fitness has been found to covary negatively with male courtship frequency which also suggests that there is a female cost resulting from male courtship (Friberg & Arnqvist, 2003). Females of the isopod, *Idotea baltica*, suffer energetic costs associated with pre-copulatory mate guarding (Jormalainen et al., 2001). Males guard females before mating to fend off other males, presumably to avoid sperm competition. Females struggle against males attempting to initiate guarding and the struggle is energetically costly for females (Jormalainen et al., 2001). Furthermore, females suffer a cost from being guarded by males in that they produce smaller eggs than unguarded females (Jormalainen et al., 2001). This may explain why females struggle against males that attempt to initiate guarding.

1.6.2 Evidence for sexual conflict arising from predation risk associated with mating

Sexual interactions can potentially increase the probability of predation by inhibiting locomotion or increasing the conspicuousness of the individuals involved. For example, in water striders, in addition to the energetic costs (section 1.6.1), a major female mating cost is an increased risk of predation during pre-mating struggles and during mating itself (Rowe, 1994). Also, in the firefly, *Photinus collustrans*, the flightless females have to leave their burrows to gain matings, to flash responses to signalling males (Wing, 1988). This exposes the females to a much higher risk of predation than when they are inhabiting their burrows (Wing, 1988). *P. collustrans* females only mate once in their lifetime which has been suggested to be an adaptation to avoid exposing themselves to predation risk more than is necessary (Wing, 1988).

1.6.3 Evidence for sexual conflict arising from morphological damage to females from male adaptations associated with mating

There is evidence from several species of insect that females can incur physical harm during pre-mating struggles or as a result of copulation. Probably the best known example is that of the yellow dung fly, *Scatophaga stercoraria*, in which females can sometimes be torn, contaminated with liquid dung or even drowned in dung as males battle with each other for access to females (Parker, 1979). In addition, a survival cost in females has been identified in *S. stercoraria* (Hosken et al., 2002b) but it is unclear whether the survival cost in this experiment was due to mating *per se* or due to other components of reproduction. Females were collected from the field and hence varied in age and only one sample of offspring production was measured (Hosken et al., 2002b). Hence, it is also unclear as yet whether there is an overall female fitness cost to mating in this species. In the common dung fly, *Sepsis cynipsea*, females can incur internal injuries during mating, caused by the armoured genitalia of males (Blanckenhorn et al., 2002). Female survival decreases with increasing numbers of copulations in this species, possibly due to the internal injuries sustained during mating (Blanckenhorn et al., 2002). However, fecundity also increases with copulation frequency and thus it is as yet unclear whether there is an overall fitness cost to multiple mating in females of this species.

Females of the bed bug, *Cimex lectularius*, experience abdominal wounding during mating. *C. lectularius* has a bizarre system of insemination whereby the male pierces the female abdominal wall with an intromittent organ and ejaculates into the female body cavity (Stutt & Siva-Jothy, 2001). This 'traumatic insemination' causes wounding in the region where the male pierces the body cavity. Females suffer fitness costs (reduced survival and lifetime reproductive success) from frequent mating, presumably related to the wounding caused by mating (Stutt & Siva-Jothy, 2001). Why this method of insemination evolved is unclear. One possibility is that it allows males to detect the ejaculates of previous mates using chemoreceptors on their intromittent organ (Siva-Jothy & Stutt, 2003). This would allow males to determine whether they will encounter sperm competition and hence adjust their ejaculate size accordingly (Siva-Jothy & Stutt, 2003). Importantly, there is evidence to suggest that females have evolved a counter-adaptation to traumatic insemination in the form of the spermalege, a specialized area of the body wall in the region where the male usually pierces (Morrow & Arnqvist, 2003). Piercing of the

spermalege, as occurs during mating, results in relatively low costs to females compared to piercing of the body cavity elsewhere (which is not pierced during normal mating), suggesting that the spermalege is an adaptation to minimise mating costs (Morrow & Arnqvist, 2003). Thus, in bed bugs, there is evidence that supports the prediction that harm caused by male adaptations should select for female adaptations to minimise the extent of the male-induced harm.

Costs associated with mating have also been investigated in two species of *Callosobruchus* beetles. In these species females will not lay eggs without a suitable substrate, and thus it may be possible to separate the costs of reproduction and the costs of male-induced harm by limiting or removing access to suitable laying substrates. For example, a survival cost of exposure to males was detected in the adzuki bean beetle, *Callosobruchus chinensis*. The cost of mating was separated from the cost of egg-laying by exposing females to either males and an egg-laying substrate or to males and no suitable egg-laying substrate (Yanagi & Miyatake, 2003). However, it is not yet known whether the male-induced costs to females were mediated though mating or non-mating interactions between the sexes (Yanagi & Miyatake, 2003). In the bean weevil, *Callosobruchus maculatus*, the male intromittent organ has strongly sclerotized spines which damage the female genital tract during copulation (Crudgington & Siva-Jothy, 2000). Females that were given limited oviposition opportunities (and therefore did not show differences in fecundity) suffered reduced survival from elevated mating rates. This suggests that there may be female fitness costs to mating in the bean weevil, resulting, at least partly, from the damage incurred to the female genital tract during mating (Crudgington & Siva-Jothy, 2000). Females appear to have evolved a form of resistance to male harm by kicking during copulation, which significantly reduces copulation duration and the amount of damage sustained (Crudgington & Siva-Jothy, 2000). Males might benefit from piercing the internal genitalia of females by facilitating the transfer of male-benefiting seminal fluid products (like those in *Drosophila melanogaster*, see section 1.7.2) into the haemolymph (Crudgington & Siva-Jothy, 2000). Alternatively, males could benefit if females responded to the damage by delaying remating to avoid further damage or by increasing current reproduction in response to reduced future reproductive value (Lessells, 1999; Johnstone & Keller, 2000, but see Morrow et al., 2003a; section 1.4.3).

In the studies of female costs associated with mating in *Callosobruchus* beetles, female egg-laying was experimentally limited (Crudgington & Siva-Jothy, 2000; Yanagi & Miyatake, 2003). However, preventing eggs from being laid does not necessarily ensure that all of the possible costs of egg-production are abolished because costs might still be incurred by females from the operation of genes acting upstream of egg-laying (e.g. genes that enable egg-laying). Hence, further investigations are required to confirm whether survival costs of mating occur independently of survival costs of reproduction in these species.

1.6.4 Evidence for sexual conflict arising from harm to females caused by male seminal fluid products

In *Drosophila melanogaster*, female mating costs are caused by primarily by the actions of male seminal fluid products. The female cost of mating (Fowler & Partridge, 1989) is independent of other male-related and reproductive costs such as courtship and egg production (Partridge et al., 1986; Partridge et al., 1987b). Fowler and Partridge (1989) experimentally reduced female mating frequency and found that 'low-mating' females lived longer than 'high-mating' females but produced eggs at the same rate, had the same egg fertility and were courted at the same rate. Thus there was a female fitness cost that came not from reproduction or courtship but from mating *per se*. A further study, utilizing transgenic males in which the main cells of the accessory glands had been ablated (Kalb et al., 1993), showed that the female cost of mating results from the actions of proteins made in the main cells of the male accessory glands (Acps) (see section 1.7.2) that are transferred to females during mating (Chapman et al., 1995). Furthermore, the quantity of Acps that females received was proportional to the effect on female fitness – the more Acps females received the lower their survival (Chapman et al., 1995). Acps have a variety of male-benefiting functions (see section 1.7.1). Thus, the Acp(s) that harms females and benefits males is likely to mediate sexual conflict. There are estimated to be approximately 80 Acps (Swanson et al., 2001a) but an Acp that causes the female mating costs and potentially underlies sexual conflict, has not yet been identified (but see Chapters 5 and 6 of this thesis). The functions of known *Drosophila melanogaster* Acps and candidate Acps for causing female mating costs are discussed in sections 1.7.3 and 1.7.4.

1.6.5 Evidence for sexual conflict arising from the transfer of sexually transmitted diseases (STDs)

A potential cost of mating for both sexes is the receipt of parasites from mates. There are many examples of STDs in the insects (approximately 182 species of host, reviewed in Knell & Webberley, 2004). One example is the podapolipid mite parasite, *Coccipolipus hippodamiae*, which infects the ladybird, *Adalia bipunctuata*, and is transferred between individuals primarily during mating (Hurst et al., 1995). This mite significantly reduces egg production rate (by about 25%) and massively decreases the viability of eggs of infected females (Hurst et al., 1995). Thus, females that mate with infected males often suffer major fitness costs. While both sexes suffer costs from the parasite (because male fitness is realised through females) males potentially gain more from mating multiply than do females (section 1.6.) and therefore the presence of STDs is predicted to exacerbate conflict over mating. However, contrary to predictions, mate choice by female *Adalia bipunctuata* is unaffected by the presence of parasites on males, presumably because there are constraints upon, or costs arising from, choosing uninfected mates (Webberley et al., 2002). For example, if females avoided mating with infected males (or *vice-versa*) then there would be strong selection on the STD to become cryptic (Knell & Webberley, 2004). More work is required on the role of STDs in causing mating costs to understand their evolutionary implications (Webberley et al., 2002).

1.6.6 Evidence for sexual conflict arising from female costs resulting from male paternity assurance

Whilst female fitness is usually maximised by a mating rate lower than that which maximises male fitness, females in many species benefit from mating more than once. For example, females can gain direct benefits from nuptial feeding (e.g. Hayashi, 1998; Vahed, 1998; Arnqvist & Nilsson, 2000) or replenishing sperm supplies (e.g. Wedell et al., 2002; Montrose et al., 2004). Also, females might gain indirect genetic benefits from mating with the best sperm competitors (e.g. Keller & Reeve, 1995; Pizzari & Birkhead, 2002), from post-copulatory mate-choice (e.g. Konior et al., 2001; Bretman et al., 2004), from avoiding genetic incompatibility (e.g. Tregenza & Wedell, 1998; Newcomer et al., 1999), from avoiding inbreeding (e.g. Tregenza & Wedell, 2002; Bretman et al., 2004), from avoiding mutated sperm (e.g. Radwan, 2003) or from increasing offspring diversity (e.g. Baer & Schmid-Hempel, 1999; Baer & Schmid-Hempel, 2001). However, it is in the interests of

males to delay or completely prevent females from remating to avoid sperm competition. Thus, there is the potential for conflict over the time until, or occurrence of, female remating. For example, in the bumble bee, *Bombus terrestris*, females benefit from receiving the sperm of multiple males because parasite load is reduced in colonies of high genetic diversity, leading to increased reproductive success (Baer & Schmid-Hempel, 1999; Baer & Schmid-Hempel, 2001). However, in nature *Bombus terrestris* females only mate once in their lifetime (Schmid-Hempel & Schmid-Hempel, 2000) because males can prevent females from remating by transferring seminal products that form a mating plug (Sauter et al., 2001). Thus, there appears to be a conflict of interest over female remating in *Bombus terrestris* and males currently appear to have the most control over the outcome of the conflict. Also, in the cockroach, *Nauphoeta cinerea*, males enforce monogamy through the insertion of a spermatophore into the bursa copulatrix of their mates which inhibits the sexual receptivity centre in the female's brain (Montrose et al., 2004). Males can become sperm depleted but retain the ability to enforce monogamy which can result in females receiving insufficient sperm to fertilize their eggs (Montrose et al., 2004). Thus, while males still benefit from avoiding sperm competition, females can suffer reduced fitness.

Males use a variety of techniques to delay female remating. In addition to mating plugs and spermatophores, the seminal fluid of male insects often contain substances that delay female remating in other ways (Gillott, 2003; see section 1.7.2). Also, the males of some species guard females before or after mating, or prolong copulation to avoid sperm competition, and this can be energetically costly to females (see section 1.6.1). Thus, it is possible that females could benefit from mating more frequently than is in the interests of their previous mates. Also, females may be inhibited from remating at their optimum frequency by mating costs which outweigh the benefits of mating frequently. This question has so far received little attention (except in *Bombus terrestris*, see above; Baer & Schmid-Hempel, 1999; Baer & Schmid-Hempel, 2001). However, with new techniques to manipulate the relevant male traits (such as genetic engineering of seminal fluid in *Drosophila*, e.g. Kalb et al., 1993; Chapman et al., 2003c; Liu & Kubli, 2003) investigations into the potential female benefits of polyandry and sexual conflict resulting from male traits that increase paternity, should be possible. For example, Civetta and Clark (2000) found that, in *D. melanogaster*, males that performed better in sperm competition and delayed

female remating for longer also induced early female mortality. Thus, males that were most effective at inhibiting female receptivity and successful in sperm competition appeared to be the most harmful to females, at least in terms of early female survival (see also section 1.7.2). More studies are required to assess the general evolutionary importance of sexual conflict over female remating and the extent to which female fitness can be reduced below its potential by male traits that delay or prevent female remating.

1.6.7 Evidence for sexual conflict arising from immunity costs associated with mating

Recently, the immunity costs resulting from mating have received attention. In the mealworm beetle, *Tenebrio molitor*, immunity is compromised for at least 24 hours after mating in both sexes (Rolff & Siva-Jothy, 2002). This is likely to be important given that this species mates approximately every two days, suggesting that the beetle spends approximately half its life with compromised immunity (Rolff & Siva-Jothy, 2002). Rolff and Siva-Jothy (2002) also showed, by transplanting the *corpora allata* of mated or unmated individuals into virgins, that the immunity suppression was, at least in part, due to juvenile hormone (JH) released form the *corpora allata* after mating, providing an important insight into an insect-wide mechanism of mating costs. In the Monarch butterfly elevated JH is associated with reduced lifespan (Herman & Tatar, 2001), and levels of JH are also low in the diapause stage (in which ageing is reduced) of several invertebrate species (Pener, 1972; Tatar et al., 2001; Tatar & Yin, 2001) suggesting that there are survival costs to the production of, or the effects of, JH. The costs of mating and reproduction were not separated in the study of Rolff and Siva-Jothy (2002) and the lifetime implications of mating-induced immunity suppression are unknown. Consequently, post-mating immunity suppression requires further investigation to determine its role in sexual conflict. Possible avenues for future research on this subject are discussed further in the General Discussion (Chapter 7), section 7.4.1.

1.6.8 Evidence for sexually antagonistic coevolution from experimental evolution studies

Experimental evolution studies have provided important insights into sexually antagonistic coevolution, particularly in *Drosophila melanogaster*. Sexual conflict is predicted to occur over mating in this species because females suffer mating costs

that result from the actions of male accessory gland proteins (Acps) (see section 1.6.4). Females are predicted to evolve resistance to the harmful effects of Acps. However, without knowledge of which Acp(s) harms females or the mechanism by which females are harmed, it has not been possible to target specific receptors that mediate the female response to male-induced harm. Consequently no studies to date have been able to provide direct evidence for sexually antagonistic coevolution in *Drosophila*. However, several experimental evolution studies provide indirect evidence. Rice (1996) used a stock containing chromosomal translocations to make a static female phenotype to which males could adapt. Females were discarded each generation, and could not counteradapt. When tested after 41 generations of selection, the adapting males had evolved higher mating rates and sired more offspring than control males (Rice, 1996). This suggested that the adapting males benefited from the inability of females to respond to the male adaptations, a finding that is consistent with the idea of sexually antagonistic evolution. However, the control males used in this experiment were created by crossing the chromosome translocation into the control male stock during the last generation of selection to give them the same genetic background as adapting males. Hence the sudden change in genetic background could potentially reduce the viability of control males (e.g., by reducing their ejaculate potency or volume). This could explain the weaker post-mating responses of control males compared to adapting males, which could confound the findings (Bangham, 2003).

In a separate experimental evolution study, Holland and Rice (1999) imposed monogamy in two replicate lines of *D. melanogaster* while in two replicate control lines, polyandry was allowed. In the monogamy lines one female was housed with one male and in the polyandry lines one female was housed with 3 males (n = 114 females for each replicate of each treatment). The males and females were housed together for 7 days and the eggs laid in the last 2 days were used to propagate the next generation. Under monogamy conditions sexual conflict is predicted to be minimal (see section 1.6.3; Holland & Rice, 1999; Hosken et al., 2001; Martin & Hosken, 2003). The results of experiments after 32-47 generations of selection (detailed below) were consistent with sexually antagonistic coevolution. Females mated once to monogamy males lived longer than females mated once to control males and females continuously housed with monogamy males produced a greater mass of eggs than females continuously housed with control males (Holland & Rice,

1999). This suggests that the monogamy males may have evolved reduced harmfulness to females compared to controls. Furthermore, monogamy females died faster than control females when housed continually with control males suggesting that monogamy females were less resistant to male-induced harm than control females (Holland & Rice, 1999). The results were consistent with the idea that monogamy males had evolved to be less harmful to females with selection for this phenomenon arising from the fact that, for monogamy males, reducing female fitness would also directly reduce their own fitness (Fisher, 1930). Consequently, selection for female resistance to male-induced harm may have been relaxed in monogamy lines. However, a potential caveat is that monogamy lines had effective population sizes of about half that of control lines and would therefore have suffered more inbreeding than controls. Inbreeding, like sexual conflict, predicts that monogamy males would evolve reduced reproductive performance and fitness, and hence would cause reduced harm to females (Sharp, 1984; Snook, 2001; Chapman et al., 2003b). Furthermore, a subsequent study found that, as predicted by inbreeding, monogamy males were significantly smaller than controls (Pitnick et al., 2001) and smaller males are also known to be less harmful to females (Pitnick & García-González, 2002; Friberg & Arnqvist, 2003). Thus, the findings that monogamy males evolved to be less harmful and that monogamy females evolved to be less resistant to harm provide only limited evidence for sexually antagonistic coevolution, because sexual conflict and inbreeding make the same predictions. Chapter 3 of this thesis uses selection lines to test for sexually antagonistic coevolution utilizing a novel experimental design that is not subject to the same potentially confounding inbreeding problem.

In *Scatophaga stercoraria*, sexual conflict is predicted to occur because females can be damaged or killed as males compete with each other for access to females, and also because of possible costs associated with mating *per se* (see section 1.6.3). An experimental evolution study in *Scatophaga stercoraria* utilised an experimental design similar to that of Holland and Rice (1999) (see above) to impose monogamy and polyandry. Hosken et al. (2001) found that both male and female characters responded to selection in the directions predicted by sexual conflict. Four replicate populations of flies were maintained in either monogamous or polyandrous conditions (Hosken et al., 2001).Theory predicts that in polyandry treatments there is a greater potential for conflict between the sexes over paternity because females

have the potential to choose which males fertilise their eggs. Also, sperm competition between different males occurs in polyandry treatments but is absent in monogamy treatments. Thus, reproductive organs that may affect paternity, such as testes in males and possibly female accessory sex glands in females, were predicted to evolve to be larger in polyandry treatments compared to monogamy treatments. As predicted by sexual conflict and sperm competition, male testis and female accessory sex glands evolved to be larger in polyandry treatments relative to monogamy treatments (Hosken et al., 2001). However, traditional sexual selection theories also predicted the same results. Females are predicted to evolve elevated choosiness in polyandry treatments relative to monogamy treatments because there is no opportunity for mate choice in monogamy treatments. Hence, if female accessory sex glands can influence paternity, then these should evolve to be larger in the polyandrous treatments. However, it is not known whether sperm or female accessory glands are involved in sexual conflict in this species. Hence, the study of Hosken et al. (2001) cannot distinguish between coevolution in response to sexual conflict and coevolution in response to other forms of sexual selection. Also, in the selection regime of Hosken et al. (2001) the same number of offspring were taken from each female at the end of each generation and therefore selection was not based on differential reproduction (Snook, 2001). Thus, differences in female reproductive success would not have affected the representation of genotypes in the subsequent generations which makes the basis for selection unclear (Snook, 2001).

A study using *Sepsis cynipsea* provided the first experimental evidence for the evolution of pre-mating reproductive isolation through sexually antagonistic coevolution resulting from sexual conflict (Martin & Hosken, 2003). Martin and Hosken (2003) employed experimental evolution to manipulate sexual conflict by varying the size and density of populations (3 replicate populations of 250 males and females per container, 3 replicate populations of 25 males and females per container and 3 replicate populations of 20 monogamous pairs). Sexual conflict was predicted to increase with increased population size/density as the number of sexual interactions should increase with increasing number of males present (Blanckenhorn et al., 2000). After 35 generations of selection, mating propensity and female reluctance to mate were reduced in smaller/less dense populations, as predicted by sexual conflict (Martin & Hosken, 2003). The effects of inbreeding are unknown in this species and it is possible that females from the smaller populations (which were

therefore predicted to be more inbred) might evolve reduced reluctance to mate for this reason. However, in other fly species it is primarily male mating propensity that is reduced through inbreeding (Sharp, 1984; Meffert & Bryant, 1991). More importantly though, in the non-monogamous populations, mating propensity was higher and female reluctance was lower in 'within-population' crosses (males and females from the same population size/density treatment and same replicate population) compared to 'between-population' crosses (males and females from the same population size/density treatment but different replicate) and this effect was stronger in the higher conflict populations (Martin & Hosken, 2003). Thus, sexual conflict appeared to drive female preference for males from their own population, hence facilitating reproductive isolation. The results were also consistent with the model of Gavrilets (2000) which suggested that reproductive isolation driven by sexual conflict should also evolve faster in larger populations (section 1.4.5). Chapter 4 of this thesis tests the generality of the prediction that sexual conflict leads to pre-mating reproductive isolation, utilising *D. melanogaster* selection lines and an experimental design based on that of Martin & Hosken (2003). Chapter 4 also tests whether females can evolve resistance to males through alterations in pre-mating willingness to mate.

1.6.9 Evidence for sexually antagonistic coevolution from comparative studies of closely related species

Theory predicts that sexually antagonistic coevolution can facilitate the rapid divergence of genes and traits involved in sexual conflict. Studies in water striders and in the family Drosophilidae have investigated morphologies or genes suspected to be involved in sexual conflict and compared those morphologies or genes across related species. In water striders there is the potential for sexual conflict over mating because females suffer energetic costs (see section 1.6.1) and increased predation risk costs (see section 1.6.2) as a result of pre-mating struggles and of mating itself. In some water strider species, males posses genital graspers and distinct body shapes that aid genital contact and female water striders possess anti-grasping spines and a downward tilting abdominal tip to thwart the attachment of males to the female genitalia (Arnqvist & Rowe, 1995; Arnqvist & Rowe, 2002b). Remarkably, the level of male and female 'armaments' are closely matched across species, suggesting that each sex evolves in response to the ability of the other to win pre-mating struggles (Arnqvist & Rowe, 2002b). Differences between the sexes in the relative level of

armaments within species result in alterations in the outcome of pre-mating struggles – if males have a higher level of armaments than females then pre-mating struggles last longer and if females have a higher level of armament then pre-mating struggles are shorter (Arnqvist & Rowe, 2002a). However, differences in the absolute level of armaments between species do not affect the duration of pre-mating struggles. This shows that it is only the relative level of armaments of interacting males and females (i.e. those of the same species) that predicts the length of contests (Arnqvist & Rowe, 2002a). Thus, the likely reason that the level armaments are closely matched across species may be that each sex adapts to the level of armaments in the opposite sex. The studies on water striders indicate that evolutionary changes in sexually antagonistic traits can lead to changes in the outcome of sexually antagonistic interactions (Arnqvist & Rowe, 2002a). Thus, there is evidence that the sexes evolve adaptations and counter-adaptations to gain advantages in sexual interactions, which provides support for the existence of sexually antagonistic coevolution.

Genes involved in sexually antagonistic coevolution are predicted to evolve rapidly and hence facilitate reproductive isolation (Rice & Holland, 1997; Rice, 1998; section 1.4.5). Sexual conflict is predicted to place selection pressure on genes encoding traits involved in the conflict. Consistent with this theory, there is evidence from several taxa that proteins involved in reproductive processes can diverge remarkably rapidly and under positive selection (reviewed in Swanson & Vacquier, 2002). For example, in abalone gastropods (Haliotidae: *Haliotis*), proteins that are essential for fertilisation, such as the sperm protein lysin (which creates a hole in the egg envelope for the sperm to enter) and the sperm protein sp18 (which is thought to mediate the fusion of the egg and sperm) are both extremely divergent in closely related species (Swanson & Vacquier, 2002). There is evidence that sp18 may evolve up to 50 times faster than the fastest known evolving mammalian proteins (Metz et al., 1998). In *Drosophila*, male reproductive proteins are estimated to evolve at twice the rate of non-reproductive proteins (Civetta & Singh, 1995). Furthermore, Acps, of which some, at least, are likely to be involved in sexual conflict (section 1.6.4) show rapid divergence driven by positive selection between closely related species such as *D. melanogaster* and *D. simulans* (Swanson et al., 2001a). Evidence for positive selection has been found in several specific Acps (Acp26Aa, Aguadé et al., 1992; Aguadé, 1998; Tsaur et al., 1998; Acp70A (sex

peptide), Cirera & Aguadé, 1997; Acp29AB, Aguadé, 1999; Acp36DE Begun et al., 2000; see section 1.7.2 for discussion of Acp functions). Also consistent with sexually antagonistic coevolution is the maintenance of genetic polymorphisms at some Acp loci (Coulthart & Singh, 1988; Clark et al., 1995; Prout & Clark, 1996). Genetic polymorphisms of Acp loci might indicate male adaptations in response to female resistance to sexually antagonistic traits (e.g. male x female interactions, Clark et al., 1999). For example, if there is non-transitivity in traits involved in success in antagonistic interactions between the sexes, that is, if the success of individuals in interactions depends on the specific genotypes of the interacting males and females, then polymorphisms can be maintained in those traits. This is because each allele may be successful in some interactions but no one allele is the best in all interactions (Prout & Clark, 1996).

The finding of high rates of divergence amongst female ovary proteins in *Drosophila melanogaster* and *Drosophila virilis* group species (Civetta & Singh, 1995) and the finding of positive selection in several female reproductive proteins in mammals (Swanson et al., 2001b) is also consistent with sexually antagonistic evolution, because sexual conflict is predicted to drive the rapid evolution of reproductive proteins in both sexes. However, evidence of interspecific divergence of reproductive proteins, positive selection on reproductive proteins, positive selection on Acps and polymorphisms in Acps, is also consistent with selection resulting from sperm competition and female post-copulatory choice (Prout & Bundgaard, 1977; Hughes, 1997; Clark et al., 2000). It is therefore difficult to separate the influence of sexual conflict and other forms of sexual selection on the pattern of evolutionary change in genes encoding male and female reproductive proteins (Bangham, 2003). Knowledge of the Acp(s) underlying sexual conflict and the mechanism by which females incur harm is required to identify the selective forces that act on *D. melanogaster* reproductive proteins.

1.6.10 Evidence for sexually antagonistic coevolution from crosses between genotypes, crosses between populations and crosses between species

Sexually antagonistic coevolution has also been investigated in crosses between specific genotypes, crosses between allopatric populations within a species, and crosses between closely related species. The hypothesis is that the males and females are predicted to respond differently to mates of different genotypes, to mates from

allopatric populations or to mates from different species than they are to mates of the same genotype, the same population or the same species. For example, two key hypotheses are 1) that female oviposition rate after mating would differ between matings with males from their own population and matings with males from other populations because this would indicate a complex signal/receiver system of the type predicted by sensory exploitation (section 1.4.2) and 2) that females will be fitter when mated to males from their own population because they have been able to evolve resistance to their manipulative traits (Rowe et al., 2003). However, there is a current lack of mathematical theory regarding population crosses that makes it impossible to predict the pattern of outcomes that would distinguish sexually antagonistic coevolution from other coevolutionary processes.

Clark et al. (1999) demonstrated that there was a significant effect of male x female genotype on the outcome of sperm competition in *D. melanogaster*, which was suggested to be consistent with sexually antagonistic coevolution of the underlying genes. This is because the interaction between male and female genotypes may affect the efficiency of sperm storage and the pattern of sperm usage and which might represent a conflict between the sexes over the control of fertilisations. Clark et al. (1999) used 6 isogenic lines of flies and performed crosses within and between lines, testing the sperm competitive ability of males from the lines again a standard 'tester male' genotype. Females used fewer sperm from males of their own lines when the sperm of tester males was also available (tester males achieved higher paternity than would be expected from random sperm use), suggesting that females were avoiding inbreeding (Clark et al., 1999). This may benefit females as they produce less inbred offspring but it reduced the fitness of males from the same lines as their mates, as the share of paternity of those males was reduced.

