Of War and Love: A Study of Sexual Conflict and Sexual Selection Using *Drosophila melanogaster* Laboratory System

A thesis

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By

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Dedicated to my teachers and those who always stood by me

DECLARATION

The work presented in this thesis has been carried out by me under the supervision of Dr.N.G.Prasad at the Indian Institute of Science Education and Research Mohali.

This work has not been submitted in part or in full for a degree, diploma or a fellowship to any other university or institute. Whenever contributions of others are involved, every effort has been made to indicate this clearly, with due acknowledgement of collaborative research and discussions. This thesis is a bonafide record of original work done by me and all sources listed within have been detailed in the bibliography.

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Date:

Place:

In my capacity as the supervisor of the candidate's thesis work, I certify that the above statements by the candidate are true to the best of my knowledge.

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List of publications

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CONTENTS

Summary

Fitness can broadly be decomposed into three components – survival, fecundity and mating/fertilization success. This last component defines "Sexual selection" and is the key to my understanding of evolution in sexually reproducing species. Unless there is a life-long monogamy, males are expected to be under strong sexual selection in terms of (a) mate-choice imposed by females and (b) competition among males for access to mates. Males in turn have been shown to evolve adaptations in response to such selection. These male adaptations have two important consequences. First, many of these male adaptations have been shown to harm females leading to antagonistic co-evolution, commonly called "Intersexual conflict". Secondly, these adaptations impose a cost of reproduction on the males, thereby selecting males to evolve mating strategies including pre- and post-copulatory mate choice. In the first part of this thesis I addressed Sexual conflict using Experimental Evolution approach using replicate populations of *Drosophila melanogaster*. In the second part, I addressed adaptive male reproductive strategies using Laboratory Island analysis with populations of *Drosophila melanogaster*.

Intersexual conflict is defined as the conflict between the evolutionary interests of the two sexes of a given species. It is an outcome of the differences in investment in reproduction by the two sexes. While males (which typically invest less in reproduction) experience intense sexual selection, evolution in females is by and large mostly influenced by fecundity and viability selection. This leads to a situation where traits are selected in opposite directions in the two sexes, giving rise to Intersexual Conflict. Sexual conflict can happen either by direct male-female antagonistic interactions (Inter-locus conflict) or by the non-sex-limited

expression of the traits which have opposing fitness consequences in males and females (Intra-locus conflict). Interlocus conflict is characterized by males seeking as many mates as possible, often coercing females to mating, whereas females trying to minimize harmful male interactions and increasing their life-time progeny production. This can potentially maintain an open ended co-evolutionary arms' race, perpetuated by male adaptations and female counter-adaptations. Such conflict has been shown to be pervasive, affecting a wide range of species and expected to affect many more. However, the evolutionary outcome of interlocus conflict, in terms of changes in reproductive behaviour of the two sexes and life-history traits, is poorly understood. In this thesis, I present the results of a laboratory experimental evolution study, where replicate populations of *Drosophila melanogaster* were subjected to altered levels of interlocus conflict. The level of conflict was varied by altering the operational sex ratio. Three sex ratio regimes were adopted – M, male biased (3:1), C, equal sex ratio and F, female biased (1:3). While male biased regime represents increased male-male competition and intersexual conflict, female biased condition represents the opposite. Equal sex ratio represents the ancestral conditions. Three replicate populations per regime were maintained. I present results of assays done between 8-60 generations of selection.

After only 8 to 12 generations of selection I observed interesting evolutionary trends. Fitness (under competitive condition) of males from the male biased and female biased regime was found to have diverged significantly. While males from the male biased (M) regime were found to be more competitive, males from the female biased regime were found to be poor at competition. I did not find a significant effect of the selection on mating latency (time taken by a virgin male and a virgin female to start copulation) when assayed against the ancestral females. This indicated that M-males did not become more efficient in inducing ancestral females to mate. However, M-males courted females more often compared to males of the

other regimes. Additionally, males from the female biased regime were found to mate for longer duration – a predicted outcome of decreased risk of sperm competition for several generations in a species with high last male sperm precedence (such as *Drosophila*). Females of male biased and female biased regimes were also found to be significantly different in terms of their fitness under competitive condition. M-females higher fitness compared to F-females when both were competed against the ancestral females. In a separate experiment, I also found a significant effect of the selection regime on mating latency between ancestral males and selected females. This indicates that ancestral males took longer time to start mating when paired with M females compared to when they were paired with C females. This possibly indicates that the M-females evolved increased resistance to mating attempts. If this is true, then it is likely that M-females had also evolved to be less affected by harmful male interactions. This might also explain the difference in competitive fitness of among the selected females. The fitness assay was done against the common back ground of ancestral males. Thus, females of all three regimes were under same amount of mate-harm (male-induced fitness reducing effects) during the assay. If M-females were more efficient at resisting such mate-harm, they are expected to have higher fitness compared the females of the other two regimes.

In interlocus conflict, males are selected to evolve traits that are adaptations to male-male competition. These traits often cause reduction in female fitness. Theories of interlocus conflict also predict that since the male-specific adaptations that evolve are likely to be costly, they will extract a cost in terms of evolution of aging and life-span of males. However, studies repeatedly showed lack of response of male traits to laboratory selection. After 40-50 generations of altered levels of interlocus conflict in my study, I found the evolution of males' ability to harm females and the consequent evolution in their life-history.

Males from the male biased (high conflict) regime evolved higher activity and courtship frequency. This was associated with their greater mate harming ability in terms of increased mortality of females mated to them. Consistent with the theories of life-history evolution, I observed an increase in rates of aging and decline in mean lifespan of males from male-biased populations. Males from the female biased regime did not evolve in terms of courtship frequency and locomotor activity. However, they were found to be less harming (less damaging to female lifetime fitness) and relatively long lived. This is the first empirical evidence, clearly showing the evolution of male reproductive traits under intersexual conflict and its life-history consequences in terms of changes in life-span and rate of aging.

Another important component of male fitness in promiscuous species, such as *Drosophila*, is sperm competitive ability and is expected to evolve in response to varying degrees of male-male competition. Several studies have documented the existence of ample genetic variation in sperm competitive ability of males. However, most experimental evolution studies have found sperm competitive ability to be unresponsive to selection. Even direct selection for increased sperm competitive ability has failed to yield any measurable changes. In my experimental evolution study, there was a systematic difference in the level of male-male competition across the three operational sex ratio regimes. While male biased operational sex ratio is thought to generate increased male-male competition compared to the equal sex ratio condition, females biased sex ratio is expected to generate the opposite condition. I observed the evolution of sperm competitive ability (sperm defence-P1, offence-P2) in the above mentioned populations of *D. melanogaster* after 55-60 generations of selection. Males from populations with female biased operational sex ratio evolved reduced P1 and P2, without any measurable change in the male reproductive behaviour. Males in the male-biased regime evolved increased P1, but there was no significant change in P2. Increase in P1 was

associated with an increase in copulation duration, possibly indicating greater ejaculate investment by these males. This study is the first empirical evidence for the evolution of sperm competitive ability of males under different operational sex ratios (and hence different levels of male-male competition).

