Costs and Consequences of Plastic Responses to the Sperm Competition Environment in *Drosophila melanogaster*

By Joshua Moatt

Thesis submitted for the degree of

Master of Science (by Research)

University of York

Department of Biology

December 2012

Abstract

Sperm Competition (SC) has been one dominant focus of evolution and behavioural ecology since its conception. Plastic SC responses evolved in response to rapid changes in SC risk. In the model organism *Drosophila melanogaster* it is known that males reared with rivals mate longer, alter seminal fluid production and transfer an ejaculate containing more seminal fluid proteins, resulting in higher fertilization success and paternity share. Here I investigate potential longevity costs for males expressing plastic SC responses, as well as any longevity cost to females mating to these males. I also explore whether males vary sperm production in response to SC risk. I demonstrate that virgin males reared in high SC environments live significantly longer than males from low SC environments; mating negates this longevity benefit. I expected males to pay a longevity cost for their plastic SC responses: the counterintuitive finding suggests that males may trade-off this cost by reducing other expensive functions. In contrast, females showed no instantaneous longevity cost or benefit to mating with males from a high SC background. In addition to the plastic behavioural and physiological responses to SC risk already reported, I demonstrate here that male *D. melanogaster* also respond by increasing both sperm quantity and quality. Despite the longstanding use of *D. melanogaster* as a model species in SC research, this result has never previously been described. The work reported here reveals two major findings: male *D. melanogaster* adjust sperm quality in response to risk of SC, and that longevity of these males increases above that of males raised alone. This research raises new questions about plasticity in response to SC in this species, in particular, if the costs of the response are so small, why do males not constantly show the elevated response? The answer is likely to lie in the precise balance of trade-offs between different life history variables.

Contents

List of Figures and Tables

Figure(F) / Table(T) **Page**

Acknowledgements

There are a number of people who need to be thanked in relation to this thesis. First I would like to thank Doctor M. D. Thom and Professor C. Dytham for the opportunity to carry out this research and their expert guidance throughout its undertaking. Also I thank them for their hard work and tireless efforts in helping to design experiments and their comments on this thesis. I would also like to thank Doctor S. Sweeny for allowing me to use his lab for the rearing and breeding of my flies and for his suggestions and criticisms of previous experimental designs. Similarly I would like to thank Doctor P. Mayhew for his advice on experimental design and thesis construction. Finally I would like to thank all my friends and family for their support throughout the undertaking of this masters, and for putting up with my constant fly talk over the last year.

Declaration

I hereby declare that all works presented in this thesis are my own. All experiments and data collection were carried out by J. P. Moatt, and this thesis was prepared solely by the author. The work presented in Chapter 3 'Virgin males live longer when reared with rivals' has been prepared and submitted for publication in the journal *Biology Letters* by J P Moatt, C Dytham and M D Thom, under the title '*Exposure to sperm competition risk improves survival of virgin males*' and is currently under review. However the chapter presented here was prepared separately and by the author alone. The work shown in Chapter 4 "Males Alter Sperm Production in Response to Rivals' is also being prepared for submission to the journal *Functional Ecology*, by authors J P Moatt, C Dytham and M D Thom, however submission has not yet taken place. Again, the chapter presented here was produced by J. P. Moatt alone and is separate from the paper currently being prepared.

Chapter 1

Introduction

The phenomenon of sperm competition (SC) was first recognised by Geoff Parker in 1970 as the competition between one or more males' sperm for the fertilization of a given set of ova (Parker 1970; Parker et al. 1996; Parker et al. 1997; Wigby and Chapman 2004). It is taxonomically widespread, seen in taxa including fish (Stockley et al. 1997; Boschetto, Gasparini, and Pilastro 2011), birds (Westneat 1987; Birkhead, Pellatt, and Hunter 1988; Sheldon 1994; Birkhead et al. 1999), mammals (Harcourt et al. 1981; Gomendio and Roldan 1991; Harcourt, Purvis, and Liles 1995; Hosken 1997; Lemaître et al. 2011; Lemaître et al. 2011) and insects (Gage and Barnard 1996; Bretman, Fricke, and Chapman 2009; Wigby et al. 2009; Fedorka, Winterhalter, and Ware 2011). Sperm competition can be divided into two variants: intra-ejaculate competition is the competition between sperm from a single male's ejaculate, inter-ejaculate competition is the competition between the sperm of different males ejaculates (Wigby and Chapman 2004).

Inter-ejaculate SC can only occur when females mate multiply, as this leads to the requisite temporal and spatial overlap of sperm. There is an accumulating body of evidence demonstrating that female multiple mating, or polyandry, is widespread, largely because of the benefits that females accrue through this behaviour. Polyandry can lead to direct benefits to the female, such as increased sperm supply, nuptial gifts and paternal care of offspring. In a meta-analysis of 122 insect species, direct benefits alone can explain the prevalence of polyandry within insects (Arnqvist and Nilsson 2000). Polyandry also leads to a number of indirect benefits such as, increased egg hatching success, shorter gestation and larger broods (reviewed Simmons 2001). The good sperm hypothesis predicts that males that are good competitors at SC are likely to be of higher genetic quality, as they can divert more resources into their testes. This means by utilizing polyandry and causing SC females are ensuring the production of fitter offspring (Simmons 2001). Similarly, by utilizing polyandry, females may be selecting males who are good SC competitors. This will produce sons who are also good at SC, thus

leading to higher numbers of grand-offspring (Simmons 2003). Multiple mating might be being utilized as a means of selecting the mate with superior genetic quality. By mating more than once the female can “trade up” to a male with the best genetic quality, thereby increasing the quality of her offspring (Simmons 2001). Finally, multiple mating could be a means of inbreeding avoidance and compatibility screening. By multiply mating the genetic diversity of sperm stores is increased. This coupled with the utilization of post-copulatory selection methods, such as cryptic female choice and SC, can ensure only sperm from compatible males achieve fertilization (Zeh and Zeh 1997). Although the reason SC occurs is unclear, it certainly has potent evolutionary effects on behaviour, morphology and physiology.

In these studies I will be looking solely at inter-ejaculate competition, which has been shown to have a diverse range of responses. In response to high levels of SC, species sperm size can evolve in one of two ways: either evolving towards ejaculates containing fewer larger, or alternatively many smaller, sperm (Snook 2005; Wigby and Chapman 2005). The advantage of smaller sperm is that an ejaculate can contain more sperm (Pitnick 1996). This fits with “fair raffle” model, where number of sperm represents tickets in a raffle, meaning more sperm increase the chance of achieving successful fertilization (Parker 1970; Parker 1990; Birkhead and Møller 1998). While the advantage of larger sperm is not fully understood, it is possible that larger sperm could displace smaller sperm (LaMunyon and Ward 1998), live longer (Parker 1993; Parker 1998) or swim faster (Gomendio and Roldan 1991). It has been proposed that sperm size is a measure of ejaculate quality (Snook 2005). Sperm quality is an important factor in deciding the outcome of competitive fertilizations, as has been shown many times (reviewed Snook 2005). Sperm of a higher quality have a fitness advantage so they can outcompete rivals (Wigby and Chapman 2004). Quality of sperm has been measured in numerous ways such as mobility (Birkhead et al. 1999), velocity (Boschetto, Gasparini, and Pilastro 2011) and longevity (Snook 2005). The most commonly seen SC response is to produce a larger ejaculate containing more sperm (Gage and Barnard 1996;

Stockley et al. 1997; Wigby and Chapman 2004; Bretman, Fricke, and Chapman 2009; Wigby et al. 2009).

SC was originally studied over evolutionary timescales. It was noted that in promiscuous species, where multiple matings are frequent, males had relatively larger sized testes than monogamous species (Harcourt et al. 1981; Harcourt, Purvis, and Liles 1995). These were lifelong changes that were outside the individual males' control. The heaviest primate species *Gorilla gorilla* (gorilla), and *Pongo pygmaeus* (orangutan), are monogamous, with a combined testis mass of 30 g and 35 g respectively. However the lighter primate, *Pan troglodytes* (chimpanzees), which are promiscuous, have a mass for both testes combined of 120 g (Harcourt et al. 1981). The authors propose that this four-fold difference in testis size is due to the differences in breeding system. Larger testis means larger ejaculate of better quality, thus better chance of fertilization success. Therefore, the risk of SC leads to the development of larger testes (Harcourt, Purvis, and Liles 1995).

However, not only do SC responses occur over evolutionary timescales, but they occur plastically as well. Phenotypic plasticity is the ability of an organism to express different phenotypes depending on the abiotic and biotic environment, including the social environment (Agrawal 2001; Bretman, Gage, and Chapman 2011a). They are short term non-permanent responses (Juliano et al. 1995; Via et al. 1995; Agrawal 2001; Bretman, Gage, and Chapman 2011a) which increase an organism's fitness in variable environments (DeWitt, Sih, and Wilson 1998). Plastic responses are important areas of study as they are attempts to maintain maximum fitness in all environments (Juliano et al. 1995; Agrawal 2001). By studying them we can gain insight into how organisms could respond to more permanent changes in their natural environment. Some plastic responses are caused by alterations in gene expression levels, in these cases the lack of genetic variation could be restricting the evolution of some behavioural traits (DeWitt, Sih, and Wilson 1998).

Plastic responses to perceived risk of SC occur in *D. melanogaster*. In this species, males detect rivals through a combination of four cues: auditory, olfactory, tactile and visual stimulation

(Bretman, Gage, and Chapman 2011b). Vision is of the least importance, but any combination of two of the other three is enough to trigger an SC response. This highlights the level of redundancy in the detection of rivals, as a male could lose any one of these sensory apparatus and still detect the presence of rivals (Bretman, Gage, and Chapman 2011b). There is a critical time of around 29 hours a male must spend with rivals in order for the response to develop (Bretman et al. 2010). However only a single rival male is needed to be present in order for the SC response to be triggered, and increasing the number of rivals does not increase the magnitude of the response (Bretman et al. 2010). If a male does detect a rival before mating, it triggers a number of behavioural and genetic responses, which improve the male's reproductive success. Firstly, gene expression levels for some genes associated with seminal fluid proteins (Sfp) in the male are altered as is Sfp sequestration to the testis (Fedorka, Winterhalter, and Ware 2011). This altering of ejaculate composition results in a much larger ejaculate being transferred to females in subsequent matings (Wigby et al. 2009). Mating duration is also significantly increased (Bretman, Fricke, and Chapman 2009); this is positively associated with time spent with rivals prior to mating (Bretman et al. 2010). Here mating duration is being used as a proxy for ejaculate investment, meaning males are strategically increasing the allocation of resources to mating. In contrast, if rivals are present in the mating area, this results in a shortening of this mating duration change (Bretman, Fricke, and Chapman 2009). This seems counterintuitive, as a possible explanation for the increased duration of mating is that it is a form of mate guarding, if that were the case it would be expected that mating duration would increase with rivals in the arena also. This unexpected result could be due to harassment of the mating pairs by rival males (Bretman, Fricke, and Chapman 2009).

Taken together, these plastic behavioural and physiological responses lead to a 7% increase in female egg laying rate and a 3% increase in egg to adult survival, thus increasing the male's reproductive success regardless of whether the male is mating in the P1, first male to mate, or P2, second male to mate, position. Bretman et. al. (2009)

propose that the increased mating duration allows for the transfer of larger volumes of key Sfps, and this results in an increased latency to remate by the female, leading in turn to the higher fertilization success. However the support for this claim is not entirely consistent. Wigby et. al. (2009) show that males transfer larger volumes of Sfps to the female. However, it has been shown that in response to rivals, males downregulate expression of certain Sfps (Fedorka, Winterhalter, and Ware 2011). Furthermore, transfer of ejaculate in this species occurs remarkably early during copulation, within 6-10 minutes (Gilchrist and Partridge 2000). There is evidence that disruption of mating after ejaculate transfer but before the natural end of copulation resulted in no loss of reproductive success but a decrease in female latency to remate (Gilchrist and Partridge 2000). However, Sfps in *D. melanogaster* have been shown to influence many female attributes including egg laying (Herndon and Wolfner 1995; Chapman et al. 2003; Liu and Kubli 2003). If the main Sfp transfer occurred after sperm transfer, and longer mating resulted in a larger transfer as proposed above, we would expect to see a decrease in reproductive success when mating is disrupted prior to the natural end. It is clear however, that these responses to rival male presence significantly impact on male mating strategy and this is likely to affect both the male and the female.

Key to the increased reproductive success seen in the above responses are Sfps. Sfps can act in a number of ways to increase a male's chance of achieving fertilization, be this through chemical action or physical prevention. In some species, Sfps form physical barriers in species known as a mating or copulatory plug. Copulatory plugs are gelatinous blobs which block the females' genital tract preventing them from remating or rival sperm reaching the ova (Shine, Olsson, and Mason 2000). They are seen in many species including: bumblebees *Bombus terrestris* (Baer et al 2000), red sided garter snakes *Thamnophis sirtalis parietalis* (Shine, Olsson, and Mason 2000), funnel web spider *Agelena limbata* (Masumoto 1993), eastern grey squirrels *Spermophilus carolinensis* (Koprowski 1992), anopheline mosquitos (Giglioli and Mason 1966) and chalcedon checkerspot butterfly

Euphydryas chalcedon (Dickinson and Rutowski 1989). The effectiveness of these mating plugs can vary from species to species, and even mating event to mating event (Koprowski 1992). Some mating plugs can be very effective, but in *S. carolinensis* many females simply remove the mating plug 30 seconds after it is formed and eat it (Koprowski 1992), due to it containing nutritious compounds (Baer et al. 2000).

Among the *Drosophila* there is a particular subset of Sfps, known as accessory proteins (Acps), which manipulate female reproductive behaviour. These play a role in sexual conflict between males and females, where males try to prevent a female remating and females attempt maximise their reproductive success (Chapman et al. 2003). However Acps may have originally evolved through sexual selection, as a means of honest signalling of ejaculate quality. It has been proposed that prior to the evolution of Acps, some matings would result in females receiving inadequate ejaculate levels from the males (Cordero 1995). Females therefore needed a way to discriminate between adequate and inadequate ejaculates; and those females which could discriminate would have a selection advantage over those who could not (Cordero 1995). As males were transferring chemicals in their ejaculates, females could use these chemicals as a measure of ejaculate quality (Cordero 1995). Acps could have spread either through the handicap principle or by Fisher's runaway selection theory (Cordero 1995; Eberhard and Cordero 1995; Cordero 1996; Cordero 1998). The handicap principle suggests that as Acps contain nitrates, a limiting resource in *D. melanogaster*, only the fittest males can produce these chemicals, and that therefore the chemicals were an honest signal of male fitness (Cordero 1995; Eberhard and Cordero 1995; Cordero 1996; Cordero 1998). Under the runaway selection model it is assumed that females who could discriminate would be able to tell if they had sufficient sperm within an ejaculate to fertilize a large number of eggs, therefore they would not have to waste time and energy remating or laying unfertilized eggs. If the trait and female preference for the trait were heritable, her offspring would also have the advantage and so the trait would spread (Cordero 1995; Eberhard and Cordero 1995; Cordero 1996; Cordero 1998).