In a population cross study using *S. stercoraria*, males originating from different populations to their mates (heteropopulation males) were found to have a fertilization advantage over males originating from the same population as their mates (conpopulation males) (Hosken et al., 2002a). These findings were suggested to be consistent with sexually antagonistic coevolution. This was based on the idea that heteropopulation males gained an advantage because females had not evolved with them and therefore the females were less able to resist heteropopulation males than conpopulation males (Hosken et al., 2002a). An alternative explanation is that

females may preferentially use the sperm of heteropopulation males over the sperm of conpopulation males to avoid inbreeding (Hosken et al., 2002a) which would still indicate conflict between the sexes over paternity in conpopulation crosses only (see also the study of Clark et al., 1999, above).

Male-female interactions were also detected in crosses between two closely-related species of desert *Drosophila* (Knowles & Markow, 2001). The character tested was the insemination reaction mass, which is a large mass that forms in the vagina after mating and is important because females do not typically oviposit or remate until the mass has subsided, which can take several days in some crosses (Knowles & Markow, 2001). The results suggested that female oviposition might be delayed for longer after interpopulation crosses than after intrapopulation crosses because the mass was larger and/or of longer duration in interpopulation crosses than in intrapopulation crosses. This infers that females were more 'compatible', in terms of the reaction mass, with males from their own species. This was also suggested to be consistent with sexually antagonistic coevolution (Knowles & Markow, 2001). However, incompatibility in reproductive processes between different species is inevitable and may not necessarily be a result of sexually antagonistic coevolution. Thus, the evidence for sexually antagonistic coevolution from this study is limited.

The outcome of intraspecific crosses between individuals from different populations has been investigated in several other species (e.g. between different strains of the housefly, *Musca domestica* Andrés & Arnqvist, 2001, between geographically isolated populations of *Callosobruchus maculatus*, Brown & Eady, 2001, and between different strains of flour beetles *Tribolium castaneum* Nilsson et al., 2002) and, although the results have been mixed, all of the studies claimed to present evidence for sexually antagonistic coevolution. One problem with studies that use crosses between genotypes, populations or closely related species is the lack of specific predictions based on sexual conflict for each situation (Chapman et al., 2003b; Pizzari & Snook, 2003; Rowe et al., 2003). In the examples mentioned above, the fitness consequences of the levels of inbreeding and the genetic distance between populations might be crucial for understanding whether females should prefer (or resist least), or be most compatible with males from their own or different populations. In addition, without knowledge of the mechanism of sexual conflict, population crosses cannot differentiate between traditional sexual selection models

and sexual conflict based models of coevolution (Chapman et al., 2003b; Pizzari & Snook, 2003; Rowe et al., 2003). A recent model suggests that the outcome of population crosses may be unpredictable (Rowe et al., 2003) and therefore yield little information about the selection processes involved in coevolutionary change in allopatric populations.

1.6.11 Evidence for the role of sexual conflict in promoting speciation

There are currently few empirical studies testing the idea that sexual conflict promotes speciation (but see the experimental evolution study of Martin & Hosken, 2003; section 1.6.8). The results of one study of speciation rates in insects were consistent with the theory that sexual conflict may promote speciation (Arnqvist et al., 2000). Arnqvist et al. used paired phylogenetic contrasts to compare closely related clades that have polyandrous or monogamous mating systems. The potential for sexual conflict is predicted to be higher in polyandrous species than in monandrous species because adaptations that increase the ability of males to gain fertilizations, even if those adaptations harm females, will be more strongly selected in males of polyandrous species than in males of monogamous species. In monogamous species the potential reproductive success of each mating male and female will be similar and therefore males should evolve to become relatively benign to their mates. The study found that polyandrous groups were more speciose than monandrous groups (Arnqvist et al., 2000) suggesting that, if the level of polyandry is a good proxy for sexual conflict, sexual conflict may promote divergence and speciation. However, similar types of study on mammals, butterflies and spiders (Gage et al., 2002) and on birds (Morrow et al., 2003b) provided no evidence that predicted levels of sexual selection or sexual conflict were associated with speciosity. In both these studies (Gage et al., 2002; Morrow et al., 2003b), the potential level of sexual selection or sexual conflict was measured by the level of sexual size dimorphism and the relative testes size of males (which is related to the degree of polyandry). Sexual dichromatism (differences between the sexes in plumage shade, colour or pattern) was an additional character used for birds (Morrow et al., 2003b). Sexual selection and sexual conflict were predicted to be higher with increased sexual size dimorphism, increased relative testis size and increased sexual dichromatism. All the measures of sexual selection and sexual conflict in these studies (Arnqvist et al., 2000; Gage et al., 2002; Morrow et al., 2003b) were proxies and it is therefore difficult to make unequivocal conclusions

from the results. Further studies are required to test the hypothesis that sexual conflict promotes speciation and they would benefit greatly by an understanding of the specific mechanisms and genes underlying sexual conflict, in order to provide a more robust predictive basis for comparisons.

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

The model organism, *Drosophila melanogaster*, that is used in this thesis is ideally suited to the study of sexual conflict and sexually antagonistic coevolution. Much is known about *Drosophila* genetics, physiology and behaviour and there is some knowledge of the proximate source and mechanisms of sexual conflict over mating. The male accessory gland proteins (Acps) cause female mating costs (section 1.6.4) and therefore one or more Acp is likely to mediate sexual conflict in this species. Acps can be genetically manipulated (e.g. Chapman et al., 2003c; Liu & Kubli, 2003) which means it should be theoretically possible to uncover the genetics that underlie sexual conflict in *D. melanogaster*. *Drosophila* are relatively cheap and easy to rear and have a short life cycle (egg-adult in 10 days) which makes them ideal for experimental evolution studies to investigate sexually antagonistic coevolution. In the following sections (1.7.1 – 1.7.4) I discuss aspects of the behaviour, physiology and genetics of *Drosophila* that are relevant to the study of sexual conflict in this species.

1.7.1 Courtship and copulation in *Drosophila melanogaster*

There is a large body of research on mating behaviour and reproductive processes in *Drosophila melanogaster*, which helps us to understand the nature of sexual conflict in this species. In *D. melanogaster*, male courtship precedes mating and plays an important role in stimulating females in to a state of receptivity. Male courtship includes orientation towards the female, foreleg tapping upon the female's abdomen, following the female if she moves around, wing vibration to produce a 'love song', genital licking and attempted copulation (Spieth, 1974; Hall, 1994). The female response to courtship depends on her state of receptivity. To accept a mating a female stops moving, assumes a particular posture and manipulates her external genitalia appropriately (Spieth, 1974; Hall, 1994). Rejection behaviours of the female include abdomen bending, ovipositor extension and kicking (Spieth, 1974; Connolly & Cook, 1973). These female behaviours are likely to influence whether mating occurs. There is a non-mating female cost of exposure to males that may

result at least partially from courtship (Partridge & Fowler, 1990; Friberg & Arnqvist, 2003) as there are likely to be energetic costs involved in avoiding and rejecting males. Thus, superfluous courtship may be a source of conflict between the sexes. Copulation duration appears to be primarily under male control (MacBean & Parsons, 1967) and typically lasts around 20 minutes in *Drosophila melanogaster.* Sperm transfer appears to be complete in most matings by 8 minutes but matings of a full duration are required to induce a full female refractory period (Gilchrist & Partridge, 2000). Components of the seminal fluid reduce female receptivity after mating (section 1.7.2 and 1.7.4), thus, the extra copulation time beyond sperm transfer may be required for the complete transfer of seminal fluid. As components of the seminal fluid also cause female mating costs (Chapman et al., 1995) the continuation of copulation after sperm transfer may be detrimental to females and could thus represent a further source of conflict between the sexes.

1.7.2 The seminal fluid proteins of male *Drosophila melanogaster*

Male seminal fluid proteins have been shown to cause behavioural and physiological effects in the females of many insect species, though by far the most extensively studied are the accessory gland proteins (Acps) of *Drosophila melanogaster* (Gillott, 2003). The seminal fluid of male *Drosophila melanogaster* is a major determinant of post-mating fitness for both males and females. Components of the seminal fluid mediate female mating costs (Chapman et al., 1995) and are therefore likely to mediate sexual conflict (see section 1.6.4). The most obvious effects of mating on females in *D. melanogaster*, as in many other insect species, are a reduction in receptivity and an increase in oviposition. However, these effects only last a few days (Liu & Kubli, 2003) and consequently females remate frequently during their lifetime (Harshman & Clark, 1998; Imhof et al., 1998), creating conditions that facilitate post-mating male-male competition. The seminal fluid of *Drosophila melanogaster* males contains proteins ranging from small peptides to large glycoproteins (Chen et al., 1988; Wolfner, 1997; Wolfner et al., 1997; Swanson et al., 2001a). The majority of seminal fluid proteins are made in the main cells of the male accessory glands (Kalb et al., 1993). Other seminal fluid proteins are made in the secondary cells of the accessory glands, the ejaculatory duct and the ejaculatory bulb (e.g. Lung & Wolfner, 2001; reviewed in Chapman, 2001). The Acps produced in the main cells of the accessory glands are essential for increasing female oviposition, reducing female receptivity after mating (other seminal fluid products

cannot replace their function; Kalb et al., 1993) and mediating the cost of mating (Chapman et al., 1995).

There are around 80 Acps (Swanson et al., 2001a) that are transferred to females during mating. Some Acps remain in the genital tract while others enter the circulatory system via the posterior vaginal wall (Lung & Wolfner, 1999). So far functions have been determined for only a few Acps (Wolfner, 1997; Chapman, 2001). Some Acps affect female behaviour and physiology, others are involved in sperm competition and its prevention, and at least one Acp harms females (section 1.6.4). The accessory gland also produces antibacterial peptides, proteases and protease inhibitors (Chapman, 2001; Lung et al., 2001; Lung et al., 2002). Acp70A, the sex peptide (SP), is involved in both the increase in egg laying and the decrease in receptivity in females after their first mating (Chen et al., 1988; Chapman et al., 2003c; Liu & Kubli, 2003; see section 1.7.4). Acp26Aa, one of the fastest evolving proteins in the *Drosophila* genome (Tsaur & Wu, 1997; Tsaur et al., 1998; Swanson & Vacquier, 2002), stimulates the release of oocytes from the ovary which leads to a small increase in egg laying for 1 day after mating (Herndon & Wolfner, 1995; Heifetz et al., 2000). Also, Dup99B, an ejaculatory duct protein, which shows a high amino acid sequence homology to SP at the C terminal end, induces a similar but much weaker response than SP in females (Saudan et al., 2002; Liu & Kubli, 2003). Females that receive Dup99B but no Acps show a brief or no reduction in receptivity (Kalb et al., 1993; Xue & Noll, 2000; Saudan et al., 2002), only a slight increase in egg production (Xue & Noll, 2000) and no increase in egg production in the absence of sperm (Kalb et al., 1993). Acp36DE is essential for normal sperm storage (Neubaum & Wolfner, 1999) and as a consequence, males that do not transfer Acp36DE (and therefore store few sperm) perform poorly in sperm competition (Chapman et al., 2000).

1.7.3 Seminal fluid proteins and the female cost of mating

One candidate Acp for mediating the female cost of mating and hence, sexual conflict, is Acp62F. Acp62F is a protease inhibitor that reduces female survival when repeatedly and ectopically expressed at more than 50 times the levels delivered during a single mating (Lung et al., 2002). Approximately 10% of the Acp62F received by females enters the haemolymph (Lung & Wolfner, 1999) and it was suggested that the toxic effect of Acp62F might be a result of it's protease

inhibitory activity, causing interference with essential proteolytic processes within the female (Lung et al., 2002). Seven other Acps that were also tested did not reduce female survival indicating that Acp62F is a candidate for contributing to the cost of mating (Lung et al., 2002) and hence sexual conflict. To determine the function of an Acp and its role in the cost of mating through normal matings, males that lack that Acp are required. However, a recent experiment utilizing such males provided no evidence that Acp62F contributes to the cost of mating (Bangham, 2003). One potential problem with the experiment of Bangham (2003) was that females were kept on low nutrition relative to previous experiments in which mating costs have been examined (e.g. Fowler & Partridge, 1989; Chapman et al., 1995) and females only suffer significant mating costs when kept on high nutrition (Chapman & Partridge, 1996a). However, there is currently no evidence for a role of Acp62F in the cost of mating and sexual conflict. Other candidate Acps need to be examined for their potential roles in the female cost of mating, as is done for SP in Chapter 5 of this thesis.

Civetta and Clark (2000) found that males that proved more successful in sperm competition and inducing female refractoriness also induce earlier female mortality (Civetta & Clark, 2000; section 1.6.6). Specifically, the proportion of progeny sired by the first male to mate, which measures the 'sperm-defence' ability, was correlated with female mortality, as was the time until females remated (Civetta & Clark, 2000). This suggests that components of the male ejaculate that contribute to the female cost of mating are the same, or are correlated with, the components that are involved in male sperm competitive ability and delay female remating. Thus candidates include Acp36DE (Neubaum & Wolfner, 1999; Chapman et al., 2000) and SP (Chapman et al., 2003c; Liu & Kubli, 2003; section 1.7.4). However, there is currently no evidence that Acp36DE is associated with female mating costs (Neubaum & Wolfner, 1999).

1.7.4 The sex peptide

The sex peptide (SP) is one of the best characterized Acps and is involved in elevating the oviposition rate of females and reducing the receptivity of females after mating. However, it was unknown whether SP plays a role in mediating female mating costs and sexual conflict, a subject that I investigate in Chapter 5 of this thesis. The function of SP was first investigated by Chen et al. (1998), who found

that injection of synthetic SP into virgin females increased egg production and reduced female receptivity for 1 to 2 days. Recent studies using males that lack SP, but produce all other seminal fluid products, confirmed the effects of SP after normal matings. Virgin females mated once to males lacking SP show a short term reduction in receptivity and were significantly more receptive than controls (and almost as receptive as virgins) by 48 hours after mating (Chapman et al., 2003c; Liu & Kubli, 2003). In addition, matings with SP-lacking males produced a weak, 1-day elevation of egg production, followed by several days of lower fecundity than controls (Chapman et al., 2003c; Liu & Kubli, 2003). The short-term effects on receptivity inhibition in the absence of SP might be due to the effects of other seminal fluid proteins such as Dup99B (Liu & Kubli, 2003; but see section 1.7.2) and the short term increase in egg production could be due to the effects of Dup99B and/or Acp26Aa (Heifetz et al., 2000; Herndon & Wolfner, 1995; Saudan et al., 2002; section 1.7.2). The effect of SP on female egg production and receptivity lasts longer after normal matings (about a week, Liu & Kubli, 2003) than after injection. This can be explained by the fact that SP binds to sperm and can be detected on sperm heads several days after deposition into the female reproductive tract (Peng et al., 2005). SP is released gradually from sperm over the days following mating (Peng et al., 2005) from where it might enter the haemolymph and act upon receptors in the female outside of the reproductive tract. When sperm are absent, the effects of SP may be shorter-lived because the slow release mechanism (i.e. sperm) is absent. Thus, SP appears to be the molecular basis for the 'sperm effect' which describes the requirement of sperm for the persistence of the post mating female responses such as elevated egg production and reduced receptivity (Manning, 1962).

The effect of SP on egg production includes the stimulation of oogenesis (maturations of eggs) and egg laying (Chen et al., 1988). Oogenesis is stimulated by SP via the *corpora allata*, a pair of small glands located behind the brain. SP appears acts on the *corpora allata* to release juvenile hormone (Moshitzky et al., 1996) which, in turn, stimulates oogenesis (Soller et al., 1999). The stimulation of oogenesis by SP does not, in turn, stimulate the ovulation, oviposition or receptivity of females which suggests that SP has other targets of action (Soller et al., 1999) including the nervous system. Such receptors in the nervous system are assumed to be involved in the inhibition of female receptivity (Kubli, 2003). The targets for SP which stimulate receptivity inhibition and oviposition are unknown but there are at

least two, one in the nervous system and another in the genital tract (Ding et al., 2003). However, it is not known whether SP reaches both these targets *in vivo* (only a target in the uterus has been confirmed *in vivo*; Ding et al., 2003; Kubli, 2003). A current hypothesis for SP action is that the target in the female nervous system begins a cascade leading to elevated egg production and reduced receptivity, and the target in the reproductive tract may represent a peptide transporter that moves SP from the genital tract into the haemolymph (Ding et al., 2003; Kubli, 2003). However, an area of the posteria vaginal wall in females is permeable and is known to allow some Acps (Acp26Aa and Acp62F) into the female haemolymph (Lung & Wolfner, 1999). Thus, small proteins and peptides (such as SP) may be able to enter into the haemolymph without the need for transporters.

SP was originally thought unlikely to be involved in female mating costs and sexual conflict for two main reasons. Firstly, SP exerts only short term responses in females without sperm (e.g. Chen et al., 1988; Kalb et al., 1993; Xue & Noll, 2000) and therefore the female cost of mating would be predicted to be reduced in matings with spermless males if SP harmed females. However, females that mate with spermless males suffer a cost of mating (Chapman, 1992). Thus, if SP harms females, it must do so free from association with sperm. Secondly, SP was thought to act as a signal that females use to their benefit (Smid, 1997) to begin laying eggs at a high rate only after mating and to avoid superfluous, costly matings while there was still fresh sperm in storage. For example, mutant *dunce* females that fail to show fully reduced receptivity in response to SP mate more often than control females and consequently suffer greater survival mating costs (Chapman et al., 1996). This suggests that SP regulates female mating rate. In addition, *dunce* females are more sensitive to 'per-mating' harm suggesting that the female cost of mating does not occur because of female responses to the receptivity-inhibiting target of SP. Hence, females mated SP-deficient males are predicted to mate more frequently than controls and therefore suffer increased mating costs. This prediction was tested in Chapter 5 of this thesis. SP is also predicted to benefit males in post-mating male-male competition because its effects on females result in delayed sperm competition and sperm displacement and an increased rate of offspring production. Hence SP is predicted to elevate the share of a male's paternity. This prediction was tested in Chapter 6 of this thesis.

1.8 Outline of thesis

The work in this thesis was funded by the Natural Environmental Research Council and was performed under the supervision of Tracey Chapman (first supervisor) and Kevin Fowler (second supervisor). All experiments were performed by the author. The aim of the thesis was to investigate the evolution and underlying genetics of sexual conflict in *Drosophila melanogaster* by using experimental evolution (Chapters 3 and 4) and genetic manipulation of males (Chapters 5 and 6).

Chapter 2 describes general materials and methods used in the experiments described in Chapters 3, 4, 5 and 6.

Chapter 3 uses experimental evolution to examine how the sexes adapt to different levels of sexual conflict. The work contributes to the understanding of the selective forces involved in sexual conflict. The work in Chapter 3 has been published in the journal *Evolution* (2004, Vol. 58(5), pages 1028-1037) with co-author Tracey Chapman (Appendix I).

Chapter 4 uses the lines of flies from Chapter 3 and tests for differences in female willingness to mate and for evidence of pre-mating isolation between populations, driven by sexually antagonistic coevolution. The work adds to our understanding of the way in which *Drosophila melanogaster* females adapt to different levels of sexual conflict.

Chapter 5 investigates the role of a single male seminal fluid protein, the sex peptide, in determining female mating costs. The sex peptide induces behavioural and physiological changes in females after mating (Chapman et al., 2003c; Liu & Kubli, 2003). I used males that lack sex peptide (Chapman et al., 2003c) to elucidate the effect of sex peptide on female fitness. The work contributes to our understanding of sexual conflict in *Drosophila melanogaster* by providing a likely genetic basis of male-induced harm. The work has been published in the journal *Current Biology* (2005, Vol. 15(4), pages 316-321) with co-author Tracey Chapman (Appendix II).

Chapter 6 investigates the role of sex peptide in determining male fitness. The work adds to the understanding of the role of sex peptide in mating frequency,

reproduction and sexual conflict by testing the prediction that sex peptide increases male post-copulatory success.

Chapter 7 is a general discussion of the findings in this thesis. The chapter includes a discussion of future directions for investigation into sexual conflict in *Drosophila melanogaster*. It also includes discussion of some issues regarding sperm competition that have been published as a primer in *Current Biology* (2004, Vol. 14(3), pages R100-R103) with co-author Tracey Chapman (Appendix III).

Chapter 2. General materials and methods

2.1 Stocks and cultures

All flies were maintained prior to and during experiments on sugar-yeast food (SY food; section 2.1.1) in non-humidified rooms at 25°C on a 12:12 hour light:dark cycle.

2.1.1 Bottles, vials and food

The glass bottles used in stock cages and bottle culture were 189ml (1/3 pint) and contained 70ml of SY food. The glass shell vials used in experiments were 73mm high by 23mm diameter and contained 7ml of SY food. The SY food consisted of 100 g autolysed yeast powder, 100 g dextrose, 20 g agar, 30 ml nipagin (10% w/v solution), 3 ml propionic acid, and 1 L water. The yeast, dextrose, agar and water was brought to boiling point and simmered for several minutes and then removed from the heat. The medium was cooled to $\leq$ 60°C before the nipagin and propionic acid was added and then the food was immediately dispensed into bottles or vials. The bottles or vials were ready for use once the food had cooled to room temperature and the excess condensation had evaporated. Nipagin is an anti-fungal agent and propionic acid is an anti-bacterial agent. In all experiments, and when required in bottle culture, live yeast granules, *Saccharomyces cerevisiae*, were sprinkled on top of the food to maximise the rate of mating and egg laying (Chapman & Partridge, 1996a; Simmons & Bradley, 1997). Yeast paste, used in some experiments and during standard density culture (section 2.2.1), was made from mixing live yeast granules with water.

2.1.2 Grape juice medium

For collecting fly eggs, a grape juice medium was used. The medium consisted of 1.1L water, 50g agar, 600ml red grape juice concentrate, 42.5ml nipagin (10% w/v solution). One litre of water and the agar were mixed and brought to the boil, the red grape juice concentrate was added and the mixture was again brought to the boil and simmered for a few minutes. The remaining 0.1L (cold) water was added and the mixture was allowed to cool to $\leq$ 60°C before the nipagin was added. The mixture was immediately dispensed into Petri dishes and was ready for use after cooling to room temperature.

2.1.3 Wild-type stock

The wild-type stock flies were collected from Dahomey, now Benin, in West Africa in 1970 and have been maintained in the lab since collection. Dahomey flies were kept in four discrete population cages (45 × 25 × 25cm) with overlapping generations in each. Each cage contained twelve bottles of SY food and every seven days the three oldest bottles were removed and replaced with three fresh bottles. Thus, each bottle stayed in the cage for 4 weeks allowing plenty of time for flies to lay eggs, larvae to develop, and pupae to eclose (egg to adult development time is approximately 10 days at 25°C).

2.1.4 *UAS-SP-IR* and *Acp26AaPGal4* stocks used to generate sex peptide-deficient ('SP-knockdown') males (Chapters 5 and 6)

RNA interference was used to knockdown the level of SP in males. Two previously described lines were used that contained *UAS* upstream of the *SP* gene in a sense-antisense orientation (Chapman et al., 2003c). The accessory gland specific *Gal4* driver line contained an *Acp26Aa* promoter fused to *Gal4* (*Acp26Aa-P-Gal4*) (Chapman et al., 2003c). These lines were maintained in bottle culture prior to experiments. Two replicate matched lines of SP knockdown and control males were generated (SP1 and C1; SP2 and C2). SP1 and SP2 knockdown males were the sons of *Acp26Aa-P-Gal4* mothers and *UAS-SP-IR1* or *UAS-SP-IR2* fathers. The *Gal4* driver was X-linked allowing control males, autosomally matched to the SP knockdown males, to be obtained from the reciprocal crosses. Hence, C1 and C2 males were the sons of *UAS-SP-IR1* or *UAS-SP-IR2* mothers and *Acp26Aa-P-Gal4* fathers. SP deficiency in the knockdown lines and SP presence in the control lines was confirmed by Western Blotting (section 2.2.7). SP was undetectable in SP knockdown males in the Western Blot analysis (section 2.2.7).

2.1.5 *sparkling poliert* stock (Chapter 6)

sparkling poliert flies are homozygous for a recessive mutation that produces small, smooth, glassy eyes which can easily be distinguished from wild-type flies when viewed under a dissecting microscope. The stock used (Chapter 6) was described in Partridge et al (1994) (the '25°C line'). The stock was maintained in bottle culture prior to experiments.

2.2 General methods

2.2.1 Standard larval density culture

Standard density culturing equalises competition between larvae. This minimises any environmentally derived differences in body size arising from competition for food between larvae and variation in food quality arising from contamination of food from larval faeces. Thus, standard larval density is essential when measuring fitness traits in adult flies and when selecting on fitness related traits (Clancy & Kennington, 2001). To collect eggs for standard density culture, Petri-dishes containing grape juice medium ('grape plates', section 2.1.2) with small drops of live yeast paste (section 2.1.1), were placed in cages for approximately 16 hours, up to a maximum of 20 hours if required. Large numbers of eggs could be obtained using this method which allowed the adult flies that were used in experiments to be collected over a short period of time after eclosion, i.e., < 8 hours. At the end of the laying period the yeast paste was removed. Two methods were used to rear flies at standard larval densities, as follows:

1) Larval picks

When relatively small numbers of experimental flies were required from a standard, low-density culture (e.g. < 1000 flies), 'larval picks' were employed. Approximately 24 hours after the mid-point of the egg-collecting period, first instar larvae were picked from the grape juice medium using a mounted needle and placed into vials containing SY food, at a density of 100 larvae per vial.

2) Egg washes

When large numbers of experimental flies were required from standard density cultures, (e.g. > 1000 flies), a method described by Clancy and Kennington (2001), referred to in this thesis as 'egg washes', was employed. This method is less labour intensive than larval picks when large numbers of flies are required from standard density cultures. Briefly, after the egg collecting period, eggs were washed, via a funnel, into a plastic tube with a conical bottom in 1× PBS solution (phosphate buffered saline) (Clancy & Kennington, 2001). The eggs were allowed to settle, the supernatant was discarded and more PBS was added (Clancy & Kennington, 2001). This was repeated until the solution became clear (so that most of the residual live yeast paste had been removed). Eggs were taken up from this solution using a 100 µl micropipette, with a 100 µl pipette tip cut approximately 6mm from the end (Clancy

& Kennington, 2001), and expelled into bottles containing SY food. I used a setting of 20µl on the pipette which resulted in uncrowded standard-density bottle cultures.

2.2.2 Fly handling and virgin collection

Unless specified otherwise, when flies had to be handled (e.g. to transfer them to fresh vials or bottles), they were anaesthetised using carbon dioxide. However, when virgin flies were required they were collected within 8 hours of eclosion using ice anaesthesia, as exposure to carbon dioxide in flies < 3hours old can cause damage, sterility or death (Calboli, 2004).

2.2.3 Manipulating sexual conflict by experimental evolution (Chapters 3 and 4)

To manipulate sexual conflict, adult sex ratio was experimentally altered in *D. melanogaster* selection lines. To obtain flies for the selection lines, eggs were collected from the four Dahomey population cages (section 2.1.3). First instar larvae were picked and placed in batches of 100 into vials (standard density culture, section 2.2.1). After all flies had eclosed, the adults were mixed, sexed under CO_2 anaesthesia, and randomly allocated to one of three selection treatments. Three replicate lines each of male biased (MB, 75 males and 25 females), equal sex (ES, 50 males and 50 females), and female biased (FB, 25 males and 75 females) sex ratio treatments were set up (i.e., nine lines in total). Each line was maintained in a plastic cage (220 × 140 × 85 mm) with a gauze-covered top. In each cage flies had access to water via two water-filled vials with cotton wool wicks. Flies were fed with two vials of SY food with added live yeast every two or three days. Live yeast was nonlimiting across all selection regimes, as indicated by excess yeast remaining in all vials each time the food was changed. Nine days after the cages were set up eggs were collected from each cage on grape plates smeared with yeast paste. The majority of eggs on the egg collection plates were allowed to hatch before larvae were collected, thus minimizing selection on early egg hatchability. Three hundred first instar larvae of each line were picked and raised at standard density (100 larvae per vial). To minimize selection on development time, all adults were allowed to eclose over two days before being allocated to the same sex-ratio treatment and replicate number as their parents. All subsequent generations were maintained using the same protocol as described above.

2.2.4 Mating and courtship observations

Mating and courtship observations were made on flies in vials or cages beginning immediately after lights-on. Courtship was defined as wing display directed towards a female or attempted mounting. For mating and courtship observations, vials were placed on a specially designed viewing rack which allowed large numbers of vials to be scanned in quick succession, at eye level.

2.2.5 Egg counts and egg to adult viability

Eggs counts were made using a dissection microscope at × 25 magnification. Vials were retained to allow offspring to emerge and after 12 days (which is more than ample for larval development, pupation and eclosion) the vials were inverted and frozen at -80°C. The adult flies in the frozen vials were counted at a later date. Inverting the vials made it easier to remove the flies for counting. Egg to adult viability was calculated as the proportion of eggs giving rise to adults.