Theories of sexual conflict also predict female counter adaptation to the male induced harm (mate-harm). Direct empirical evidence for the evolution of female resistance to male induced harm is rare. Those that do show female adaptation, mostly, fail to mention life-history consequence and mechanism of it. I present results of assays after 40-50 generations of selection, suggesting that females from populations experiencing higher level of intersexual conflict evolved increased resistance to mate harm, in terms of both longevity and fitness. The rate of aging analysis (using age specific mortality observations) revealed that increase in resistance to mate harm came at the cost of slightly increased rate of aging. Females from the populations with low conflict were significantly more susceptible to mate harm, suffering greater fitness depression upon continuous exposure to the males. However, these females produced more progeny upon single mating and had significantly higher longevity in absence of any male exposure – an indication of trade-off between resistance related traits and other life-history traits, such as longevity and fecundity. Alternatively, increase in body size of these females can lead to such observations as increased body size also represents increase in the available resources. I also report two additional findings which are novel and of great interest. I found tentative evidence of an increased male cost of interacting with more resistant females, a novel finding, which was hitherto expected theoretically but lacked empirical support. In addition, I found females of the M-regime to be more active. However, at this point it is difficult to predict whether this evolved as a direct

response to the selection on females or is a correlated response of the selection on male-activity.

After 40-55 generations of selection I quantified the effect of the adaptation to sexual antagonism on three important life-history traits – development time, larval survivorship and starvation resistance. I found pre-adult development to be significantly slower in flies from the female biased line, possibly explaining the increase in body size (both sexes) mentioned earlier. Selection regime did not have a significant effect on larval survivorship. Starvation resistance of the selected males and females was not different across the three selection regimes when measured soon after eclosion. However, after 4 days of adult life, including 2 days of interaction with the mates, substantial differences in starvation resistance were formed among the different regimes. Four days of adult life did not make a significant difference in the starvation resistance of the F-males, while a significant negative effect was observed in the males of the other two regimes. As was previously mentioned F-males were found to be significantly larger than the males of the other two regimes. This possibly means that F-males had higher amount of resources that could be utilized under starved condition and therefore even if they didn't have reduced reproductive activities (as was observed in a separate assay) they could resist starvation better. F-females however, had a stronger negative effect of the 4-days of adult life in terms of their starvation resistance, a likely consequence of their increased susceptibility to antagonistic male interactions (during the four days of adult life). This, along with the longevity and rate of aging results discussed earlier, is one of the very few attempts to correlate intersexual conflict and life-history evolution.

Besides, sexual conflict, sexual selection on males has been predicted to have another important consequence – adaptive male reproductive strategies. Particularly, males' ability to show any form of mate choice has received very limited attention over the past several decades of research in sexual selection. One reason for this is the seemingly trivial cost of reproduction for males. However, in the past few decades, there is a growing body of evidence suggesting male cost to sexual reproduction to be non-trivial. Even the results presented in the first part of this thesis suggest that males evolve in response to sexual conflict by evolving their reproductive behaviour and/or physiology but at the cost to their life-history traits (e.g., life-span and aging rate). Thus, if the cost of reproduction is real, then it is important to test whether males are capable of showing mate choice, either in the form of pre-copulatory mating decisions or post-copulatory ejaculate adjustment.

Theories suggest that when cost of reproduction is high, males should show adaptive mate choice if there is sufficient variance in female quality. I tested this prediction using "laboratory island analysis". Essentially, I did a set of experiments using the outbred populations of *D. melanogaster* which have been adapted to the laboratory conditions for hundreds of generations. I have shown that sperm limited males preferentially mated with young and/or well fed females. The preferred females had higher reproductive output – direct evidence of adaptive precopulatory male mate choice. The most striking finding in this study of mine was the strong positive correlation between the degree of mating bias showed by the males and the variance in the fitness of the females. I did not find any evidence suggesting post copulatory mate choice in this experiment. I discuss the possible mechanism for such adaptive male mate choice and propose that such choice has important consequences with respect to the existing understanding of the mating system and the evolution of aging.

Even though I did not find any evidence for post copulatory mate choice in the previous study, it is likely that such form choice is expressed only under certain conditions, where pre-copulatory choice is not optimal strategy. For example, when a mate is available it pays the male to mate with her. But if there is a possibility for this female to undergo another mating very soon (a frequently encountered situation in many studied organisms), the first male's ejaculate will have to compete with the second male's ejaculate for fertilization opportunity. It would be advantageous for a male to adjust his investment depending on the risk of having to face such competition. Though multiple lines of evidence suggest that early adult life is very important in shaping reproductive behaviour of males, few studies have looked at the fitness consequences of the variation in reproductive behavior induced by differences in early life experience of males. This is important as males suffer from a nontrivial cost of reproduction and they are thus expected to evolve strategies to maximize the potential benefit by choosing the optimum investment per female. I tested this prediction using a long term laboratory adapted population of *D. melanogaster*, an approach known as laboratory island analysis. I found early life experience, in terms of co-inhabitant numbers, to affect male mating behavior and at least one fitness component. I found a non-linear relationship between early life experience and fitness components. In my study, co-inhabitant number had a significant effect on copulation duration and sperm defence. Both these traits showed a sharp increase as the co-inhabitant numbers changed from 1 to 16. However, there was a decline in the trait values as the co-inhabitant number increased further. My results are very important empirical support for the theories of sperm competition and only the second test such evidence. In addition, the nonlinear relationship mentioned above is a novel finding pointing to the incompleteness of the theories.

Chapter 1

Introduction

One of the main reasons for the remarkable diversity and richness of life on earth is certainly 'sex' and the consequent sexual selection. Sexual selection "..depends, not on a struggle for existence, but on a struggle between males for possession of females; the result is not *death* to the unsuccessful competitor, but *few or no offspring*" (Darwin 1859). Although Charles Darwin went on to suggest that sexual selection is "less rigorous" than natural selection and limited mainly within one sex, generally males (Darwin 1859), we now know that sexual selection can be much more rigorous than it was initially thought to be (see Kirkpatrik 1982 for the analytical model) and affects both sexes, albeit, to different degrees. Even Darwin himself emphasized the importance of sexual selection by devoting one full book discussing its role in organic evolution (Darwin 1871). Darwin defined sexual selection as "the advantage which certain individuals have over others of the same sex and species solely in respect of reproduction" (Darwin 1871). Till today this is the best working definition of sexual selection available to us. Essentially, the concept can be summarized by decomposing fitness into three components – survival, fecundity and mating/fertilization success (Arnqvist and Rowe 2005). The last component is the representative of sexual selection. Even though many people, especially early investigators, tended to sharply differentiate between sexual and natural selection, such demarcation is now thought to be unnecessary and even erroneous (Arnqvist and Rowe 2005).