There are many Acps predicted from the study of sequence data, these include: peptides/prohormone precursors, glycoproteins, modifying enzymes (proteases, protease inhibitors, lipases) and novel proteins (Wolfner 2002). Acps have an effect on many areas of female activity (for full review see Chapman 2001): The most studied Acp is Acp70A, or sex peptide. Sex peptide causes female unreceptivity to mating which lasts for 24-48 hours (Chapman et al. 2003). It also increases oviposition in females which lasts for a similar amount of time (Herndon and Wolfner 1995; Tracey Chapman et al. 2003; Liu and Kubli 2003). Another key accessory protein, Acp26Aa, also stimulates egg laying, but its action is earlier in the oviposition pathway (Herndon and Wolfner 1995; Heifetz et al. 2000). Acp36DE is primarily utilized in sperm storage, increasing efficiency of storage two fold (Qazi and Wolfner 2003).

A side effect of Acp action is toxicity leading to a reduction in longevity. Spermless males which only transfer Acps cause a serious reduction in female longevity (Chapman 1992; Chapman, Hutchings, and Partridge 1993; Chapman et al. 1995). Females mated with males who could not produce these main cell products live significantly longer than those mated with wild type males (Chapman et al. 1995). The conundrum regarding Acps is why males have such control over female mating and egg laying (Chapman 2001). As shown above, males, through the action of Acps, are able to manipulate female post mating behaviour to increase their reproductive success. This often occurs at the expense of what is considered optimal behaviour for the female. This level of control by males does not seem in a female's best interest.

It has been hypothesized that Acp toxicity occurs as an attempt to limit the number of rematings a female can undertake (Chapman 1992; Chapman, Hutchings, and Partridge 1993; Chapman et al. 1995). This follows the adaptive harm hypothesis which predicts that as males try to manipulate female behaviour, they will harm the female (Teuschl, Hosken, and Blanckenhorn 2007). Harm could be through injury, toxic compounds or disease transmission (Chapman 1992). Males are trying to make the female use more of their sperm in fertilization of the ovum. But this usually is by harming or damaging the female so much that they will

not re-mate (Teuschl, Hosken, and Blanckenhorn 2007). It is predicted that *D. melanogaster* Acp toxicity is designed to cause a female such harm that subsequent matings could prove fatal (Chapman 1992; Wigby and Chapman 2005). Harm could also be caused inadvertently: *D. melanogaster* males respond to SC by increasing their mating duration (Bretman, Fricke, and Chapman 2009; Bretman et al. 2010), which increases both male and females risk from predation. Although this has not been shown in *Drosophila*, female *Photinus collustrans* have altered mating behaviour in response to this risk: they have fewer, shorter matings to minimise chances of predation. This is not in a males' best interest so they try to prolong mating, thereby increasing the risk of predation (Wing 1988). Mating, therefore, poses a significant cost to both sexes, a cost which usually ends with an early death or loss of fecundity (Fowler and Partridge 1989).

The toxic effects of Acps are examples of reproductive costs. Costs of reproduction are seen when the act of mating negatively impacts on either sex (Fowler and Partridge 1989). The costs of mating, for the majority of species, are usually caused by gamete production (Smith 1958), courtship (Cordts and Partridge 1996) and injury or physical damage (Chapman 1992). The outcome of reproductive costs is usually a reduction in longevity and fertility (Partridge and Andrews 1985). Both males and females of many species have a reduced lifespan if they mate (Partridge et al. 1986; Partridge and Fowler 1990; Chapman et al. 1995; Prowse and Partridge 1997). A positive correlation exists between an early cessation of reproductive activity and life expectancy (Partridge and Andrews 1985).

Gamete production is the most obvious cost to mating. An individual ovum costs significantly more to produce than a single sperm. A study of *Drosophila subobscura* females highlights the high cost of ovum production. In this species, females that laid eggs have significantly lower longevity than females that could not lay eggs (Smith 1958). However, there is no correlation between increasing egg laying rates and decreasing longevity; rather if any egg laying occurs, a reduction in longevity will be seen (Partridge, Green, and Fowler 1987). This cost is

increased in species such as *D. melanogaster* where males can actively increase egg production rates by the female (Partridge, Green, and Fowler 1987; Wigby and Chapman 2005). However, the view that ovum production is far more costly than sperm production is misguided. We cannot compare a single ovum to a single sperm as an ejaculate contains millions of sperm and associated products, we therefore must consider the value of a whole ejaculate when considering the cost this poses to a male (Dewsbury 1982). Again, work on *Drosophila* species has demonstrated the cost of sperm production. Studies in a number of *Drosophila* species have shown that dry body mass is positively correlated with amount and length of sperm produced (Pitnick 1996). This means males, with more resources, can produce better quality ejaculates: if the production of sperm was energetically negligible, this would not be the case. The cost of ejaculate production in male *Drosophila* is not just in the production of sperm. Acps, as discussed above, significantly enhance a male's chance of achieving successful fertilization. However, Acps are made of rare nutrients and are therefore costly for the male to produce (Cordero 1995; Eberhard and Cordero 1995; Cordero 1996; Cordero 1998). This further increases the cost per ejaculate, again highlighting the error of considering the production of a single ovum to a single sperm (Dewsbury 1982).

The life-history theory hypothesizes that organisms are undergoing many trade-offs, such as between reproduction and longevity, and that these trade-offs are shaping the characteristics and timings of individuals behaviour through natural selection (Roff 1993). Sexual examples of trade-offs are extremely common. Pre-/post-copulatory mate guarding is aimed at increasing reproductive success, by either ensuring a male is the first to mate with a female or preventing the female re-mating (Parker 1974). The reproductive trade-off here is the benefits of being the first or only male to mate with a female out-weighing the costs of finding a new mate (Parker 1974). So long as the benefits outweigh the costs, mate guarding is the optimal strategy. This is directly relevant to this study as the increased mating time shown by *Drosophila* in response to SC (Bretman, Fricke, and Chapman 2009; Bretman et al. 2010), has been

proposed to be a form of mate guarding, and the effects of Acps on latency to mate, is also thought to be a form of chemical mate guarding (Herndon and Wolfner 1995; Chapman et al. 2003; Liu and Kubli 2003). Trade-offs evolving around mate guarding are not uncommon: in the amphipod *Gammarus lawrencianus* increased mate guarding leads to a reduction in feeding and therefore a reduction in size (Robinson and Doyle 1985). The trade-off represented here being between mate guarding, and body size. However, a mate guarding trade-off is not the only potential trade-off occurring in this use of plastic SC response. Often, trade-offs can arise between the costly production of ejaculates and other expensive traits. In the male field cricket, for instance, a trade-off between immunity and ejaculate production occurs: crickets that were immunologically challenged as juveniles had poorer ejaculate quality than males that were unchallenged (Simmons 2012). Alternatively, a trade-off between sperm quality and costly tissues can occur: echo-locating bats face a trade-off between brain size and sperm production (Hosken 1997; Lemaître et al. 2011). Additionally, the mounting of a plastic response itself could form the basis of a life-history trade-off. Plastic responses themselves are thought to be costly (reviewed DeWitt, Sih and Wilson 1998; Relyea 2002), indicating that a life history trade-off is inevitable whenever plasticity occurs. Potential trade-offs associated with plastic SC responses will form the main research of this study.

The literature presented here highlights fundamental deficiencies in our current understanding of plastic SC responses. This is surprising as SC research has been at the forefront of ecological research, from both a behavioural and an evolutionary standpoint, since its identification in 1970. In this thesis I describe a series of experiments investigating life-history trade-offs of plastic responses to SC in the model organism *Drosophila melanogaster.* This species is well studied in regards to SC, however it has not been utilized to its full potential to investigate possible life-history trade-offs within SC. First, I hypothesize that the plastic SC responses in *D. melanogaster* will require significantly more investment by the male, leading to increased reproductive costs. I propose these costs will form a trade-off between expressing the plastic SC response

and male longevity and suspect that males utilizing the plastic SC response will suffer decreased longevity as a result of the increased expenditure. Second, I predict that the use of these plastic SC responses by males, will also negatively impact upon female longevity. I hypothesize that the increase in toxic components and time spent mating will lead to a reduction in female longevity. Although there has been much research into how males alter ejaculate composition according to their perception of SC, particularly in *D. melanogaster*, as yet there is no measure of how this species alters sperm production in response to SC. Finally, I propose that males will increase both quality and quantity of sperm within their ejaculates in response to SC. By investigating the above, I aim to advance current understand of how SC responses impact upon both males and females alike.

Chapter 2

No Reduction in Longevity for Females Mated to Males Reared with Rivals.

2.1 Introduction

Sperm competition (SC) is a major evolutionary force shaping physiology, morphology and behaviour (Gage and Barnard 1996; Stockley et al. 1997). SC has been shown to affect organisms on the evolutionary scale (Harcourt et al. 1981; Harcourt, Purvis, and Liles 1995). However, SC can also affect organisms in the short term, plastic scale (Bretman et al. 2012).

There have been several recent studies providing insight into plastic SC responses in *Drosophila melanogaster*. It has been shown that when male *D. melanogster* are kept with rivals for 72 hours prior to mating, they mate significantly longer and transfer larger ejacuates than males reared in isolation (Bretman, Fricke, and Chapman 2009; Wigby et al. 2009; Bretman et al. 2010). This response is shown to be due to multiple redundant cues; any one of these cues alone cannot trigger the SC response but equally the loss of any one of these senses will not inhibit the SC response being implemented (Bretman, Gage, and Chapman 2011b). This high level of redundancy allows males to employ the optimal response for a variety of environments. It has also been shown that male *D. melanogaster* will alter the ratio and production of sperm related products, such as seminal fluid proteins (Sfps), in response to rivals in the premating environment (Fedorka, Winterhalter, and Ware 2011). These responses have been shown to increase both egg-laying rates of females (7% difference in means) and egg to adult survival (3% higher survival) when the male expressing the SC response is the fist male to mate (P1) (Bretman, Fricke, and Chapman 2009). Also, by increasing female latency to remate, males expressing this response achieve higher fertilization success, when mating in the P1 position (Bretman, Fricke, and Chapman 2009). Despite these advancements in our understanding, there are still many unanswered questions; such as what is the cost to a female mating to male expressing this response? And what is the costs to males themselves?

A cost of mating is when the act of mating negatively impacts on either sex, this can be by reducing fertility or longevity (Fowler and Partridge 1989). The most common costs associated with mating are due to gamete production (Smith 1958), courtship (Prowse and Partridge 1997) or mechanical damage (Chapman 1992). However, in species such as *D. melanogaster*, mating costs for females are imposed by male ejaculates. Ejaculates from this species contain accessory proteins (Acps): Acps cause changes within a female which increase a male's fertilization success (Chapman, Hutchings, and Partridge 1993; Chapman et al. 1995). However, Acp activity leads to increased ovulation rate (Herndon and Wolfner 1995; Heifetz et al. 2000) which likely poses increased costs for females. Furthermore, Acps are toxic: this has been shown multiple times in a variety of mutant studies, where females receiving a full dose of Acps had lower lifespan (median 16 days) than females receiving no Acps (24 days) (Chapman 1992; Chapman, Hutchings, and Partridge 1993; Chapman et al. 1995). This is further supported in that females mated to males which could not produce main cell products suffered no longevity cost (Chapman et al. 1995).

I propose that a reproductive trade-off will be occurring in females mating to males from high SC backgrounds. Males displaying the SC response mate significantly longer and transfer a larger ejaculate (Bretman, Fricke, and Chapman 2009; Wigby et al. 2009; Bretman et al. 2010), the main cell products of the ejaculate are also altered resulting in more Acps being transferred to the female (Wigby et al. 2009; Fedorka, Winterhalter, and Ware 2011). These Acps are highly toxic to females leading to a reduced longevity (Chapman 1992; Chapman, Hutchings, and Partridge 1993; Chapman et al. 1995). These responses to SC probably increase reproductive costs imposed on females. This may be as a byproduct of alterations to Acp levels intended to increase paternity, also increasing toxicity. Alternatively this may be a strategy to prevent females from remating by making the cost of mating so high that subsequent matings would prove fatal. Therefore, I predict that females mated to males from high SC backgrounds will have lower longevity than those females mated to males from a low SC background. I assayed the

longevity costs to females mated to males from high SC backgrounds and females mated to males from a low SC background. This was performed on *D. melanogaster* owing to its short life cycle, defined mating and courtship composed of several discrete steps and the relative ease to which the social environment can be manipulated.

2.2 Method

All flies were kept in a 25^0C 12hr L: 12hr D cycle controlled temperature room, on standard sugar yeast agar medium (medium recipe used throughout was 50g sugar, 25g yeast and 7.5g agar per 500ml distilled water; fungicides used were Nipagin and Bavistin). Experimental individuals were F1 generation of laboratory maintained Oregon-R females x Canton-S males. Flies were sexed under light CO_2 anesthesia up to six hours post eclosion.

Preliminary Experiment.

Prior to undertaking the female longevity experiment I ran a number of preliminary experiments to test the feasibility of such an experimental design. In both preliminary experiments, virgin females were kept in isolation for six to seven days post eclosion. Virgin males were separated into two treatments: either high SC (3M) treatment consisting of three males in a single breeding tube, or low SC (1M) treatment consisting of a single male. Males were maintained in treatment for seven days. In the first preliminary experiment males were removed on day seven and given a two hour opportunity to mate with a virgin female, mating duration records were kept. Immediately post-mating females were removed and placed in isolation in a 40ml breeding vial containing 7ml of standard sugar yeast agar medium, males were discarded. Females were moved to a fresh vial every seven days and checked twice a day until dead. 32 females were tested in this experiment, 16 mated to 1M males and 16 to 2M males. In the second preliminary experiment, I examined the rates of female rematings in this species. Females were given a six-hour window to mate with males. Immediately post-mating, or after the six-hour opportunity, females were placed in isolation for 48

hours. This was repeated so that each female had a six-hour mating window on five occasions spaced 48 hours apart. For each opportunity observations were made of the start and the end of mating and the mating duration was calculated. Males and females were then discarded.