2.2.6 Body size, testes size and accessory gland size measurements

All body size, testes size and accessory gland size measurements were made using the NIH Object Image program (version 1.62n3, by Norbert Vischer, available at http://simon.bio.uva.nl/object-image.html) on a Macintosh computer attached to a compound microscope (× 100). For body size, I used a measure of wing area (Gilchrist & Partridge, 1999; Calboli, 2004). The right-hand wings of the flies to be measured were mounted on glass slides using propanol and Aquamount (Calboli, 2004). Wing perimeter was measured as described in Gilchrist and Partridge (1999) by calculating the area within 6 landmark points around the edge of the wing (Fig. 2.1, green line). For testes and accessory gland measurements, males were dissected as previously described, in PBS (phosphate buffered saline), on a glass slide (Bangham et al., 2002). The polygon tool of the NIH Object Image program was used to measure the perimeter and area of testes and accessory glands (Fig. 2.2). Where possible, both accessory glands and testes from each individual were measured and the mean was used in subsequent analyses. If only one testis or accessory gland was measurable (e.g. if the other was damaged during dissection) then a single measurement was made.

2.2.7 Western blotting to determine sex peptide (SP) levels (Chapters 5 and 6)

To confirm that sex peptide (SP) knockdown males were deficient in SP and that control males produced SP (Chapter 5 and 6), Western blotting was performed as previously described (Chapman et al., 2003c). Briefly, males were placed in groups of 5 into Eppendorf tubes with 40µl of homogenization buffer (50 mM Tris·HCl, pH 7.5/10 mM EDTA, pH 8) and partially homogenized. 40µl of 2× sample buffer (125 mM Tris·HCl, pH 6.8/20% (vol/vol) glycerol/4% SDS/0.01% bromophenol blue/10% (vol/vol) 2-mercaptoethanol) was added, and the samples were fully homogenized, boiled for 4 min, transferred to ice for 2 min, centrifuged at 100 x *g* for 5 min at 4°C, and snap-frozen in liquid nitrogen. An equal amount of protein extract for each line was loaded on an SDS/polyacrylamide gel (15% acrylamide/bisacrylamide) and subjected to electrophoresis at 120V for approximately 1 hour. The gel was blotted with Towbin buffer on Hybond ECL nitrocellulose membrane (Amersham Pharmacia). The membrane was washed in blocking solution (5% low-fat dry milk in PBS/0.1% Tween 20 (PBS-T)) for 1 hour and incubated for 1.5 hours with the primary antibody (anti-SP rabbit antibodies, donated by Eric Kubli, University of Zurich). After washing with PBS-T solution, the membrane was incubated with peroxidase-labeled anti-rabbit secondary antibody (Amersham Pharmacia) for 1 hour, and then treated with the ECL Western blotting detection system (Amersham Pharmacia), according to the manufacturer's instructions. The Western blots showed that SP knockdown males produced no detectable SP and control males produced SP (Fig. 2.3).

2.2.8 Statistical analyses

Except where indicated, all statistical analyses were performed using the JMP software package (versions 5.0 or 5.1) for the Macintosh or PC (SAS, 1989-2002).

Figure 2.1 The wing of a male *Drosophila melanogaster* (× 100) mounted on a slide as described in section 2.2.6. The green line joins the 6 points used to measure wing area.

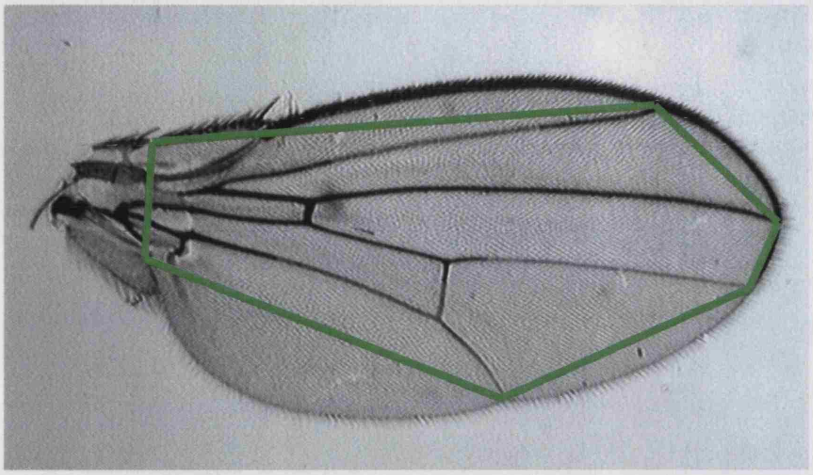
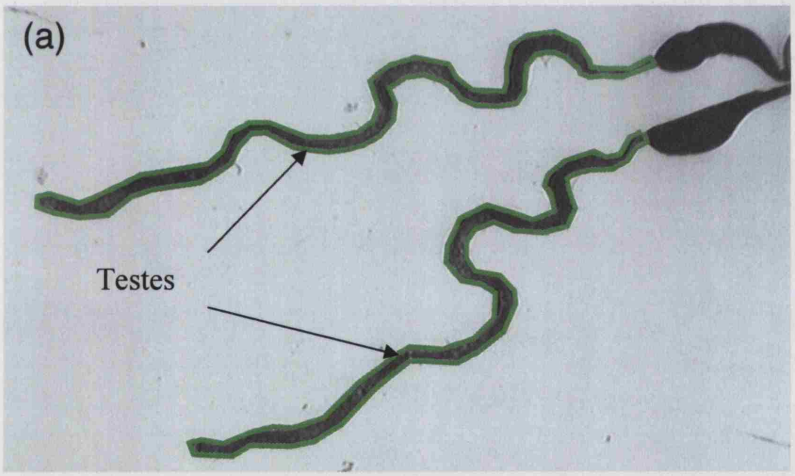


Figure 2.2 The (a) testes and (b) accessory glands of male *Drosophila melanogaster* (× 100). The green lines indicate the measurements made (see section 2.2.6 for more details).



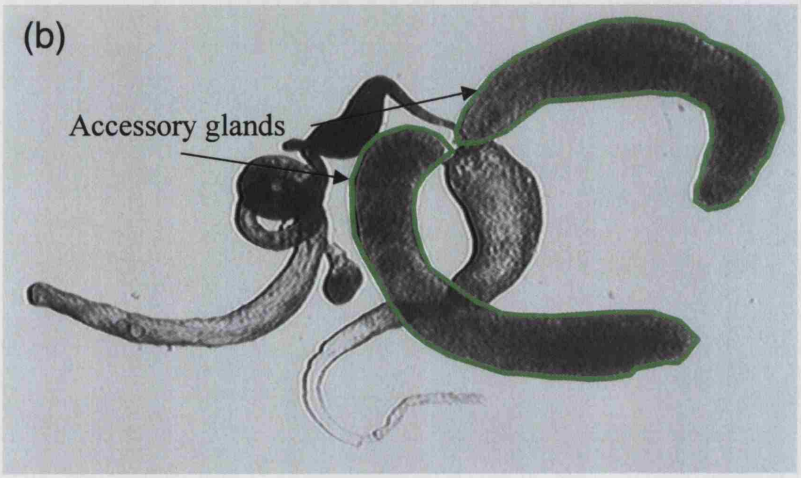
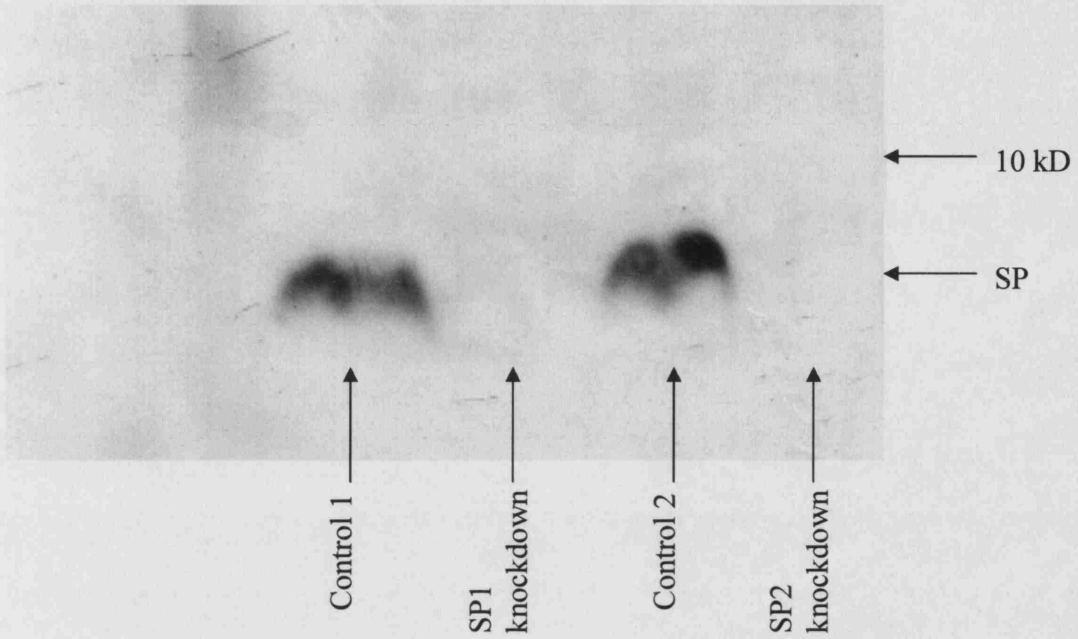


Figure 2.3 The results of Western blotting testing for the presence of SP in SP knockdown and control males used in the experiment in Chapter 5. The results were the same for Western blotting using experimental males from Chapter 6 (not shown).



Chapter 3. Female resistance to male harm evolves in response to the manipulation of sexual conflict

3.1 Abstract

The interests of males and females over reproduction rarely coincide and conflicts between the sexes over mate choice, mating frequency, reproductive investment, and parental care are common in many taxa. In *Drosophila melanogaster*, the optimum mating frequency is higher for males than it is for females. Furthermore, females that mate at high frequencies suffer significant mating costs due to the actions of male seminal fluid proteins. Sexual conflict is predicted to lead to sexually antagonistic coevolution, in which selection for adaptations that benefit males but harm females is balanced by counterselection in females to minimize the extent of male-induced harm. In this chapter, I tested the prediction that elevated sexual conflict should select for increased female resistance to male-induced harm and *vice versa*. I manipulated the intensity of sexual conflict by experimentally altering the adult sex ratio. I created replicated lines of *D. melanogaster* in which the adult sex ratio was male-biased (high sexual conflict lines), equal (intermediate conflict lines), or female-biased (low sexual conflict lines). As predicted, females from high sexual conflict lines lived significantly longer in the presence of wild-type males than did females from low conflict lines. This suggests that females from high sexual conflict lines had evolved higher resistance to male-induced harm than females from low sexual conflict lines. The conclusion that the evolutionary response in females was to the level of male-induced harm is supported by the finding that there were no female longevity differences between lines in the absence of males. Differences between selection line males in female-harming ability were not detected. This suggests that the response in selection line females was primarily to differences between selection treatments in mating frequency, and not to differences in male harmfulness itself.

3.2 Introduction

Sexual conflict is predicted to drive rapid evolutionary change (sexually antagonistic coevolution, section 1.4.1) which has the potential to lead to reproductive isolation and speciation in allopatric or even sympatric populations (sections 1.4.5 and 1.6.11). The techniques used in the few studies to date to investigate sexually antagonistic coevolution, include experimental evolution (section 1.6.8), morphological and genetic comparisons across related species and within species (section 1.6.9) and experimental intraspecific crosses between specific genotypes, intraspecific crosses between allopatric populations or interspecific crosses between closely related species (section 1.6.10). Experimental evolution has proved particularly useful for detecting sexually antagonistic coevolution (section 1.6.4). For example, in several studies sexually antagonistic coevolution has been investigated by imposing monogamy in polyandrous species (Holland & Rice, 1999; Hosken et al., 2001; Martin & Hosken, 2003; see section 1.6.8). In these studies the level of sexual conflict was predicted to be reduced under monogamy and consequently males in monogamy lines were predicted to evolve increased benignness to females. A potential problem with some of these studies (e.g. Holland & Rice, 1999; Hosken et al., 2001) is that the effective population size of monogamy lines was smaller than that of control, polyandry lines. Some findings, for example, reduced male competitiveness and reduced male harm to females under monogamy are predicted not only by sexual conflict, but also by the higher inbreeding and reduced body size that may be found in monogamy lines (section 1.6.8; Sharp, 1984; Pitnick et al., 2001; Snook, 2001; Pitnick & García-González, 2002; Chapman et al., 2003b). This makes some of the conclusions of these studies equivocal (see section 1.6.8).

In this chapter, I used a novel experimental evolution design to test whether manipulating the level of sexual conflict led to predictable changes in the level of female resistance to male-induced harm. I manipulated sexual conflict in *D. melanogaster* by varying the adult sex ratio, and produced three replicate lines each of male-biased (MB, 75 males and 25 females per replicate), equal-sex (ES, 50 males and 50 females per replicate) and female-biased (FB, 25 males and 75 females per replicate) sex-ratio treatments. Adult flies were maintained in these sex ratios for 9 days before eggs were collected to propagate the next generation (see section 2.2.3 in the General Methods for more details). This allowed plenty of time for multiple

behavioural interactions between males and females, and multiple mating by females was permitted in all treatments which is characteristic of natural populations (Harshman & Clark, 1998; Imhof et al., 1998). All lines were derived from a laboratory-adapted, outbred wild-type population (section 2.1.3.). I predicted that during selection, females from MB lines would be subject to frequent mating attempts from males and therefore should mate at a higher frequency than ES females, who in turn should mate more frequently than FB females. Thus, sexual conflict during selection was predicted to be most intense in the MB lines, intermediate in the ES lines, and least intense in the FB lines. Females from the MB lines were therefore predicted to evolve the strongest resistance to male-induced harm, followed by females from the ES and then FB lines. I also expected males from the MB lines to evolve greater harmfulness to females than males from the ES followed by FB lines. I tested female resistance to male-induced harm by measuring the survival of females from the selection treatments when kept with wild-type males. To check that the survival of selection line females was determined by the effects of continual exposure to males, I also measured the survival of once-mated females in the absence of males. I tested the harmfulness of selection line males by housing them continuously with wild-type females and measuring the survival of those females.

3.3 Methods

The 9 selection lines (3 replicates each of MB, FB and ES) were set up and maintained as described in the general methods (section 2.2.3).

3.3.1 Courtship and mating frequencies during selection

Courtship and mating frequencies within the selection cages were measured in a series of snapshot observations in generation 31. During the morning of each of six days spread evenly throughout the 9-day selection period, 10 observations were made on each of the 9 cages (with at least a 20-min gap between each observation to avoid counting matings twice). During each observation every cage was viewed from three sides in turn. This method minimized the double counting of matings and provided a good index of the level of courtship activity. The number of matings and courtship bouts (see section 2.2.4) was recorded for each cage. Any dead flies were removed and counted, to ensure that correct sample sizes were used in calculations of courtship and mating frequencies. Courtship and remating frequencies observed

were thus a representative sample of total courtship activity and total remating frequency.

3.3.2 Response to selection in females

Longevity, mating frequency, fecundity and egg to adult viability of selection line females continually housed with wild-type males.

Female resistance to male-induced harm was tested after 18 generations of selection by measuring the longevity of selection females housed with wild-type males. Eggs from all selection line and wild-type Dahomey cages (section 2.1.3) were collected and reared at a standard density using egg washes (section 2.2.1; Clancy & Kennington, 2001). Dahomey eggs were collected every seven days throughout the experiment to provide fresh, replacement wild-type males. Selection line eggs were allowed to hatch and 400 first instar larvae from each replicate of each selection treatment were placed into SY vials in batches of 100 ('larval picks', section 2.2.1). One hundred virgin females from each replicate of each selection treatment and 900 virgin Dahomey males were collected (section 2.2.2). The virgin flies were placed in vials in single sex batches of 10. One bottle of Dahomey flies was allowed to emerge and was maintained in bottle culture with added live yeast, to provide spare males to replace any that died. One day after eclosion, selection females were split into batches of five and placed into fresh vials. Five Dahomey males were added to each of the vials already containing five selection females (20 vials, n = 100, for each replicate of each selection treatment). Flies were transferred onto fresh food every two or three days throughout the experiment and an equal sex ratio was maintained in each vial, using spare Dahomey males if necessary. As females died, vials were combined to maintain fly density at five females and five males per vial. Dead flies were removed each time flies were transferred to new food and every seven days all males were discarded and replaced with fresh, 1 day-old Dahomey males. Female deaths were recorded six days a week until almost all (>98%) of the females had died. The mating frequencies of experimental flies were recorded twice a week from 10 observations made on each vial at least 20 min apart. The fecundity of a sample of females from each replicate of each selection line was also measured twice a week. In each sample, 15 females were taken from each line and placed singly with one male each in a vial for 24 h, after which they were returned to their original groups. Eggs laid in the 24 h period were immediately counted and the vials retained in order to count progeny. Egg to adult viability was calculated as described

in section 2.2.5. For subsequent fecundity measures, a different set of 15 females was chosen from each line (returning to the first sample when all females within a line had been used). The use of different groups of flies for each fecundity measure minimized differential treatment of experimental females. After 22 generations of selection, a replicate longevity experiment was performed, using double the sample size of females (n = 200 for each replicate of each selection treatment, method as described above).

Longevity of once-mated selection line females in the absence of males.
To determine whether differences in female longevity were attributable to resistance to male-induced harm or instead reflected intrinsic differences in survival between the selection treatments, I measured the longevity of once-mated selection line females, after 26 generations of selection. Egg collection, rearing of larvae, and adult virgin collection were as described above, except that Dahomey virgin males were placed in groups of 50 into yeasted SY bottles. One day after eclosion, selection females were placed in groups of 50 into the bottles containing 50 Dahomey males and were left for 48h to allow all females to mate and initiate egg laying (Partridge & Fowler, 1990). Selection females were then placed in groups of 10 into yeasted SY vials and the males were discarded. Females were transferred into fresh, yeasted SY vials every two or three days for the duration of their life and dead flies were removed at each transfer. Deaths were recorded six days a week and vials were combined as females died to keep density at 10 females per vial whenever possible.

3.3.3 Response to selection in males

Longevity, mating frequency, fecundity and egg to adult viability of wild-type females continually housed with selection line males.
To measure the extent of male-induced harm to females, the longevity of wild-type females housed with selection males was tested after 33 generations of selection. Initial egg collection and larval rearing were as described above. The collection of selection line eggs was repeated every seven days throughout the experiment to provide fresh replacement selection males. The experiment was conducted as above, except that selection males and Dahomey females were used and courtship bouts were recorded in addition to matings.

Body size, accessory gland and testis size of selected flies.

To document any morphological differences between the selection treatments, I measured the body size, accessory gland size, and testis size of flies reared at standard densities from generation 32 of selection. Approximately 50 flies of each sex from each replicate of each selection treatment were placed in yeasted SY bottles and allowed to mate for four days. The sexes were then separated and females were immediately frozen at -80°C. Males were maintained in groups of five in yeasted SY vials for 10–12 days to allow sufficient time for replenishment of the contents of testes and accessory glands (Bangham et al., 2002). Measurements were taken as described in the general methods, section 2.2.6.

3.3.4 Statistical analyses

Normality and homogeneity of variances of all raw data and residuals from models were checked by Shapiro-Wilk (Shapiro & Wilk, 1965) and Bartlett's tests (Zar, 1999). Where required, data were transformed (log, inverse log, power, or a combination of these) to homogenize variances and/or normalise data. The numbers of matings and courtship bouts were summed across observation days and compared using a one-way analysis of variance (ANOVA) (Zar, 1999). When required, multiple comparisons were made using Student-Newman-Keuls (SNK) tests (Zar, 1999). To determine accurate probabilities from q-values for SNK tests I used the software program 'R' (Ihaka & Gentleman, 1996). To compare the longevity of flies from different selection treatments, a Cox's proportional hazards regression (Cox, 1972) was performed on survival data to provide unbiased estimates of survival risk for each line. Survival risk-ratios were then compared across selection treatments using one-way ANOVA followed, where necessary, by SNK tests. Where longevity was assayed in two replicate experiments, probabilities across experiments were combined (Sokal & Rohlf, 1995). I analyzed fecundity and egg to adult viability data with Kruskal-Wallis tests (Kruskal & Wallis, 1952), excluding data from lines where the number of surviving females was ≤ 5. Critical P-values for fecundity and egg to adult viability analyses were corrected for multiple comparisons using the sequential Bonferroni method (Rice, 1989). Where differences occurred, nested ANOVAs were performed (Zar, 1999). The two effects in the model were selection treatment (fixed effect) and replicate (random effect) nested within selection

treatment (restricted maximum-likelihood method). Mean absolute body size, accessory gland, and testis sizes were compared between selection treatments using one-way ANOVAs. Allometry between testis and body size and accessory gland and body size was also compared across selection treatments, by comparing the mean values from replicates between treatments using a one-way ANOVA.

3.4 Results

3.4.1 Courtship and mating frequencies during selection

Females.

During selection the number of matings per female and the number of courtship bouts received per female were highest in the MB, followed by ES, followed by FB lines, as expected. There were significant differences between selection treatments in the total number of matings per female over the 6 observation days (log transformed) (Fig. 3.1(a), $F_{2,6} = 8.25$, $P = 0.019$). Females in MB lines were mated significantly more than females in FB lines ($\overline{X} \pm SE$, MB = 0.67 ± 0.16 matings per female, FB = 0.23 ± 0.01 matings per female, $P = 0.018$) and ES lines ($\overline{X} \pm SE$, ES = 0.30 ± 0.04 matings per female, $P = 0.027$). There were no significant differences in the numbers of matings between ES and FB lines ($P = 0.35$). There were significantly more total courtships per female (log transformed) observed in the MB compared to the ES followed by the FB lines ($F_{2,6} = 51.04$, $P = 0.0002$; $\overline{X} \pm SE$, MB = 20.80 ± 3.04 courtship bouts per female, ES = 8.37 ± 0.80 courtship bouts per female, FB = 4.18 ± 0.18 courtship bouts per female: MB *versus* FB, $P = 0.0001$; MB *versus* ES, $P = 0.001$; ES *versus* FB, $P = 0.005$). These results indicate that the predicted intensity of sexual conflict varied as anticipated during selection, being most intense in the MB lines, followed by the ES then FB lines.

Males.

As expected from the above analysis, the number of matings per male and the number of courtship bouts per male during selection were highest in the FB, followed by ES, followed by MB lines. There were significant differences between selection treatments in the total number of matings per male over the 6 observation days (Fig. 3.1(b) $F_{2,6} = 31.77$, $P = 0.0006$). Males in MB lines mated significantly less often than males in FB lines ($\overline{X} \pm SE$, MB = 0.22 ± 0.05, FB = 0.69 ± 0.04, $P = 0.0007$) but not significantly less than ES lines ($\overline{X} \pm SE$, ES = 0.30 ± 0.04, $P = 0.26$)

and males in ES lines mated significantly less often than males in FB lines (P = 0.0008). There were significant differences in the number of courtship bouts delivered per male ($F_{2,6}$ = 13.41, P = 0.006). The number of courtship bouts per male in MB lines was significantly lower than in FB lines ($\overline{X} \pm SE$, MB = 6.98 ± 0.99, FB = 12.73 ± 0.50, P = 0.006). The number of courtship bouts per male in ES lines was significantly lower than in FB lines ($\overline{X} \pm SE$, ES = 8.43 ± 0.88 P = 0.01). There was no significant difference in the number of courtship bouts per male between MB and ES lines (P = 0.26).

3.4.2 Response to selection in females

Longevity, mating frequency, fecundity and egg to adult viability of selection line females continually housed with wild-type males.

There were significant, repeatable differences in female survival in the presence of wild-type males across both replicate survival experiments (Fig. 3.2, combined probabilities from both survival experiments, χ^2_4 = 12.97, P = 0.011). MB and ES line females both had significantly higher survival (i.e. lower risk-ratios) than FB line females (combined probabilities from both survival experiments, MB *versus* FB, χ^2_4 = 13.52, P = 0.009; ES *versus* FB, χ^2_4 = 9.58, P = 0.048). MB line females had higher, but not significantly higher, survival (i.e. lower risk-ratios) than ES line females (combined probabilities from both survival experiments, χ^2_4 = 6.39, P = 0.17). Mean female survival values (days ± SE) for the first and second experiments respectively were MB1 28.25 ± 0.74, 27.26 ± 0.58; MB2 24.49 ± 0.81, 25.03 ± 0.64; MB3 31.07 ± 0.82, 27.59 ± 0.62; ES1 26.80 ± 0.63, 25.54 ± 0.56; ES2 27.09 ± 0.78, 24.05 ± 0.60; ES3 26.35 ± 0.76, 24.93 ± 0.60; FB1 24.10 ± 0.84, 24.27 ± 0.59; FB2 24.28 ± 0.69, 22.73 ± 0.51; FB3 24.68 ± 0.77, 24.01 ± 0.53. These differences in female survival could not be explained by variation in mating frequencies as no differences between treatments were found (measured during the first experiment at generation 18, $F_{2,6}$ = 0.24, P = 0.79). Female survival is reduced by frequent mating (Fowler & Partridge, 1989) due to the actions of male seminal fluid proteins produced in the accessory glands (Acps) (Chapman et al., 1995; section 1.6.4). Thus, the survival results presented here provide evidence for a significant increase in resistance to male-induced harm in females from the MB relative to the FB treatments, with intermediate resistance in the ES females.

The life-history measurements from the first replicate experiment at generation 18 show that there were no significant differences in fecundity attributable to selection effects (Fig. 3.3(a)). Significant differences in fecundity on days 3 and 5 (Kruskal-Wallis tests; day 3, $\chi^2_8 = 42.86$, $P < 0.0001$, critical $P = 0.0056$; day 5, $\chi^2_8 = 25.03$, $P = 0.0015$, critical $P = 0.0063$) were attributable to replicate and not selection treatment differences (nested ANOVA model on square transformed data: day 3, selection effect, $F_{2,125} = 1.0426$, $P = 0.41$; day 5, selection effect, $F_{2,120} = 0.52$, $P = 0.62$). day 3 median (lower and upper quartile) fecundity values were MB1 91.5 (87.75, 100.25); MB2 68 (50, 74); MB3 81 (61, 86); ES1 91 (75, 99); ES2 83 (66, 85); ES3 98 (88, 107); FB1 88 (82, 90); FB2 92 (89, 99); FB3 88 (73, 104). Day 5 median (+ lower and upper quartile) fecundity values were MB1 86 (80, 95); MB2 84.5 (77, 94); MB3 73.5 (65.5, 79.5); ES1 90.5 (82.75, 100); ES2 76 (59.5, 89.25); ES3 96.5 (87.5, 111.5); FB1 91 (59, 99); FB2 89 (78, 99); FB3 88.5 (75.5, 97.5). There was also no significant effect of selection on egg to adult viability (i.e. number of progeny/number of eggs, Fig. 3.3(b)). The one significant difference on day 5 (Kruskal-Wallis test, $\chi^2_8 = 23.24$, $P = 0.0031$, critical $P = 0.01$) was attributable to variation between replicates and not to differences between selection treatments (nested ANOVA on day 5 egg to adult viability data twice inverse log transformed and raised to the power of 1.5; selection effect, $F_{2,119} = 2.14$, $P = 0.20$). Day 5 median (lower and upper quartile) egg to adult viability values were MB1 0.90 (0.77, 0.93); MB2 0.72 (0.67, 0.81); MB3 0.76 (0.70, 0.87); ES1 0.82 (0.74, 0.91); ES2 0.91 (0.85, 0.94); ES3 0.88 (0.83, 0.92); FB1 0.86 (0.73, 0.95); FB2 0.90 (0.87, 0.95); FB3 0.92 (0.62, 0.94). The results show that the longevity differences between females from the different selection treatments were not accompanied by detectable differences in mating frequency, age-specific fecundity or egg to adult viability.

Longevity of once-mated females in the absence of males.

There were no significant differences between selection treatments in the survival risk-ratios of once-mated females in the absence of males (risk-ratios, $\overline{X} \pm SE$, MB = 0.99 ± 0.34, ES = 1.30 ± 0.29, FB = 0.92 ± 0.09; $F_{2,6} = 0.58$, $P = 0.59$). Mean survival values (days ± *SE*) were MB1 36.35 ± 0.80; MB2 43.32 ± 1.29; MB3 43.81 ± 1.37; ES1 42.36 ± 0.97; ES2 33.37 ± 1.07; ES3 38.47 ± 1.34; FB1 38.24 ± 1.29; FB2 41.29 ± 0.93; FB3 42.93 ± 1.19. This finding supports the interpretation that the

differential longevity of selection females in the presence of males (Fig. 3.2) is a specific response to the effects of males and does not stem from differences between treatments in intrinsic female survival.