This special form of "selection" solved, or at least attempted to solve, one central problem – how do traits which have no survival benefits, such as, bright colouration, costly courtship, horns and antlers etc., evolve? In addition to defining sexual selection, Darwin also noted that

sexual selection can act in two ways (a) competition among the same sex for access to mate (Intra-sexual selection) and (b) mate-choice imposed by one sex on the other sex. He added that usually males fight for access to mates and females are choosy. This was explained in terms of the apparent disparity of investment made by the two sexes – a deep rooted notion that is still debated (see Arnqvist and Rowe 2005 for a review of the idea).

Traditional models of sexual selection

Darwin's concept of sexual selection had to wait till 1930 for further advancement, when Ronald A. Fisher provided the first model for the origin and evolution of female preference and the male sexual traits. Fisher (1930) suggested a male-female co-evolutionary dynamics, wherein female preference drove the evolution of male sexual traits and female preference itself evolved as the male traits evolve in the population. According to this model, males are thought to be selected for expressing the traits that are preferred by females during mate-choice. Assuming a positive correlation between the trait value and male mating success, such situation can lead to the increase in male trait value over generations. Females benefitted by preferring males with increased trait by producing sons which are assumed to inherit their father's 'attractive trait'. Importantly, the progeny not only is expected to inherit the male trait, but also their mother's preference trait. This association between female-preference trait and male sexual trait could, in principle, lead to the exaggeration of the male sexual trait. This 'run-away' exaggeration of male traits is checked by the opposing effects of viability selection. This was the first of the now widely known "genetic-benefit" group of theories of sexual selection. An alternate group of theories, "direct benefit", suggested that 'sexy son' benefit is not sufficient to explain the evolution of female preference and male traits (Kirkpatrik 1985). Female preference, according to this theory, could only have evolved if there was an immediate fitness benefit to the females for showing preference.

One of the problems in the theory was the initial origin and spread of the preference gene in females. Fisher (1930) suggested an 'initial advantage' of preference gene. According to this, preference can spread in a population, if the preferred male trait is connected, even loosely, to fitness of the males and can be inherited to the progeny. This means that the paternal 'sexy' trait and the fitness connection will be inherited to the offspring along with the maternal preference gene. After the initial spread of the preference and male trait, male-female co-evolution can ensue.

An alternative to these co-evolutionary models was proposed in the form of a non-co-evolutionary model – "sensory exploitation" model (Ryan 1990, Basolo 1990, 1995). Sinervo and Basolo (1996) showed female preference to have evolved before the preferred male trait in a sword tail fish species (*Xyphosura*). It was proposed that female preference (or sensory bias for certain male traits) is likely to be a by-product of viability selection on the sensory system of the females. Hence, males at this point can be expected to be selected for exploiting the pre-existing sensory bias in females in terms of inducing affinity in females for mating.

Rise of the sexual conflict theory

By the second half of the twentieth century there was a growing body of empirical evidence, which suggested sexual reproduction to be anything but a cooperative venture between the two sexes. A. J. Bateman (1948) showed that optimum mating frequency is different in the two sexes in fruit flies. This idea was expanded to a great extent by Robert Trivers (1972) to suggest a general difference between the male and female strategies to derive fitness. Parker (1979) formalized and further expanded the theory and showed how competition among males (i.e., intra-sexual selection) can cause harmful side effects to their mates leading to

what we now call Intersexual conflict. Subsequent work, such as those of Rice (1984, 1986, 1987, 1996), Arnqvist (1989a, b, 1992) and Arnqvist and Rowe (1995), introduced a new paradigm in our understanding of male-female co-evolution – sexually antagonistic co-evolution (Rice 2000, Arnqvist and Rowe 2005). In contrast to 'preference', females according to this paradigm are selected to evolve 'resistance' to the male persistence. Such female resistance is now well studied in model organisms such as, bed bugs, water strider and fruit flies (Crudgington and Siva-Jothi 2000, Reinhardt et al. 2007, Arnqvist and Rowe 1995, Rice et al. 2006, Linder and Rice 2005). In addition, Holland and Rice (1998) proposed that many apparent anomalous examples of sexual selection hitherto reported could represent such female resistance. The authors also provided a thought provoking model of 'chase-away selection' (Holland and Rice 1998). The beauty of this theory was that it involved no complicated assumptions. Any male trait that makes females mate at a higher rate is selected for in males. This can be simple behavioural coercion or some cryptic form of manipulation (for example, sensory bias suggested by Basolo 1990). The suboptimal rate of mating is expected to decrease female fitness thereby selecting females to evolve resistance to male stimulation and/or coercion. This is thought to initiate intersexual antagonistic co-evolution (Rice 2000).

Sexual conflict and two decades of experimental research

Intersexual conflict (or simply, Sexual conflict) is defined as the discord between the evolutionary interests of the two sexes (Parker 1979, Chapman et al. 2003). Two forms of such conflict has been identified – intralocus and interlocus (Chapman et al. 2003, Rice et al. 2006). Intralocus conflict happens when a given allele has opposite fitness effects on the two sexes and is not sex limited in terms of its expression. Given that sexes share a common gene pool, this leads to a situation where the gene, in question, is selected in opposite directions

(i.e., favoured in one sex and selected against in the other) in the two sexes (Rice and Chippindale 2001, Prasad et al. 2007). It creates a 'gender load' (Rice 1996a, Prasad et al. 2007) and has been shown be important in chromosome evolution (Rice 1996b). Under promiscuity, the correlation between fitness of males and their mates is very often less than unity (Rice 2000). Under such a situation sexes can evolve strategies to maximise their fitness at the expanse of the fitness of the opposite sex, leading to Interlocus conflict (Holland and Rice 1998, Chapman et al. 2003, Rice et al. 2006). This form of sexual conflict was originally described by Parker (1979) citing mating behaviour in dung beetles. It is characterised by male adaptation to intrasexual selection (i.e., male-male competition) that reduces female fitness as a by-product. This further leads to female counter adaptation to overcome male induced fitness decreasing effects (Rice 2000, Arnqvist and Rowe 2002, Chapman et al. 2003). Female adaptation in turn might reduce male fitness, thereby starting an open ended arms' race – reminiscent of the co-evolution between prey-predator or host-parasite (Holland and Rice 1998, Rice 2000, Arnqvist and Rowe 2005, Rice et al. 2006). It is also thought to be capable of facilitating the process of speciation (Rice 1996, Parker and Partridge 1998, Arnqvist et al. 2000, Gavrilets 2000, Martin and Hosken 2003, Gavrilets and Hayashi 2005).