In the first preliminary experiment, mating duration was significantly different between high SC males (3M; 18.56 ± 0.68 min) and low SC males (1M; 15.18 ± 0.58 min; GLM; n=16,16; F= 14.43; *P*<< 0.001). However no difference was detected in survival between females mated to high SC males (3M; mean survival 37.38 ± 2.09 days) and females mated to low SC males (1M; mean survival 35.25 ± 1.92; Coxph; n=16,16; Likelihood ratio test =1.94; *P*= 0.166).

In the second preliminary experiment I encountered great difficulty in achieving remating in females (table 1). The data presented in table 1 shows that despite a 48 hour period between matings 20% of females mated only once and no females mated on every occasion.

	Number of Matings				
	1	2	3	4	Total
3M	3	6	3	1	13
1M	3	9	6	0	18
Total	6	15	9	1	31

Table 1. Number of females mating for second preliminary experiment. 3M- high SC treatment, three males in single breeding vial. 1M-low SC treatment, single male in breeding vial.

The preliminary experiments are included as they helped shape the final outline of the female longevity experiment. These two experiments raised some problems that I designed solutions for in the later experiments, including those in this chapter and those in chapter 3. Firstly and most surprisingly was the finding that female remating was not as high as expected. Only 32% of females mated on three or more occasions (table 1). It is known that achieving remating in female *D. melanogaster* is difficult, however it has been used successfully in the

past (for example, Bretman, Fricke, and Chapman 2009), so I did not expect the remating rates shown here. However, my preliminary test made it clear that for any future experiments to work efficiently multiple mating was not an option. Secondly, the preliminary analysis highlighted that placing a female in isolation in a plentiful supply of nutrients resulted in no cost of mating being detected. This is probably due to any cost of mating being an instantaneous cost, and is supported by findings that early cessation of mating increases longevity (Partridge and Andrews 1985). As these females were living on average between 35-37 days, this instantaneous cost would not be detectable. It is also possible that there was a difference in survival, however as the time of deaths here were only to the nearest 6 hours, analyses were unable to detect it. The preliminary experiments led me to consider the immediate effect of a single mating on a female, and utilizing a *Drosophila* Activity monitor (DAM2, Trikinetics, Massachusetts USA) to accurately measure time of death. This apparatus records separately for each vial in a 32 vial set up every occasion when a fly crosses an infrared beam. Recordings can be taken anything up to every one second, thereby giving a much more accurate time of death than previously possible.

Female Longevity Experiment

There were three female treatments in this experiment, females mated to males from high SC backgrounds (2M), females mated to males from low SC backgrounds (1M) and unmated females (0M). High risk of SC males were two males placed in a single vial separated by a permeable divide (2M), low risk of SC males were a single male in a single vial on one side of a divide (1M; figure 1). Males were kept in these treatments until mating. Male ages ranged between six and eight days at the time of mating. Virgin females were placed in isolation in individual breeding vials immediately post sexing. Females were kept in isolation for seven days until mating. On day seven, a single female and a single male from either the high or low SC treatment were aspirated into 7 ml bijou tube containing standard sugar yeast medium, with supplementary yeast granules, and a male from the appropriate SC background. They were

given a two-hour window of opportunity to mate. Latency to mate was calculated, measured as time until onset of mating. Mating duration was measured as time from honest of mating until the pair was completely separated. Unmated females remained in isolation for 7 days until transfer to the *Drosophila* Activity monitor (DAM). Immediately post mating mated and unmated females were transferred to 5 mm glass tubes plugged with technical agar (Fluka Analytical, 1.5% agar-agar) and nonabsorbent cotton wool, and placed in a DAM which records separately for each vial every occasion when the female crosses an infrared beam. Data were collected every 5 seconds until all females had died. These were transformed into relative activity (number of activations per hour) and survival time, measured as number of hours in vials until final activation. Activity was measured so as to provide possible insight into any difference in survival rates detected. Males were discarded (figure 2). A total of 51-0M, 47-1M and 44-2M females were tested.

Statistical Analysis

Data was analyzed using R (version 2.13.1), survival data was analyzed using Cox's Proportional hazard through the R package Survival (version 2.36-12) and relative activity, mating duration and latency to mate were measured using linear models.

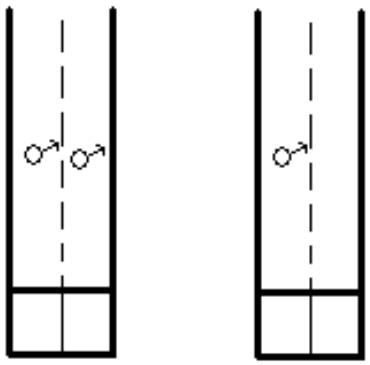

Figure 1. Male treatment vials. High SC background (left) two males separated by a permeable divide. Low SC background (right) single male on one side of a permeable divide. 40 ml tube containing 7 ml of standard medium, permeable divide is a rigid plastic sheet containing 21 holes above the medium, bunged with non-absorbent cotton wool.

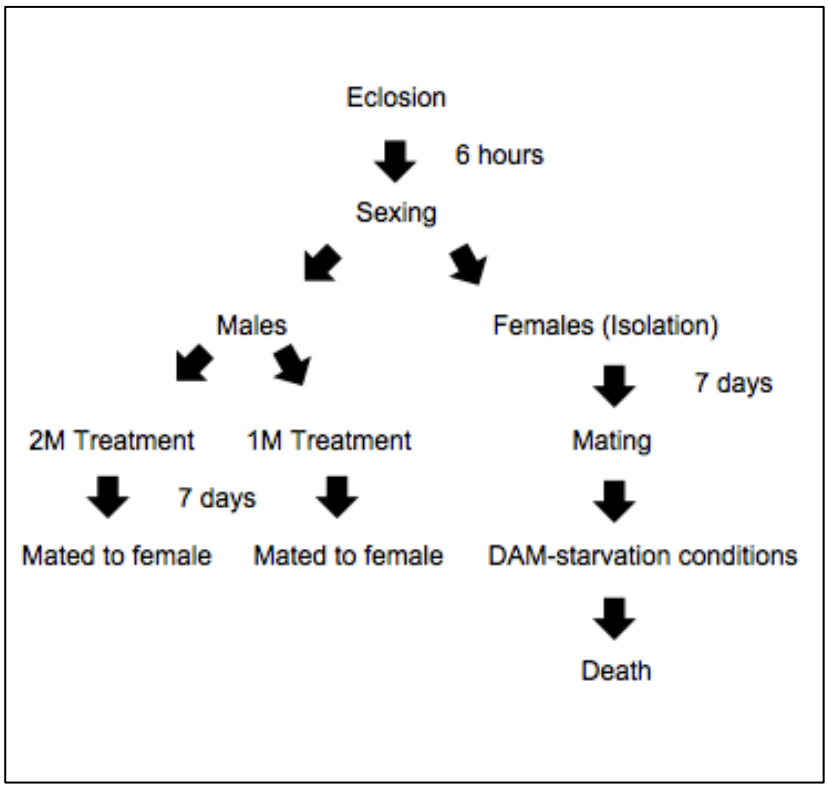

Figure 2. Practical outline for female longevity experiment. Effect on longevity of mating to males from a high SC environment. 2M= high SC environment, 1M= low SC environment, 0M= unmated, straight from isolation into DAM.

2.3 Results

Latency to Mate and Mating Duration.

Latency to mate between the high (2M, mean 12.56 ± s.e.1.73 minutes) and low (1M, 11.36 ± 1.69) SC groups did not differ significantly (GLM; n= 44 2M, 47 1M; $F_{1, 89}$= 0.246; *P*= 0.6208). However, mating duration was significantly longer in high SC (13.52 ± 0.23) than low (11.92 ± 0.34), (GLM; n=44 2M, 47 1M; $F_{1,89}$= 14.55; *P*<< 0.001).

Relative Activity.

Relative activity did not differ between any of the treatments (0M (n= 51) mean 65.12 ± s.e. 2.35 activations/hour; 1M (n= 47) 63.02 ± 2.93: 2M (n= 44) 64.76 ± 3.03). This was not significant between mated and unmated (figure 3; GLM; n= 51 unmated, 91 mated; $F_{1,140}$= 0.145; P= 0.704). It was also non-significant between 1M and 2M mated females (GLM; n= 44 2M, 47 1M; $F_{1,89}$= 0.169; *P*= 0.681).

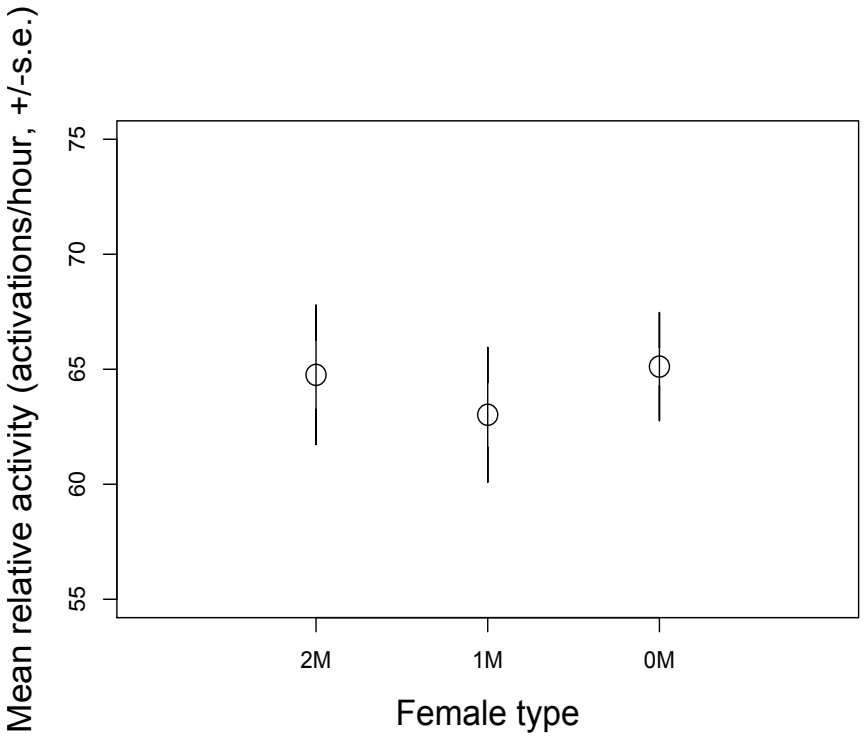

Figure 3. Mean relative activity (number of activation per hour). 0M= unmated females, 1M= females mated to low SC males, 2M= females mated to high SC males. Error bars are one standard error. Y axis from 55 activations/hour to 75 activations/hour.

Female Survival.

Mated females did not live significantly longer than unmated females (mated (n= 91) = 69.26 hours ± 1.80; unmated (n= 51)= 68.27± 3.20; figure 4; CoxPH; Likelihood ratio test= 0.18; *P*= 0.668; resulting in mated females having an 0.8% increase in survival hazard). A comparison of survival for mated females showed no significant difference between those mated to high SC males (2M; 68.75± 2.84 hours) and those mated to low SC males (1M; 69.74± 2.27; figure 4; CoxPH; n= 44, 47; Likelihood ratio test= 0.05, *P*= 0.819; resulting in a 4.8% reduction in survival hazard for 2M mated females)

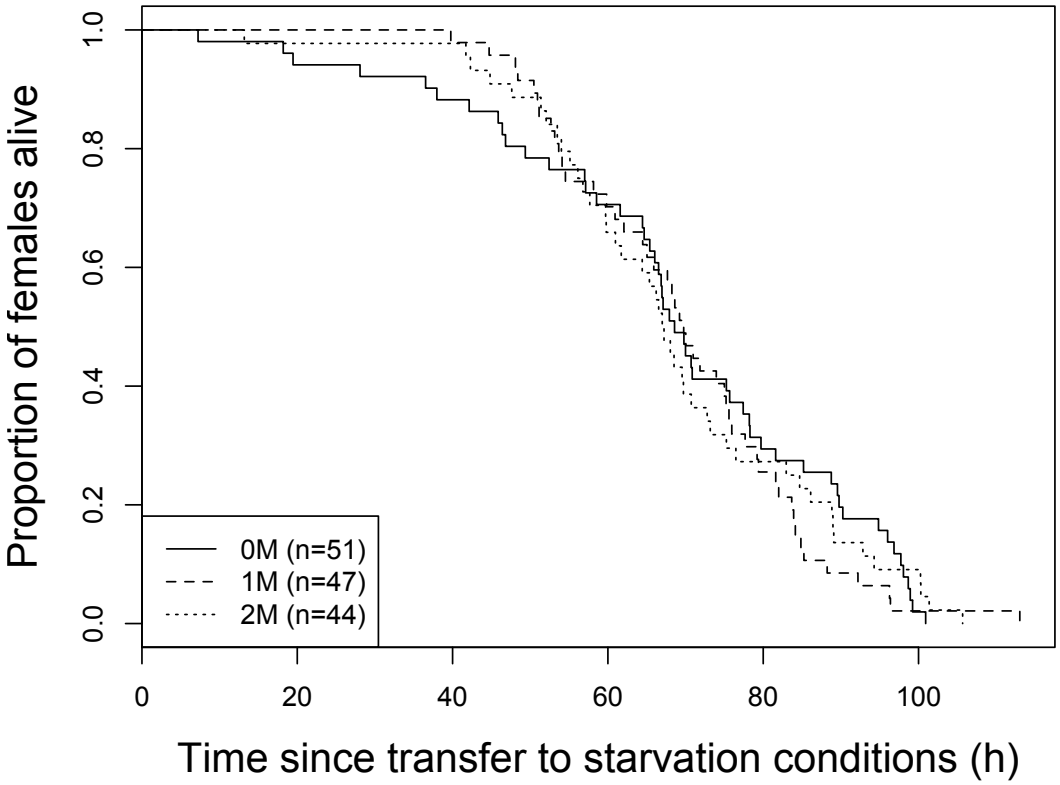

Figure 4. Survival plot for females in DAM. 0M= Unmated females, 1M= females mated to low SC males, 2M= females mated to high SC males. Created using the Survfit function of the survival package.

2.4 Discussion

Female Longevity Experiment

Utilizing the correct SC response is highly beneficial to males, perhaps explaining the value placed on SC detection by males and the plastic nature of the response (Bretman, Gage, and Chapman 2011b; Bretman et al. 2012). When mating in the P1 position, males expressing the plastic SC response receive significant advantage in both egg-laying rates of females and egg to adult survival over males not expressing the response (Bretman, Fricke, and Chapman 2009). It has also been shown that males expressing this response, when mating in the P1 position, can increase a females' latency to remate by 10 minutes, and this significantly increased the number of offspring they sire (Bretman, Fricke, and

Chapman 2009). This means males expressing plastic SC responses achieve much higher fertilization success.