3.4.3 Response to selection in males

Longevity, mating frequency, fecundity and egg to adult viability of wild-type females continually housed with selection line males.

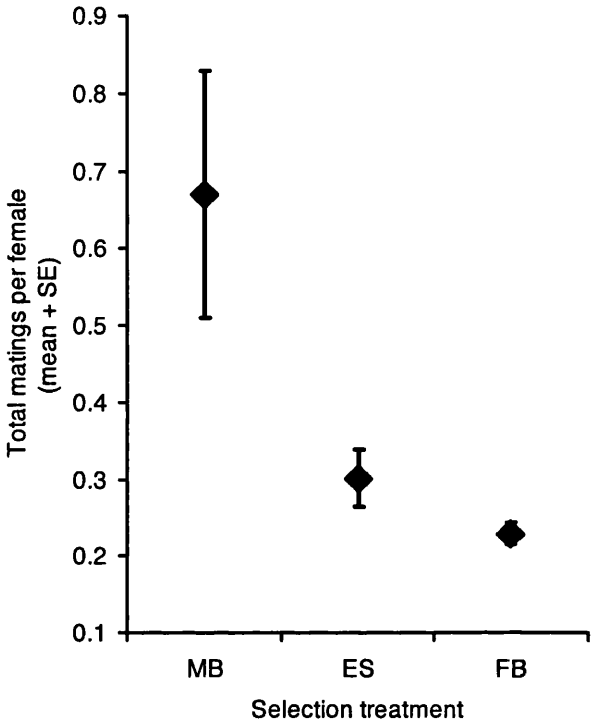
There were no significant differences between the survival of wild-type females housed with males from the different selection treatments (Fig. 3.4, risk-ratios, $\overline{X} \pm SE$, MB = 1.09 ± 0.13, ES = 0.87 ± 0.13, FB = 1.11 ± 0.09; $F_{2,6} = 1.51$, $P = 0.29$). Mean survival values (days ± *SE*) were MB1 22.31 ± 0.59; MB2 21.17 ± 0.56; MB3 23.55 ± 0.59; ES1 22.24 ± 0.58; ES2 23.90 ± 0.69; ES3 24.28 ± 0.62; FB1 21.63 ± 0.58; FB2 22.17 ± 0.56; FB3 22.78 ± 0.61. There were significant differences between selection treatments in the total number of matings per female ($F_{2,6} = 12.12$, $P = 0.008$). Females housed with MB males mated significantly more frequently than females housed with ES males ($\overline{X} \pm SE$, MB = 0.065 ± 0.007 matings per female, ES = 0.039 ± 0.003 matings per female; $P = 0.006$). Females housed with MB males also mated more, but not significantly more, than females housed with FB males ($\overline{X} \pm SE$, FB = 0.053 ± 0.008 matings per female; $P = 0.063$). Females housed with FB males mated significantly more than females housed with ES males ($P = 0.039$). However, these mating frequency differences did not result in significant differences in longevity. There were no significant differences between the selection treatments in the total number of courtship bouts received per female ($F_{2,6} = 2.36$, $P = 0.18$). There also were no significant differences in age-specific fecundity (Fig. 3.5(a)) between treatments on any day ($\chi^2_8 < 10.76$, $P > 0.21$, critical P = 0.0083) or in egg to adult viability (Fig. 3.5(b)) between treatments on any day ($\chi^2_8 < 15.15$, $P > 0.056$, critical $P = 0.0083$). The results show that, contrary to expectation, there were no differences in the ability of selection males to harm wild-type females. This suggests that the evolution of female resistance was achieved by a response in females to differences in the number of matings received and not to differences between males in their ability to harm females.

Body size, accessory gland and testis size of selected flies.

There were no significant differences between the selection treatments in any of the morphological characters measured. There were no significant differences between selection treatments in mean male (cube transformed, $F_{2,6} = 0.79$, $P = 0.50$) or female wing areas (Table 3.1, $F_{2,6} = 0.12$, $P = 0.89$). There were also no significant differences between selection treatments in mean male accessory gland area (Table 3.1, $F_{2,6} = 0.50$, $P = 0.63$) or mean testis area (Table 3.1, $F_{2,6} = 2.21$, $P = 0.19$). Nor were there any significant differences in allometry between selection treatments in mean accessory gland and wing area (log transformed, $F_{2,6} = 0.27$, $P = 0.77$) or mean testis and wing area ($F_{2,6} = 1.33$, $P = 0.33$).

Figure 3.1 The mean (± *SE*) total number of matings (a) per female and (b) per male observed during selection. Matings were observed on 6 days out of the 9 day selection period. 10 observations were made on each cage on each day, with at least a 20 min gap between each to avoid double counting matings.

(a)



(b)

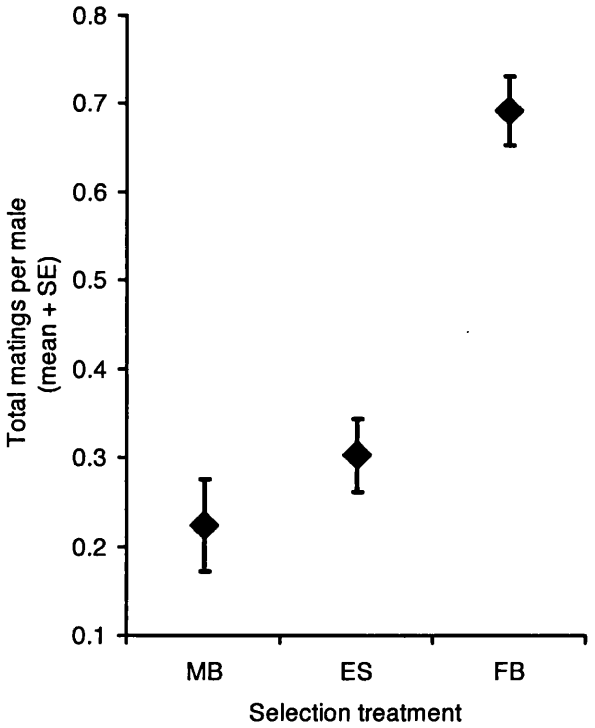


Figure 3.2 Survival risk-ratios (mean ± *SE*) of selection line females continually housed with wild-type males. Results of two replicated experiments performed at generations 18 (a) and 22 (b) are shown. Note that high risk-ratio values indicate low survival.

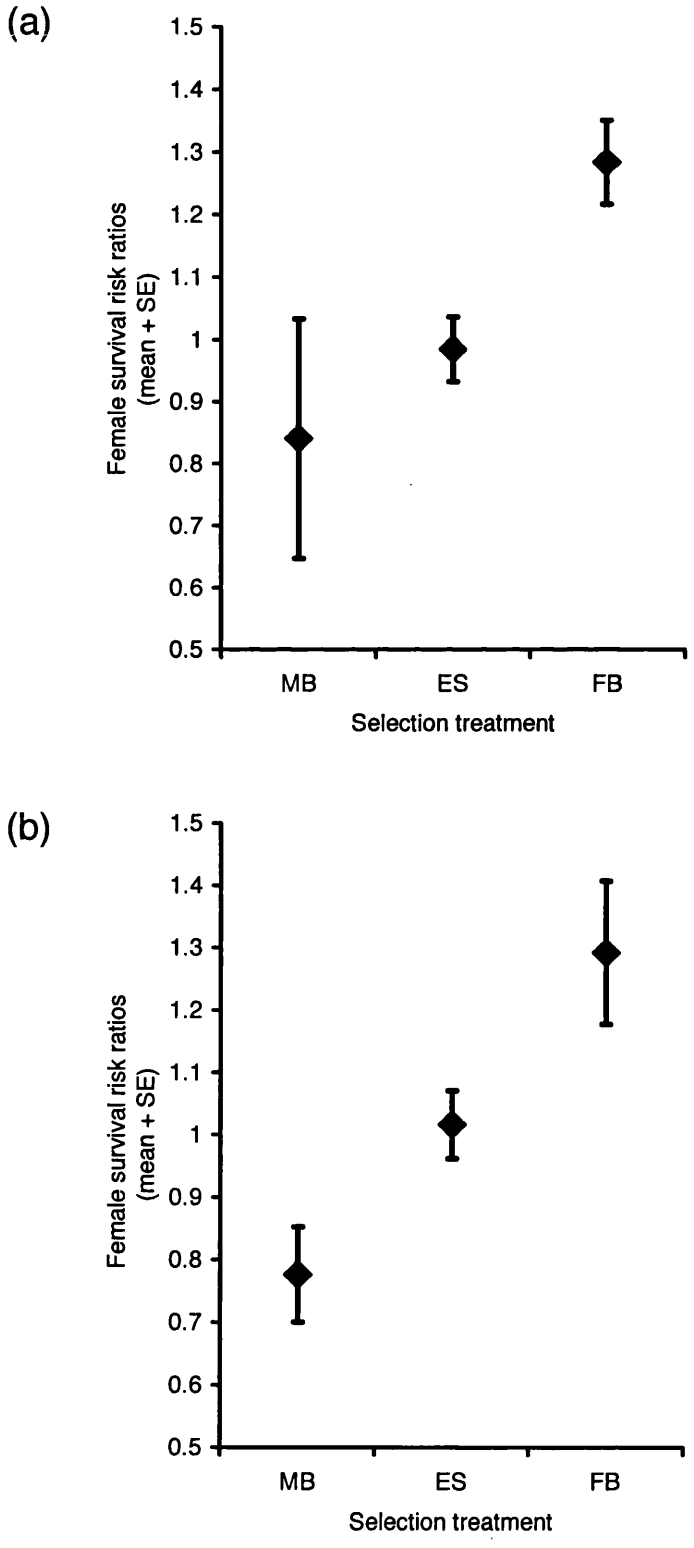
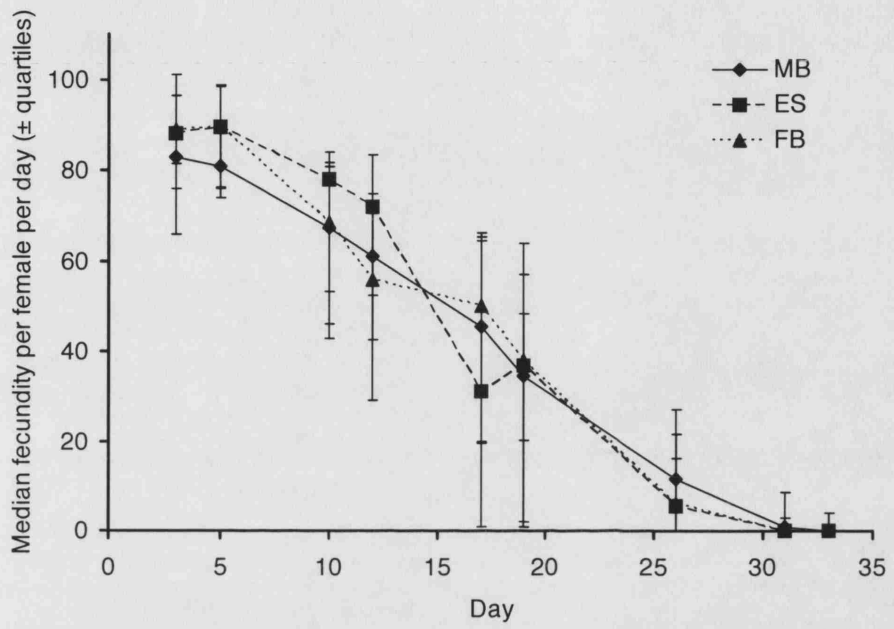


Figure 3.3. Median (± interquartile range) (a) fecundity per female per day and (b) viability of eggs per female per day (number of progeny/number of eggs) of selection line females (replicates combined) continually housed with wild-type males after 18 generations of selection.

(a)



(b)

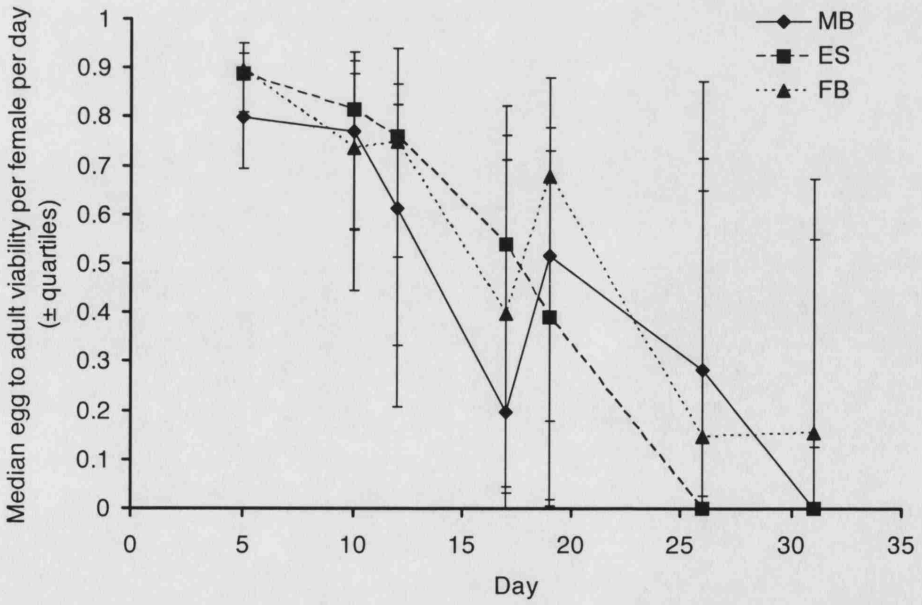


Figure 3.4 Survival risk-ratios (mean ± *SE*) of wild-type females continually housed with selection line males after 33 generations of selection. Note that high risk-ratio values indicate low survival.

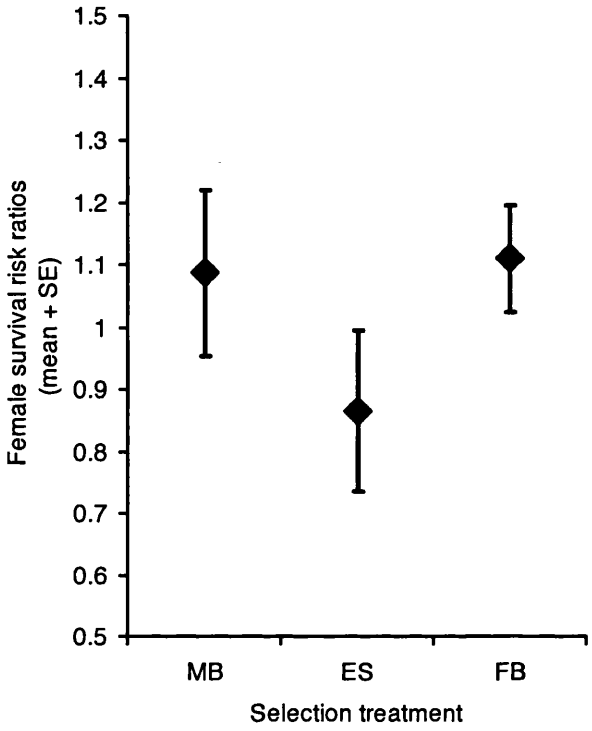
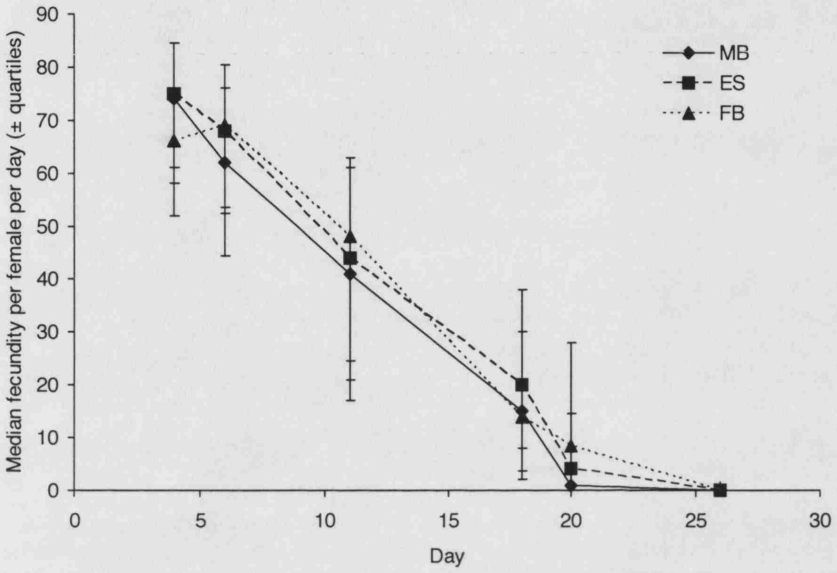


Figure 3.5 Median (± interquartile range) (a) fecundity per female per day and (b) egg to adult viability per female per day (number of progeny/number of eggs) of wild-type females (replicates combined) continually housed with selection males after 33 generations of selection.

(a)



(b)

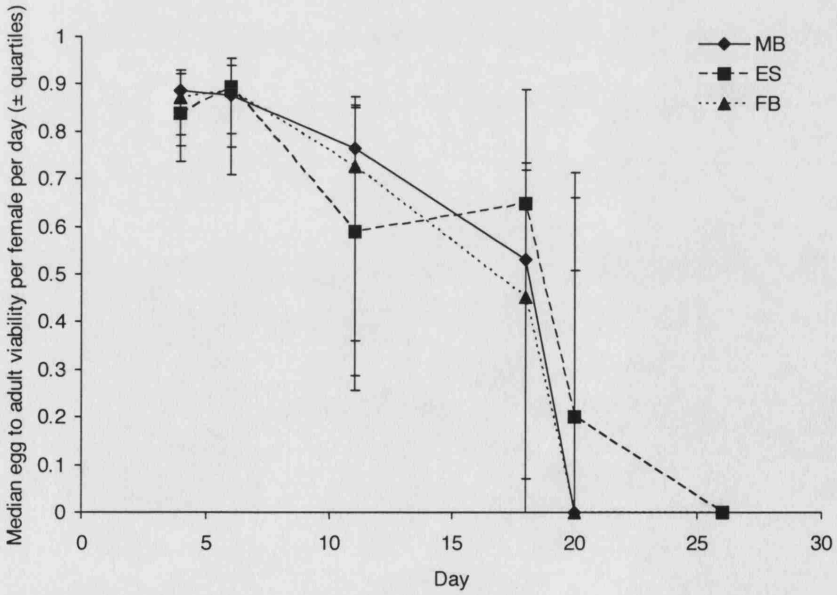


Table 3.1 Body size (wing area), accessory gland area and testis area of the selected flies. Mean area ± *SE*.

	Mean area (mm^2) ± *SE*			
Selection treatment	Female wing	Male wing	Accessory gland	Testis
MB	1.334 ± 0.023	1.043 ± 0.016	0.231 ± 0.013	0.146 ± 0.005
ES	1.343 ± 0.011	1.063 ± 0.003	0.232 ± 0.002	0.165 ± 0.010
FB	1.330 ± 0.020	1.049 ± 0.014	0.242 ± 0.005	0.151 ± 0.004

3.5 Discussion

3.5.1 Female resistance to male-induced harm and male harmfulness of selected flies

The experimental evolution imposed by the alterations in adult sex ratio manipulated sexual conflict as predicted. During selection, females were courted and mated most frequently in MB populations, at intermediate frequency in ES populations, and least frequently in FB populations (Fig. 3.1). Hence, during selection, females from the MB lines should have experienced greater male-induced harm than females from ES lines, with FB females experiencing the least harm. The most striking finding from the experiments described in this chapter was that, as predicted by sexual conflict theory, females evolved in response to differences in the risk of male-induced harm. Females from the MB lines showed significant and repeatable longer lifespan in the presence of wild-type males compared to FB females, with females from the ES treatments showing intermediate longevity (Fig. 3.2). No such differences in survival were detected in the absence of males, consistent with the response in females being specific and directed towards altering resistance to male-induced harm. Thus, higher intensities of sexual conflict selected for more resistant female phenotypes and *vice versa.* These differences in female survival occurred in spite of, rather than being confounded by, inbreeding which can result in decreased fitness (Sharp, 1984; Miller et al., 1993; Garcia et al., 1994) and can represent a problem if experimental and inbreeding predictions are the same (e.g. in Holland & Rice, 1999; Hosken et al., 2001). In the selection lines used in this study, the highest effective population size (Wright, 1938) was in the ES (N_e = 100), followed by the FB and MB lines (N_e = 75 for both,). However, the MB lines are actually likely to be more inbred than the FB lines because the variance in mating success (and therefore reproductive success) in males in a population tends to be greater than the variance in reproductive success in females (e.g. Joshi et al., 1999). Thus, in MB populations, because there are more males than in FB populations, there are likely to be fewer individuals contributing genes to subsequent generations. In the tests, female survival was highest in MB, followed by ES then FB females, a pattern opposed by inbreeding differences. The evolution of female resistance to male-induced harm in this study was not accompanied by detectable trade-offs in the other fitness-related traits measured, i.e., fecundity and eggs to adult viability. Such costs of resistance are expected and it will be interesting to examine other traits in these females that can affect fitness, such as immunity and larval competitive ability.

The results showed, contrary to expectation, no evidence that males from the MB treatments had evolved to become more harmful to females. This suggests that females from these lines responded predominately to differences in mating frequency between the sex ratio treatments during selection, and not to differences between the harmfulness of males. It will be interesting to confirm this by examining the responses of females from these lines to males with deficient ejaculate components (e.g. Chapman et al., 2003c; Liu & Kubli, 2003).

3.5.2 Morphological traits of selected flies

Large *D. melanogaster* males are observed to be more harmful to females than are small males (Pitnick & García-González, 2002). The lack of significant body size differences between selection treatment males in this study is therefore consistent with the idea that the evolution of female resistance was to differences in the number of matings received and not to differences between males in their ability to harm females. I also detected no significant differences in male testis or accessory gland size between treatments. This is perhaps unexpected, in light of the fact that the intensity of sperm competition should differ between the sex ratio treatments (predicted to be most intense in the MB lines, intermediate in ES lines and least intense in FB lines). Male mating frequency also differed between sex ratio treatments (highest in the FB lines, intermediate in ES lines and lowest in MB lines). Thus, the number of mating opportunities per male during selection was greatest for FB males, least for MB males and intermediate for ES males. Theory predicts that males should partition investment in ejaculates between matings (Parker, 1998) such that males that have many mating opportunities should invest less in each mating than males that have fewer mating opportunities (discussed further in Chapter 4). From this theory, FB males would be predicted to show the most ejaculate partitioning, ES males intermediately and MB males would be predicted to invest heavily in every mating opportunity. Traits that are likely to be involved in sperm competition and investment in ejaculates would perhaps have been expected to respond to the differences between treatments (see Hosken et al., 2001; Pitnick et al., 2001). For example males from the FB lines might be expected to invest more in sperm production than MB males, and males from the MB lines to invest more in Acp production and body size than FB males. However, it is difficult to make robust predictions of the direction of responses in these traits to the predicted differences in sperm competition intensity. Male strategies will vary depending on the exact level

of competition, for example, it may pay a male to increase investment in traits that ensure sperm competitive success (e.g., large body size, large testis size or large accessory gland size Parker, 1998). However, this would only hold up to a certain level of competition, beyond which the risk of sperm usurpation would be so high that increased investment could be selected against (Parker et al., 1996; Parker et al., 1997). Under such potentially conflicting selection pressures, it is unclear whether the prediction is for larger or smaller body/accessory gland/testis size under the MB, ES or FB regimes. Accurate predictions rely on identifying the exact selective pressures involved. For example, males from FB lines may exert similar amounts of energy in their frequent courtship bouts and matings as males from MB lines exert in male-male competition and their less frequent matings. Thus investment in competitive activity and body size could constrain the evolution of larger accessory glands. It would be interesting to probe for potential alternative strategies employed by males from the different selection lines (e.g. by testing single mating productivity, induction of female non-receptivity, ejaculate replenishment and changes in sperm characteristics).

Lack of responses to selection could be explained if all flies were released from selection for harm and counter harm upon introduction to the novel selection line culturing regime. However, in the stock populations, under cage culture, selection on early fecundity is expected, if anything, to be even stronger and adult lifespan shorter than for the selection line flies used here, during selection. This would not then relax selection in the manner proposed. In addition, this argument would not explain why females responded strongly to differences in mating rate, but in males a potential response in harming ability was masked by adaptation to the culturing conditions (which both sexes encountered). I therefore conclude that responses in males did not include differences in harming ability *per se*.

3.5.3 Conclusions

In summary, the results presented in this chapter provide evidence that sexual conflict can be experimentally manipulated and that females can adapt to alterations in the intensity of sexual conflict by evolving increased or decreased resistance to male-induced harm. The results also make further predictions. For example, 'susceptible' females from the FB lines would be expected to alter their current reproductive output according to their residual reproductive value (Fisher, 1930),

and hence increase investment into current as opposed to future reproduction (Lessells, 1999). In wild populations, one would also expect selection pressure in favour of the evolution of female resistance to male harm to track closely the operational sex ratio. Further work is now required to investigate this, to confirm the mechanism by which females evolve resistance to male-induced harm, to investigate the direction of responses relative to the base stock and to elucidate whether males harm females to gain direct fitness benefits or whether females are harmed as a side-effect of male adaptations that confer increased reproductive success.

Chapter 4. No evidence that mating probability or copulation duration evolves in response to manipulation of sexual conflict, sperm competition or male mating opportunities in *Drosophila melanogaster*

4.1 Abstract

When females mate multiply there is the potential for both sexual conflict and sperm competition to occur. A prediction from sexual conflict theory is that increased levels of sexual conflict should select for a reduced willingness of females to mate to avoid mating costs, and that this could drive reproductive isolation by decreasing the probability of matings between different, allopatric populations. Elevated levels of sperm competition and low numbers of mating opportunities for males are predicted to select for increased 'per-mating' male investment in ejaculates. Larger ejaculates may take longer to transfer to females and in *Drosophila melanogaster* copulation duration appears to be largely under male control. Therefore, increased levels of sperm competition and low numbers of mating opportunities may select for extended copulation times in males. To test these predictions from sexual conflict and sperm competition theory I used replicate lines of *Drosophila melanogaster* that had been selected under different levels of sexual conflict and sperm competition. The females from these lines exhibited differences in the level of resistance to male-induced harm (Chapter 3). I tested the time until mating and the copulation duration of pairs of virgin flies. The pairs were comprised of either a male and a female from the same replicate of the same treatment, or a male and a female from different replicates of the same treatment. The mating probability of pairs of virgin flies from high conflict lines was not different from the mating probability of pairs of virgin flies from low conflict lines, and the mating probability of pairs consisting of males and females from the same replicate was not different from the mating probability of pairs in which the male and female were from different replicates. Also, differences in copulation duration were not in the directions predicted by sperm competition. The results did not support the predictions that sexual conflict drives population divergence via changes in female willingness to mate or that sperm competition selects for extended copulation duration in *Drosophila melanogaster*. Furthermore, the results did not support the prediction that females evolve pre-mating resistance to males by reducing their willingness to mate, but instead the results supported the idea that females can resist male-induced harm via post-mating mechanisms.

4.2 Introduction

4.2.1 Sexual conflict and reproductive isolation

Theory predicts that sexual conflict has the potential to drive reproductive isolation and speciation (Parker & Partridge, 1998; Gavrilets, 2000; Gavrilets et al., 2001; Martin & Hosken, 2003). Several studies provide evidence that sexual conflict can drive the coevolution of antagonistic traits between the sexes (Rice, 1996; Holland & Rice, 1999; Knowles & Markow, 2001; Arnqvist & Rowe, 2002a; Rowe & Arnqvist, 2002). However, only one study to date has provided empirical evidence for reproductive isolation through sexual conflict (Martin and Hosken 2003; see section 1.6.8). Martin and Hosken (2003) used experimental evolution to investigate the role of sexual conflict in the evolution of female resistance to male mating attempts in the dung fly *Sepsis cynipsea*. Three replicate populations each of 3 treatments, monogamous, low density and high density were set up. Increasing density was assumed to lead to higher levels of sexual conflict, as mating rates are known to increase with increasing numbers of males (Blanckenhorn et al., 2000). The results showed that females that had evolved in higher density populations resisted male mating attempts significantly more than females that had evolved in lower density populations, consistent with the idea that females had evolved resistance to males by decreasing their receptivity to mating. Females that evolved at high density also resisted mating attempts from males from different populations significantly more than they resisted mating attempts from same-population males. In contrast, the willingness to mate of monogamous females was independent of the origin of their mating partner. This is consistent with the suggestion of higher divergence and hence stronger reproductive isolation in the high density (high sexual conflict) populations (Martin & Hosken, 2003).