Interlocus conflict is pervasive and has been documented in many species across different taxa (Rice 2000, Arnqvist and Rowe 2005, Koene 2012). In *D. melanogaster*, males' ability to succeed in siring progeny critically depends on their ability to successfully mate with available females and then their sperm competitive abilities. As a result males have evolved a suit of relevant traits that, as a by-product (Civetta and Clark 2000, Rice 2000), bring down their mates' fitness (Chapman et al. 1995, Rice et al. 2006). These effects can be precopulatory (i.e., behavioural), such as persistent courtship (Fowler and Partridge 1989,

Partridge and Fowler 1990, Kuijper et al. 2006), or postcopulatory, mediated by the physiological effects of seminal fluid or Accessory Gland Proteins (Chapman et al. 1995, Wolfner 1997). Combination of all the harmful effects of males on female fitness is referred to as Mate-harm (Jiang et al. 2011). As mentioned earlier, because mate-harm brings down female fitness, females experience natural selection to evolve resistance to mate-harm. In *D. melanogaster*, this is thought to involve frequent mate-rejection, extrusion of genitalia and some unknown physiologic mechanisms (Connolly and Cook 1973, Rice et al. 2006, Wolfner 2009). Thus interlocus sexual conflict can be studied as the co-evolution between mate-harm and resistance to mate-harm. A number of studies using laboratory model systems have contributed to our understanding of the process of evolution under intersexual conflict (both intra-locus and inter-locus) to a large extent. Here I will give a brief overview of these studies with emphasis on the studies involving laboratory experimental evolution. In Chapter 2, I will also provide a review of the research in sexual conflict using a *Drosophila* laboratory system (LH).

An ingenious method of 'male limited evolution' was adopted by Rice (1996, 1998) where only males were allowed to evolve in a set of populations without any female counter adaptation. The result was the evolution of males' increased mate-harming ability, associated with an increase in male competitive fitness including sperm competitive ability (Rice 1996, 1998). Taking the same approach, Jiang et al. (2011) found no evidence of the evolution of mate-harm and sperm competitive ability in males, though male fitness increased compared to the controls (Prasad et al. 2007). This result was even more striking as both the studies used the same base population. Jiang et al. (2011) argued that long term laboratory domestication has the potential to erode additive genetic variation with respect to the relevant traits through strong directional selection on the male fitness components. This corroborated

well with one previous study, which also used the same base population and selected directly for increased sperm competitive ability (Bjork et al. 2007). In this study, Bjork et al. (2007) failed to see any measurable response to the imposed selection and pointed two possible factors – (a) lack of sufficient additive genetic variation in the right direction and (b) complex interactions between males, their mates and their competitors with respect to the outcome of sperm competition (Bjork et al. 2007). However, sperm competitive ability and related male reproductive traits were found to be responsive to experimental relaxation of sexual selection through enforced monogamy across a number of studies using different model systems (Pitnick et al. 2001, Hosken et al. 2001, Simmons and Garcia-Gonzalez 2008, Firman and Simmons 2011).

Another approach was to evolve populations under experimentally enforced monogamous (thereby relaxing sexual conflict) and polyandrous / polygynous / promiscuous (thereby retaining sexual conflict) mating system. *D. melanogaster* populations subjected to monogamy evolved males with less toxic seminal fluid and females that are more susceptible to mate-harm (Rice and Holland 1999). Dung fly (*Sypsis cynapsea*) populations were subjected to monogamy and polyandry for 29 generations, where yet again monogamous males were observed to be relatively benign while females from the same regime were found to be less resistant to mate-harm (Martin and Hosken 2003). Crudgington et al. (2005, 2009) found similar evidence of evolution of male and female specific traits using *D. pseudoobscura*.

A third approach to address sexual conflict is to manipulate the operational sex ratio in the population and there by alter the level of male-male competition and male-female encounter rate in the population. This, as a result, is expected to alter the intensity of interlocus sexual

conflict. Male biased condition is thought to represent high conflict, female biased sex ratio – low conflict and equal sex ratio is the standard ancestral condition. Wigby and Chapman (2004) subjected replicate populations of *D. melanogaster* to such alterations of operational sex ratio. After 18-33 generations of selection, the only significant response to selection was seen in the female biased regime in the form of significantly decreased resistance to mate-harm in females (Wigby and Chapman 2004). The experimenters also could not detect any change in males (Wigby and Chapman 2004). There was no evidence to suggest primordial stages of speciation even after 41 generations of selection (Wigby and Chapman 2006). However, after 60-67 generations, males from the female biased regime were found to have evolved slower ejaculate depletion pattern (Linklater et al. 2007). Another relatively recent study altered operational sex ratio in flour beetles – *Tribolium castaneum* and after 20 generations of selection it was observed that females from the females biased regime were susceptible to multiple mating leading to decline in fitness (mate-harm), those from the male biased regime did not show any such effect (Michalczyk et al. 2010). Although they did not directly quantify the harming ability of the males, the competitive fitness of the males from male-biased regime was found to be significantly higher than those from the female biased regime (Michalczyk et al. 2010). The divergence between the populations experiencing male biased and female biased operational sex ratio is quite evident in this study. However, it is difficult to predict the exact evolutionary trajectory – whether populations under both the regimes evolved or only one of them did.

Thus in conclusion, studies using laboratory systems have indeed proved to be very fruitful. However, there are areas that are still insufficiently explored or widely debated. For example, there are only few studies addressing evolution of males' harming ability and females' resistance in response to the alteration of the levels of sexual conflict in the population. While

sperm competitive ability has been shown to evolve in response to the removal of sexual selection in a number of studies, it is not clear whether populations evolve in similar way in response to the alteration of the level of sexual selection as opposed to complete removal of it. In addition, as I will describe in the next section, our understanding of the correlation between sexual conflict and evolution of life-history traits (e.g. life-span and aging rate, stress resistance etc.) is still far from being complete.