This study set out to discover any potential longevity cost for females mating to males utilizing these plastic SC responses. Since males transfer a larger volume of Acps (Wigby et al. 2009) when expressing the plastic SC response, I hypothesized this would increase the toxicity of females mating to these males, thereby reducing their longevity. This could either have been a byproduct of males altering Acp levels to increase paternity, thereby increasing toxicity within the ejaculate. Alternatively it could have been a male stratagem to increase the cost of mating, therefore making it fatal for a females to remate. Furthermore, Acp action leads to increased ovulation (Herndon and Wolfner 1995; Heifetz et al. 2000), this probably leads to increased costs. The action of increased mating duration (Bretman, Fricke, and Chapman 2009) is also likely to increase female costs. However, no longevity cost was detected (figure 4). There are several possible explanations for this. First, there may be a longevity cost, which is only detectable with multiple matings. Secondly, there actually is no increased longevity cost of mating with a male from a high SC background. Finally, it is possible that there was a cost to females, which we failed to detect.

It has long been known that mating is costly to females (Partridge et al. 1986; Fowler and Partridge 1989; Prowse and Partridge 1997) and this cost is due primarily to the toxicity of Acps (Chapman 1992; Chapman, Hutchings, and Partridge 1993; Chapman et al. 1995). However, this decrease in longevity is increased through multiple matings. It is possible that, although males from high SC backgrounds are altering their Acp levels, this change only negatively impacts upon females after multiple matings. If this were the case, it would explain why no longevity decrease was detected here. The second preliminary experiment shows that obtaining high rates of female rematings is difficult, with only 32% of females mating on three or more occasions. With rates as low as this, obtaining enough replicates for longevity studies would be problematic.

It is also possible that there really is no longevity cost to females mating with males from a high SC background. At present there is still confusion as to how males alter their Acp production, we have conflicting evidence from two recent studies. One has shown that males from high SC backgrounds increase Acp transfer to females (Wigby et al. 2009). However, it has also been shown that males from high SC backgrounds downregulate Acp production (Fedorka, Winterhalter, and Ware 2011). This confusion is most likely caused by both studies failing to isolate SC risk and intensity: a high risk environment is one where the chance of encountering SC is high, for example the presence of a rival male, while a high intensity environment is one where there is a high number of competing ejaculates. In this case a single rival male represents a high risk low intensity environment (Engqvist and Reinhold 2005). Since risk and intensity have opposing responses (Hodgson and Hosken 2006) this is a problem. But if males are decreasing Acp production, then it is possible that females are suffering less toxicity from Acps, therefore it is likely that no longevity cost is imposed on females. However, if males are transferring more Acps, females mated to males from high SC backgrounds should suffer a decrease in longevity owing to the increased levels of toxic Acps. However, until we have clarification on how exactly male Acp levels are altered in response to high SC backgrounds, we will not be able to know whether this response is as expected, or is not.

Finally it is possible that there is a flaw in the design of this experiment. Previous work has suggested that when nutrients are limited and encounter rates are low, the cost of mating can be reduced or disappear entirely (Chapman and Partridge 1996). Another recent discovery has shown that the toxic effect of Acps is reduced and even completely negated with nutrient stress (Fricke, Bretman, and Chapman 2010). I placed females into nutrient lacking environments immediately post mating. It is possible that this has resulted in any Acp toxicity not affecting female longevity in any way. This is somewhat supported in that we failed to detect any longevity difference between mated and unmated females, which is a long known cost of mating (Partridge et al. 1986; Fowler and Partridge 1989; Chapman 1992; Chapman, Hutchings, and

Partridge 1993; Chapman et al. 1995; Prowse and Partridge 1997). Again however, carrying out this experiment another way is not practical. The preliminary experiments indicate that plentiful nutrient supply and only one mating event results in no detectable longevity difference between females meted to males from high SC backgrounds, and those mated to males from low SC backgrounds. This ties in with findings that early cessation of mating leads to a reduction in longevity costs of mating (Partridge and Andrews 1985). This is likely due to the cost of mating being caused by instantaneous costs, therefore if females are allowed to live for any length of time post mating, we would not detect any effect of mating.

This study has revealed there are some significant areas of our knowledge that further studies must address. Firstly, this experiment could be redesigned so as to remove the potential design flaw described above. By using an alternative means of stressing the females, such as temperature, we could potentially solve the confusion surrounding whether or not Acps are affecting females. This would give us more certainty in our findings. Secondly, it is clear that in order to properly understand our findings, we need clarification on how males alter their Acp levels in response to SC, the only way this would be possible is for SC risk and intensity to be looked at independently from one another. Until this is done, we will not know how this response will affect females mating to males from high SC backgrounds.

It is clear that with this study we cannot answer with certainty whether there are any longevity costs to females mating with males from high SC backgrounds. This could be because a single mating event is not sufficient to cause an increased cost to females when mating to high SC males: in previous longevity studies females were reared with groups of males and allowed to mate multiply. It is also possible that the use of starvation conditions represents a significant design flaw, as this negates the activity of Acp toxicity on females: previous longevity studies have used optimal conditions. However, it is also possible that there really is no increased cost to females mating to high SC males, yet owing to gaps in our present knowledge we cannot conclude this with any certainty.

Chapter 3

Virgin Males Live Longer when Reared with Rivals

3.1 Introduction

Plastic responses are fast changes which attune an individual to rapid environmental changes (Agrawal 2001; Bretman, Gage, and Chapman 2011a). It is likely that phenotypic plasticity developed to give an organism the highest fitness possible in an ever-changing environment (Agrawal 2001), as a single phenotype will not be the best in all situations (Via et al. 1995). Plastic SC responses in *Drosophila melanogaster* have been shown to alter mating duration (Bretman, Fricke, and Chapman 2009; Bretman et al. 2010; Bretman et al. 2012), accessory protein (Acp) gene expression (Fedorka, Winterhalter, and Ware 2011) and ejaculate size (Wigby et al. 2009). These responses can be triggered by a single rival's presence for 29 hours (Bretman et al. 2010) and are fully plastic, indicating that there must be a high cost for utilizing the wrong SC strategy (Bretman et al. 2012). These responses result in increased female oviposition rate and increased egg-adult survival (Bretman, Fricke, and Chapman 2009). However, the cost to the male expressing this response has never been fully explored.

D. melanogaster costs of mating begin before copulation, since courtship has been shown to be the most costly aspect of mating in this species (Cordts and Partridge 1996; Prowse and Partridge 1997; Wolfner 2002). Sperm production costs are often considered to be low, but the production of high numbers of sperm per ejaculate, and ejaculate components, do pose significant costs to males (Dewsbury 1982). Male *D. melanogaster* produce a cocktail of Acps which manipulate female behaviour such as egg laying rate (Herndon and Wolfner 1995; Chapman 2001; Wolfner 2002; Chapman et al. 2003), sperm storage (Neubaum and Wolfner 1999; Chapman 2001; Qazi and Wolfner 2003), release of oocytes (Heifetz et al. 2000) and female receptivity (Chapman 2001; Chapman et al. 2003). These Acps could have evolved as honest signals of male quality and therefore are costly to produce (Cordero 1995; Cordero 1996; Cordero C. 1998). It has been shown that in *D. melanogaster*, males who frequently mate, suffer from a reduction in

longevity (Prowse and Partridge 1997), likely a result of the high costs mating poses. Therefore the costs of mating can never be considered as negligible to a male.

Males often face reproductive trade-offs, for example the trade-off between sperm size and number (Immler et al. 2011). Time investment strategy indicates a trade-off between courting unreceptive females, and the searching time for finding a receptive female (Parker 1974). Field Crickets, *Teleogryllus oceanicus*, suffer a trade-off between sperm quality and immune function (Simmons 2012). *T. oceanicus* males that up regulate immune function do so at the expense of reproductive success (Simmons 2012). A trade-off between testis size and brain tissue is also present in some species (Pitnick, Jones, and Wilkinson 2006; Lemaître et al. 2009). However, a potential trade-off associated with mounting an SC response in *D. melanogaster* is as yet, unexplored. This represents a serious gap in our current understanding. I hypothesized that male *D. melanogaster* exhibiting the SC response will suffer a reduction in longevity in response to the cost of mounting the plastic SC response.

Using an experimental method, I examined the costs posed on male *D. melanogaster* who exhibit the above SC response. I performed the test using the model organism *D. melanogaster* owing to its regimented mating routine, fast generation time and the ease with which I could manipulate their social environment.

3.2 Method

Drosophila melanogaster stocks were maintained in 12:12 L:D cycle, 25^0C CT room on standard sugar yeast medium (see chapter 2). Experimental individuals were F1 generation obtained from Oregon-R (OR) females crossed with Canton-S (CS) males. F1 were collected up to six hours post eclosion, thereby ensuring virgin mating status. Females were kept for 5-10 days in vials containing approximately 20 virgin females of the same age. Males were kept in one of two treatments for seven days post eclosion. The high SC treatment (2M) contained two males isolated by a permeable divide; this allows for individual male identification but will still cause the SC response to be initiated (Bretman,

Gage, and Chapman 2011b). Keeping the males isolated from one another is vital as males are known to suffer a reduction in longevity when in physical contact with other males. Both males from the 2M treatment vials were used in experiments; therefore each male was coded to allow individual recognition. The low SC (1M) treatment was a single male kept on one side of a divide (figure 5).

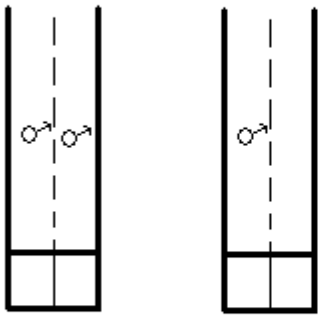

Figure 5. Male treatment vials. High SC background (left) two males separated by a permeable divide (2M). Low SC background (right) single male on one side of a permeable divide (1M). 40 ml tube containing 7 ml of standard medium (see chapter 2) permeable divide is a rigid plastic sheet containing 21 holes above the medium, bunged with non-absorbent cotton wool.

Longevity of Rearing in High SC Environment.

Males were left for 12 days in treatment vials, then placed into 5mm diameter glass vials, plugged at one end with technical agar (Fluka Analytical, 1.5% agar-agar) and non-absorbent cotton wool at the other. These vials were then placed in a *Drosophila* Activity Monitor (DAM; DAM2, Trikinetics, Massachusetts USA). This utilizes an infrared beam to record for each vial when the fly breaks the beam, thus enabling an accurate estimation of the time of death to be recorded. Males were left in these conditions until dead (figure 6). A total of 48 1M and 53 2M males were tested.

Experiment 2: Effect on Longevity of Mating for High SC males.

Matings were carried out in 5ml bijou tubes containing standard sugar-yeast medium with supplementary yeast granules in a 25^0C CT room. Males were offered 5-10 day old virgin females once a day for five consecutive days. Mating opportunities lasted two hours, or until end of copulation. During this time measurements were taken of, time to onset of mating and duration of mating. At end of mating opportunity, or end of copulation, the male was returned to the treatment vial until the next mating opportunity. At the conclusion of the fifth mating opportunity, males were moved into DAM (DAM2, Trikinetics, Massachusetts USA),

under the same conditions described above and left until dead (figure 7). During the experiment 53 1M and 48 2M males were tested.

Statistical Analysis

All results will be analysed using R v 2.13.1. Latency to mate was analysed using a general linear model, as was male activity measured as number of activations per hour. Male mating duration was analysed using a linear mixed effects model, with all terms of interest nested within individual males. Male survival was analysed using Cox's proportional hazard analysis.

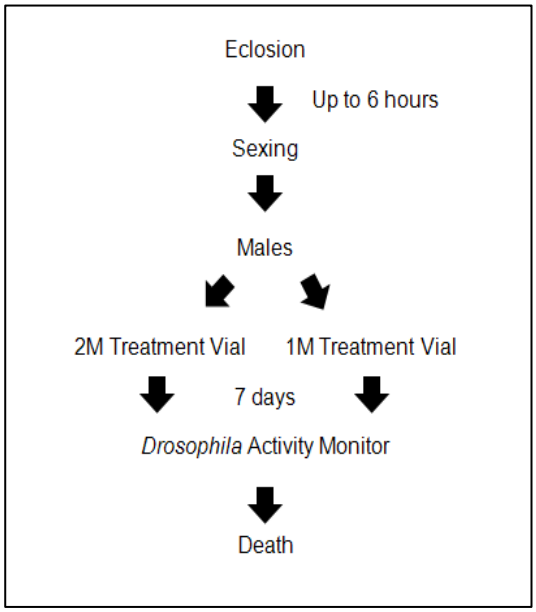

Figure 6. Practical outline experiment 1: Effect on longevity of rearing in high SC environment. High SC males (2M) two males separated by permeable divide, Low SC males (1M) one male on one side of permeable divide.

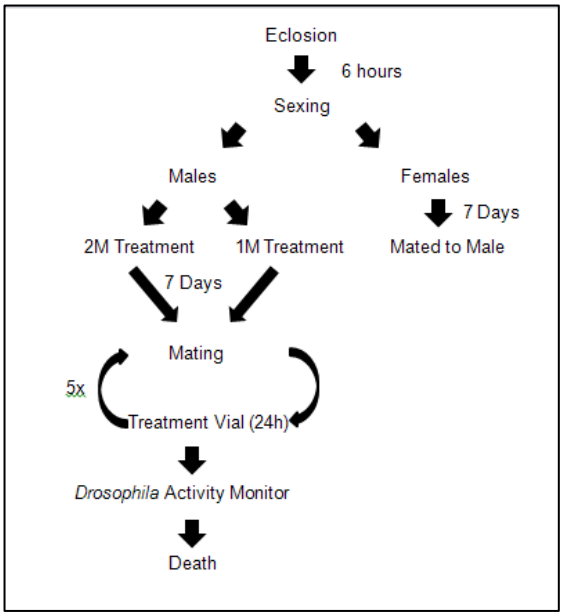

Figure 7. Practical outline of experiment 2: Effect on longevity of mating for high SC males. High SC males (2M) two males separated by permeable divide, Low SC males (1M) one male on one side of permeable divide.