It is not yet clear whether increased divergence under strong sexual conflict is likely to be a general phenomenon (e.g. Parker and Partridge 1998), nor whether similar traits are likely to be involved across different species with different mating systems. To address these issues, I tested the prediction of greater pre-mating reproductive isolation under high levels of sexual conflict, using *D. melanogaster*. The hypothesis was that females that evolve under experimentally elevated levels of sexual conflict should evolve a reduced willingness to mate, but have a higher probability of mating with males from their own population than with males from different populations. Females can exhibit a variety of mating avoidance behaviours including abdomen

bending, ovipositor extension and kicking (Connolly & Cook, 1973) and thus have the potential ability to reduce the probability of copulation.

4.2.2 Sperm competition, copulation duration and investment in ejaculates in *D. melanogaster*

The adaptive significance of copulation duration in *Drosophila melanogaster* is relatively poorly understood. Copulation duration appears to be mainly under male control (MacBean & Parsons, 1967) and extended copulation (over 8 minutes) is essential to induce a full refractory period in females (Gilchrist & Partridge, 2000). Thus, copulation duration over 8 minutes may be necessary to allow receptivity-inhibiting substances such as the sex peptide (Chen et al., 1988; Chapman et al., 2003c; Liu & Kubli, 2003) and possibly DUP99B (Saudan et al., 2002) to be fully effective. Copulation duration may therefore be associated with the intensity of sperm competition (Parker et al., 1996), long matings and thus strong receptivity inhibition being strongly selected when the intensity of sperm competition is high and selection on long matings being relaxed when the intensity of sperm competition is lower. This is because, in general, investment in ejaculates is predicted to increase with increased sperm competition (Parker, 1998, but see Parker et al., 1996) and copulation duration is expected to increase with increased ejaculate investment if larger ejaculates take longer to transfer. Thus, I predict that males that evolve under high levels of sperm competition may evolve to copulate for longer than males that evolve under low levels of sperm competition.

Male investment in ejaculates is also likely to reflect the number of mating opportunities that males experience. Theory predicts that males should partition how much they invest in each ejaculation (Parker, 1998) because, if there are limits to the quantity of ejaculate that males can produce, partitioning of ejaculates may be advantageous and may avoid ejaculate depletion. Males that have evolved under conditions of many mating opportunities are predicted invest less in each mating than males that have evolved with fewer mating opportunities. For example, in *D. melanogaster*, large males have higher success in gaining matings than do small males (Partridge & Farquhar, 1983; Partridge et al., 1987a; Partridge et al., 1987c) and thus the number of mating opportunities is greater for large males than it is for small males. Small males copulate for longer than large males which is consistent with the idea that small males invest more ejaculate in each mating than large males

because the number of mating opportunities for small males is lower than the number of mating opportunities for large males (Pitnick, 1991). Thus, males that have many mating opportunities are predicted to invest less each mating than males that have fewer mating opportunities.

4.2.3 Testing predictions about sexual conflict and sperm competition using experimental evolution in *D. melanogaster*

To test the predictions outlined above (in sections 4.2.1 and 4.2.2) that sexual conflict promotes pre-mating reproductive isolation, and that sperm competition selects for increased copulation duration, I used flies from the selection lines described in Chapter 3 in which the adult sex ratio was manipulated. There were 3 replicates each of male-biased (MB), female-biased (FB) and equal sex-ratio (ES) lines. These lines were shown, during selection, to differ in male and female mating rates and hence in the intensity of sexual conflict (Chapter 3). Females in the selection lines responded to the differences in the levels of sexual conflict by evolving differences in the level of resistance to male-induced harm (Chapter 3). Because mating rates differed between treatments during selection, the intensity of sperm competition was predicted to differ, being highest in the lines in which mating rate of females was highest. During selection, female mating frequency, the predicted intensity of sperm competition and sexual conflict was highest in the MB lines, intermediate in the ES lines and lowest in the FB lines (Chapter 3). Female resistance to male-induced harm also evolved to be highest in the MB lines, intermediate in the ES lines and lowest in the FB lines (Chapter 3). Male mating rates during selection were lowest in the MB lines, intermediate in the ES lines and highest in the female-biased FB lines (Chapter 3).

To test the hypotheses about sexual conflict and sperm competition, the time until mating and copulation duration of pairs of virgin flies was measured within replicates and between replicates for each selection treatment. Pairs of flies consisting of a male and female from the same replicate and same treatment ('within' replicate pairs) and pairs consisting of a male and female from different replicates of the same selection treatment ('between' replicate pairs) were tested. Sexual conflict theory predicts that females that evolve under high levels of sexual conflict may evolve a reduced willingness to mate (to avoid the costs of mating) relative to females that evolve under low levels of sexual conflict. In addition, if

sexual conflict leads to increased divergence in pre-mating traits then theory predicts that females that evolve under high levels of sexual conflict may show a stronger preference for within-replicate males over males from other replicates, than would be shown by females that evolve under low levels of sexual conflict. This is based on the idea that there may be some fitness benefits to females for mating with males from the same replicate as opposed to mating with males from other replicates from which they may have genetically diverged (Martin & Hosken, 2003), e.g., increased genetic compatibility and fertility. Thus, it was predicted that MB females would be the least willing to mate and would show the strongest preference for within-replicate males over between-replicate males. FB females were predicted to be most willing to mate and show the least preferences for within-replicate males over between-replicate males. ES females were expected to have values intermediate between MB and FB. Thus, the probability of mating was predicted to be higher in FB pairs than in MB pairs and intermediate in ES pairs. In addition, under the hypothesis that sexual conflict leads to increased pre-mating reproductive isolation, the probability of mating was predicted to be higher in within-replicate matings than in between-replicate matings and this difference was predicted to be most apparent in MB lines, least apparent in FB lines and intermediate in ES lines. From the differences in the predicted intensity of sperm competition and actual male mating opportunities between lines, the prediction was that the longest copulation durations should occur in the MB lines, intermediate copulation durations in ES lines and shortest copulation durations in FB lines.

4.3 Methods

4.3.1 Time until copula and copulation duration of pairs of virgin flies from the selection lines

To measure the probability of mating, the time until copula of pairs of virgin flies was measured after 41 generations of selection (selection regime described in section 2.2.3). The copulation duration of these pairs of flies was also measured. The experimental design was based on that of Martin and Hosken (2003). Eggs from all selection lines were collected on grape plates over a 16 h period (section 2.2.1). One thousand first instar larvae from each replicate of each selection treatment were placed into SY vials in batches of 100 ('larval picks', section 2.2.1). One hundred virgin females and 100 virgin males from each replicate of each selection treatment were collected (section 2.2.2) and flies were placed into SY vials with added live

yeast granules (section 2.1.1). Females were housed individually and males were housed in batches of 5. Four days after eclosion, single males from the same ('within') or different ('between') replicate populations of the same selection treatment were added, without anaesthesia, to the vials containing single females. Fifty within-replicate vials and 50 between-replicate vials were created for each replicate (e.g. replicate MB1; within replicate mating, 50 MB1 females × 50 MB1 males; between replicate mating, 25 MB1 females × 25 MB2 males + 25 MB1 females × 25 MB3 males). The time (to the nearest minute) until copulation for each pair was recorded for 1 hour after the flies were placed together. The copulation duration of pairs that began mating within that hour was recorded.

4.3.2 Statistical analysis

Normality and homogeneity of variances of all raw data and residuals from models were checked by Shapiro-Wilk (Shapiro & Wilk, 1965) and Bartlett's tests (Zar, 1999). To analyse the mating probability of pairs of flies I chose the time point at which the greatest deviation in the proportion of pairs mating between treatments occurred (Fig. 4.1, 19 minutes). The proportion of pairs mating after 19 minutes and the mean copulation duration of all pairs that mated within 1 hour were compared between mating types (within- and between-replicates) and between treatments (MB, ES and FB) using a two-way ANOVA (Zar, 1999). To compare between pairs of treatments one-way ANOVAs (Zar, 1999) were performed followed by Student-Newman-Keuls (SNK) tests (Zar, 1999). To determine accurate probabilities from q-values for SNK tests, the software program 'R' (Ihaka & Gentleman, 1996) was used.

4.4 Results

4.4.1 Mating probability of pairs of virgin selection line flies

There were significant differences in the proportion of matings at 19 minutes between selection treatments (MB, ES and FB) but no significant differences between mating types (within- and between- replicate pairings) and no significant interaction between selection treatment and mating type (selection treatment, $F_{2,21} = 7.36$, $P < 0.005$; mating type, $F_{1,21} = 0.03$, $P > 0.85$; selection treatment × mating type: $F_{2,21} = 1.02$, $P > 0.37$; Fig. 4.2). A significantly higher proportion of ES pairs mated at 19 minutes than FB pairs ($\overline{X} \pm SE$, ES = 0.629 ± 0.025, FB = 0.479 ±

0.029, $P < 0.005$) or MB pairs ($\overline{X} \pm SE$, MB = 0.536 ± 0.023, $P < 0.05$). There was no significant difference in the proportion of pairs mating at 19 minutes between MB and FB treatments ($P > 0.12$). These results show that differences in the level of sexual conflict did not predict the observed pattern of differences in mating probability (the prediction was that mating probability would be highest in FB pairs followed by ES pairs and lowest in MB pairs). Furthermore flies in all treatments did not show a higher probability of mating in within-replicate pairings as compared to between-replicate pairings.

4.4.2 Copulation duration of pairs of virgin selection line flies

There were significant differences in duration of copulation between selection treatments but no significant differences between mating types and no significant interaction between mating type and selection treatment (Fig. 4.3, selection treatment, $F_{2,21} = 3.61$, $P < 0.05$; mating type, $F_{1,21} = 0.002$, $P > 0.96$; selection treatment × mating type, $F_{2,21} = 0.42$, $P > 0.83$). Copulation duration was significantly longer in MB pairs compared to ES pairs ($\overline{X} \pm SE$, MB = 18.39 ± 0.20, ES = 16.86 ± 0.83, $P < 0.05$). There was a marginally non-significant difference in copulation duration between FB and ES selection treatments ($\overline{X} \pm SE$, FB = 17.81 ± 1.48, $P = 0.067$) and no significant difference between MB and FB selection treatments ($P > 0.24$). The results show that differences in the intensity of sperm competition during selection between treatments did not predict the observed pattern of differences in copulation duration (the prediction was for the longest copulation durations in MB pairs followed by ES pairs and the shortest copulation durations in FB pairs). Also, pairs did not differ in copulation duration between within-, or between-replicate matings.

Figure 4.1 Proportion of pairs mating over time (replicates and mating types (within- and between- replicates) combined). The greatest divergence between any two treatments occurred 19 minutes after placing flies together.

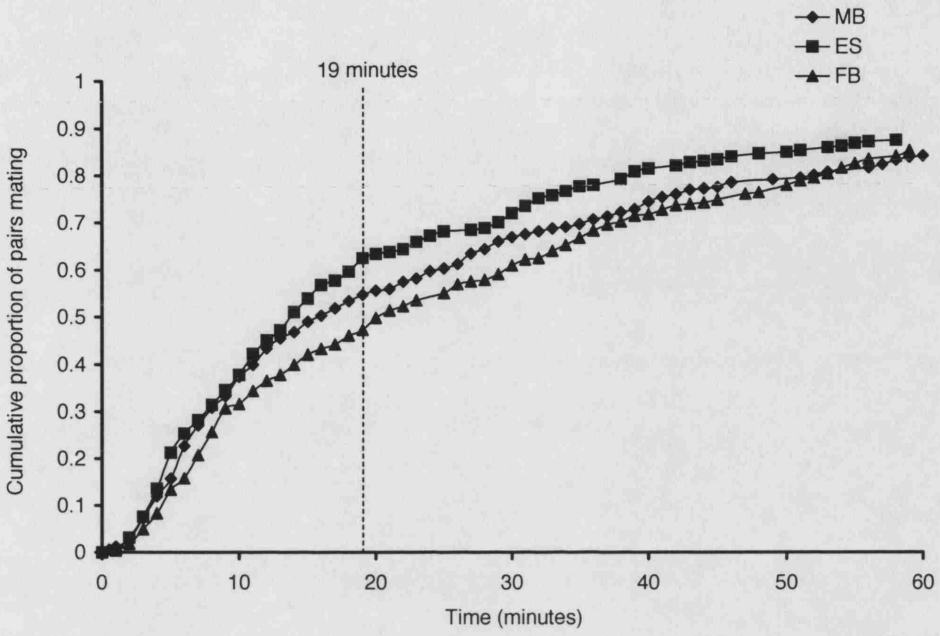


Figure 4.2 Mean proportion of pairs mating after 19 minutes (±*SE*) in within- and between- replicate matings.

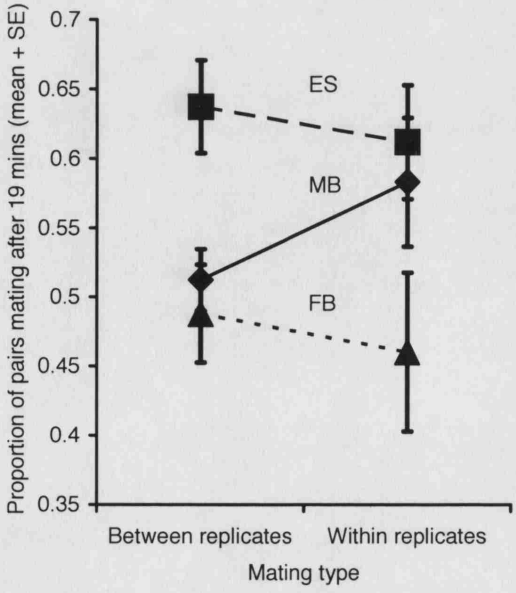
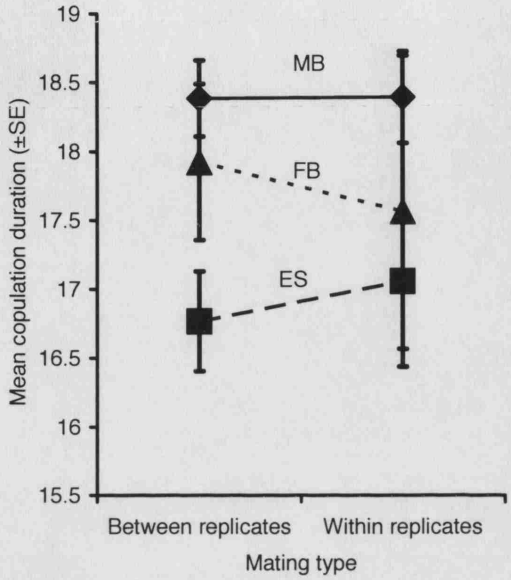


Figure 4.3 Mean copulation duration (± *SE*) of pairs of selected flies that mated within an hour of introduction in within- and between- replicate matings.



4.5 Discussion

4.5.1 Mating probability of pairs of virgin selection line flies

The pattern of mating probability results was not as predicted by sexual conflict. The mating probability of pairs of flies evolving under higher levels of sexual conflict was not lower than the mating probability of pairs of flies evolving under lower levels of sexual conflict. Also, mating probabilities were not higher in within-replicate crosses than in between-replicate crosses in any selection treatment. Hence, there was no indication that in high levels of sexual conflict led to increased divergence in pre-mating traits between replicate populations in the traits measured, in contrast to the results obtained in *Sepsis cynipsea* (Martin and Hosken 2003). The data presented in this chapter provide no support for the hypothesis that sexual conflict drives pre-mating reproductive isolation via female willingness to mate in *D. melanogaster*.

One explanation for the difference in the two studies is that increased sexual conflict may not inevitably lead to increased divergence in these particular traits. The nature

of pre- and post-mating selection arising from sexual conflict in the two species (*Sepsis cynipsea* and *Drosophila melanogaster*) may be very different. The results of this study show that *D. melanogaster* females may not be able to decrease the level of harm inflicted upon them by evolving decreased willingness to mate. Instead, the results from this chapter support the results from Chapter 3 for the interpretation that the evolved female resistance to male-induced harm may be primarily via post-mating responses that decrease the harmful effects of Acps or responses that decrease the harmful effects of courtship. Although insufficient number of generations or insufficient strength of selection cannot be excluded as potential explanations for the lack of divergence in the predicted directions, these explanations seem unlikely given that differences in female resistance to male-induced harm evolved rapidly between treatments and were apparent after 18 generations of selection.

It is possible that measuring the probability of mating of pairs of virgin flies may not accurately reflect the overall willingness of females to mate. In addition, if males differed between treatments in their ability to gain matings (e.g. if they differed in the intensity or frequency of courtship) this could confound this measure of female willingness to mate. When wild-type females were housed continuously with selection males, no differences in the number of courtship bouts received per female were found (Chapter 3, section 3.4.3). This suggests that there are no differences in courtship frequency attributable to the selection line males. However, in the same experiments there were differences in mating frequency between treatments (Chapter 3, section 3.4.3). This suggests that there is some difference, aside from courtship frequency between selection treatments that causes differences in the ability of males to gain mates or differences in the ability to reduce female receptivity. Further work is required to test these hypotheses.

The results are unlikely to be confounded by differences in the level of inbreeding between treatments. ES lines are predicted to be less inbred than MB and FB lines and MB lines are likely to be more inbred than FB (discussed in Chapter 3, section 3.5.1). If males in FB and MB lines were less effective at courtship due to inbreeding then this could explain why more ES pairs mated than MB or FB pairs but it cannot explain the lack of differences between the MB and FB treatments. In addition, there are no body size differences among the males from these lines (see

Chapter 3, Table 3.1) and so the measures in this chapter are also unlikely to be confounded by size-related variation among males (such as differences in courtship intensity, or female preference for large males, e.g. Partridge & Farquhar, 1983; Partridge et al., 1987a; Partridge et al., 1987c). However, the possibility that there were unidentified differences in male behaviour or morphology between treatments that could have affected the results cannot be excluded.

4.5.2 Copulation duration of pairs of virgin selection line flies

The second trait that was measured was copulation duration. The pattern of differences in copulation duration was not as predicted by the risk of sperm competition experienced by males during selection (Fig. 4.3). The prediction was that copulation duration would follow the predicted intensity of sperm competition during selection, being longest in MB lines, intermediate in ES lines and shortest in FB lines. However, the results were that MB and FB pairs both copulated for longer than did ES pairs (there was no difference between MB and FB pairs). It is possible that simply measuring total copulation duration is not the correct measure to use as it ignores any differences in the rate of sperm and seminal fluid transfer. In addition these measurements were made on virgin females, and during selection, as well as in the wild, most matings are likely to occur with non-virgin females (Harshman & Clark, 1998; Imhof et al., 1998). However, it seems reasonable that males should make their ejaculate allocation based on future intensity of sperm competition (e.g. Parker et al., 1996) even when mating to virgin females.

Male investment in each copulation will depend on the exact intensity of sperm competition. Across species, ejaculate investment (at least in terms of sperm number) is predicted to increase with increasing sperm competition risk (Parker, 1998). However, within species, if sperm competition intensity is very high then, in certain situations, increased investment may be selected against (Parker et al., 1996; Parker et al., 1997). This theory is based on a situation in which males share the same probability of gaining future matings. However, in the lines used this chapter (and Chapter 3) the mating frequency of males during selection differed between treatments, being highest in the FB lines, lowest in MB lines and intermediate in the ES lines (Chapter 3, section 3.4.1). Thus, the investment in single ejaculates by MB males is predicted to evolve to be higher than ES males followed by FB males, because the probability of gaining additional matings during selection would be

lowest in MB males, followed by ES males, followed by FB males. This prediction is similar to the prediction of 'the terminal investment' in offspring production in individuals whose future reproductive value is low (e.g. Lessells, 1999).

During selection, eggs were collected to form the following generation after 9 days of interaction between the adult flies. Therefore, it is possible that during selection there was little adaptive advantage for males to be successful in early matings (e.g. during the first few days of selection). This because most or all of the sperm from those matings might be depleted, displaced or heavily outnumbered before eggs that contributed to the next generation of selection were produced. This would affect MB lines most strongly because the predicted intensity of sperm competition was highest in these lines, it would affect FB lines most weakly and it would affect ES lines intermediately. Thus, the selection against investment in ejaculates early during the selection period would act in opposition to the predicted selection for increased investment in ejaculates in lines with higher levels of sperm competition. Selection against investment in large ejaculates would diminish over time during the 9-day selection period as the probability of sperm transferred at matings contributing to the next generation increased. Therefore, it would be interesting to test the ejaculate investment of non-virgin, aged males who have undergone conditions that mimic the selection environment.

4.5.3 Conclusions

In conclusion, the results presented in this chapter provide no evidence that sexual conflict had a predictable effect on the evolution of female willingness to mate or that sperm competition or mating opportunities had a predictable effect on the evolution of copulation duration in *D. melanogaster*. The mating probability of pairs of virgin flies did not differ between within- and between-replicate matings. These results suggest that pre-mating female mate discrimination is unlikely to be a universal mechanism by which sexual conflict may drive reproductive isolation. Alternative mechanisms by which sexual conflict could drive reproductive isolation might be through variation in female post-mating responses such as egg production, egg fertility, refractory period and response to male-induced harm.

Chapter 5. Sex peptide causes mating costs in female *Drosophila melanogaster*

5.1 Abstract

Conflicts between females and males over reproductive decisions are common. In *Drosophila*, as in many other organisms, there is a conflict over how often to mate. Male fitness increases with increasing numbers of matings. Female fitness, however, can be reduced by mating too frequently. High mating rates shorten female lifespan and lower the reproductive success of females, a cost which is mediated by male ejaculate accessory gland proteins (Acps). In this chapter, I demonstrate that a single Acp, the sex peptide (SP or Acp70A), which decreases female receptivity and stimulates egg production in the first matings of virgin females, is a major contributor to Acp-mediated mating costs in females. Females continuously exposed to SP-deficient males (that produce no detectable SP) had significantly higher fitness and higher lifetime reproductive success than control females. Hence rather than benefiting both sexes, receipt of SP decreases female fitness, making *SP* the first gene identified that is likely to play a central role in sexual conflict.

5.2 Introduction

In many species there is the potential for disparity in the optimum mating frequency for males and females. Selection for frequent matings is predicted to be stronger in males than in females, because males gain fitness from each extra mating they obtain, whereas, as mating frequency increases, female fitness gains may cease (Bateman, 1948) and then reverse (e.g. Fowler & Partridge, 1989). Hence the presence of female mating costs may reflect the action of sexual conflict over mating (Parker, 1979), in which males have evolved traits that increase their fitness relative to other males but that decrease the fitness of females with which they mate, or attempt to mate.

In *Drosophila melanogaster*, the proximate mechanism underlying mating costs in females has been explored. Females that mate at high frequencies suffer longevity and reproductive fitness costs (Fowler & Partridge, 1989) due to the actions of male seminal fluid accessory gland proteins (Acps) (Chapman et al., 1995; see sections 1.7.2 and 1.7.3). This Acp-mediated mating cost of mating is potentially large and is incurred in addition to costs of reproduction such as those due to egg production (Partridge et al., 1987b; Sgrò & Partridge, 1999) and to other non-mating activities (Partridge & Fowler, 1990). Acps mediate a variety of effects that benefit males, such as stimulating female egg production (Herndon & Wolfner, 1995; Heifetz et al., 2000; Saudan et al., 2002; Chapman et al., 2003c; Liu & Kubli, 2003), reducing female receptivity (Saudan et al., 2002; Chapman et al., 2003c; Liu & Kubli, 2003), ensuring effective sperm storage (Tram & Wolfner, 1999; Neubaum & Wolfner, 1999) and promoting male success in sperm competition (Clark et al., 1995; Chapman et al., 2000). The female cost of mating arising from Acp transfer by males may be a side-effect of Acp function (Chapman et al., 1995; Lung et al., 2002) or a direct, sexually selected effect to reduce the likelihood of female remating and/or increase current investment in reproduction (Crudgington & Siva-Jothy, 2000; Johnstone & Keller, 2000).

I investigated whether a single Acp, the sex peptide (SP or Acp70A, Chen et al., 1988; section 1.7.4), is responsible for mating costs in females. SP decreases female receptivity and stimulates egg production following the first matings of virgin females (Chapman et al., 2003c; Liu & Kubli, 2003) and was generally assumed to benefit both sexes, acting as a signal to initiate high reproductive rate in successfully

mated females and as a mechanism for increasing paternity in males. Wild-type females were exposed throughout life to SP knockdown males (that produced no detectable SP, Chapman et al., 2003c; see Western blots, Fig. 2.3) or to control males, matched for autosomal genetic background (Chapman et al., 2003c). I used two independent replicate pairs of SP knockdown and control male lines (SP1 knockdown and C1, and SP2 knockdown and C2). 110 females for each treatment of each line were kept in groups of 5 with 5 males. Female survival was measured and female mating frequency, egg production and egg to adult viability were sampled throughout the experiments. Female survival and age-specific offspring production data were used to calculate fitness (an index of 'r', the intrinsic rate of population increase, Gotelli, 2001), for each treatment of each line and I also calculated indices of lifetime egg production per female and lifetime offspring production per female (see section 5.3.4, below).

Females continuously exposed to SP knockdown males were predicted to mate significantly more frequently than females continuously exposed to control males, because SP-induced receptivity inhibition would be absent in mates of SP knockdown males (Chapman et al., 2003c; Liu & Kubli, 2003). I predicted that this difference in mating frequency would lead to higher survival mating costs in females mated to SP knockdown males, provided there was a difference in mating frequency of a least 2.2-fold over that of the control females (previously shown to be sufficient to cause mating costs in females, Fowler & Partridge, 1989). I also predicted that females exposed to SP knockdown males would produce fewer eggs than controls, as SP stimulates egg production in first matings by virgin females (Chapman et al., 2003c; Liu & Kubli, 2003). I measured the survival of females exposed to SP knockdown or control males for just 48 hours, to check that the survival of females continuously exposed to males was determined by male-derived reproductive costs.

5.3 Methods

5.3.1 Generation and culturing of experimental flies

Experimental males (SP knockdown and control) were generated as described in section 2.1.4. Experimental males were reared at low density to prevent larval competition, collected using CO_2 anaesthesia within 2 days of eclosion and aged for 5 days in groups of 10 per vial. A few vials of males and females were maintained to provide replacement males for any that died. Wild-type Dahomey females (section

2.1.3) were reared in bottles using a standard density culturing method ('egg washes', section 2.2.1). Virgin females were collected (section 2.2.2), aged for 3 days in groups of 5 per vial and randomly allocated to 4 groups of 110 females, for continuous exposure to males, or 4 groups of 60, for 48-hour exposure to males.

5.3.2 Mating rate, longevity, egg production, egg to adult viability and offspring production of wild-type females continuously exposed to SP knockdown or control males

Five experimental males were added to each vial of 5 wild-type females (22 vials, n = 110 for each treatment of each line). Flies were transferred onto fresh food every 1 to 3 days and any dead flies were removed. An equal sex ratio was maintained by adding spare males and removing males when necessary and vials were combined to maintain fly density at 5 females per vial. Vials were combined in this way to eliminate potentially significant, confounding effects on survival due to progressive reductions over time in fly density. The use of this procedure meant that, whilst it was possible to calculate indices of fitness, lifetime egg production and lifetime offspring production for each treatment of each line, it was not possible to calculate lifetime reproductive success for individual females or individual vials. Experimental males were replaced 10 and 24 days into the experiment with 2 to 5 day-old males reared and collected as described above. Female deaths were recorded until the first line expired. Mating and courtship were recorded twice a week in 10 observations of each vial, made at least 20 minutes apart. Mating takes approximately 20 minutes in *D. melanogaster* and so this procedure avoids the double counting of matings. Egg production was sampled twice a week (9 times in total throughout the experiment) by transferring flies into fresh food vials with a standard drop of dried yeast paste on the surface. Flies were allowed to lay eggs for several hours (3.5 hours on day 2 increasing to 22 hours on day 30 to adjust for decreasing egg production) before being transferred to fresh food. Controlling the time allowed for egg laying allowed us to count eggs accurately, to ensure that females did not run out of oviposition sites (which would be confounding because they would cease egg laying), and that larvae were not overcrowded (which could confound egg to adult viability and fitness measures due to larval death). All egg production comparisons were age-specific, hence they were not confounded by any non-linearity in egg production resulting from varying the egg laying periods. The number of eggs counted was standardised by dividing by the number of females

alive in the vial and the laying time in hours. Vials were retained and adult offspring were counted. I calculated indices of lifetime egg production and lifetime offspring production for each treatment of each line. I first calculated the mean number of eggs or offspring produced per female per sample for each treatment of each line. To take into account censored females (1 experimental female was censored before the final sample was taken) the number of females used for each treatment of each line for each sample was 110 (the number at the start of the experiment) minus the number of females censored in the given treatment of the given line before the sample was obtained. The index of lifetime egg or offspring production was the mean number of eggs or offspring produced per female per sample summed across all samples. Thus, a single value was obtained for each treatment of each line. I calculated an index of fitness, the Malthusian parameter 'r' (the intrinsic rate of population increase), for each treatment of each line from mean age-specific progeny production per female and survival values (Gotelli, 2001). 'r' is the solution to the Euler equation: $1 = {}_0\int^w l_x m_x e^{-rx} dx$, where x = age, l_x = survival probability to age x, m_x = the number of offspring produced at age x and w = age at last breeding (Gotelli, 2001). As I sampled progeny values from the fertility samples taken throughout the experiment, the values obtained represent an index of r.