Sexual conflict and life-history evolution

Scheduling of reproduction over the entire life-span of an organism is termed "life-history" of the organism. Central to our understanding of organic evolution is the knowledge of how organisms evolve life-history traits – life-span, aging rate, time to first reproduction, duration of reproductive phase, amount of reproduction etc. This is because fitness of an organism comes through life-history. As it is evident from the above discussion, intersexual conflict plays a vital role in shaping the fitness of both the sexes, essentially by influencing the life-history of both sexes. Males harm their mates by increasing their mortality and/or decreasing females' total life-time progeny production (see the above discussion on sexual conflict). As discussed above, this also selects females to evolve resistance-traits, which in turn selects males to invest more in reproduction related activities (see Rice 2000, Arnqvist and Rowe 2005). Sexual conflict is thus predicted to have two major effects on the life-history of an organism – (a) it is expected to increase extrinsic mortality in the population, either in one or both sexes (reviewed in Bonduriansky et al. 2008, Koene 2012, also see Adler and Bonduriansky 2011) and (b) it is likely to cause changes in the pattern of resource allocation by selecting for increased investment in reproductive behaviour and/or physiology (Wedell et al. 2006, Chapman and Edward 2011).

These consequences of sexual conflict have been predicted to influence evolution of life-span and aging rate (Promislow and Pletcher 2002, Promislow 2003, Bonduriansky et al. 2008). While evolution of aging is traditionally explained in terms of Medawar's mutation accumulation (Medawar 1952) and Williams' antagonistic pleiotropy (Williams 1957), sexual conflict provides a mechanism through which these theories can operate (Promislow and Pletcher 2002). By increasing mortality rates (either due to increased investment in reproductive activities and/or higher extrinsic mortality), sexual conflict has been predicted to select for increased intrinsic rate of senescence through a process similar to mutation accumulation and/or antagonistic pleiotropy (Promislow 2003, Bonduriansky et al. 2008). However, there are very few empirical tests of this theoretical prediction.

When replicate populations of seed beetles (*Callosobruchus maculates*) were subjected to experimentally enforced monogamy and polygamy for 35 generations, Maklakov et al. (2007) found a significant effect of selection on female aging rate and life-span. Polygynous females aged at a faster rate and lived longer (Maklakov et al. 2007). However, there was no change in male aging parameters. Their analyses also revealed that females from the two regimes did not differ in their age-dependent mortality rates but did differ in their age-independent mortality rate (Maklakov et al. 2007). To the best of my knowledge, this is the only experimental evolution study that directly tests the sexual conflict theory of aging. Adler and Bonduriansky (2011) found that female neriid flies (*Telostylinus angusticollis*) aged significantly faster under male biased condition, whereas males aged faster under female biased condition. This possibly indicated that male interaction increases aging rate in females and mating rate increases rate of aging in males.

With respect to the other life-history related traits, such as, stress resistance, development time etc., almost no empirical work has been done to connect them with sexual conflict. However, experimental evolution studies on life-history traits have occasionally hinted at some changes in the sexual behaviour and/or physiology as correlated response (Service and Vossbrink 1996, Chippindale et al. 1997).

In pursuit of a deeper in-sight: introduction to the thesis

Thus, given the multitude of experimental evidences, the wide spread occurrence of interlocus conflict is unquestionable. However, much of the mentioned studies, especially those involving experimental evolution, are plagued with inconsistency across different studies. In addition, the role of intersexual conflict in reference to the evolution of life-history traits (such as aging rate, life-span, and stress resistance) is poorly studied.

In the first part of this thesis, I intend to bridge these gaps in our understanding. I have taken laboratory experimental evolution approach to do this. I have used laboratory adapted populations of *Drosophila melanogaster* as the model system. I subjected replicate populations to different levels of sexual conflict, similar to the approach adopted by Wigby and Chapman (2004, 2006) but with critical modifications (discussed in the Experimental System section). I then observed the response to the selection imposed in terms of male's ability in causing mate-harm, male and female reproductive behaviour, male and female fitness under competitive condition, females' resistance to mate-harm, aging and life span in both the sexes, males' sperm competitive ability and life-history related traits such as larval viability, developmental time and starvation resistance.

In the second part of the thesis, I address two consequences of intrasexual selection in males – precopulatory male mate choice and post copulatory strategic ejaculate adjustment. Starting from Darwin, for over a century, females were widely believed to be the choosy sex and males being more interested in just increasing the number of mates without any form of choice. The underlying notion that gave rise to this concept was the satisfactory explanation – male investment in sexual reproduction being trivial compared to that of the females, male fitness is never limited by gametes and ejaculate investment. However, this idea has recently received criticism from many investigators (Dewsbury 1982, Pitnick and Markow 1994a, Bonduriansky 2001, Byrne and Rice 2006, Edward and Chapman 2011). Over the past few decades, empirical studies have shown non-trivial nature of male mating cost (Partridge and Farquhar 1981, Dewsbury 1982, Chippindale et al. 1997, Galvani and Johnston 1998, Pitnick and Markow 1994a, b, Judge and Brooks 2001, Bonduriansky 2001, Byrne and Rice 2006). Hence, it is now important to know the male response to such cost of mating. In this part of the thesis I addressed two specific questions. (a) Do males show adaptive mate choice? If they do, how does mate quality variance affect their behaviour? (b) Do males show adaptive post-copulatory ejaculate adjustment at the face of different risks of sperm competition? I address these questions by taking the "laboratory island analysis" (Rice et al. 2006) approach using longterm laboratory adapted populations of *D. melanogaster*. In one study, I manipulated female quality by varying their age and nutritional status and then empirically assessed male behaviour to such females. In another study, I subjected males to different early-life experience in terms of different numbers of co-inhabitant males and then assessed their reproductive behaviour and fitness components.

Chapter 2

Experimental system

The enormous complexity of factors that affect ecological phenomena led many early investigators to think that handling such complexity and the apparent unpredictability is beyond the scope of laboratory investigation. Consequently, traditionally the study of ecology and evolution (and relatively recent field of behavioural ecology) has been mostly field based. While theoreticians often got around this problem by building models with carefully chosen assumptions and then relaxing the assumptions to accommodate complexity, complexity was a particularly intriguing problem for empiricists. Especially while testing an existing hypothesis, empiricists came across several additional factors that the hypothesis assumed to be non-existent or negligible, thereby making the test meaningless. In addition, many studies attempted to provide evolutionary explanations to different traits by relying on phenotypic observations only, completely ignoring the genetics of the organism – indeed a considerably non-trivial compromise. Apart from all these, statistical power (replication, sample size etc.) of the field studies has always been a cause of concern. Thus, how to tackle the complexity of the biological world and still stick to the frame work of theory has been a long standing issue of debate.

Much of the difficulties in carrying out field experiments are easily (relatively) overcome by working with the laboratory systems. However, laboratory systems are not devoid of limitations. Many studies, for example have used laboratory strains of organisms to tackle problems in ecology and evolution. These laboratory strains (e.g., Canton S, Origon R etc.) have a history of being maintained under severely curtailed population size, leading to strong inbreeding effects and loss of genetic variation. An alternate approach was adopted by using

laboratory adapted, outbred populations of organisms (such as, *Drosophila melanogaster*). Typically, such populations are started with a large founding group collected from the wild and thereafter the population is propagated using large viable size that reduces the effect of inbreeding. Experiments are done after the population has adapted to the laboratory condition for several generations (Rose 1984).