3.3 Results

Male Mating Duration

As part of this analysis, mean latency to mate for the first mating event was analysed, but no difference was found between high and low SC males (GLM; $F_{1,101}$= 0.774, *P*= 0.381). Males from high SC (2M) backgrounds mated significantly longer than low SC (1M) males on each of the five mating occasions (figure 8; linear mixed effects model n= 49, 52; χ^2= 46.04; *P*<< 0.001), owing to the non-independence of this data, mating duration at each event was nested within individual male. There was a decline in mating duration over the five consecutive matings within treatments (SC treatment, linear mixed effects model n= 49; χ^2= 10.05; *P*= 0.0015; No SC treatment, linear mixed effects model, n= 52; χ^2= 20.72; *P*<< 0.001). However the difference in mating duration between 2M and 1M males did not decrease over the course of the five matings (linear mixed effects model, n=49, 52; χ^2= 0.054, *P*= 0.817).

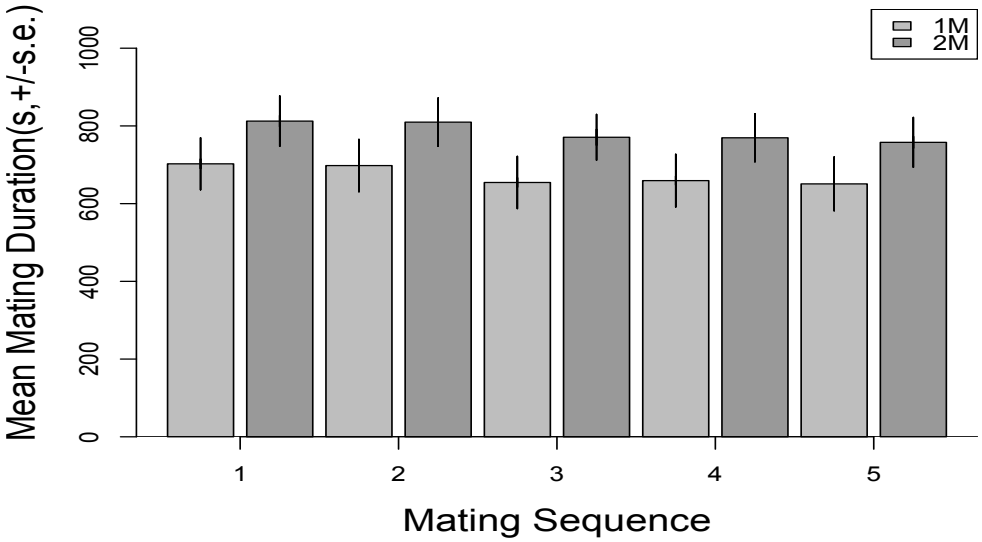

Figure 8. Mean mating duration for each mating event. High SC males (2M) two males separated by permeable divide, Low SC males (1M) one male on one side of permeable divide..1M events 1-5 n= 53, 53, 53, 52, 46. 2M events 1-5 n= 48, 48, 48, 44, 42. Error bars represent ± one standard error.

Male Activity

Overall, high SC (2M) males activated the sensor significantly fewer times than low SC (1M) males (general linear model $F_{1,198} = 5.44$, $p = 0.021$). However there was no similar result for analysis on mating status ($F_{1,198} = 0.78$, $p = 0.377$). Among the unmated group, greater activity caused a larger decline in longevity for 2M males than for 1M males (Cox proportional hazards model, sperm competition treatment x activity interaction term: $Z = 1.96$, $p = 0.05$).

Male Survival

Mated male longevity was significantly shorter than unmated male longevity (mated= 31.42 ± 0.67 h unmated= 33.47 ± 0.79 h; Cox Proportional Hazards model; survival hazard of mating: 38.22% increase; n= 101, 101; Likelihood ratio test= 5.11, *P*= 0.0236). Counter intuitively, among the unmated males, we found a striking difference: high SC (2M) males survived significantly longer (35.31 ± 1.26 h), than did low SC (1M) males (31.44 ± 0.85 h; figure 9: Cox Proportional Hazards Model; survival hazard associated with being high SC male: 49.4% reduction; n= 53, 48 respectively; Likelihood ratio test= 10.6; *P*= 0.00151). However, there was no significant difference in survival between high SC males and low SC males in the mated male treatment (Cox Proportional Hazards model; survival hazard associated with being high SC male: 4.7% reduction; n= 53, 48; Likelihood ratio test= 0.06; *P*= 0.812).

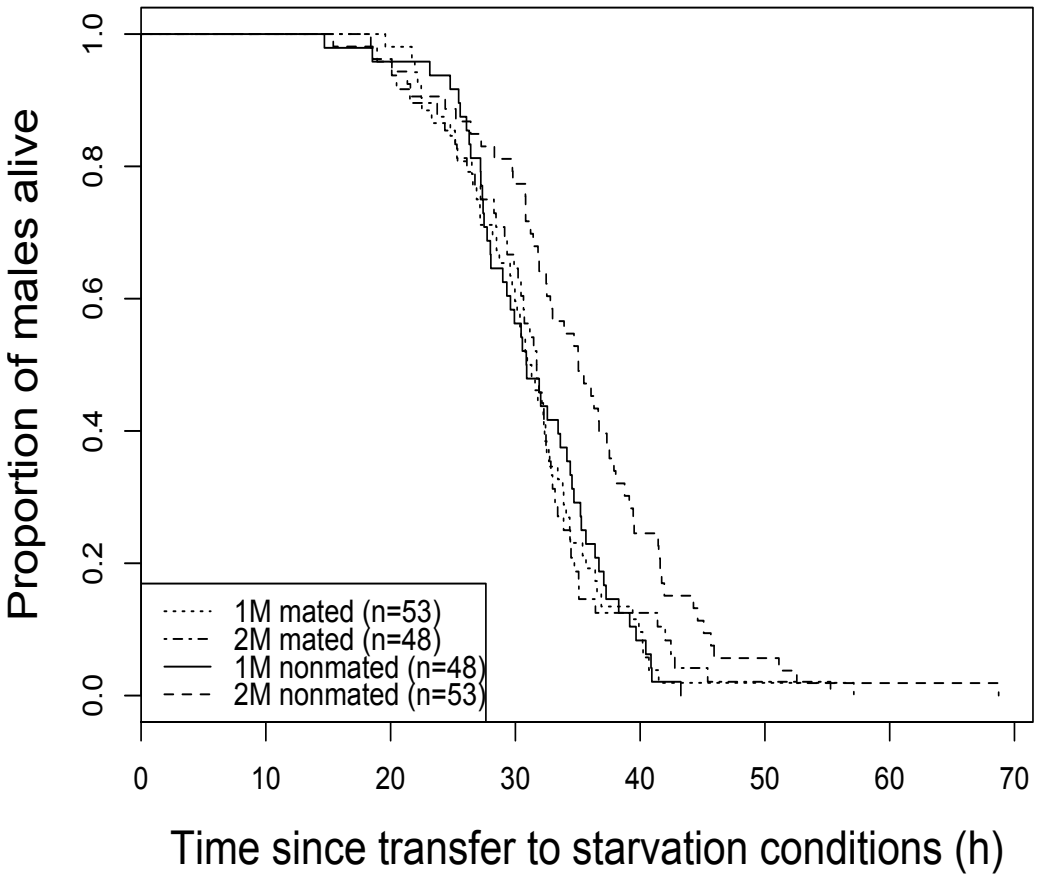

Figure 9. Male survival (hours) in starvation conditions. High SC males (2M) two males separated by permeable divide, Low SC males (1M) one male on one side of permeable divide.

3.4 Discussion

Mean Mating Duration Across Five Mating Events

In highly polyandrous species, such as *D. melanogaster*, the chance of encountering SC is high, thus the ability to express plastic SC responses. However previously it was never known if this was a one off response, or whether males could maintain this response even after matings. From this study we can conclude that males have the ability to maintain their plastic SC responses over subsequent matings. Mean mating duration was 2 minutes longer, averaged across all five mating events, in high SC (2M) males than low SC (1M) males. As we have shown (figure 8) at each mating event 2M males mated longer. Also we

have shown that, although mean mating duration for both high and low SC males decreases over subsequent matings, the magnitude of the difference in mating time remains remarkably fixed throughout the mating sequence.

Effects of Sperm Competition responses on Male Longevity

The main finding of this study is counterintuitive; unmated high SC males lived significantly longer (approximately 4 hours) than low SC males (figure 9). This finding is the opposite of what I would expect. Why unmated high SC males live longer than low SC is not easy to explain. We know that in response to SC intensity, males will downregulate Acp production (Fedorka, Winterhalter, and Ware 2011). It has also been suggested that males will reduce seminal fluid production in response to SC intensity (reviewed Hodgson and Hosken 2006). This could explain the result we have. Acps form a large proportion of ejaculates in male *D. melanogaster*; they are costly to produce (Cordero 1995; Cordero 1996; Cordero C. 1998). If males are downregulating Acp gene expression, it is likely they are suffering less Acp production costs. There are numerous possible explanations for this. Firstly, males could be reducing seminal fluid and Acps because, when mating in high SC environments, the chance of mating with a virgin female is low. Therefore there is going to be more seminal fluid of previous males in the female reproductive tract. Males thereby can reduce their own reproductive costs by investing less in seminal fluid and utilizing previous males' ejaculate components to their own advantage (reviewed Hodgson and Hosken 2006) and increased longevity is a by-product of this response. However, it could be that the increase in longevity is the main outcome of the response not a by-product. If SC is high, the chance of a male finding a virgin or being the last male to mate is small. Therefore it is advantageous for a male to live as long as possible to find the optimal mate. A high risk of SC could indicate a large population size, meaning encounter rate will be high. Therefore males can afford to be less active, increasing the lifespan, without suffering a reduced encounter rate. To increase longevity and therefore their overall searching time is advantageous to the male. Thus male responses to SC increase their

longevity to this end. However we cannot say for certain without further study.

My results indicate that there is no significant difference in survival between high and low SC mated males. However this does not necessarily indicate that high SC males are not facing increased costs. Mated males from high SC backgrounds mated significantly longer across all five matings (total increased mating time of 8.65 minutes). I propose this increase in mating duration completely negates the benefit of being a virgin high SC male discussed previously. Thus explaining why no longevity difference is detected between high and low SC males. However it is also possible that courtship and mating in this species, both of which are known to be costly (Partridge & Andrews 1985; Cordts & Partridge 1996), is so traumatic that any cost or benefit of being a high SC male has no overall impact on male longevity.

There is one final outcome of this study that must be discussed. My research suggests that males expressing plastic SC responses suffer no longevity cost when mating yet live significantly longer when remaining virgin. However previous work shows males expressing these responses achieve higher fertilization success (Bretman, Fricke, and Chapman 2009). This would suggest that constant expression of the plastic SC response would be the optimal strategy for a male to pursue. However this behaviour exists only as a plastic response to the presence of rival males. It is likely that some other cost or trade-off is occurring, such as with immunity (Simmons 2012) or other costly tissues (Pitnick, Jones, and Wilkinson 2006; Lemaître et al. 2009). At present this is an evolutionary conundrum.

This study has raised many questions that will shape future research. Firstly we must uncover why this behaviour is not the normal behaviour of *D. melanogaster*. It would seem like the optimal strategy for reproductive success, therefore there must be a substantial cost we have yet to discover. We know males downregulate Acps in response to intensity, but we do not know the response to risk and how this affects the ejaculate of males. A microarray should be performed on Acp gene expression comparing males from high and low SC backgrounds, to

finally answer how Acp production varies depending on SC background. Finally we still have much to uncover about the nature of the mating response. We have shown that with continuous stimuli, males will continue to express plastic SC responses over five subsequent matings. However it is not yet known how long the response lasts with the removal of the stimulus and for how many consecutive matings a male can continue to express the response without a break. These questions will undoubtedly form the cusp of much future research in SC.

This study, set out to uncover any longevity costs associated with the production, maintenance and effect of mating with a plastic SC response. I have shown that males from high SC backgrounds can express plastic SC responses for five consecutive matings. The key finding of this study is that high SC virgin males live significantly longer when remaining unmated. This is probably due to decreased Acp production costs or decreased toxicity owing to lower levels of Acps in the seminal fluid. Or through lower activity levels in high SC males. Also I have shown that males from high SC backgrounds must be suffering increased reproductive costs, as there is no detectable difference in longevity between high and low SC males. This indicates that the increased duration of copulation is sufficient to reduce high SC male survival to that of low SC males, thereby negating the longevity benefit of being a high SC male, seen in the virgin males. While providing useful information on the effect of expressing plastic SC responses, this study also raises one major question: why is this response not continually expressed in male *D. melanogaster*?

Chapter 4

Males Alter Sperm Production in Response to Rivals

4.1 Introduction

The "fair raffle" model of sperm competition (SC) predicts that as the risk of encountering SC increases, males should evolve increased sperm production rates and the number of sperm per ejaculate (Birkhead and Møller 1998). The empirical evidence for this is seen throughout nature. Number of sperm positively influences the outcome of SC in a number of fish species (Stockley et al. 1997; Stoltz and Neff 2006; Boschetto, Gasparini, and Pilastro 2011), crickets (Gage and Barnard 1996), passerine birds (Immler et al. 2011) and butterflies (Wedell and Cook 1999). Because measuring sperm volume is complicated, testes size is often used as a proxy for ejaculate investment: larger testes are assumed to allow the production of higher numbers of sperm per ejaculate. In one of the first studies to use this method, Harcourt et al (1981) showed that promiscuous primate species have consistently larger testes than monogamous species.

Although the affect of SC on primates, discussed above, is an example of an evolutionary response, SC can lead to changes within an individuals life time as well: these are plastic responses. In *Drosophila melanogaster*, it has been shown that with a high risk of SC, males will increase mating duration (Bretman, Fricke, and Chapman 2009), downregulate production of some Sfps (Fedorka, Winterhalter, and Ware 2011) and transfer an ejaculate containing a larger quantity of Sfps (Wigby et al. 2009). These responses increase a male's reproductive share when competition for fertilization occurs (Bretman, Fricke, and Chapman 2009). However little is known about the numbers of sperm transferred by male *D. melanogaster* who experience a high risk of SC. Previous papers have focused on Sfp adjustment alone rather than investigating how SC risk impacts upon sperm quantity. Recent work has looked at the mechanisms of SC in *D. melanogaster* to explain the last male to mate precedence seen in this species. It has been shown that sperm from the first male to mate (P1) is retained for a short time, when a female remates (Civetta 1999). However, the male mating second (P2) is

able to influence the outcome of SC within the female reproductive tract. Males mating in the P2 position are able to displace a previous male's sperm (Civetta 1999; Manier et al. 2010) thereby reducing the previous male's paternity share. It has been hypothesized that male *D. melanogaster* ejaculate many more sperm than can be stored by the female for this reason, and that this is a response to SC risk that is driving the evolution of ever larger ejaculates (Manier et al. 2010). A study of a number of *Drosophila* species has shown that the main evolutionary SC response in this genus is to increase sperm size (Immler et al. 2011). Larger sperm size is often seen in smaller species as a method of displacing rival males sperm (Wigby and Chapman 2004), but is also seen in larger mammals such as primates (Gomendio and Roldan 1991). Furthermore, ejaculate characteristics are often limited by the size of the testes. Testes can hold a set volume of ejaculate, increasing sperm size may lead to a reduction in sperm number and vice versa: species therefore often face a trade-off between sperm size and number (Immler et al. 2011).