5.3.3 Longevity of wild-type females exposed to SP knockdown or control males for 48 hours only

Virgin females were allocated to groups of 30 in culture bottles into which 40 experimental males were added (2 bottles, n = 60 females for each treatment of each line). 48 hours later, males were discarded and females were placed in vials in groups of 10. Flies were transferred onto new food every 2 to 3 days and vials were combined to maintain density at 10 flies per vial. Dead flies were removed at each transfer and female deaths were recorded until the end of the continuous exposure experiment.

5.3.4 Statistical analyses

The two SP RNAi lines used to obtain the SP knockdown and control males were independently derived and the lines were hence compared separately for mating, courtship, survival, age-specific egg production and age-specific egg to adult viability analyses. For the population measures (fitness, lifetime egg production per female and lifetime offspring production per female) I analysed the difference

between treatments across both lines using a one-way ANOVA (Zar, 1999). With one degree of freedom, these were conservative tests. Mating and courtship data for the continuously exposed females were analysed in contingency tables using Chi-square tests (Zar, 1999). Courtship opportunities were defined as the total number of observations (10 per sampling day) summed over all sampling days multiplied by the number of females present on each sampling day. Mating opportunities were defined as the number of observation days multiplied by the number of females present on each sampling day (as in previous studies, Fowler & Partridge, 1989; Chapman et al., 1995) and I assumed that each female would only mate once during the 10 observations (~3 hours) made on a sampling day. Age-specific egg production and egg to adult viability data were analysed using Kruskal-Wallis tests (Kruskal & Wallis, 1952) (vials were used as the unit of replication and values were adjusted for the number of females alive per vial at the time of sampling). Female survival curves were compared using Log Rank tests (Peto & Peto, 1972).

5.4 Results

5.4.1 Mating rate and longevity of wild-type females continuously exposed to SP knockdown or control males

Females continuously exposed to SP knockdown males mated significantly more frequently than did females exposed to control males (Chi-square tests; line 1, 15.2-fold difference, $\chi^2_1 = 126.0$, $P < 0.0001$; line 2, 12.5-fold difference, $\chi^2_1 = 156.6$, $P < 0.0001$; Table 5.1.). Females continuously exposed to SP knockdown males were also courted significantly more often than were control females (Chi-square tests; line 1, 1.56-fold difference, $\chi^2_1 = 105.9$, $P < 0.0001$; line 2, 1.62-fold difference, $\chi^2_1 = 99.5$, $P < 0.0001$; Table 5.1.). Thus, costs resulting from mating and courtship were expected to be higher in females continuously exposed to SP knockdown males than in control females. However, the survival of females continuously exposed to SP knockdown males was significantly longer than that of controls in line 1, and in line 2 there were no significant differences in survival between treatments (median survival, in days, from the first day of exposure to males (lower and upper quartile), SP1 knockdown = 24 (21, 31), control 1 = 22 (18, 28), Log Rank test, $\chi^2_1 = 4.35$, $P = 0.037$; SP2 knockdown= 24 (21, 29), control 2 = 24 (19, 29), Log Rank test, $\chi^2_1 = 0.63$, $P = 0.43$). This suggests that matings with SP-knockdown males were

significantly less costly in terms of female survival than were matings with control males.

5.4.2 Egg production, egg to adult viability, offspring production and fitness of wild-type females continuously exposed to SP knockdown or control males

Contrary to expectation, females continuously exposed to SP knockdown males laid significantly more eggs than females exposed to control males in some early samples, i.e. on days 2 and 9 in line 1 (Kruskal-Wallis tests; $\chi^2_1 > 6.63$, $P < 0.01$, all other days $\chi^2_1 < 0.84$, $P > 0.35$; Fig. 5.1.(a)) and on days 2, 4 and 9 in line 2 ($\chi^2_1 > 7.50$, $P < 0.01$, all other days $\chi^2_1 < 1.80$, $P > 0.18$; Fig. 5.1.(a)). Eggs laid by females exposed to SP knockdown males had significantly higher egg to adult viability than those of females exposed to control males on day 16 in both lines (Kruskal-Wallis tests; line 1, $\chi^2_1 = 14.40$, $P < 0.0001$, all other days $\chi^2_1 < 2.65$, $P > 0.10$; line 2, $\chi^2_1 = 4.56$, $P = 0.033$; Fig. 5.1.(b)) and marginally non-significantly higher on day 18 in line 2 ($\chi^2_1 = 3.15$, $P = 0.076$, all other days $\chi^2_1 < 2.08$, $P > 0.14$; Fig. 5.1.(b)). Females continuously exposed to SP knockdown males had significantly higher values of 'r' (ANOVA; $F_{1,2} = 26.51$, $P = 0.036$), and lifetime offspring production ($F_{1,2} = 29.46$, $P = 0.032$) than females exposed to control males (Fig. 5.2 (a) and (b)). Females continuously exposed to SP knockdown males had marginally significantly higher values for lifetime egg production than females exposed to control males ($F_{1,2} = 18.15$, $P = 0.051$; Fig. 5.2 (c)). These results consistently indicate that females continuously exposed to SP knockdown males were fitter than control females. This suggests that the presence of SP in the ejaculates of control males is costly to females and reduces female fitness.

5.4.3 Longevity of wild-type females exposed to males for 48 hours only

The survival of females exposed to males for 48 hours only was significantly higher than the survival of females continuously exposed to males for both treatments of both lines (Log Rank test, $\chi^2_1 > 14.0$, $p < 0.0003$). There were no differences in the survival of females exposed to SP knockdown or control males for 48 hours in either line (median lifespan in days (and lower and upper quartile) SP1 knockdown = 30 (24, 36), control 1 = 30 (23, 36), SP2 knockdown = 30 (22, 36), control 2 = 31 (23, 36), Log Rank test, $\chi^2_1 < 0.01$, $p > 0.92$ both lines).

Figure 5.1 Median (and inter-quartile range) (a) number of eggs laid per hour by females continuously exposed to males and (b) egg to adult viability for the eggs laid by the females shown in (a).

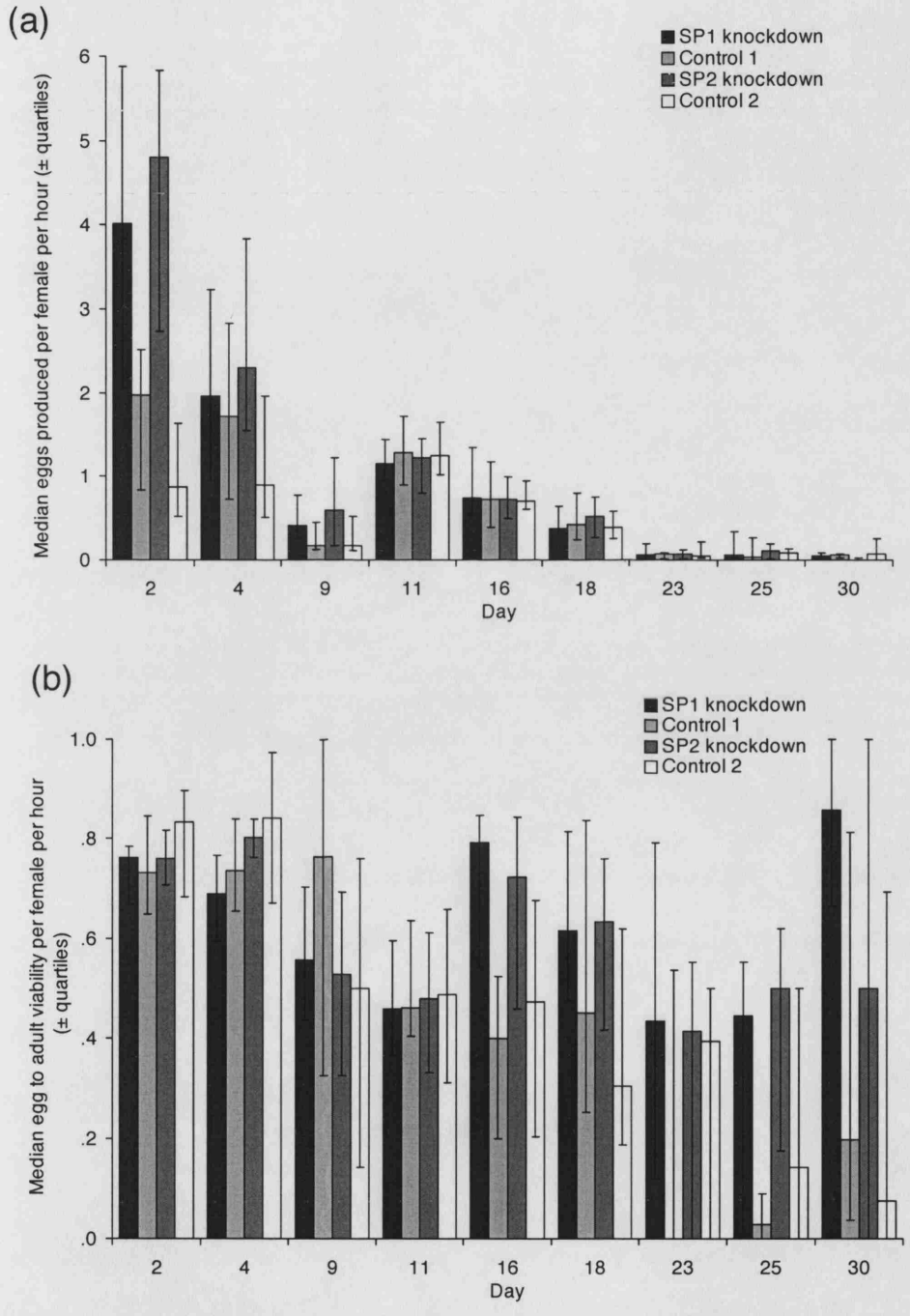


Figure 5.2 Indices of (a) fitness, 'r', (b) lifetime offspring production and (c) lifetime egg production for females continuously exposed to males. The percentage increase in females mated to SP knockdown males above those mated to control males for fitness ('r') was 55.7% and 127.7% (lines 1 and 2 respectively), for lifetime % offspring production per female was 41.2% and 71.4% and for lifetime egg production per female was 24.5% and 43.0%.

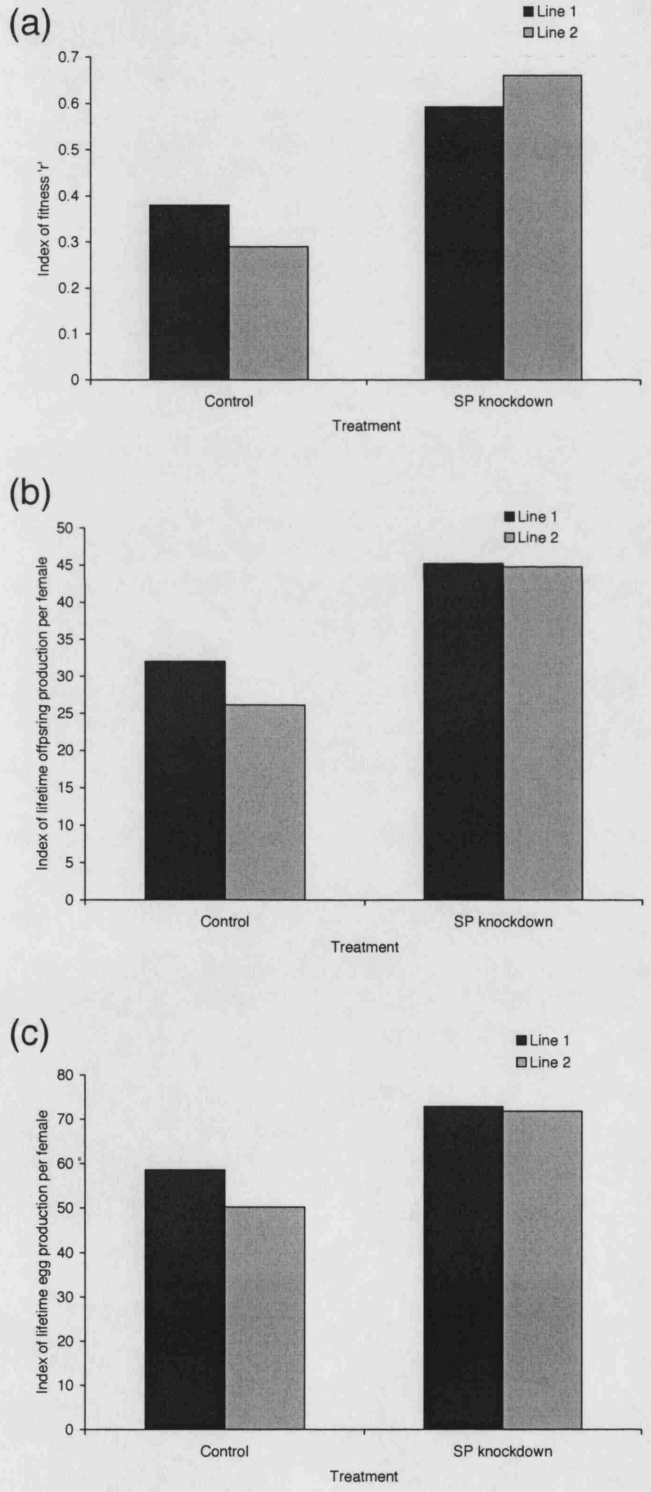


Table 5.1 Total number of mating and courtship opportunities taken or not taken under continuous exposure to SP knockdown or control males.

	Mating frequency			Courtship frequency		
	Opportunities Taken	Opportunities Not Taken	% Taken	Opportunities Taken	Opportunities Not Taken	% Taken
SP knockdown 1	154	671	18.67%	1266	6984	15.35%
Control 1	9	724	1.23%	721	6609	9.84%
SP knockdown 2	180	605	22.93%	962	6888	12.25%
Control 2	14	747	1.84%	568	7042	7.46%

5.5 Discussion

5.5.1 Mating frequency and female survival of wild-type females continuously exposed to SP knockdown or control males

As expected, females continuously exposed to SP knockdown males mated significantly more frequently than did females exposed to control males (Table 5.1). Females exposed to SP knockdown males were also courted significantly more often than were control females (Table 5.1). However, despite mating more than 12 times as frequently, and receiving significantly elevated levels of courtship, females continuously exposed to SP knockdown males did not have reduced survival compared to controls (the prediction if substances other than SP cause mating costs). Instead, the mates of SP knockdown males lived at least as long as (line 2), or even significantly longer (line 1) than females continuously exposed to control males. The difference in mating rate between females exposed to SP knockdown and control males far exceeded that previously shown to cause female survival mating costs (Fowler & Partridge, 1989). The results presented in this chapter therefore indicate that, purely in terms of female survival, matings with SP knockdown males were largely 'mating cost free'. Females exposed to control males mated at a lower frequency (% mating opportunities taken: C1 = 1.2%; C2 = 1.8%; see Table 5.1) than was observed in similar assays of mating frequency in a previous study of female mating costs ('Low-mating' = 2.5% 'High-mating' = 5.4%; Fowler & Partridge, 1989). This would have led to relatively low mating costs in females exposed to control males. However, females mated to SP knockdown males mated at much higher frequencies (mating opportunities taken: SP1 = 18.7%, SP2 = 22.9%; see Table 5.1.) than did the 'High-mating' females from the previous study, in which

significant mating costs were observed (Fowler & Partridge, 1989). Hence the chances of detecting survival mating costs in females exposed to SP knockdown males, had they been present, were maximised. Of course survival measures alone do not necessarily indicate the existence of reproductive costs, and to address whether SP is a contributor to Acp-mediated mating costs, survival is considered together with reproductive success (see fitness and reproductive success, section 5.5.3, below).

5.5.2 Egg production and viability of eggs of wild-type females continuously exposed to SP knockdown or control males

In further contrast to the predictions, I found that females continuously exposed to SP knockdown males laid significantly more eggs than females continuously exposed to control males on days 2 and 9 in line 1 and on days 2, 4 and 9 in line 2 (Fig. 5.1(a)). Also, females continuously exposed to SP knockdown males had marginally significantly higher indices of lifetime egg production than controls (Fig. 5.2(c)). Previous work has shown that virgin females mated for the first time to SP knockdown males show significantly lower egg production than females mated once to control males (Chapman et al., 2003c; Liu & Kubli, 2003). It was therefore unexpected to find significantly higher early egg production in females continuously exposed to SP-deficient males in this study. This observation is not attributable to a low stimulation of egg production in females mated to the control males. The same control male genotype stimulates egg production above that of the SP knockdown males following single matings (Chapman et al., 2003c) and in assays where males and females are housed in individual pairs (Wigby, S., Crossman, A., and Chapman, T., unpublished data). The increased early egg production in females continuously exposed to SP knockdown males is consistent with a gene × mating frequency interaction. At low mating frequencies, the receipt of other ovulation- and oviposition-stimulating seminal fluid proteins such as Acp26Aa (Herndon & Wolfner, 1995), and possibly Dup99B (Saudan et al., 2002) may be insufficient to offset the lack of SP, leading to low egg production in mates of SP knockdown males. At higher frequencies of mating however, the receipt of Acp26Aa and Dup99B may be at a sufficiently high level to result in increased egg production relative to control females (that receive lower levels of these other Acps). This is consistent with functional redundancy among Acps that stimulate egg production. An alternative explanation is that the higher egg production in females continuously

exposed to SP knockdown males is the result of an improvement in female health, due to the absence of SP. As egg production is known to contribute to reproductive costs (Partridge et al., 1986; Partridge et al., 1987b), the finding that the magnitude of differences in egg production was less, and occurred over a shorter time, in line 1 compared to line 2 (Fig. 5.1(a)), might explain why females continuously exposed to SP knockdown males lived significantly longer than their controls in line 1, but not line 2. The eggs laid by females mated to males of both lines generally showed no differences in egg to adult viability, although mates of SP knockdown males had significantly higher egg to adult viability in one of the later samples of the experiment (Fig. 5.1(b)).

5.5.3 Fitness and lifetime reproductive success of wild-type females continuously exposed to SP knockdown or control males

The most striking effect in the study presented in this chapter was that females continuously exposed to SP knockdown males had significantly higher indices of fitness and lifetime offspring production (Fig. 5.3(a) and (b)), and marginally significantly higher indices of lifetime egg production than controls (Fig. 5.3.(c)). Fitness, 'r' (Gotelli, 2001) was calculated from age-specific progeny and survival values. Measures based on 'r' are more directly related to fitness than lifetime reproductive success, particularly so for *D. melanogaster* which probably does much of its reproduction in expanding populations (Charlesworth, 1980). Having said this, the measures of lifetime egg production and reproductive success are entirely consistent with the fitness measures - they all indicate that females continuously exposed to SP knockdown males had higher fitness, and higher lifetime reproductive success than did females mated to control males. Large and significant Acp-mediated costs of mating can be observed in females even when other costly activities such as egg production and exposure to courting males are held constant (Chapman et al., 1995). In this study, females exposed to SP knockdown males had significantly higher exposure to courtship and significantly higher early egg production than did control females. Yet despite this, these females mated at least 12 times as often as control females and still had significantly higher fitness and lifetime reproductive success. SP is therefore responsible for at least a major part of Acp-mediated female mating costs in *D. melanogaster.*

5.5.4 Other reproductive costs in mated females

As expected, the survival of females exposed to males for 48 hours only was significantly higher than the survival of females continuously exposed to males for both treatments of both lines. The lower survival of females continuously exposed to males, compared to females exposed to males for 48 hours, is likely to be due to higher reproductive costs such as those arising from egg production (Partridge et al., 1987b) and the receipt of courtship (Partridge & Fowler, 1990). In addition, there could also be contributions to reproductive and mating costs from other potentially harmful Acps (such as Acp62F which reduces female survival when ectopically expressed at >50 times natural levels; Lung et al., 2002). It is not possible to exclude the possibility therefore that Acps other than SP contribute to reproductive costs. As expected, because mating costs are detectable only against a background of frequent mating in this species (Chapman & Partridge, 1996a), there were no differences in the survival of females exposed to SP knockdown or control males for 48 hours in either line.

X chromosome differences between SP knockdown and control males could have contributed to differences in male behaviour (e.g. courtship and mating frequency) and hence female reproductive success. However, differences in X chromosome constitution are not likely to confound the results through any potential effects on Acp levels, because the genes encoding all the Acps responsible for mating costs in females (Chapman et al., 1995) are autosomal (Swanson et al., 2001a), unless, that is, there are X-linked, trans-acting genes that modulate Acp function (e.g., enzymes that regulate Acp potency).

Mating and courtship rates of the control males in the experiments described in this chapter are broadly comparable with the range seen in the population cage environment from which the experimental females were drawn. Even in the wild, females are subject to very intense bombardment from males (Partridge et al., 1987c) and multiple mating is common (Imhof et al., 1998). The mating and courtship rates observed were also comparable with those seen in previous experiments (Fowler & Partridge, 1989). If mating and courtship were artificially high in my experimental set up, it would make the lack of cost seen in females mating to SP knockdown males all the more remarkable.

5.5.5 SP and sexual conflict

Males are predicted to gain from SP transfer because, even though it ultimately reduces the fitness of their mates, SP also induces a refractory period (Chapman et al., 2003c; Liu & Kubli, 2003), which significantly increases 'per-mating' paternity (Chapter 6). Hence SP is likely to increase male fitness. The results of this study indicate that, rather than benefiting both sexes, receipt of SP decreases female fitness. I would predict therefore that females with elevated SP, via ectopic SP-induction (Aigaki et al., 1991), or via matings with males that produce and transfer elevated levels of SP, should incur increased mating costs. The results are also consistent with the finding, from a large scale study of the effects of variation in male sperm competitive ability on females, of positive correlations between the length of female refractoriness (i.e. remating interval) and early female mortality (Civetta & Clark, 2000). This might suggest that males that can induce longer remating intervals also cause higher female survival mating costs. The results of the experiments in this chapter highlight SP as an obvious candidate mechanism.

Females could gain indirect genetic benefits from mating with SP transferring males if their male offspring had higher mating success. However, such benefits are likely to be small in comparison to direct costs incurred from the receipt of SP (e.g. Kirkpatrick & Barton, 1997; Cameron et al., 2003). Females could also benefit directly from receipt of SP if mating opportunities were limited to one or a very few matings, through increased egg production (Chapman et al., 2003c; Liu & Kubli, 2003). However, multiple mating is the norm in *D. melanogaster* both in the laboratory and in the wild (e.g. Harshman & Clark, 1998; Imhof et al., 1998), and as I have shown here, with frequent mating, fecundity benefits through receipt of SP may not occur. It is therefore unlikely that females often benefit from the receipt of SP. Consequently the *SP* gene is likely to play a role in sexual conflict rather than cooperation.

Females may initially have been subject to natural selection to evolve sensitivity to substances such as SP, to allow them to modulate egg production and receptivity adaptively following sperm transfer (Chapman, 2001). The demonstration of direct costs due to receipt of SP in this chapter are however consistent with a scenario whereby SP is under the influence of sexual conflict and other forms of sexual selection, which may have selected for SP activity that increased male reproductive

success regardless of the effect upon females. If SP is subject to sexual conflict, then theory predicts that it should show relatively rapid evolutionary change. Although the *SP* gene C terminus appears relatively conserved in the melanogaster species subgroup, *D. subobscura* (Cirera & Aguadé, 1998) and *D. suzukii* (Schmidt et al., 1993b), the N terminal region is somewhat divergent (Schmidt et al., 1993a; Cirera & Aguadé, 1998) and significant departures from neutrality have been detected in the region flanking the 5' end of the *SP* gene (Cirera & Aguadé, 1997). It is not clear whether SP alone is responsible for female mating costs, or whether harm is caused by the interaction of SP with other ejaculate molecules. SP binds to sperm and can be detected on sperm heads several days after its deposition in the female reproductive tract (Peng et al., 2005). There is no reduction in the cost of mating in females continuously exposed to spermless males (Chapman et al., 1993), which suggests that the SP which is harming females must be free from association with sperm. SP appears to stimulate egg production by causing the release of juvenile hormone (JH) BIII from the *corpora allata* (Moshitzky et al., 1996), which stimulates oocyte progression in the ovary (Soller et al., 1999). Increased JH levels are negatively associated with lifespan in other insects (Herman & Tatar, 2001). Hence costs resulting from the effects of increased JH, such as immunity suppression (Rolff & Siva-Jothy, 2002), are candidate mechanisms for future study (see section 7.4.1).

5.5.6 Conclusions

In summary the data presented in this chapter provide evidence that SP is a major contributor to the Acp-mediated female cost of mating in *Drosophila melanogaster*. Acps other than SP can be at most only weakly harmful to females in the absence of SP, because females continuously exposed to SP knockdown males received all other Acps more than 12 times as often as control females but did not suffer higher survival or reproductive costs. Instead, females that did not receive SP had significantly higher fitness than control females despite mating more than 12 times as frequently. SP induces a refractory period in females and is therefore predicted to benefit males by delaying the onset of sperm competition and sperm displacement. Thus, SP may be beneficial to males (see Chapter 6) but harmful to females, making *SP* the first gene identified that is likely to play a central role in sexual conflict. It will be interesting in future studies to investigate the mechanism by which SP harms females (see section 7.4.1), whether SP harms females by itself or in conjunction

with other Acps, and how SP-induced harm to females depends on environmental factors such as mating rate and nutrition level (see section 7.4.2).

Chapter 6. The effect of sex peptide on male reproductive success in *Drosophila melanogaster*

6.1 Abstract

In many taxa sexual selection continues after mating. Female *Drosophila melanogaster* mate multiply and simultaneously store the sperm of two or more males, thus creating an arena for sperm competition and post-mating sexual selection. Male *D. melanogaster* vary in post-mating competitive ability, a process which is influenced by male accessory gland proteins (Acps). Acps are transferred to females in the seminal fluid, during mating. Acp70A, the sex peptide (SP), stimulates egg production and reduces receptivity after a female's first mating. SP is also a major contributor to the female cost of mating and reduces female fitness after multiple matings (Chapter 5). In this chapter I investigate the effect of SP on male fitness in an environment in which males have to compete both for access to females and for access to fertilizations. I found that the ability of SP-deficient males to gain matings was significantly higher than that of control males but that this did not consistently lead to higher reproductive success. This is because the 'per-mating' paternity of SP knockdown males was significantly reduced compared to control males. This result is likely to be due to the delayed remating in mates of SP transferring males compared to females mated to SP-deficient males. Hence, SP ensures greater paternity by delaying the onset of sperm competition and sperm displacement.

6.2 Introduction

Sexual selection has been a major subject of scientific investigation for more than a century. For much of this time researchers focussed on pre-copulatory sexual selection. However since the work of Parker (1970) the importance of post-mating sexual selection has been realised, particularly for species in which females mate multiply. When females are promiscuous (such as in *Drosophila melanogaster*; Imhof et al., 1998; Harshman & Clark, 1998), and simultaneously store the sperm of two or more males then sperm competition can occur (Parker, 1970). *Drosophila melanogaster* males can vary greatly in their post-copulatory competitive ability (e.g. Prout & Bundgaard, 1977; Clark et al., 1995; Fiumera et al., 2005) but typically the most recent male to mate with a female sires the majority of her subsequent progeny (Kaufman & Demerec, 1942; Prout & Bundgaard, 1977; Scott & Richmond, 1990; Scott & Williams, 1993). Thus, it is in the interests of males to induce a refractory period in their mates, to delay the onset on sperm competition and sperm displacement.