For an experimenter interested in studying organic evolution, an ideal system will be a closed (isolated from other populations) population (a group of individuals breeding together) of some decent size (number of individuals in the population) having ample genetic variation, whose organic and inorganic effectors can be manipulated by the wishful experimenter. Laboratory adapted populations of *D. melanogaster* provides just this. As a consequence, several investigators have used this system to get deep in-sights into several issues in life-history evolution and sexual conflict. While simple phenotypic manipulations reveal fitness consequence of important traits, laboratory experimental evolution provides the ultimate experimental tool to dissect the phenotypic and genetic correlation between different traits (Prasad and Joshi 2003). In addition, the well defined genetics (both classical and molecular) and biochemistry of the *D. melanogaster* makes complicated proximal level understanding of different evolutionary phenomena (e.g., trade-off) possible (for example, Flatt and Kawecki 2007). In this chapter, I will introduce the model system. I will discuss the general life-cycle of the flies, the laboratory population and the experimental approaches adopted. I will also outline the populations used in this thesis and their history.

Drosophila melanogaster is a holometabolous dipteran insect, belonging to the family Drosophilidae. Their life cycle passes through four distinct stages – egg, larva, pupa and adult. At the usual condition of the laboratory, i.e., under 25^{o}C temperature, 60-90% relative

humidity, their metamorphosis follows the pattern described here in brief (Figure 2.1). Eggs usually take around 18 hours to hatch. The larval stage passes through three sub-stages or instars – first, second and third. During larval stage they dig into the food (the fly media in case of laboratory cultures) and actively feed upon the available food. Upon reaching a "critical size" they become committed to the post larval development. The late third instar larva stops feeding, comes out of the food and becomes stationary on some suitable substrate (vial/bottle wall or the cotton plug for laboratory cultures) and forms pupa secreting a chitinous covering. The larval stage lasts for approximately 4-5 days. The pupa completes development in about 4-5 days. The adult fly comes out of the pupal shell – a process commonly referred to as 'eclosion'. The entire preadult development takes about 8-10 days. The adult males and females do not eclose as reproductively active individuals. They usually take 8-10 hours to start reproductive activity. Females might start laying eggs by 24 hours of eclosion. Female fecundity heavily depends upon their ability to forage for live Yeast (Prasad and Joshi 2001, Stewart et al. 2005, Nandy et al. 2012). Females mate with multiple males and store sperms in an organ called spermatheca for future use (Lefevre & Jonsson 1962). Ability to find mate and success in sperm competition are two of the most important determinants of male fitness (Fricke et al. 2010). Specific aspects of the life-history of both the sexes for the experimental system are given below along with the details of laboratory population.

Laboratory adapted population: LH and derivatives

Typically, a laboratory population is started with a number of wild caught mated females and allowing them to produce progeny in the lab. The next generation onwards, a large group (hereafter referred to as 'population') of interbreeding individuals are maintained under a

more or less fixed maintenance regime (temperature, humidity, food, cage, density etc.). These populations are used for experimental purpose only after they have adapted to the

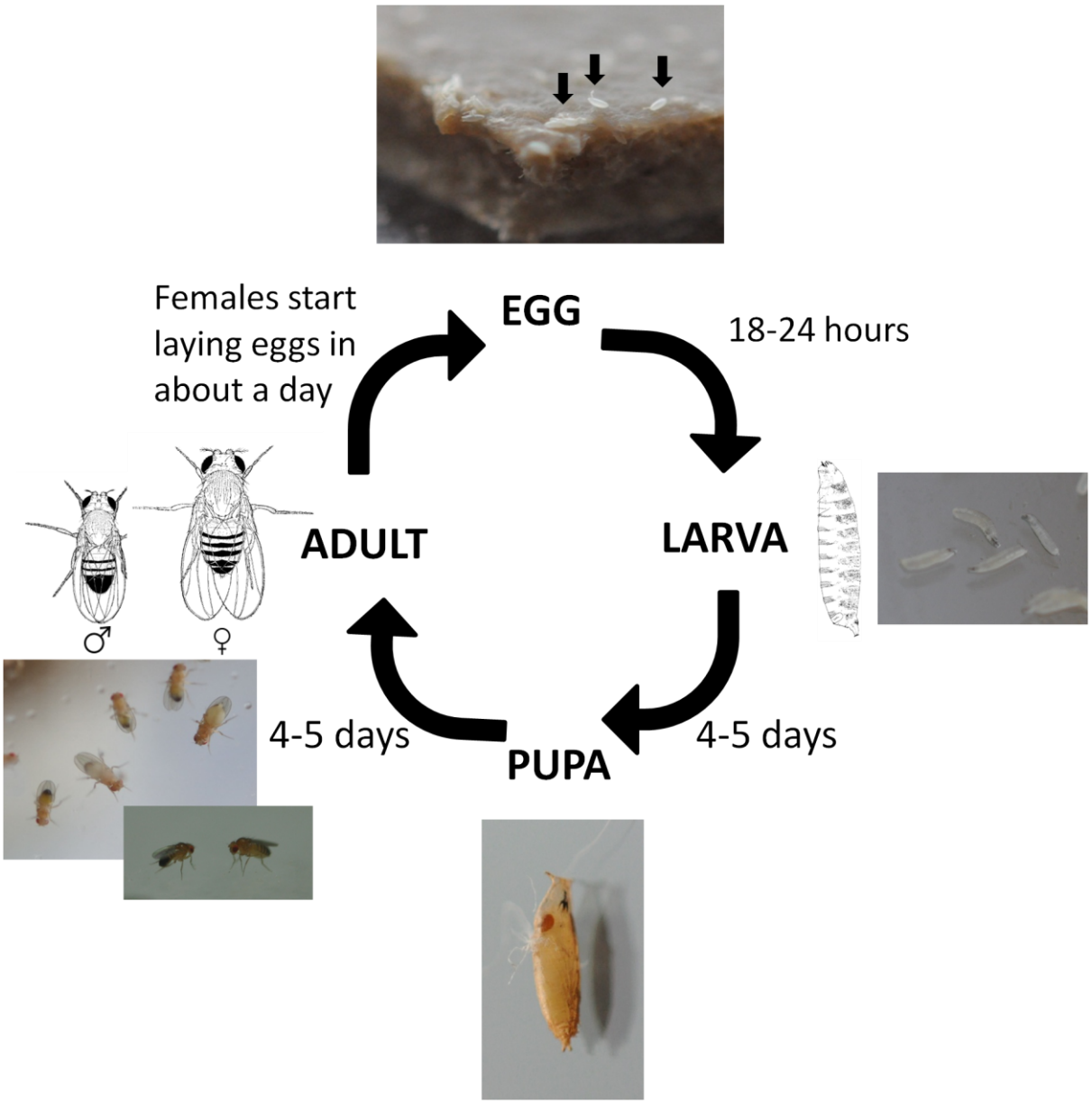

Figure 2.1: Life cycle of fruit flies (*Drosophila melanogaster*). Generation time corresponds to the typical fly life cycle under laboratory condition (25^{o}C temperature, 60% RH).