As well as increasing number of sperm and altering sperm size, it is expected that males will also increase sperm quality in response to SC (Wigby and Chapman 2004). Ejaculate quality plays in important role in fertility success. The influence of sperm quality on the outcome of competitive fertilizations has been shown many times (reviewed Snook 2005) and can be measured in many ways such as mobility (Birkhead et al. 1999), velocity (Boschetto, Gasparini, and Pilastro 2011) and longevity (Snook 2005). But often these traits influence one another: longer sperm tend to be more mobile and longer-lived. Ejaculates of high quality are costly to produce, often forming trade-offs with other expensive characteristics such as immunity (Simmons 2012). However little is known about how a high risk of SC will affect male *D. melanogaster* ejaculate quality.

Our understanding into the effect of SC on ejaculate composition in *D. melanogaster* is far from complete. The most recent evidence indicates that males downregulate production of key Sfps in response to SC (Fedorka, Winterhalter, and Ware 2011). However, this seems to

conflict with previous findings that males transfer an ejaculate containing higher levels of Sex Peptide and Ovulin, two important Sfps (Wigby et al. 2009). This confusion is unsurprising, as in both studies SC risk and SC intensity are confounded. A high SC risk environment is one where the chance of encountering SC is high: this could be detected through the presence a rival male or the encountering of many non-virgin females (Engqvist and Reinhold 2005). A high intensity environment is one where there is a high number of competing ejaculates (Engqvist and Reinhold 2005). While facing high a risk of SC males should increase ejaculate investment. However when facing a high intensity of SC the opposite effect is predicted: it is hypothesized that males will decrease investment in ejaculate as this provides no advantage to a male when competing in a high intensity SC environment (Parker et al. 1996). It has also been suggested that by decreasing ejaculate expenditure in response to SC intensity, males will utilize a previous males' Sfps to increase the survival of their sperm in the female reproductive tract (Hodgson and Hosken 2006). The confounding of these two principles is an obvious limitation in both experimental designs as it is evident that they ellicit opposing responses in males. It is clear therefore that we must attempt to answer how risk and intensity influence ejaculate composition in isolation from each other in this species before we can wholly understand how males react to each.

I assayed male *Drosophila* sperm production in high and low risk SC environments. Sperm quality was quantified by using the proportion of live and dead sperm, and I also quantified the total number of sperm produced. I predicted that males would strategically increase both sperm number and sperm quality in accordance with current SC risk theory (Hodgson and Hosken 2006).

4.2 Method

Drosophila melanogaster were reared on standard sugar yeast agar medium (see chapter 2), in 12hr:12hr L:D cycle, 25^0C controlled temperature room. All experimental males were Oregon-R x Canton-S F1 generation. F1 Males were collected within 6 hours of eclosion, using light CO_2 anaesthesia, and placed immediately in one of two treatments: the

high SC treatment (2M) contained two males separated by a permeable divide; and low SC treatment (1M) containing one male on one side of a permeable divide (figure 10). A total of 20 males for each treatment were tested. Males were then left in treatment vials for 7 days. After 7 days, males were killed using ether exposure and both testes dissected out in ice cold PBS buffer solution. Testes were transferred to staining solution (per 1ml- 870µl PBS, 120µl Hoechst, 10µl Propidium Iodine (PI)) on a microscope slide. Hoechst stains live sperm blue and PI stains dead sperm red. While in sperm staining solution, the sperm was teased out of each of the testes. The maximum quantity of testis tissue possible was then removed and a cover slip placed on the microscope slide and left to incubate for 15 minutes. In some cases not all the testes tissue was removed as this would have removed some of the sperm also.

Sperm were visualised using an upright microscope (Zeiss model 510 Meta -confocal 3). Owing to the difficulty in getting random dispersal of *Drosophila* sperm, 2 images were taken for each slide using the 20x optic. The images were taken of the two areas with the highest sperm density. Although this method is not optimal it has been used successfully in the past to control for the difficulties in getting *Drosphila* sperm randomly dispersed (Snook and Hosken 2004). Images were analysed using Zeiss software (ZEN Lite 2011), they were enhanced using the sharpen tool and saved as separate images of live and dead sperm for each region of intensity.

To eliminate observer bias during sperm counting, all images were given a randomly assigned numerical code, then randomly recoded and re-ordered by an independent observer blind to the treatment type (figure 11). These coded images were counted using a 6 x 6 cell grid superimposed over the image, with each cell being counted individually, then all cells combined for the final total count. The number of individual live and dead sperm were counted for each image. Also the coverage for each image was counted, this was measured as number of grid squares containing sperm. In some cases grid squares were partially or wholly covered in testis tissue thereby obscuring some or all of the sperm contained within that square. Grid squares that were over 50% obscured

were marked as uncountable and noted for each image. All sperm present in each image were counted, not just sperm with elongated heads. I tested the repeatability of the sperm quantification method by re-counting the live, dead, and total sperm numbers for a random sub-set of 10 images 2 months after the original recount. First and second counts were highly and significantly correlated (Spearman's Correlation; Total sperm number; R= 0.92, $P << 0.001$; Proportion of live sperm; R= 0.98, $P << 0.001$).

All analyses were carried out using R v 2.13.2. Total sperm number and proportion of live sperm were analysed using a general linear model. Proportion of live sperm were arcsine square-root transformed and in both analyses terms of interest were nested within individual male.

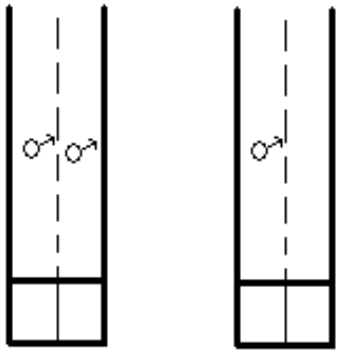

Figure 10. Male treatment vials. High SC background (left) two males separated by a permeable divide (2M). Low SC background (right) single male on one side of a permeable divide (1M). 40 ml tube containing 7 ml of standard medium permeable divide is a rigid plastic sheet containing 21 holes above the medium, bunged with non-absorbent cotton wool.

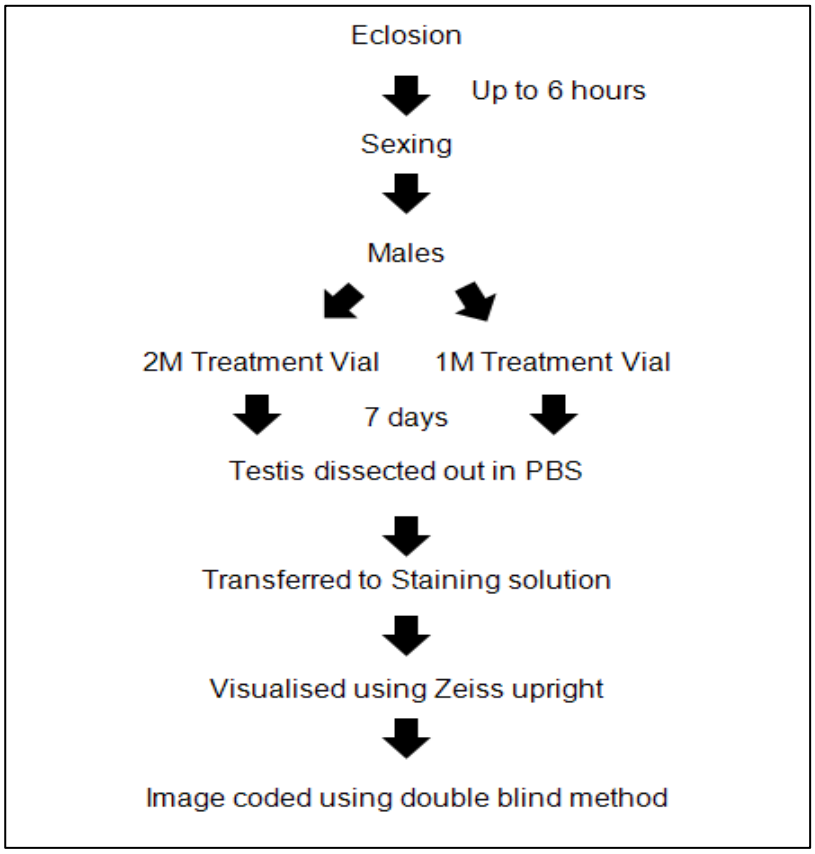

Figure 11. Experimental schedule for sperm staining experiment. Staining solution contains: 870µl PBS, 120µl Hoechst, 10µl Propidium Iodine (PI) per 1ml. Hoechst stains live cells blue. PI stains dead cells red.

4.3 Results

Total Sperm Number

High SC (2M) males produced more sperm (mean number of individual sperm per male 251.83 ± 23.65) than low SC (1M) males (198.83 ± 19.78), which was significantly different when the number of sperm containing squares was included as a covariate (figure 12; General Linear Model; n= 20,20; $F_{1, 38}$= 5.72; *P*= 0.022). This is justified as total sperm number is highly correlated with number of sperm containing squares (Spearman's Rank; *P*<< 0.001).

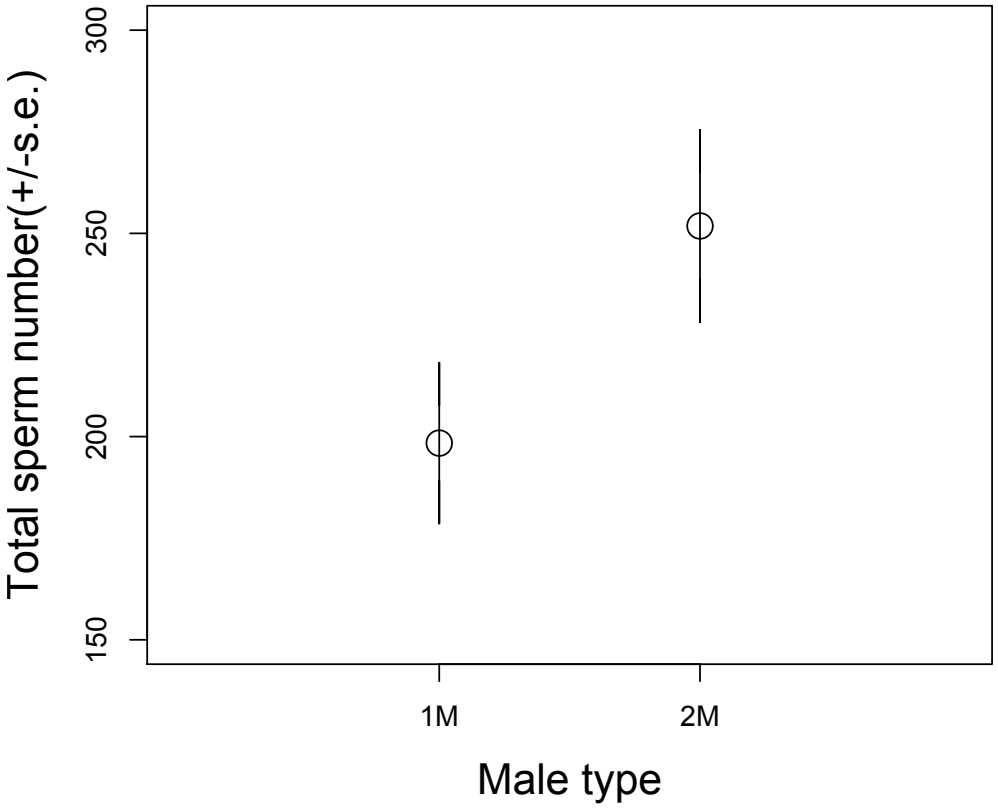

Figure 12. Mean number of total sperm per image, two images per individual. High SC males (2M) two males separated by permeable divide, low SC males (1M) 1 male on one side of a permeable divide. Error bars= ±1 standard error. X axis scale 150 – 300 sperm.

Proportion of Live Sperm

In high SC (2M) males there was a higher proportion of live sperm (median 0.763, range 0.482-0.879) than in low SC (1M) males (0.612, 0.234-0.967). This is strongly significant (figure 13; General Linear Model type/male/number of sperm squares with total sperm included as a covariate; n= 20,20: $F_{1, 38}$= 21.78, *P*<< 0.001).

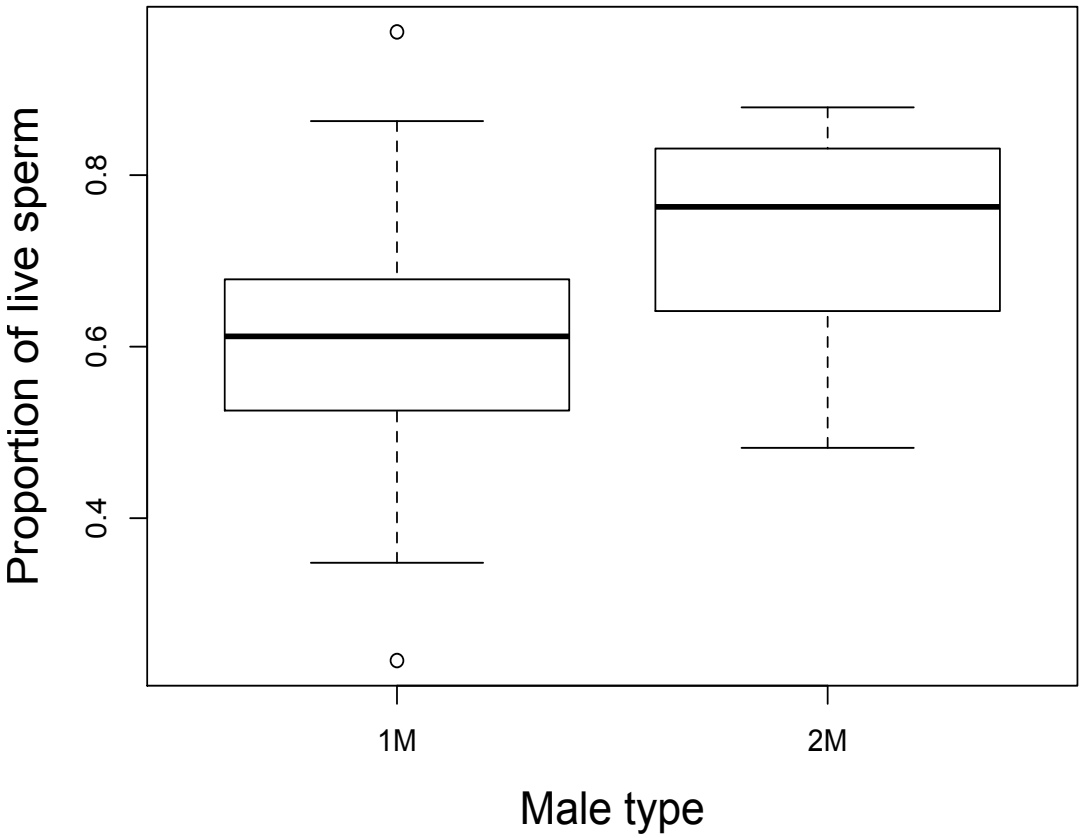

Figure 13. Proportion of live sperm per image, two images per male. High SC males (2M) two males separated by a permeable divide; low SC males (1M) 1 male on one side of a permeable divide.