In *Drosophila melanogaster* male post-mating success is strongly influenced by accessory gland proteins (Acps) transferred during mating. When males mate to non-virgin females, Acps appear to facilitate the displacement of, or disable, the stored sperm of previous males (Harshman & Prout, 1994; Prout & Clark, 2000). Associations between specific Acp alleles and components of sperm competitive ability such as sperm displacement (Clark et al., 1995), female refractoriness, and P1 and P2 (the proportion of offspring sired by the first and second male to mate respectively) have been found (Fiumera et al., 2005). In addition, the outcome of sperm competition can depend on the genotype of the female (Clark & Begun, 1998) and on interactions between male and female genotypes (Clark et al., 1999). Sperm precedence lacks transitivity (Clark et al., 2000); that is, males cannot be arranged in a linear order of sperm competitive ability but instead the outcome of sperm competition depends on the specific genotypes of the competing pair of males. The non-transitivity of sperm precedence is predicted to maintain polymorphisms in traits that affect sperm precedence (Prout & Bundgaard, 1977). In addition, such polymorphisms could be maintained by pleiotropy between fecundity and mating success (Prout & Clark, 1996). The high levels of polymorphisms in Acps (e.g. Coulthart & Singh, 1988; Begun et al., 2000; reviewed in Chapman, 2001) could be due to these explanations.

In addition to facilitating sperm storage and sperm displacement Acps also increase female egg-laying rate and reduce female receptivity (Chen et al., 1988; Herndon & Wolfner, 1995; Chapman et al., 2003c; Liu & Kubli, 2003). Furthermore, the frequent receipt of Acps, specifically sex peptide (SP), causes female fitness costs by reducing survival and lifetime reproductive success (Chapman et al., 1995; Chapter 5). SP is a major contributor to the female cost of mating (Chapter 5). SP reduces female receptivity and stimulates egg production after the first matings of virgin females (Chapman et al., 2003c; Liu & Kubli, 2003). By reducing female receptivity after first matings, SP increases the time until remating (Chapman et al., 2003c; Liu & Kubli, 2003) and hence is predicted to delay the onset of sperm competition. Also, by increasing the rate of offspring production in females after first matings, males that transfer SP may increase their 'per-mating' reproductive success. SP is therefore expected to benefit males by increasing post-mating success. Because of the predicted benefits to males, and the finding that SP reduces female fitness, the *SP* gene is likely to play a role in sexual conflict. Under a sexual conflict scenario, adaptations that benefit one sex can persist despite reducing the fitness of their mates (Parker, 1979). Thus SP, which harms females, could mediate sexual conflict if it also benefits males. However, the repeated receipt of SP decreases the rate of female offspring production (Chapter 5) which could potentially offset the predicted male benefits of SP. It is therefore important to test whether SP benefits males in an environment in which females are permitted to mate multiply and in which males encounter competition for fertilisations (the norm for laboratory and wild-populations of *D. melanogaster*, e.g., Harshman & Clark, 1998; Imhof et al., 1998).

In this chapter I tested the prediction that SP benefits males in an environment in which males have to compete for fertilizations. I housed SP knockdown males or control males (Chapman et al., 2003c) with competitor males and then introduced females. Females were exposed to males throughout life. The competitor males and the females carried a recessive mutation that displayed an eye phenotype, *sparkling poliert* (Chapter 2, section 2.1.5), which allowed competitor males and their offspring to be differentiated from experimental (SP knockdown or control) males and their offspring. I measured the pre-mating success (the ability to gain matings) and the post-mating reproductive success (the 'per-mating' share of paternity) of

experimental (SP knockdown or control) males relative to competitor males. I predicted that SP knockdown males would have reduced post-mating reproductive success compared to controls. This is because the mates of SP knockdown males would remate sooner than the mates of control males and therefore the sperm of SP knockdown males would encounter competition and sperm displacement sooner, resulting in reduced paternity.

6.3 Methods

6.3.1 Generation and culture of flies

SP knockdown and control males were generated as described in the general methods (section 2.1.4.). Females and competitor males were obtained from a stock bearing the recessive mutation *sparkling poliert* (section 2.1.5). I checked that *sparkling poliert* males produced SP by performing Western blots (section 2.2.7). *sparkling poliert* males were found to produce SP at levels similar to that of control males. Experimental males (SP knockdown and control) were reared at low density to prevent larval competition, collected within 2 days of eclosion and aged for 3 days in groups of 10 per vial. Females and competitor males (both *sparkling poliert*) were reared in bottles using the 'egg washes' standard density culturing method (section 2.2.1), collected as virgins (section 2.2.2) and aged for 3 days after collection. A few vials of experimental males and females and competitor males and females were maintained to provide spare males to replace any that died.

6.3.2 Female survival, mating rates, mate choice, offspring production and offspring paternity

Two competitor males (*sparkling poliert*) and 2 experimental males (SP knockdown or control) were placed into 20 vials for each treatment of each line (i.e. 80 vials in total). One day later, 2 *sparkling poliert* females were aspirated, without anaesthesia, into each vial. Immediately following the introduction of females, the vials were observed continuously for 4 hours and all matings were recorded. Vials containing mating pairs were inspected under a dissecting microscope to determine the eye phenotype (and genotype) of the mating males. The cessation of matings was recorded to allow us to record any further matings in that vial during the observation period (i.e. if the 2 females present did not mate simultaneously). The first matings of 125 out of 160 females were observed. Matings were recorded on a further 13 days spread throughout the experiment. On each of these days 10 observations were

made on each vial, at least 20 minutes apart to avoid counting matings twice. For each mating observed, the genotype of mating male was determined using the method described above.

Flies were transferred to fresh vials every 1 or 2 days until day 17 and every 3 days from then on. Any dead males were replaced with spare males and all males were replaced on day 14 with fresh 3-day old males. All vials were retained and the offspring were allowed to develop for 12 days and eclose before being frozen at -80°C. Twelve days at 25°C was ample time for all offspring to emerge. The number of offspring of competitor and experimental males from the frozen samples was then counted. The offspring of competitor males had the *sparkling poliert* eye phenotype while the offspring of experimental males had eyes that were wild-type in appearance. Female deaths were recorded 6 days a week until > 90% of females in all lines had died. When a female died, 2 males (1 experimental and 1 competitor male) were removed from that vial to maintain the sex ratio at 2:1, male:female.

6.3.3 Statistical analysis

Except where stated, the analyses were performed separately for each line. Mating data were analysed in contingency tables using Chi-squared tests (Zar, 1999). First, overall mating frequency was analysed by comparing the total number of matings taken in each group to the total number of mating opportunities not taken. Mating opportunities were defined as in Chapter 5 (section 5.3.4.). To determine whether there were differences between treatments in the number of matings with experimental or competitor males, while taking into account the total number of mating opportunities for each treatment of each line, a further analysis was conducted. The categories used in the contingency table for this analysis were the numbers of mating opportunities not taken, the number of matings with experimental males and the number of matings with competitor males (Table 6.1).

Female survival curves were compared between treatments using Log Rank tests (Peto & Peto, 1972). The total lifetime offspring numbers (experimental plus competitor) per vial were compared between treatments using Kruskal-Wallis tests (Kruskal & Wallis, 1952). To analyse the proportion of paternity achieved by experimental males, the total number of offspring from experimental and competitor males and the percentage fathered by experimental males was calculated for each

vial. The percentage of offspring fathered by experimental males in each vial was compared between treatments using Kruskal-Wallis tests. Also, the total number of offspring was summed across all vials to provide a single pair of values (one for experimental offspring, one for competitor offspring) for each treatment of each line. These values were compared between treatments in contingency tables using Chi-square tests (Table 6.2).

To analyse male post-mating reproductive success, a 'per-mating' measure of paternity was calculated. First, the mean lifetime number of offspring per female fathered by of each type of male was calculated (Table 6.2). Then, the mean lifetime number of matings per female with each type of male was calculated. From these values the percentage of offspring fathered by, and matings with, experimental males was calculated for each treatment of each line. The mean % lifetime number of offspring per female fathered by experimental males was then divided by the mean % lifetime number of matings per female with experimental males. This gave a single value for each treatment of each line, an index of male-post-mating reproductive success (Table 6.3). The indices of male post-mating reproductive success were compared across treatments of both lines in a one-way ANOVA (Zar, 1999). The use of populations instead of vials as the unit of replication in this analysis of male post-mating success created a conservative test with one degree of freedom. Too few matings were observed in each individual vial to perform an effective analysis using 'per-vial' data.

6.4 Results

6.4.1 Mating frequencies of SP knockdown males, control males and competitor (*sparkling poliert*) males

There were marginally no significant differences between treatments in the overall mating frequencies in either line (Chi-square tests; Line 1, $\chi^2_1 = 1.077$, $P = 0.299$; Line 2, $\chi^2_1 = 3.709$, $P = 0.054$). However, there were significant differences between treatments in both lines when the genotype of the mating males (experimental, control and competitor) were included in the analysis (Chi-square tests; Line 1, $\chi^2_1 = 25.90$, $P < 0.0001$; Line 2, $\chi^2_1 = 11.146$, $P = 0.0038$; Table 6.1). The analysis shows that in both lines the SP knockdown males gained significantly more matings relative to competitor males than did control males.

6.4.2. Female survival and offspring production

Female survival was not significantly different for either line (median lifespan in days from the first day of exposure to males (lower and upper quartile), SP1 knockdown = 21 (20, 23), control 1 = 21 (20, 23), Log Rank tests $\chi^2_1 = 0.007$, $P = 0.93$; SP2 knockdown= 20 (17, 23), control 2 = 22 (20, 27), $\chi^2_1 = 2.95$, $P = 0.086$). Total offspring production was not significantly different for either line (median offspring number per vial (lower and upper quartile), SP1 knockdown = 999.5 (835.25, 1159), control 1 = 844.5 (565.25, 1127.75), Kruskal-Wallis test, $\chi^2_1 = 1.90$, $P = 0.17$; SP2 knockdown= 992.5 (789, 1286), control 2 = 1026.5 (762, 1102.25), Kruskal-Wallis test, $\chi^2_1 = 0.026$, $P = 0.87$).

6.4.3 Paternity of SP knockdown and control males and post-mating reproductive success relative to competitor males

There was no significant difference in the percentage of offspring per vial fathered by SP knockdown or control males in either line (Line 1, Kruskal-Wallis tests, $\chi^2_1 = 1.1707$, $P = 0.2793$; Line 2, $\chi^2_1 = 2.2948$, $P = 0.1298$; Fig. 6.1). However, when the total frequencies of offspring of experimental and competitor males for each line (not for each individual vial) were compared between treatments using Chi-square tests, significant differences were detected. These differences were in opposite directions in the two lines (Line 1, SP knockdown > control, Chi-square tests, $\chi^2_1 = 560.57$, $P < 0.0001$; Line 2, SP knockdown < control, $\chi^2_1 = 2000.52$, $P < 0.0001$; Table 6.2).

The index of male post-mating reproductive success (Table 6.3), 'per-mating' paternity, was significantly higher for control males than for SP knockdown males (ANOVA, $F_{1,2} = 31.02$, $P = 0.031$; Table 6.3).

Figure 6.1 Median (± interquartile range) percentage of offspring per vial fathered by experimental (SP knockdown or control) males relative to competitor males.

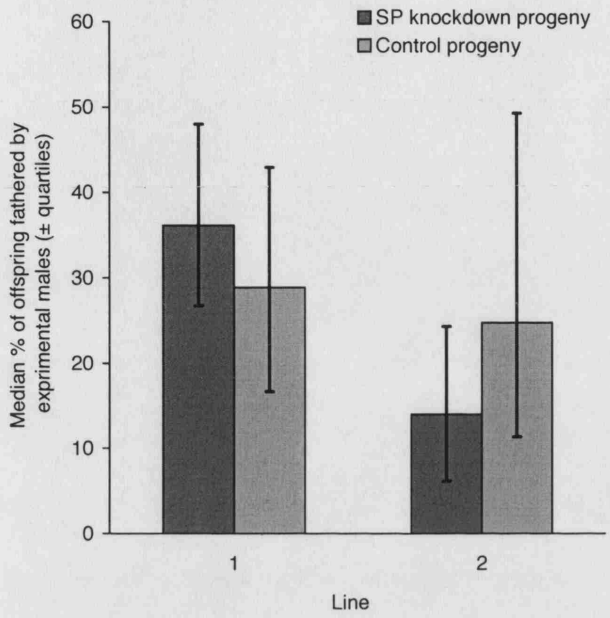


Table 6.1 Lifetime number of mating opportunities taken, and not taken, with experimental or competitor males. Percentages are given in below the values, in parentheses.

Treatment	Line	Mating opportunities taken		Mating opportunities not taken
		Experimental males	Competitor males	
Control	1	13	44	458
		(2.52%)	(8.54%)	(88.93%)
	2	13	43	476
		(2.44%)	(8.08%)	(89.47%)
SP knockdown	1	46	22	448
		(8.91%)	(4.26%)	(86.82%)
	2	35	40	444
		(6.74%)	(7.71%)	(85.55%)

Table 6.2 Mean lifetime number of offspring per female fathered by experimental and competitor males. Percentages are given below the values, in parentheses.

Treatment	Line	Mean offspring per female	
		Experimental males	Competitor males
Control	1	132.38	306.27
		(30.18%)	(69.82%)
	2	164.79	325.03
		(33.64%)	(66.36%)
SP knockdown	1	215.33	299.10
		(41.86%)	(58.14%)
	2	74.49	429.84
		(14.77%)	(85.23%)

Table 6.3 Post-mating reproductive success of experimental males calculated as the mean % lifetime number of offspring per female fathered by experimental males divided by the mean % number of matings per female with experimental males.

Treatment	Line	Mean % no. offspring per female fathered by experimental males / Mean % no. matings per female observed with experimental males
Control	1	1.35
	2	1.48
SP knockdown	1	0.62
	2	0.29

6.5 Discussion

6.5.1 Ability of SP knockdown and control males to gain matings

There were no significant differences in overall mating frequencies between treatments although there was a marginally non-significant trend towards more matings in the SP knockdown treatments in both lines. This suggests that, despite not receiving SP from matings with SP knockdown males, the frequency of matings

with competitor males was sufficient to inhibit the overall mating rate to a level similar to that of control treatments. Alternatively, the frequency of observations may have provided too little data to be able to detect these more subtle differences in overall mating frequency. When the genotype of mating males was added to the Chi-square analysis however, significant departures from independence were found indicating that SP knockdown males gained a significantly greater number of matings relative to competitor males than did control males in both treatments (Table 6.1). This is consistent with the finding in Chapter 5 that, when continuously housed with females, SP knockdown males courted females and mated with females significantly more often than did control males (section 5.4). However, all females in this study were not alone with SP knockdown or control males but had access also to *sparkling poliert* competitor males (which produce the same levels of SP as control males), whereas in the experiment of Chapter 5, females in SP knockdown treatments would never have received SP. Thus, in Chapter 5 the higher frequency of mating and courtship in SP knockdown treatments compared to control treatments was consistent with the increased receptivity of females that did not receive SP. However, the differences in mating success in this experiment are relative to competitor males and therefore can only be attributed to differences in female receptivity if there is an interaction between female receptivity and the relative success of experimental and competitor males at gaining matings (e.g. if SP knockdown males responded more strongly to receptive females than did control males).

The differences in mating success between SP knockdown and control males relative to competitor males could potentially result from the presence or absence of SP. For example, if there was a trade-off between SP production and courtship effort. However, investigating the cost of SP production itself for males is not possible with SP knockdown males produced by RNAi. This is because RNA production is not halted in SP knockdown males and hence not all the potentially costly aspects of SP production can be abolished. However, the cost of SP production would have to be large to offset the post-mating reproductive benefits that males derive from transferring SP (see section 6.5.3, below).

Alternatively, the differences in the ability to gain mates may be a result of eye colour variation between SP knockdown and control males. The *UAS-SP-IR* lines

and *Acp26Aa-P-Gal4* line were both created in white-eyed genetic backgrounds and the transgenes were marked with w^+ to allow detection of the transgene (Chapman et al., 2003c). SP knockdown males carried both the *UAS-SP-IR* and the *Acp26Aa-P-Gal4* transgenes and therefore carried two copies of the w^+ marker resulting in eyes of relatively dark, wild-type appearance. Control males, however carried a single w^+ marker from the *UAS-SP-IR* contruct which resulted in a paler eye phenotype. Eye colour is reported to be associated with both male courtship bout length and the time until mating. For example, when placed with wild-type females, white-eyed males demonstrate reduced courtship bout length and increased time until mating compared to wild-type males (Willmund & Ewing, 1982). This may be partly due to the defective vision of white-eyed males which prevents them from following females effectively (Willmund & Ewing, 1982; Schaffel & Willmund, 1985). Thus, if the pale eye colour of control males results in visual impairment then this might explain the observed reduced courtship frequency. Furthermore, there is evidence that is consistent with the hypothesis that females are less receptive to white-eyed males than to red-eyed males and are consequently less likely to mate with white-eyed males (Willmund & Ewing, 1982; Schaffel & Willmund, 1985). Thus, the pale eyes of control males could have been less successful at overcoming female receptivity inhibition which could have contributed towards the reduced ability of control males to gain matings. SP knockdown and control males also differ in their X chromosomes and X-linked genes that affect male mating success may also have contributed to the differences in pre-mating success.

6.5.2. Share of paternity gained by SP knockdown and control males relative to competitor (*sparkling poliert*) males

Despite achieving a greater proportion of matings (relative to competitor males) than control males (Table 6.1), SP knockdown males did not father a greater percentage of offspring per vial than controls in either line (Fig. 6.1). This suggests that, the 'per-mating' production of offspring was lower for SP knockdown males than for control males (see section 6.5.3, below). Chi-square analysis of the total offspring counts per treatment of each line showed significant differences between treatments in both lines but in opposite directions (Table 6.2). In line 1, SP knockdown males fathered a greater proportion of offspring relative to competitor males than did control males. Conversely, in line 2, control males fathered a greater proportion of offspring relative to competitor males than did SP knockdown males. These

opposing results may reflect the differences between lines in the relative mating success of SP knockdown compared to control males. The mating success of SP knockdown males relative to competitor males was approximately 3 times higher than control males in line 1 but only 2 times higher in line 2 (Table 6.1). However, the only way to interpret these differences and to accurately analyse the post-mating reproductive success of SP knockdown and control males is to standardise for the number of matings obtained by each type of male (section 6.5.3, below).

6.5.3. Post-mating reproductive success of SP knockdown and control males

The most important result from this chapter is the finding that post-mating success, the 'per-mating' number of offspring contributing to fitness, was significantly higher for control males than it was for SP knockdown males. The post-mating success of control males was more than double that of SP knockdown males in line 1 and over 5 times higher in line 2 (Table 6.3). The most parsimonious explanation for this result is that control males inhibited their mates from remating for longer than did SP knockdown males (as they do after first matings to virgin females; Chapman et al., 2003c; Liu & Kubli, 2003). This would have delayed the onset of sperm competition and sperm displacement, and increase the share of paternity of SP transferring males. SP could also potentially affect sperm storage, sperm competitive ability or resistance to sperm displacement but these possibilities remain to be tested.

It is possible that females might preferentially use the sperm of SP transferring males for fertilizations (i.e. display cryptic female choice; e.g. Thornhill, 1983; Eberhard, 1998). Females might preferentially use the sperm of SP-transferring males to gain indirect genetic benefits (Kirkpatrick & Barton, 1997; Cameron et al., 2003). For example the SP-transferring male offspring of females that mate with control or competitor males would have high post-mating reproductive success relative to SP knockdown males. This higher post-mating reproductive success of SP-transferring sons would confer indirect genetic benefits on their mother. However, females also suffer a large, direct fitness cost from the receipt of SP (Chapter 5). To elucidate whether it is advantageous for females to use sperm from SP-transferring males it is necessary to understand the way in which SP harms females. SP binds to sperm and is released from sperm during the days after mating (Peng et al., 2005). Free SP is hypothesised to then enter the female haemolymph

(Monsma et al., 1990; Kubli, 2003). The SP that harms females appears to be free from sperm as there is no reduction in the cost of mating with males that do not transfer sperm (Chapman, 1992). Thus, if SP is released from sperm when the sperm is utilized, then preferential use of sperm transferred with SP might not be beneficial because it could increase the amount of SP entering the female haemolymph and hence elevate SP-induced harm. However, SP appears to be released from sperm even when sperm is not utilized (Peng et al., 2005) in which case females will be harmed regardless of whether they use the sperm of their SP-transferring mate. In this situation females would always gain from the preferential use of the sperm of SP transferring males to gain indirect genetic benefits.

6.5.4. Female survival and reproductive success

Females that mate repeatedly to males that do not transfer SP have elevated fitness compared to females that mate to control males (Chapter 5). However, in the experiment described in this chapter, females encountered SP-producing competitor males in both treatments and therefore females exposed to SP knockdown males may not have encountered the full fitness benefits of SP reduction. Female survival and total offspring production did not differ significantly between treatments in either line. Further studies are required to determine if females gain benefits from mating with SP knockdown males when they have the opportunity to mate with both SP knockdown and SP transferring males during their lifetime. This could potentially depend on several factors such as the frequency of mating with each type of male, at what age females mate with a particular type of male, at what life history stage SP harms females, whether SP exerts harm immediately or with a time delay and whether females can recover from SP induced harm.

6.5.5. Conclusions

In this chapter I found that SP knockdown males were significantly more successful at gaining matings than control males but, despite this, SP knockdown males did not consistently father a greater percentage of offspring than control males. When the mating rate was standardised, it was found that SP knockdown males had significantly lower 'per-mating' post-mating reproductive success than control males. The differences in pre-mating success could be explained by the differences in SP levels, eye colour or X chromosome constitution between SP knockdown and control males. Further studies are required to differentiate between these

possibilities. The paternity results show that, when in competition with other males for access to fertilizations, SP benefits males by elevating post-mating reproductive success. The occurrence of male-male competition is the norm in laboratory stock populations and in wild-populations, because females typically mate with multiple males (Harshman & Clark, 1998; Imhof et al., 1998). Hence, the results suggest that male paternity will be usually be elevated by SP because it delays female remating and therefore delays the onset of sperm competition and sperm displacement. Thus, even if males are successful at gaining matings, SP is also required to maximise per-mating fitness. In addition to benefiting males, SP reduces female fitness (Chapter 5). Therefore, SP is a likely candidate for mediating sexual conflict in *D. melanogaster*. It will be important to investigate the mechanism of SP action and to determine the way in which it harms females. This would help us to understand the selection pressures placed on the sexes by sexual conflict and would begin to allow precise predictions to be made for tests to identify sexually antagonistic coevolution.

Chapter 7. General discussion of the thesis

7.1 Introduction

This thesis investigates sexual conflict in *Drosophila melanogaster*. Sexual conflict occurs over mating in this species because the optimum mating rate of males exceeds the optimum mating rate of females and, in addition, females suffer fitness costs from mating frequently (Fowler & Partridge, 1989) due to the action of male seminal fluid proteins (Chapman et al., 1995). The finding that females adapted to different levels of sexual conflict in a predictable way, by evolving different levels of resistance to male-induced harm (Chapter 3), provides support for the idea that sexual conflict is a potent evolutionary force in shaping life-history traits (e.g. Parker, 1979; Chapman & Partridge, 1996b; Rice, 1996; Rice, 1998). The results presented in Chapter 3 showed that females that had evolved under high levels of sexual conflict lived longer in the continuous presence of males than females that had evolved under low levels of sexual conflict. Unexpectedly, and in contrast to previous experimental evolution studies in *D. melanogaster* (Rice, 1996; Holland & Rice, 1999), I detected no differences in male harmfulness to females between the selection lines. This suggests that female resistance to males had evolved in response to differences in mating frequency during selection rather than to differences in 'per-mating' harm.

Sexually antagonistic coevolution resulting from sexual conflict is predicted to drive rapid divergence in allopatric populations which could potentially facilitate reproductive isolation and speciation (Chapter 1, section 1.4.5). However, the data in Chapter 4 provides no evidence that sexual conflict drives pre-mating reproductive isolation through evolved changes in female willingness to mate in *Drosophila melanogaster*, in contrast to a previous study in the dung fly, *Sepsis cynipsea* (Martin & Hosken, 2003; section 1.6.8). This, in addition to the finding in Chapter 3 that there were no differences in mating frequency between treatments when selection line females were continuously exposed to wild-type males, suggests that selection line females did not resist male-induced harm by avoiding mating. Instead, the data in Chapters 3 and 4 suggest that differences in the resistance of selection line females to male-induced harm occurred via mechanisms that operate after mating, for example, through variation in the resistance of females to the harmful effects of male Acps. In Chapter 4 I also found no evidence that males evolve

increased copulation duration in response to increased predicted levels of sperm competition and decreased numbers of mating opportunities.

Male *D. melanogaster* transfer, in addition to sperm, approximately 80 different seminal fluid proteins to females during mating (Swanson et al., 2001a) and these proteins induce behavioural and physiological changes in females (Gillott, 2003). The best characterised of these proteins is an accessory gland protein (Acp), the sex peptide (SP) (Chen et al., 1988). SP reduces female receptivity and increases female egg laying after the first matings of virgin females (Chapman et al., 2003c; Liu & Kubli, 2003). The results from Chapter 5 showed that SP is also a major contributor to the female cost of mating, because multiply mated females that did not receive SP had significantly higher fitness than control females that did receive SP. Also, females that did not receive SP mated more than 12 times as often as SP-receiving females but did not have reduced survival or reduced reproductive success. This indicates that the removal of SP makes matings effectively 'cost free' for females. SP is predicted to benefit males because it reduces female receptivity which delays female remating and is therefore likely to delay the onset of sperm competition and sperm displacement. This should increase the share of paternity for males that transfer SP. Chapter 6 confirmed this prediction - when in competition with other males for fertilisations, the 'per-mating' offspring production by SP-transferring (control) males was significantly higher than the 'per-mating' offspring production by SP-deficient males. Because SP decreases female fitness whilst elevating male post-mating reproductive success the *SP* gene is a strong candidate for mediating sexual conflict in *Drosophila melanogaster*.

In the following sections I describe in more detail the results of the experiments in Chapters 3 to 6, discuss their implications and discuss further questions posed by the findings. I also discuss some directions for future investigation that could help to provide further insights into the evolution of sexual conflict and sexually antagonistic coevolution in *Drosophila*.

7.2 Female resistance to male harm evolves in response to the manipulation of sexual conflict (Chapter 3)

7.2.1 Manipulation of sexual conflict by experimental bias of the adult sex ratio

To manipulate sexual conflict using experimental evolution, I altered the adult sex ratio of replicate lines of flies for successive generations. Selection before adulthood was minimised by standardising larval density and allowing plenty of time for flies from all lines to develop. Altering the adult sex ratio was found to alter the number of matings and the number of courtship bouts per female and per male. Hence the manipulation altered the potential for sexual conflict between treatments. During selection, the frequency of matings and courtship bouts per female, and therefore the potential for sexual conflict, was highest in male-biased (MB) lines, lowest in the female-biased (FB) lines and intermediate in the equal sex-ratio (ES) lines. Conversely, during selection, mating and courtship bouts per male were least frequent in the MB lines, most frequent in the FB lines and intermediate in the ES lines.

7.2.2 Resistance of selection line females to male-induced harm

As predicted by theory, females adapted to sexual conflict by evolving higher resistance to male-induced harm in the MB lines (high conflict) than in the FB lines (low conflict) and intermediate in the ES lines (intermediate conflict). The resistance was measured as the survival of females when continually exposed to wild-type males. An experimental evolution study by Holland and Rice (1999) previously found that *Drosophila melanogaster* females that had evolved under reduced predicted levels of sexual conflict (by imposing monogamy for 32 generations) survived for significantly less time when exposed to males than did control females that had evolved under higher predicted levels of sexual conflict (i.e. polyandry; see section 1.6.8; Holland & Rice, 1999). This suggested that monogamy females had evolved reduced resistance to male-induced harm relative to control females. One potential problem with the study by Holland and Rice (1999) was that inbreeding also predicted the result that monogamy females should be less resistant to male-induced harm (Chapman et al., 2003b; Snook, 2001). The results from Chapter 3 of this thesis were not confounded by inbreeding (which opposed the predictions from sexual conflict theory) confirming that alterations in the level of sexual conflict can lead to predictable and rapid evolutionary changes in the resistance of females to male-induced harm.

It will be interesting to discover whether the resistance of females to male-induced harm is to the harmful effects of male Acps or to other potentially female-harming factors such as the receipt of male courtship. The finding that the Acp sex peptide (SP) is a major contributor to the female cost of mating (see Chapter 5), presents us with an opportunity to test whether the evolution of female resistance in the selection lines utilised in Chapter 3 (and Chapter 4) was a response by females to the harmful effects of SP. When continuously exposed to control (SP-producing) males the survival of selection line females would be predicted to follow the pattern of results in Chapter 3 (i.e. MB females should be longest lived followed by ES females followed by FB females). If selection line females have evolved differential resistance to the harmful effects of SP, then selection line females continuously exposed to SP-deficient males would be predicted to show no differences in survival between sex ratio treatments (MB, ES or FB). This is because in the absence of SP, and the harm it causes to females, the basis for differential female survival between the treatments should be removed. The prediction that selection line females would not differ in survival when continuously exposed to SP-deficient males is consistent with the finding in Chapter 3 that there were no differences in female survival between selection treatments when mating costs were absent (i.e. in females that were exposed to males for 48-hours only).