4.4 Discussion

The key finding of this study is that males from high risk SC environments increase both sperm number and sperm quality, measured as proportion of live sperm, which fits with current theory of response to SC risk. The inclusion of coverage makes total number between the two treatments highly significant. This result fits with the growing evidence that SC often conforms to the "fair raffle" model (Parker 1990) and that increasing sperm number is a main response to risk of encountering SC (reviewed Wedell *et al.* 2002).

Low SC environment males produce far more sperm per ejaculate than can be effectively stored by females (Manier et al. 2010). Therefore the increase in sperm number seen in high SC males cannot simply be due to the "fair raffle" model. It is most likely due to male sperm displacement in this species. It has been shown in *D.* melanogaster that a male's sperm will be displaced from the sperm storage organ by subsequent mating males (Civetta 1999). This response of increased number of sperm is likely to be a response to this. By increasing sperm number, and transferring larger ejaculate (Garbaczewska, Billeter and Lavine 2013), males will be able to displace a greater amount of previous males' sperm. It will also increase the likelihood and volume of a male's sperm remaining if the female's storage organ after subsequent matings.

Sperm quality is often seen to increase in response to SC (reviewed Snook 2005): however, this has never been shown in the model organism *D. melanogaster*, despite its frequent use in SC research: this is probably due to the difficulty in extracting and analyzing the sperm bundle in this species. Total sperm number is highly correlated with proportion of live sperm. This is perhaps caused by males attempting to produce the maximum number of high quality sperm in response to SC, which would also result in an overall increase in total number of sperm produced. This improvement in sperm quality has been measured as mobility (Birkhead et al. 1999), velocity (Boschetto, Gasparini, and Pilastro 2011) and longevity (Snook 2005), all of which improve a male's competitive ability in respect to sperm competition. By transferring a larger ejaculate containing a higher proportion of live sperm, a male is

likely to drastically increase its chances of achieving higher fertilization success.

Previous studies have shown that high SC males achieve higher fertilization success regardless of mating order (Bretman *et al.* 2009). Our findings show that males from a high risk SC environment will transfer a larger ejaculate containing more live sperm. If this male is mating in the P2 position, this increased size of ejaculate will displace more of any previous male's sperm and mean a higher proportion of live sperm in the storage organ, thus leading to the higher P2 paternity share seen (Bretman, Fricke, and Chapman 2009). If the high SC male is mating in the P1 position, the larger ejaculate and higher number of live sperm in the storage organ results in a larger number of viable sperm remaining in the storage organ after displacement by a rival male has occurred, thereby explaining the higher P1 paternity share observed previously (Bretman, Fricke, and Chapman 2009).

One area needing more research is the question of whether this is truly a response to perceived risk of SC. The current findings fit with SC theory; the presence of rival males should lead to an increase in sperm production and quality. However the response observed here could equally be a response to density. The presence of an individual prior to mating would indicate high density, regardless of whether this was a male or female. High density would mean more females and therefore more mating opportunities. This itself could lead to an increase in sperm production. An interesting line of future research would be to test whether any of the responses previously seen can be triggered by the presence of females rather than a male.

This study set out to identify how male *D. melanogaster* alter ejaculate characteristic in response to SC intensity. I successfully showed that males from high SC environments increase total number of sperm as well as increasing sperm quality in terms of number of live sperm within the ejaculate. This is the first time such a study has been carried out on this species, perhaps owing to the difficulty of teasing out the *D. melanogaster* sperm bundle. Also I successfully studied SC risk in isolation of intensity, something that has not been achieved previously

when considering male ejaculates in this species. But there is still the question of why males would downregulate production of Acps yet transfer a larger ejaculate containing more Acps. Future research into the potential for this being a density dependent response is also needed so that our understanding can be complete.

Chapter 5

General Discussion

Since its conception, sperm competition (SC) has been an area of research at the forefront of evolution and behavioural ecology. SC has profound impacts on male morphology, physiology and behaviour over both evolutionary and the plastic time scales, for example, the first widely considered mechanism for SC success is through increasing ejaculate volume. In support of this prediction it has been noted that promiscuous primate species have larger testes than monogamous species (Harcourt et al. 1981; Harcourt, Purvis, and Liles 1995). This is thought to have evolved because promiscuous species are more likely to encounter SC. Therefore by having larger testes a male can produce a larger ejaculate, providing a higher chance of achieving fertilization. This has been supported by studies of numerous other species. A common morphological response to SC seen across many taxa is increasing sperm size and alterations to sperm morphology (Stockley et al. 1997), although this has many different outcomes. One of the more extreme responses to SC is males physically damaging the female to reduce remating rates: in primate species where males have penile spines, females have relatively short periods of sexual receptivity (Stockley 2002). It is hypothesized that these spines developed to deliberately damage females, thereby preventing them from remating.

One of the main model organisms for the study of SC responses has been *Drosophila melanogaster*. In this species males exhibit a number of evolutionary responses to SC such as: large ejaculates, sperm displacement and the use of seminal fluid proteins (Manier et al. 2010; Civetta 1999; reviewed in Chapman 2001). Male *D. melanogaster* transfer an ejaculate with many more sperm than can be stored by the female (Manier et al. 2010). This not only increases a males fertilization success, but also displaces any previous male's sperm (Civetta 1999). Male *D. melanogaster* ejaculates contain a cocktail of seminal fluid proteins, called accessory proteins (Acps), which manipulate female behaviour (reviewed in Chapman 2001) including: egg laying rate

(Herndon and Wolfner 1995; Chapman 2001; Wolfner 2002; Chapman et al. 2003), sperm storage (Neubaum and Wolfner 1999; Chapman 2001; Qazi and Wolfner 2003), release of oocytes (Heifetz et al. 2000) and female receptivity (Chapman 2001; Chapman et al. 2003). These Acps are toxic which significantly reduces female longevity (Chapman 1992; Chapman, Hutchings, and Partridge 1993; Chapman et al. 1995): this is believed to be an attempt to manipulate female remating behaviour.

Drosophila melanogaster males have also been shown to react plastically to the presence of one or more rival males (Bretman, Fricke, and Chapman 2009). If a *D. melanogaster* male is exposed to a rival for a minimum of 29 hours (Bretman et al. 2010) they will mate for longer (Bretman, Fricke, and Chapman 2009), downregulate Acp production (Fedorka, Winterhalter, and Ware 2011) and transfer an ejaculate containing more of two key Acps: sex peptide and ovulin (Wigby et al. 2009). These responses increase a male's fertilization success, leading to a higher paternity share (Bretman, Fricke, and Chapman 2009). The work presented in chapter four shows that males also increase both sperm quality and quantity in response to rivals. Increasing sperm quantity fits with the "fair raffle" model of SC (Birkhead and Møller 1998), which predicts that as risk of encountering SC increases males should increase their number of sperm per ejaculate. Increasing sperm quality is a response seen numerous times in other species with numerous adaptations such as: sperm mobility in domestic fowl (Birkhead et al. 1999), sperm velocity in the guppy, *Poecilia reticulata* (Boschetto, Gasparini, and Pilastro 2011) and longevity (reviewed Snook 2005). Although both these findings have been seen many times, they have never been shown in *D. melanogaster*, likely owing to the difficulty in extracting and teasing apart the densely packed sperm. My finding also explains the increased paternity share previously shown (Bretman, Fricke, and Chapman 2009): if a male is ejaculating a higher quantity of sperm with more live sperm per ejaculate, the fair raffle principle predicts that they will achieve higher fertilization success and paternity.

A key area of research for any evolutionary or behavioural response are life-history trade-offs which are thought to be shaping

organisms characteristics and behaviour through natural selection (Roff 1993). In the field of SC trade-offs are common, occurring between sperm size and sperm number (Immler et al. 2011), immunity and sperm quality, (*Teleogryllus oceanicus*: Simmons 2012) and sperm production and costly tissues such as the brain (bats and other mammals: Hosken 1997; Lemaître et al. 2009). The key to understanding any trade-off is the costs of the behaviour. Mating in *Drosophila* is well known to be costly to both males (Cordts and Partridge 1996; Prowse and Partridge 1997) and females (Chapman 1992; Chapman, Hutchings, and Partridge 1993: 1993; Chapman et al. 1995). Despite this understanding, prior to this study, little work had been carried out to investigate the costs of plastic SC responses in *D. melanogaster* or any potential trade-offs occurring.

The findings presented in chapter three are surprising. They show that far from being costly, when remaining virgin, plastic SC responses are of benefit to the male resulting in a 12% increase in survival time, equating to a 49% reduction in hourly hazard of death. But when mating occurs this benefit is completely negated. The increase in survival can be partly explained by a reduction in activity by males. However, there are a number of other possibilities such as the known down-regulation of Acp production (Fedorka, Winterhalter, and Ware 2011). Acps are well known to be costly to produce for males (Cordero 1995; Eberhard and Cordero 1995; Cordero 1996; Cordero C. 1998), therefore a downregulation of Acp production is likely to lead to a decrease in male costs and subsequently to an increase in male longevity. Alternatively it is possible that this increased longevity is not a response to SC, but to a response to perceived changes in density. If there is a high population density, the chances of encountering a female are higher. It could be possible that the reduction in activity leading to an increase in longevity is merely a response to this: males reduce activity as they have a higher chance of encountering a female. Higher density would also result in higher risk and intensity of SC, resulting in the observed SC responses. It is clear however, that much more work is needed in this area.

The study presented in chapter two revealed no increased longevity cost to females mating to males from a high SC background.

This is possibly due to the increased cost of mating to a high SC male being insignificant for one mating event. If the females mated more than once it is more likely that a cost would be uncovered. However, as shown in my preliminary experiments, achieving rematings in a short period in female *D. melanogaster* is far from simple. It is possible that the use of a nutrient limited environment reduced the impact of Acp toxicity on females (Fricke, Bretman, and Chapman 2010). Support for this comes from my failure to identify the known reduction in longevity that mating has on females (shown in: Chapman 1992; Chapman, Hutchings, and Partridge 1993; Chapman et al. 1995).

The work presented here has furthered our understanding of plastic SC responses and raised some interesting questions that will shape the direction of future research. *D. melanogaster* is a species with large sperm: a common trade-off is between sperm size and number (Immler et al. 2011). An interesting line of future research would be to examine how the increased number of sperm, shown in chapter four, affects the size of sperm in this species. It is possible that as sperm number is increased the size of the sperm decreases. It would also be interesting to examine if any other changes to sperm morphology occur, such as motility. Much future work needs to examine the costs to females mating to males from high SC backgrounds. A mutant strain of *Drosophila*, such as the Sex Peptide Receptor knockdown strain, could be used. This strain is deficient in receptors to a key Acp responsible for increasing female latency to remate (Chapman 2001; Chapman et al. 2003). By using a strain like this, multiple matings should be achievable. This would show how multiple matings to high SC males affect the longevity of females. Alternatively, the experiment described in chapter two could be adapted with an alternate method of stressing the females, such as temperature. This would rule out any chance of the nutritional status of the female having an impact. The work of chapter three raises the most interesting question, males expressing plastic SC responses receive no reduction in longevity when mating, have increased longevity when remaining virgin and receive higher numbers of offspring. Why is this behaviour only a plastic response to rivals and not normal behaviour?

It seems the optimal behaviour for a male to utilize. Future research must examine this puzzling conundrum. It is possible that some other cost or trade-off is occurring, such as reduced investment in costly tissues (Hosken 1997; Lemaître et al. 2009) or immunity (Simmons 2012). If so, this would explain why males do not pursue this behaviour all the time. This work raises another interesting possibility. Are these responses really SC responses? As discussed above, the responses discovered here could actually be responses to density rather than rival males presence. An interesting experiment would be to place a male in a vial with a female on the other side of a divide. Then assess if this triggers any of the observed responses. Finally, to assess the impact of Acp downregulation on male longevity, the experiment described in chapter three could be repeated using males which cannot produce Acps. Males unable to produce Acps have been used successfully in the past (Chapman et al. 1995), and this would show if any increase in male longevity (shown in chapter three) is due to the downregulation of Acps.

The work presented here has tackled some of the bigger questions in the field of SC. The finding that males increase sperm quality and quantity has linked a number of previous findings, explaining why males receive higher fertilization success, as well as fitting with theoretical models. The study in chapter two shows that females mating to males from high SC backgrounds suffer no increased longevity cost when only mating once. Finally, by identifying a potential benefit for males expressing plastic SC responses and showing no costs, I have raised the question: why is this behaviour only a plastic response? This question is likely to lead to a number of studies looking at any other costs for these males and could change how we view the costs of expressing plastic SC responses.

Reference List

In the style of the journal *Behavioural Ecology*.

Agrawal AA. 2001. Phenotypic plasticity in the interactions and evolution of species. Science 294:321 –326.

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

Birkhead TR, Martinez JG, Burke T, Froman DP. 1999. Sperm mobility determines the outcome of sperm competition in the domestic fowl. Proceedings of the Royal Society B: Biological Sciences 266:1759 –1764.
Birkhead TR, Møller AP. 1998a. Sperm competition and sexual selection. Academic Press. London.

Birkhead TR, Pellatt J, Hunter FM. 1988. Extra-pair copulation and sperm competition in the zebra finch. Nature 334:60–62.