In Chapter 3, the differences in survival of selection line females when they were housed continuously with wild-type males were not accompanied by changes in age-specific reproductive output. Resistance to male-induced harm is predicted to involve costs which are traded off against other life history traits such as reproduction. It is possible that the measures of egg production and adult offspring production used in Chapter 3 were not sensitive enough to detect subtle life-history differences between selection treatments. It might also be that the resistance to male-induced harm of selection line females is traded off against life-history traits that were not measured in the experiments in Chapter 3, such as immunity or larval competitive ability. Immunity and larval competitive ability are known to be involved in evolved life-history trade-offs, for example, a trade-off between parasitoid resistance and larval competitive ability was demonstrated in lines that had been artificially selected for parasitoid resistance (Kraaijeveld & Godfray, 1997). Thus, in future studies, the immunity and larval competitive ability of

selection line females could be assayed to investigate if either of these traits is traded-off against resistance to male-induced harm.

7.2.3 Harmfulness of selection line males

Holland and Rice (1999) found that monogamy males (which evolved under predicted low sexual conflict) evolved to be less harmful to females than polyandry males (where high sexual conflict was predicted). This was consistent with the idea that male-induced harm is associated with the effects of male-male competition for fertilizations, because this competition was absent in the monogamy lines. In contrast to the finding of Holland and Rice (1999), I did not detect differences in male harmfulness between selection treatments. This suggests that female resistance to male-induced harm may have evolved primarily in response to the differences in mating frequency (i.e. due to the differences in adult sex-ratio) between treatments during selection and not to differences in the degree to which selection line males harm females. A potential caveat of the study of Holland and Rice (1999) was that inbreeding also predicted that monogamy females would become more benign to females (Snook, 2001; Chapman et al., 2003b). It is therefore unclear as to whether the decreased harmfulness of monogamy males to females in was response to reduced sexual conflict or in response to inbreeding in their study.

It is possible that the protocols of the experiments in Chapter 3 were insufficient to detect differences predicted by sexual conflict in the harmfulness of males between treatments. In addition, insufficient selection pressure or number of selection generations cannot be excluded as potential explanations for the absence of an effect in males. However, females clearly did evolve in response to sexual conflict so it is unclear as to why males did not. It may be that the strength of the selection on males to evolve differences in traits that harm females was less strong than the selection on female resistance, or that males evolve harming traits more slowly than females evolve resistance. There could also be genetic constraints on adaptation in males. The potential costs of adaptation might also exceed the benefits of evolving increased female-harming traits. An alternative explanation is that the differences in inbreeding between treatments might have prevented or slowed adaptive change in males. For example, MB males were predicted to evolve to be most harmful to females but MB lines were also likely to be most inbred. Increased inbreeding predicts that MB males would become more benign. The net result therefore could

have been little or no change in male harmfulness. One possible way to test for the ability of males to evolve increased or decreased harmfulness would be to select on that trait directly (i.e. by artificial selection on the ability of males to reduce female fitness). However, one potential problem with this would be that selecting males that are more benign to females could potentially just select for unfit males (i.e. those males that lack or have reduced levels of traits that benefit males but harm females, such as Acps or courtship). Selecting only for males that have elevated harm relative to a control population would avoid this problem.

7.2.4 Accessory gland size and testis size of selected male flies

Male testis and/or accessory glands were predicted to evolve in response to selection for two reasons. 1) There were differences in female mating frequencies between treatments during selection which means that there were predicted to be differences between treatments in the intensity of sperm competition, being highest in MB lines, lowest in FB lines and intermediate in ES lines. 2) There were differences in male mating frequencies between the treatments during selection and therefore the average number of mating opportunities for males differed between treatments, being lowest in the MB lines, highest in the FB lines, and intermediate in the ES lines. Both sperm competition intensity and the number of male mating opportunities predict that investment in single ejaculates, and therefore perhaps the size of testes and accessory glands, should be greatest in MB lines, lowest in FB lines and intermediate in ES lines (e.g. Parker, 1998). However, no differences between treatments were found in male accessory gland or testis size, or the allometry between male body size and accessory gland or testis size. The opposing affects of inbreeding (as described above in reference to male harmfulness) may have also prevented a net change in the measured morphological traits. Alternatively the measurement of testes and accessory gland size may not necessarily correlate with investment in ejaculates. For example, males could vary in the rate of ejaculate production and the rate of transfer of ejaculates to females during mating without associated changes in the size of reproductive structures.

The accessory glands of male *Drosophila melanogaster* can become exhausted from repeated mating which can lead to matings that fail to fully stimulate egg production and fertilization in females (Hihara, 1981). Thus, if males mate very frequently it may be adaptive to partition investment in ejaculates to avoid depletion of the

accessory glands. Based on this idea, FB males, that have many mating opportunities during selection, are predicted to have evolved to partition their ejaculates between matings more than MB males, who have far fewer mating opportunities during selection and are thus predicted to invest heavily in each single ejaculate. One way to test this hypothesis would be to mate males successively to virgin females until the males were exhausted, and to look at the pattern and rate of offspring emergence in their mates. FB males would be predicted to partition ejaculates and to transfer fertile sperm for several successive matings without becoming exhausted. However, MB males would be predicted to invest heavily in each mating and perhaps become exhausted after fewer matings than for FB males. Additional ways to test for male investment in ejaculates could be to perform quantitative Western blots to test for the level of certain Acps for which there are antibodies (such as sex peptide; Chapman et al., 2003c; Liu & Kubli, 2003, and Acp62F; Bangham, 2003) in the accessory glands of males or to perform quantitative PCRs to assay the levels of transcription for specific Acps. These tests would provide comparisons of the potential quantity of Acps that males could transfer to females. Ideally the quantity of Acps actually transferred to females during mating would be assayed. However, detecting Acps quantitatively in females would be likely to prove very difficult, and detection could additionally be hindered by the movement of many Acps out of the female reproductive tract (e.g. into the female haemolymph) and the possible cleavage of Acps. However, the level of response of females to specific Acps (e.g. the rate of female egg laying rate in response to the receipt of SP) could provide phenotypic assays of the quantity of specific Acps that males transfer.

In conclusion, the results of the experiments in Chapter 3 are consistent with the hypothesis that females can evolve resistance to male-induced harm. The results suggest that females can evolve different levels of resistance to the harmful effects of mating such as the harmful actions of male Acps. This could be confirmed by testing the hypothesis that there would be no differences between selection treatments in the survival or fecundity of selection females when continuously exposed to males that lack harmful Acps, such as SP.

7.3 No evidence that mating probability or copulation duration evolves in response to manipulation of sexual conflict, sperm competition or male mating opportunities in *Drosophila melanogaster* (Chapter 4)

7.3.1 Sexual conflict and reproductive isolation in selected flies

Traits involved in sexual conflict are predicted to evolve rapidly and promote divergence in allopatric (Gavrilets, 2000) or sympatric populations (Gavrilets & Waxman, 2002). As traits involved in sexual conflict are related to mating and reproduction, the rapid divergence of such traits is predicted to drive reproductive isolation and speciation (e.g. Parker & Partridge, 1998). Empirical evidence for reproductive isolation through sexual conflict comes from a recent experimental evolution study in *Sepsis cynipsea* which suggested that sexual conflict leads to pre-mating isolation through female willingness to mate (Martin & Hosken, 2003; see Chapter 1, section 1.6.8). In Chapter 4, I tested the generality of this hypothesis using the *Drosophila melanogaster* selection lines from Chapter 3 in which females had been shown to evolve resistance to male induced harm in response to sexual conflict. I tested for pre-mating isolation between the selection lines by measuring the mating probability of pairs of flies from either the same treatment and same replicate (within-replicate matings) or the same treatment but different replicate (between-replicate matings). The results provided no evidence of differences in the mating probability of pairs of flies between within-replicate or between-replicate matings, in any treatment. This suggested that there were no differences in the willingness of females to mate when presented with males from the same or different replicates within the same treatment and thus no evidence for pre-mating reproductive isolation. The results also provide no evidence that selection line females differed in resistance to the costs of mating by evolving differences in willingness to mate. The lack of the predicted divergence in mating probability of pairs of selection line flies could be due to insufficient selection pressure or time for divergence. However, female resistance to male-induced harm evolved as predicted by sexual conflict (Chapter 3) which suggests that there was sufficient selection pressure to drive adaptive change in females. The data from Chapter 3 and 4 instead indicate that *Drosophila* females evolve resistance to males through post-mating rather than pre-mating changes in traits. Future investigations into reproductive isolation via sexual conflict in *Drosophila* might also assay post-mating traits such as female sperm usage, genetic compatibility between gametes and the length of refractory period after mating. These traits have the potential to affect the proportion

of offspring produced from within-population relative to between-population crosses and thus could be mechanisms by which reproductive isolation evolves.

In the experiment in Chapter 4 I used virgin flies from the selection lines. It may be that the willingness to mate of virgin females does not vary between treatments but that females vary between treatments in their resistance to subsequent matings. However, when selection line females were housed continuously with wild-type males (Chapter 3) no differences in mating frequency were detected. The finding in Chapter 4 that virgin females did not vary between treatments in their willingness to remate is therefore consistent with the mating frequency results from Chapter 3. Together, the results from Chapters 3 and 4 provide no evidence for evolved differences between treatments in the willingness of virgin or non-virgin females to mate.

FB females are predicted to have suffered the lowest mating costs during selection (as they mated least often) and MB females should have suffered the highest mating costs during selection (as they mated frequently). Thus, selection on females to resist mating is predicted to have been least strong in FB lines and strongest in MB lines. Therefore, the probability of mating was predicted to be highest in FB pairs (FB females were predicted to be most willing to mate) followed by ES females followed by MB females (MB females were predicted to be least willing to mate). However, whilst there were differences in the probability of mating between selection treatments, they were not in the direction predicted by sexual conflict. Instead, the probability of pairs mating was higher in ES lines than both MB and FB lines. One factor that could potentially affect mating probability is inbreeding, which negatively impacts male mating ability (Sharp, 1984). This could potentially explain the pattern of mating probabilities between treatments because inbreeding was predicted to be greater in MB and FB lines than in ES lines. However, MB lines were also predicted to be more inbred than FB lines (see section 3.5.1) but no difference between these treatments in mating probability was detected. In Chapter 3, when wild-type males were continuously housed with selection line males, no differences in the courtship frequency of males were detected between treatments, and the mating frequency of ES males was found to be lower than that of MB and FB males. This suggests that the higher mating probability of ES pairs in the experiment in Chapter 4 was a result of higher willingness to mate in ES females

and not because ES males were more successful at mating or less inbred than MB and FB males. Therefore, inbreeding seems unlikely to have caused the pattern of mating probability results. However, it is unclear why virgin females from the ES lines should be more willing to mate than MB or FB females.

7.3.2 Sperm competition, male mating opportunities and copulation duration

The mating rate results from Chapter 3 lead to the prediction that sperm competition was most intense in MB followed by ES followed by FB lines. Also, male mating opportunities were most scarce in MB followed by ES followed by FB lines. Thus, MB males were predicted to have evolved to invest most in single ejaculates and FB males least in single ejaculates (see above, section 7.2.4). Copulation duration is under male control and is heritable (MacBean & Parsons, 1967). Thus, if larger ejaculates take longer to transfer then copulation duration would be expected to be longest in MB males, shortest in FB males and intermediate in ES males. However the results of Chapter 4 did not follow this pattern. Although MB pairs copulated for longest, ES pairs had the shortest copulations and FB pairs had intermediate length copulations.

One explanation for the copulation duration results could be that elevated sperm competition intensity might not always lead to increased investment in ejaculates: Parker et al. (1996) found that above a certain intensity it can pay males to invest less in each ejaculate. However, Parker et al.'s (1996) model assumed that there were the same number of mating opportunities for each male, whereas in the selection lines used in this thesis there were significant differences between treatments during selection in the average number of mating opportunities per male. Thus, the prediction for reduced investment in ejaculates when sperm competition is above a certain intensity may not hold for populations in which the sex ratio is biased such as the selection lines used in Chapter 3 and 4. One potential reason why copulation duration did not evolve to be longer with increased predicted sperm competition is that copulation duration might not correlate with ejaculate investment in *Drosophila melanogaster.* For example, males may be able transfer sperm and seminal fluids at different rates with the rate of ejaculate transfer being unrelated to the overall length of copulation duration. It will thus be interesting to investigate how selection line males partition their investment in ejaculates across successive matings (see description above, section 7.2.1).

Another potential reason for the lack of predicted differences in copulation duration is that the optimal ejaculate investment of males when mating to virgin females may not reflect the optimal ejaculate investment when mating to previously mated females. During selection, eggs were collected for subsequent generations of selection approximately 10 days after eclosion. This means that there was a high probability of sperm competition and sperm usurpation after matings to virgin females because females were likely to remate at least once before producing eggs that would contribute to the next generation. Thus, future investigations could look at male investment in ejaculates after males have experienced conditions that approximate those of the selection period (e.g. in non-virgin 8 day-old males that are mated to non-virgin females).

7.4 Sex peptide causes mating costs in female *Drosophila melanogaster* (Chapter 5)

Female mating costs in *Drosophila melanogaster* are mediated by male Acps (Chapman et al., 1995). The results from Chapter 5 show that SP is a major contributor to these costs. Females housed with SP-deficient males mated more that 12 times as often as females housed with SP-transferring control males but did not suffer higher survival costs, higher reproductive costs or higher overall fitness costs than controls. Instead, females that did not receive SP laid marginally significantly more eggs and produced significantly more offspring, leading to significantly higher fitness, than SP-receiving females. The results further suggest that, when SP is absent, other Acps cause little or no harm to females. This is because females housed with SP-deficient males, that received Acps other than SP more than 12 times as often as control females suffered lower, rather than higher, mating costs. However, I cannot rule out the possibility that SP interacts with other ejaculate substances in causing harm to females and hence that other unknown substances are also required for full expression of female harm. To determine whether SP alone causes mating costs would require the production of males that transfer no Acps except SP, or the measurement of mating costs in females injected with SP and mated to Acp-deficient males.

The finding that SP is involved in mating costs is important because factors that cause female mating costs are likely to be involved in sexual conflict. To understand

the possible role of SP in sexual conflict there are potentially two major paths of investigation: 1) investigating the mechanism by which SP harms females and 2) investigating interactions between SP-induced harm and the environment. These factors will need to be studied to allow precise predictions about how, and in what circumstances, specific traits involved in sexual conflict should evolve. For example, knowledge of the mechanism by which SP harms females is required to identify genes in females that would be predicted to evolve in response to changes in SP-induced harm. Knowledge of female-specific genes that are involved in sexual conflict is required to make predictions about the results of investigations to differentiate between the operation of sexual conflict or other forms of sexual selection. For example, sexual conflict predicts that the products of genes that determine female resistance to male-induced harm should be selected to increase in quantity or quality in response to increased male-induced harm. This type of adaptation is not predicted by traditional sexual selection theory. A demonstration of the correlated evolution of male-harming genes and female resistance genes would provide strong support for sexually antagonistic coevolution. Also, in cross-species or cross-population comparative studies, the specific genes involved in sexual conflict could be targeted and the rate of divergence compared to that of genes not involved in sexual conflict. This type of study would provide a much more powerful test than is currently possible of the prediction that sexual conflict drives rapid divergence in allopatric populations. The following sections discuss potential mechanisms for the operation of sexual conflict in *Drosophila* which may provide the first opportunity to probe such mechanisms in detail. I examine below the hypotheses for the mechanism of SP-induced harm and ways in which the effects of SP may interact with the environment.

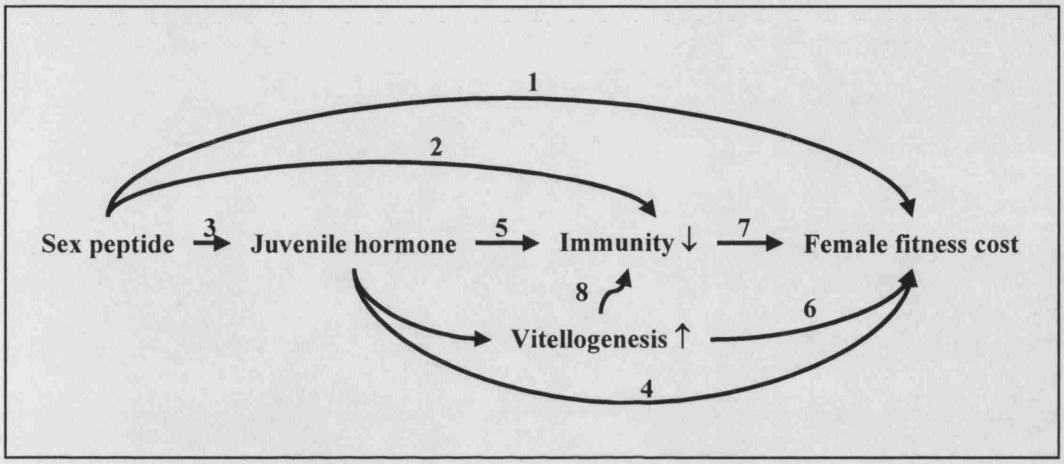
7.4.1 Future investigation into the mechanism by which sex peptide harms females: juvenile hormone and the immune system

The sex peptide elevates oogenesis after the first matings of virgin females by stimulating the *corpora allata* to release juvenile hormone (JH) (specifically juvenile hormone III-bisepoxide (JHB(3)); Moshitzky et al., 1996) which in turn stimulates oogenesis (Soller et al., 1999; see section 1.7.4). Therefore females could be harmed by SP via the actions of JH. JH has been implicated in an immunity cost resulting from mating in *Tenebrio molitor* (Rolff & Siva-Jothy, 2002; see section 1.6.7). In the study by Rolff and Siva-Jothy (2002), male and female *T. molitor* were

found to have lowered immunity 24h after mating. By transplanting the *corpora allata* (which synthesise JH) of mated and non-mated individuals into virgins, the lowered immunity after mating was shown to be attributable to the substances released from the *corpora allata* (Rolff & Siva-Jothy, 2002). Furthermore, when *corpora allata* which were removed from mated individuals were inhibited from synthesising JH before being transplanted in virgins, no immunity suppression was detected. This indicated that it was the action of JH released from the *corpora allata* that lowered immunity after mating. Lowered immunity is potentially costly because it increases the susceptibility of individuals to infection by pathogens. Infection is likely to lead to reduced fitness if it is costly to fight pathogens or if pathogens cause sterility or death. JH is elevated in *D. melanogaster* females after the receipt of SP, and if JH acts as an immunity suppressor in this species then it could potentially mediate SP-induced female mating costs. In addition, there is evidence from a study on the Monarch butterfly that high levels of JH reduce lifespan (Herman & Tatar, 2001) and reduced lifespan can represent a fitness cost if reproductive rates are not higher in shorter lived individuals. Thus, JH-induced immunity suppression is an obvious candidate path by which SP causes female mating costs.

There are several potential pathways by which SP and/or JH and/or immunity suppression could harm females (Fig. 7.1). These pathways could be tested by using the existing SP RNAi lines and by constructing transgenic female flies in which JH is suppressed or over expressed. The first problem to address would be whether mating compromises immunity in female *Drosophila melanogaster* and, if so, whether the immunity suppression is a result of SP and/or JH. If a link between SP and/or JH and immunity was found then it would be important to investigate whether immunity is traded-off against reproduction or whether immunity is unrelated to reproduction. This would be required to differentiate between the costs of reproduction and the costs of mating itself. This is important because even if survival is compromised by investment in reproduction, this might not necessarily result in an overall reduction in fitness. For example, if short-lived individuals produce more offspring than long-lived individuals then the short-lived individuals will have higher fitness. Thus, reduced survival resulting from SP or JH might not therefore necessarily indicate the operation of sexual conflict.

Figure 7.1 A model for the potential mechanisms of SP-mediated female costs of mating. SP could harm females directly (path 1), or it could decrease fitness through suppression of the immune response (path 2). SP-induced mating costs could be exerted via JH (path 3), which could, itself, be directly costly (path 4), or could compromise immunity (path 5/7) and/or incur costs through increased vitellogenesis (the incorporation of yolk into the eggs) (path 6/8).



Identification of the mechanism by which SP harms females will be an important step towards the identification of female genes involved in resistance to SP-induced harm. For example, if SP is found to harm females directly then the female-specific genes involved in resistance to SP might be receptors that interact with SP or substances that neutralise, destroy or remove SP. Alternatively, if SP-induced harm occurs via JH then genes involved in the regulation of JH may be important. Furthermore, if JH-induced immunity suppression is a female cost of SP receipt, then genes involved in the immune system and its regulation may be important factors in resisting the harmful effects of SP. Only by understanding how SP harms females can hypotheses be generated and subsequently tested about which genes are involved in female resistance.

7.4.2 Future investigation into interactions between sex peptide-induced female costs and the environment

The extent to which SP is involved in sexual conflict is predicted to depend upon the mating rate of females. The mating rate of females can in turn depend upon the level of nutrition available to females and their encounter rate with males (Chapman &

Partridge, 1996a). When females mate only once, the rate of offspring production of females that receive SP exceeds the rate of offspring production of SP-lacking females. Thus, when mating opportunities are severely limited, SP may benefit females by elevating offspring production. Environments in which female mating opportunities could be severely limited include low density populations, female-biased sex ratio populations (e.g. from the effects of male-killing micro-organisms that have been found in natural populations of several *Drosophila* species, including *D. melanogaster*; Montenegro et al., 2000) and/or under conditions where there is poor food availability.

Chapter 4 showed that when females are mated frequently, SP-receiving females have lower fitness than SP-lacking females. Evidence suggests that multiple mating is common in the field (Harshman & Clark, 1998; Imhof et al., 1998) and it is also predicted to be the norm in laboratory maintained populations. Thus, SP may be involved in sexual conflict more often than it is involved in sexual cooperation. However, this will depend on the precise mating rates of females and the relationship between female mating rate and SP-induced harm. For example, if harm caused by SP was increasingly cumulative and there was a threshold below which negligible costs or even no costs occurred then only very high rates of mating might be detrimental to females. This is because only with high mating rates will the receipt of SP become significantly and increasingly harmful to females. Alternatively, if SP-induced harm was correlated in a linear manner with the mating rate of females, then SP would decrease female fitness even at relatively low mating rates. Thus, it will be crucial to test the effect of SP on female fitness by varying mating mate. This could be achieved by manipulating female exposure to mating opportunities and/or varying the level of nutrition available.

Other factors that may be important in developing an understanding the role of SP in sexual conflict or cooperation might include at what age SP harms females and whether harm is reversible (i.e. can females recover from SP-induced harm?). This could be investigated in 'switch' experiments in which females are exposed to SP-deficient males for a period of time and at a given age the males are switched to control males or *vice versa*. By measuring the age-specific mortality and the age-specific offspring production of females in these females, the level of female-induced harm incurred at specific ages could be assayed. In addition the results of

switch experiments would indicate whether females can recover from SP-induced harm. Females could be shown to recover if, after the removal of exposure to SP transferring males, the mortality and reproductive rates of those females became comparable to those of females that had not received SP.

7.5 The effect of sex peptide on male reproductive success in *Drosophila melanogaster* (Chapter 6)

Sexual conflict is predicted to be generated by the expression of genes in individuals of one sex that benefit those individuals but that harm individuals of the opposite sex with which they interact. The results from Chapter 5 indicate that the expression of the *SP* gene in males can result in harm to females, which is consistent with SP playing a role in sexual conflict. However, to confirm the role of SP in sexual conflict requires that males simultaneously benefit from transferring SP. Chapter 6 confirmed the prediction that males that transfer SP have higher post-mating success ('per-mating' share of paternity) than SP-deficient males when in competition for fertilizations with other SP-transferring males. This is presumably because females that receive SP delay remating (Chapman et al., 2003c; Liu & Kubli, 2003) and therefore mate less frequently (Chapter 5) which delays the onset of sperm competition and sperm displacement, increasing the share of paternity for SP-transferring males. Thus, the results from Chapter 6 provides evidence that SP is beneficial to males. This finding, together with the demonstration that SP is a major contributor to female mating costs (Chapter 5) provides evidence that SP is likely to mediate sexual conflict in *Drosophila melanogaster*.

The SP-deficient males utilised in Chapters 5 and 6 (Chapman et al., 2003c) were more successful at gaining mates in a competitive environment than were control, SP-transferring males. This could potentially be related to the absence of SP (e.g. if the costs of SP production are traded off against courtship) or the difference in eye colour between SP-deficient and control males. To test whether there is a cost to SP production for males (e.g. in terms of mating ability) and to identify any trade-offs, other SP-deficient lines would need to be utilised. The SP-deficient males made by RNAi and utilised in Chapman et al. (2003) and Chapters 5 and 6 are not the best reagents to use to test for any costs associated with SP production in males, because the SP-deficient males transcribe SP RNA which could be a part of the cost of SP production. Better lines to use to test for life-history trade-offs associated with SP

production would be with those with an *SP* gene knockout (e.g., Liu and Kubli, 2003).

The results of Chapter 6 showed that males benefit from transferring SP. Hence females could gain indirect genetic benefits from mating with SP-transferring males. This is because females that have SP-producing male offspring will gain inclusive fitness benefits through the elevated reproductive success of their sons. Indirect genetic benefits in females, if large enough in comparison to the direct costs of SP, could select for female preference for SP-transferring males. However, theory suggests that such indirect benefits are likely to be small in comparison to direct costs (Kirkpatrick & Barton, 1997; Cameron et al., 2003). This means that the importance of indirect genetic benefits is likely to depend on how SP interacts with the environment in determining the level of direct costs to females (see section 7.3.2, above). For example, females of the bulb mite, *Rhizoglyphus robini*, that suffer reduced lifetime fecundity as a result of frequent exposure to males (Kolodziejczyk & Radwan, 2003) have more fecund female offspring if they are permitted to be polyandrous than if they are forced to be monogamous (Konior et al., 2001). Thus, females of this species gain indirect genetic benefits from multiple mating. This indirect benefit is smaller than that required to offset the costs of mating, but Kolodziejczyk and Radwan (2003) note that the magnitude of mating costs will vary depending on the environment. The studies on *R. robini* highlight the potential importance of environmental effects in studies of sexual conflict and highlight the need for investigations into gene × environment interactions. It will be important to consider the indirect benefits of SP to females in future studies on SP such as those discussed in sections 7.3.1 and 7.3.2. However, weighing up the net costs and benefits of SP receipt to females may depend upon interactions of the effects of SP with the environment which could alter the costs and benefits of SP for both sexes.

7.6 Summary

This thesis has focussed on investigating the evolution and underlying genetics of sexual conflict in *Drosophila melanogaster.* The data presented indicate that females can evolve resistance to male-induced harm (Chapter 3). However there was no evidence that the evolutionary changes in female resistance are associated with any pre-mating reproductive isolation between populations with contrasting evolutionary histories of sexual conflict (Chapter 4). There was also no evidence of changes in

male harmfulness in response to manipulated levels of sexual conflict (Chapter 3) which suggests that female resistance evolved in response to the differences in mating frequency that females experienced during selection. I found no evidence that higher predicted intensities of sperm competition and decreased numbers of male mating opportunities in selected lines, led to the evolution of larger testes or accessory glands in males (Chapter 3) or to the evolution of increased copulation duration (Chapter 4). I presented evidence that the repeated receipt of sex peptide (SP) decreases female fitness (Chapter 5) and that SP benefits males by increasing their post-mating reproductive success (Chapter 6). Thus, SP is likely to mediate sexual conflict in this species.

For over 30 years sexual conflict has been proposed to explain the evolution of traits involved in interactions between the sexes. Whilst sexual conflict clearly has the potential to drive coevolution between the sexes the relative importance of sexual conflict compared to other forms of sexual selection is unclear. However, knowledge of the genetics underlying sexual conflict promises to improve the predictive ability of researchers in differentiating between sexual conflict and sexual selection. To do this effectively, researchers will also need to identify genes in females that are involved in sexually antagonistic interactions.

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Appendix I. Wigby, S. & Chapman, T. 2004. Female resistance to male harm evolves in response to manipulation of sexual conflict. *Evolution*, **58**(5), 1028-1037.

Appendix II. Wigby, S. & Chapman, T. 2005. Sex peptide causes mating costs in female *Drosophila melanogaster*. *Curr. Biol.*, **15**(4), 316-321.

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