Boschetto C, Gasparini C, Pilastro A. 2011. Sperm number and velocity affect sperm competition success in the guppy. Behavioral Ecology and Sociobiology 65:813–821.

Bretman A, Fricke C, Chapman T. 2009. Plastic responses of male *Drosophila melanogaster* to the level of sperm competition increase male reproductive fitness. Proceedings of the Royal Society B: Biological Sciences 276:1705 –1711.

Bretman A, Fricke C, Hetherington P, Stone R, Chapman T. 2010. Exposure to rivals and plastic responses to sperm competition in *Drosophila melanogaster*. Behavioral Ecology 21:317 –321.

Bretman A, Gage MJG, Chapman T. 2011a. Quick-change artists: male plastic behavioural responses to rivals. Trends in Ecology & Evolution 26:467–473.

Bretman A, Gage MJG, Chapman T. 2011b. Males use multiple, redundant cues to detect mating rivals. Current Biology 21:617–622.

Bretman A, Westmancoat JD, Gage MJG, Chapman T. 2012. Individual plastic responses by males to rivals reveal mismatches between behaviour and fitness outcomes. Proceedings of the Royal Society B: Biological Sciences 279:2868–2876.

Chapman T, Arnqvist G, Bangham J, Rowe L. 2003. Sexual conflict. Trends in Ecology & Evolution 18:41–47.

Chapman T, Bangham J, Vinti G, Seifried B, Lung O, Wolfner MF, Smith HK, Partridge L. 2003. The sex peptide of *Drosophila melanogaster*: female post-mating responses analyzed by using RNA interference. Proceedings of the National Academy of Sciences 100:9923 –9928.

Chapman T, Hutchings J, Partridge L. 1993. No reduction in the cost of mating for *Drosophila melanogaster* females mating with spermless males. Proceedings of the Royal Society B: Biological Sciences 253:211–217.

Chapman T, Liddle LF, Kalb JM, Wolfner MF, Partridge L. 1995. Cost of mating in *Drosophila melanogaster* females is mediated by male accessory gland products. Nature 373:241–244.

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

Chapman T. 1992. A cost of mating with males that do not transfer sperm in female *Drosophila melanogaster*. Journal of Insect Physiology 38:223–227.

Chapman T. 2001. Seminal fluid-mediated fitness traits in *Drosophila*. Heredity 87:511–521.

Civetta A. 1999. Direct visualization of sperm competition and sperm storage in *Drosophila*. Current Biology 9:841–844.

Cordero C. 1995. Ejaculate substances that affect female insect reproductive physiology and behavior: honest or arbitrary traits? Journal of Theoretical Biology 174:453–461.

Cordero C. 1996. On the evolutionary origin of nuptial seminal gifts in insects. Journal of Insect Behavior 9:969–974.

Cordero C. 1998. Chemical ornaments of semen. Journal of Theoretical Biology 192:581–584.

Cordts R, Partridge L. 1996. Courtship reduces longevity of male *Drosophila melanogaster*. Animal Behaviour 52:269–278.

DeWitt TJ, Sih A, Wilson DS. 1998. Costs and limits of phenotypic plasticity. Trends in Ecology & Evolution 13:77–81.

Dewsbury DA. 1982. Ejaculate cost and male choice. The American Naturalist 119:601–610.

Dickinson JL, Rutowski RL. 1989. The function of the mating plug in the chalcedon checkerspot butterfly. Animal Behaviour 38:154–162.

Eberhard WG, Cordero C. 1995. Sexual selection by cryptic female choice on male seminal products - a new bridge between sexual selection and reproductive physiology. Trends in Ecology & Evolution 10:493–496.

Engqvist L, Reinhold K. 2005. Pitfalls in experiments testing predictions from sperm competition theory. Journal of Evolutionary Biology 18:116–123.

Fedorka KM, Winterhalter WE, Ware B. 2011. Perceived sperm competition intensity influences seminal fluid protein production prior to courtship and mating. Evolution 65:584–590.

Fowler K, Partridge L. 1989. A cost of mating in female fruitflies. Nature 338:760–761.

Fricke C, Bretman A, Chapman T. 2010. Female nutritional status determines the magnitude and sign of responses to a male ejaculate signal in *Drosophila melanogaster*. Journal of Evolutionary Biology 23:157–165.

Gage AR, Barnard CJ. 1996. Male crickets increase sperm number in relation to competition and female size. Behavioral Ecology and Sociobiology 38:349–353.

Garbaczewska M, Billeter J-C, Lavine JD. 2013. *Drosophila melanogaster* males increase the number of sperm in their ejaculate when perceiving rival males. Journal of Insect Physiology. 59:306–310.

Giglioli MEC, Mason GF. 1966. The mating plug in anopheline mosquitoes. Proceedings of the Royal Entomological Society of London. Series A, General Entomology 41:123–129.

Gilchrist AS, Partridge L. 2000. Why it is difficult to model sperm displacement in *Drosophila* melanogaster: the relation between sperm transfer and copulation duration. Evolution 54:534–542.

Gomendio M, Roldan ERS. 1991. Sperm competition influences sperm size in mammals. Proceedings of the Royal Society B: Biological Sciences 243:181–185.

Harcourt AH, Harvey PH, Larson SG, Short RV. 1981. Testis weight, body weight and breeding system in primates. Nature 293:55–57.

Harcourt AH, Purvis A, Liles L. 1995. Sperm competition: mating system, not breeding season, affects testes size of primates. Functional Ecology 9:468–476.

Heifetz Y, Lung O, Frongillo Jr. EA, Wolfner MF. 2000. The *Drosophila* seminal fluid protein Acp26Aa stimulates release of oocytes by the ovary. Current Biology 10:99–102.

Herndon LA, Wolfner MF. 1995. A Drosophila seminal fluid protein, Acp26Aa, stimulates egg laying in females for 1 day after mating. Proceedings of the National Academy of Sciences 92:10114 –10118.

Hodgson DJ, Hosken DJ. 2006. Sperm competition promotes the exploitation of rival ejaculates. Journal of Theoretical Biology 243:230–234.

Hosken DJ. 1997. Sperm competition in bats. Proceedings of the Royal Society B: Biological Sciences 264:385–392.

Immler S, Pitnick S, Parker GA, Durrant KL, Lüpold S, Calhim S, Birkhead TR. 2011. Resolving variation in the reproductive tradeoff between sperm size and number. Proceedings of the National Academy of Sciences 108:5325 –5330.

Juliano S, Gomulkiewicz R, De Jong G, Scheiner SM, Schlichting CD, Van Tienderen PH. 1995. Adaptive phenotypic plasticity: consensus and controversy. Trends in Ecology & Evolution 10:212–217.

Koprowski JL. 1992. Removal of copulatory plugs by female tree squirrels. Journal of Mammalogy 73:572–576.

LaMunyon CW, Ward S. 1998. Larger sperm outcompete smaller sperm in the nematode *Caenorhabditis elegans*. Proceedings of the Royal Society B: Biological Sciences 265:1997–2002.

Lemaître J-F, Ramm SA, Barton RA, Stockley P. 2009. Sperm competition and brain size evolution in mammals. Journal of Evolutionary Biology 22:2215–2221.

Lemaître J-F, Ramm SA, Hurst JL, Stockley P. 2011. Social cues of sperm competition influence accessory reproductive gland size in a promiscuous mammal. Proceedings of the Royal Society B: Biological Sciences 278:1171–1176.

Liu H, Kubli E. 2003. Sex-peptide is the molecular basis of the sperm effect in *Drosophila melanogaster*. Proceedings of the National Academy of Sciences 100:9929 –9933.

Manier MK, Belote JM, Berben KS, Novikov D, Stuart WT, Pitnick S. 2010. Resolving mechanisms of competitive fertilization success in *Drosophila melanogaster*. Science 328:354–357.

Masumoto T. 1993. The effect of the copulatory plug in the funnel-web spider, *Agelena limbata* (Araneae: Agelenidae). Journal of Arachnology 21:55–59.

Neubaum DM, Wolfner MF. 1999. Mated *Drosophila melanogaster* females require a seminal fluid protein, Acp36DE, to store sperm efficiently. Genetics 153:845 –857.

Parker GA, Ball MA, Stockley P, Gage MJG. 1996. Sperm competition games: individual assessment of sperm competition intensity by group spawners. Proceedings of the Royal Society B: Biological Sciences 263:1291–1297.

Parker GA, Ball MA, Stockley P, Gage MJG. 1997. Sperm competition games: a prospective analysis of risk assessment. Proceedings of the Royal Society B: Biological Sciences 264:1793 –1802.

Parker GA. 1970. Sperm competition and its evolutionary consequences in the insects. Biological Reviews 45:525–567.

Parker GA. 1974. Courtship persistence and female-guarding as male time investment strategies. Behaviour 48:157–184.

Parker GA. 1990. Sperm competition games: raffles and roles. Proceedings of the Royal Society B: Biological Sciences 242:120–126.

Parker GA. 1993. Sperm competition games: sperm size and sperm number under adult control. Proceedings of the Royal Society B: Biological Sciences 253:245–254.

Partridge L, Andrews R. 1985. The effect of reproductive activity on the longevity of male *Drosophila melanogaster* is not caused by an acceleration of ageing. Journal of Insect Physiology 31:393–395.

Partridge L, Fowler K, Trevitt S, Sharp W. 1986. An examination of the effects of males on the survival and egg-production rates of female *Drosophila melanogaster*. Journal of Insect Physiology 32:925–929.

Partridge L, Fowler K. 1990. Non-mating costs of exposure to males in female *Drosophila melanogaster*. Journal of Insect Physiology 36:419–425.

Partridge L, Green A, Fowler K. 1987. Effects of egg-production and of exposure to males on female survival in *Drosophila melanogaster*. Journal of Insect Physiology 33:745–749.

Pitnick S, Jones KE, Wilkinson GS. 2006. Mating system and brain size in bats. Proceedings of the Royal Society B: Biological Sciences 273:719–724.

Pitnick S. 1996. Investment in testes and the cost of making long sperm in Drosophila. The American Naturalist 148:57–80.

Prowse N, Partridge L. 1997. The effects of reproduction on longevity and fertility in male *Drosophila melanogaster*. Journal of Insect Physiology 43:501–512.

Qazi MCB, Wolfner MF. 2003. An early role for the *Drosophila melanogaster* male seminal protein Acp36DE in female sperm storage. Journal of Experimental Biology 206:3521 –3528.

Relyea R. 2002. Costs of phenotypic plasticity. The American Naturalist 159:272–282.

Robinson BW, Doyle RW. 1985. Trade-off between male reproduction (Amplexus) and growth in the Amphipod *Gammarus lawrencianus*. Biological Bulletin 168:482–488.

Roff DA. 1993. Evolution of life histories: theory and analysis. Springer. New York.

Sheldon BC. 1994. Male phenotype, fertility, and the pursuit of extra-pair copulations by female birds. Proceedings of the Royal Society B: Biological Sciences 257:25 –30.

Shine R, Olsson MM, Mason RT. 2000. Chastity belts in gartersnakes: the functional significance of mating plugs. Biological Journal of the Linnean Society 70:377–390.

Simmons LW. 2001. Sperm competition and its evolutionary consequences in the insects. Princeton University Press. New Jersey.

Simmons LW. 2001. The evolution of polyandry: an examination of the genetic incompatibility and good-sperm hypotheses. Journal of Evolutionary Biology 14:585–594.

Simmons LW. 2003. The evolution of polyandry: patterns of genotypic variation in female mating frequency, male fertilization success and a test of the sexy-sperm hypothesis. Journal of Evolutionary Biology 16:624–634.

Simmons LW. 2012. Resource allocation trade-off between sperm quality and immunity in the field cricket, *Teleogryllus oceanicus*. Behavioral Ecology 23:168-173

Smith JM. 1958. The effects of temperature and of egg-laying on the longevity of *Drosophila subobscura*. Journal of Experimental Biology 35:832 –842.

Snook RR, Hosken DJ. 2004. Sperm death and dumping in *Drosophila*. Nature 428:939–941.

Snook RR. 2005. Sperm in competition: not playing by the numbers. Trends in Ecology & Evolution 20:46–53.

Stockley P, Gage MJG, Parker GA, Møller AP. 1997. Sperm competition in fishes: the evolution of testis size and ejaculate characteristics. The American Naturalist 149:933–954.

Stockley P. 2002. Sperm competition risk and male genital anatomy: comparative evidence for reduced duration of female sexual receptivity in primates with penile spines. Evolutionary Ecology 16:123–137.

Stoltz JA, Neff BD. 2006. Sperm competition in a fish with external fertilization: the contribution of sperm number, speed and length. Journal of Evolutionary Biology 19:1873–1881.

Teuschl Y, Hosken D, Blanckenhorn W. 2007. Is reduced female survival after mating a by-product of male-male competition in the dung fly *Sepsis cynipsea*? BMC Evolutionary Biology 7:194.

Via S, Gomulkiewicz R, De Jong G, Scheiner SM, Schlichting CD, Van Tienderen PH. 1995. Adaptive phenotypic plasticity: consensus and controversy. Trends in Ecology & Evolution 10:212–217.

Wedell N, Cook PA. 1999. Butterflies tailor their ejaculate in response to sperm competition risk and intensity. Proceedings of the Royal Society B: Biological Sciences 266:1033–1039.

Wedell N, Gage MJG, Parker GA. 2002. Sperm competition, male prudence and sperm-limited females. Trends in Ecology & Evolution 17:313–320.

Westneat DF. 1987. Extra-pair copulations in a predominantly monogamous bird: observations of behaviour. Animal Behaviour 35:865–876.

Wigby S, Chapman T. 2004. Sperm competition (Primer). Current Biology 14:100 – 103.

Wigby S, Chapman T. 2005. Sex peptide causes mating costs in female *Drosophila melanogaster*. Current Biology 15:316–321.

Wigby S, Sirot LK, Linklater JR, Buehner N, Calboli FCF, Bretman A, Wolfner MF, Chapman T. 2009. Seminal fluid protein allocation and male reproductive success. Current Biology 19:751–757.

Wing SR. 1988. Cost of mating for female insects: risk of predation in *Photinus collustrans* (Coleoptera: Lampyridae). The American Naturalist 131:139–142.

Wolfner MF. 2002. The gifts that keep on giving: physiological functions and evolutionary dynamics of male seminal proteins in *Drosophila*. Heredity 88:85–93.

Zeh JA, Zeh DW. 1997. The evolution of polyandry II: post–copulatory defenses against genetic incompatibility. Proceedings of the Royal Society B: Biological Sciences 264:69–75.