laboratory conditions for several of generations. This system overcomes two major problems most commonly encountered by laboratory studies – (a) usually traits studied are not confounded by effects of maintaining small number of individuals – inbreeding, and thus can be extrapolated to natural populations. (b) As the populations are given time to adapt to the laboratory conditions and the experiments are performed under similar conditions (i.e., natural condition for these populations), interaction between experimental condition and natural condition can thus be handled through careful experimental design. In addition to these, as these populations are maintained as persistently large effective size, genetic variation is preserved, allowing the experimenter to undertake 'laboratory experimental evolution' studies, not possible with most laboratory stocks.

All the experiments described in this thesis are carried out using the base population LH (named after the original founder, Larry Harshman) and a derived population – LH_{st}. LH population was founded with 400 wild caught females from central California, USA in 1991 (Chippindale and Rice 2001). Since then the population is maintained under 14-day discrete generation cycle at 25^{o}C temperature, 60-80% relative humidity and 12-h light / 12-h dark, on standard cornmeal-Yeast-molasses fly food (composition described in the end of this chapter). The population is maintained in 8-dram vials (25mm diameter × 90mm height). A total of 60 vials constitute the population. Every generation, larvae are grown under moderate density (around 150 eggs per 8-10ml of food in 8-dram vials) in 'juvenile competition vials'. On 12th day post egg collection, adult flies across different vials are mixed and distributed (16 males and 16 females per vial) in fresh vials (=60) having food seeded with limited amount of live Yeast. This is done under light CO_2-anaesthesia (exposure time < 3 minutes). These vials are referred to as 'adult competition vials'. They are left undisturbed for two days and on 14th day they are transferred to 'oviposition vials' having 8-10ml of fresh food without the

use of anaesthesia. They are allowed to oviposit for 18 hours, following which the flies are discarded. The egg densities in these vials are trimmed to around 150 per vial and these now become the juvenile competition vials for the next generation. This maintenance regime of LH-population is described in Figure 2.2.

LH_{st} was derived from LH base population by introducing the reseccive-autosomal trait scarlet-eye ('st') by repeated back crosses (Prasad et al. 2007). LH_{st} is maintained under identical conditions, except that the population is made up of 30 vials. LH_{st} is periodically backcrossed with LH to maintain the genetic uniformity across the two populations.

By the time the work of this thesis was started the LH population had already completed around 400 generations of laboratory maintenance. The essence of this system lies in the well defined and standardized measure of male and female fitness in these populations. Since these populations have been maintained under same laboratory conditions for more than 400 generations, they are expected to have adapted to the laboratory regime. This allows us to identify a set of time windows which are important in determining the fitness of the individuals in the population. For example, selection of adult traits is strongest during the life in adult competition vials and oviposition vials. Here females compete to get access to limited amount of live Yeast and resist male coercion, whereas males compete among themselves both at the precopulatory and the postcopulatory stage. Flies are discarded after 4-5days of adult life (after they have produced the eggs for the next generation) and hence any trait expressed after this period does not contribute to the fitness of the flies in the regular maintenance regime. These late affecting traits are thus beyond the scope of selection under this regime.

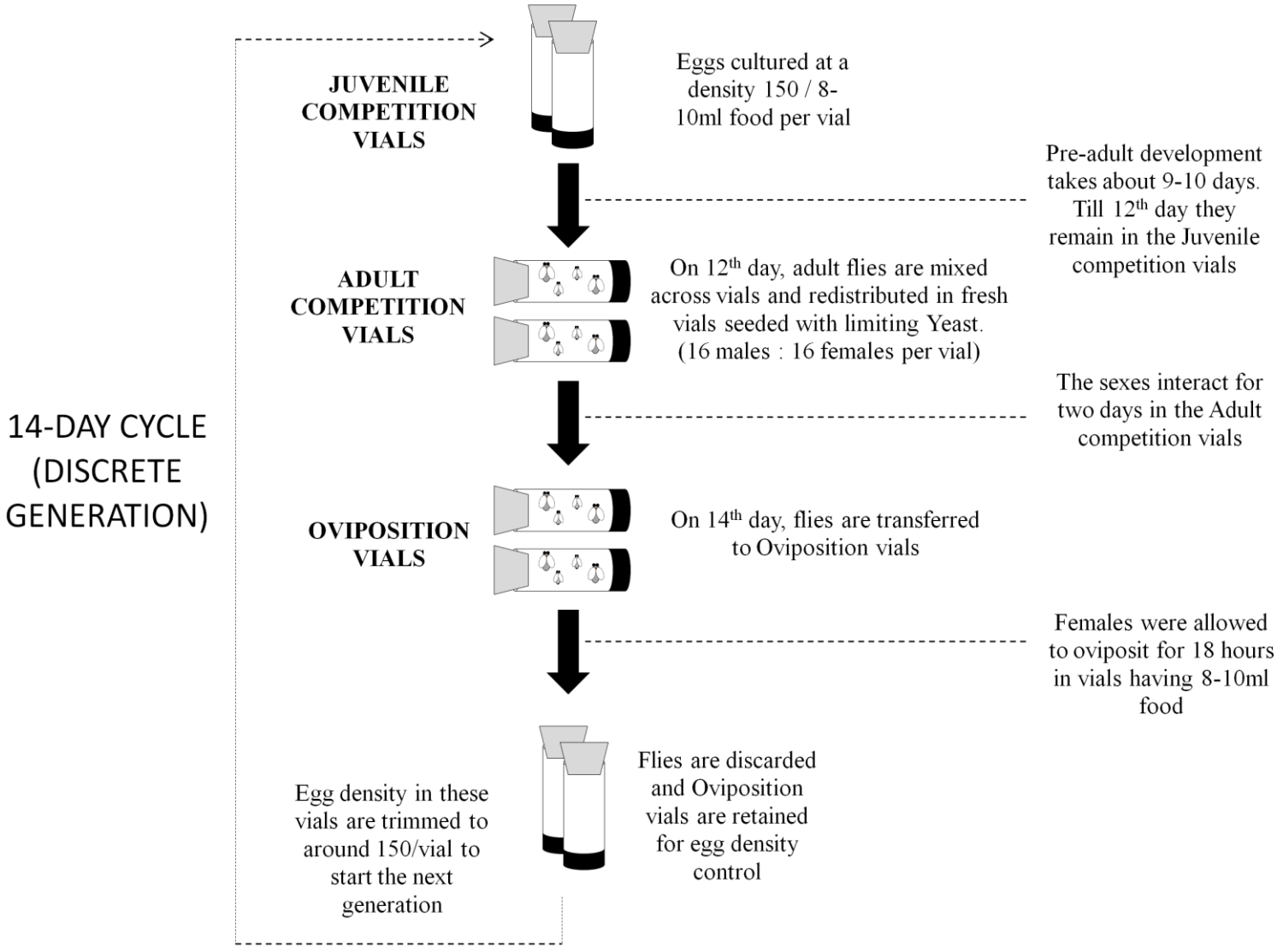

Figure 2.2: Maintenance protocol for the LHpopulation

Laboratory experimental evolution:

Dubbed "experimental wonderland" (Rose 1996) for an evolutionary biologist, laboratory experimental evolution is arguably the most powerful tool for studying evolution under laboratory conditions. In this approach, an experimenter subjects a set of laboratory adapted populations (described above) to some alterations in the physical or biological property of the environment relevant to the precise question and tracks the resulting evolutionary response of the population through generations. The greatest attribute of this approach is that, not only one directly observes evolution under the carefully designed laboratory environment but also can replicate the same condition as many times as he/she wants. The replicable trait response of a population to the specific manipulation (i.e., selection) allows the experimenter to come to robust conclusion regarding the direct and correlated response to the given selection (reviewed in Rose 1996, Harshman and Hoffman 2000, Garland 2003, Bennett 2003, Prasad and Joshi 2003, Meuller et al. 2005, Rose and Garland 2009).

Although, rudiments of this approach can be found in the traditional breeding programmes, the rigorous use of this technique in Evolutionary Biology is relatively new. It is somewhat surprising to find the delay in realisation of the power of this approach by scientists. Some scientists attribute this delay to the legacy that Darwin himself left. Rose and Garland (2009) suggested that Darwin's idea that "natural selection will always act very slowly, often only at long intervals of time, and generally on only a very few of the inhabitants of the same region at the same time" (Darwin 1859), led many of the later evolutionary thinkers to adopt a 'gradualist' prejudice, writing off such powerful methods as experimental evolution. Ironically, Darwin himself took up breeding programme very seriously and one chapter in his 'Origin of species' was devoted to the discussion of such animal breeding and its relevance to

the natural process of evolution. Nonetheless, practice of this method now is indeed not rare, specifically when it comes to research in sexual selection and life-history evolution.

Modern selection experiments however differ from the traditional breeding programmes by one very crucial attribute. In breeding programme, the breeder carefully selects to breed a particular phenotype and thereby improves the stock with respect to that very phenotype. In the experimental evolution, particularly laboratory experimental evolution, the experimenter chooses to alter some physical/biological parameter of the environment and then the population is left to evolve in any direction. Different response to the selection across replicate populations is a possible outcome. After all, playing the tape of evolution over and over again does not necessarily yield the same play.

One criticism against this approach is the difference in the laboratory and the 'field' condition limiting the generality of the experimental findings (Sgro and Partridge 2000, also see Harshman and Hoffman 2000). Mueller et al. (2005) suggested that whereas field condition indeed is different from that of the laboratory, looking into the 'field' does not really solve the problem of generality as there is no real unified 'field' condition. Populations of organism are generally distributed across different places having very different conditions. Therefore looking into one of them does not provide a unified solution. However, the conditions in the laboratory are at least manageable and this allows the experimenter the freedom to unentangle the complex interaction between different factors by looking at one (or few) factors at a time. The differences between laboratory and field condition therefore should be taken as challenges to the field rather than limitation.

However powerful the technique might be, it comes with several precautions. Rose (1996) showed that result of experimental evolution can potentially be plagued with the 'Cheshire cat' phenomena (the appearance and disappearance of phenomena, such as trade-off, due to genotype × environment interactions across different studies) and 'Mad Hatter Syndrome' (inbreeding affecting the result of a selection experiment). Although these cautions are discussed in detail a number of times (Rose 1996, Chippindale 2006, Meuller et al. 2005), I here briefly mention them for the context of the thesis. The interaction between gene and environment is well known for a long time. The same interaction can play major role in selection experiments as well, particularly when it comes to the actual assays for different traits after the population has been selected for a number of generations. For example, well known trade-off between longevity and earl-life fecundity was found to be difficult to reproduce. Leroi et al. (1994), using a systematic assays, concluded that demonstration of trade-off was only possible under the original population maintenance regime and not in other conditions, as was done by Chippindale et al. (1993). Thus even in this thesis, different assays were done under conditions that closely matched the selection conditions. Deviations from such protocol and the possible effects are discussed in the respective places. Even while doing Laboratory Island Analyses (discussed later) using laboratory adapted populations, experiments were done under conditions that very close mimicked that of the usual maintenance regime for the populations, thus controlling for the G×E interaction.

Another important factor that can ruin an experimental evolution study is the effective population size of the selected populations. If effective population size of the different populations is not controlled, i.e., kept sufficiently high to avoid severe inbreeding, inbreeding depression can creep in to the populations, differentially affecting the populations under study. Inbreeding depression has the potential to produce unexpected fitness

consequences, thereby ruining the study. One example is the study by Partridge and Fowler (1992) selecting populations for early and late-life reproduction, where the late-reproducing populations suffered from severe inbreeding depression (discussed in Rose 1996) thus confounding the entire study. The base-line populations (LH and LH_{st}) used in the present thesis, are all maintained under sufficiently high effective population size to avoid inbreeding. Even in the experimental evolution discussed in this thesis, all populations were maintained under same effective populations size (N_e = 450) to avoid differential levels of inbreeding across different populations (see detailed discussion on N_e of the experimental populations later in this chapter). Additionally, signs of inbreeding were checked every few generations and discussed wherever necessary.

In addition, success of an experimental evolution in correctly deciphering the evolutionary process in question depends critically on the selection design. Selection designs are expected to be fine-tuned as to not impose any inadvertent selection on some trait.

Phenotypic manipulation - 'Laboratory island analysis'

The other approach adopted in this thesis is to directly manipulate a set of conditions in an experimental set of flies and observe the organisms' behaviour. The system also allows one to measure fitness under this experimental condition. This allows the experimenter to infer the fitness correlation of a given trait under study. Rice et al. (2006) broadly referred to this approach as "laboratory island analysis". The laboratory adapted populations described above have several similarities to island populations (Rice et al. 2006, 2005) and can similarly be used to test general principles of evolution. This needs to be emphasised that the results in such island analyses should not be directly extrapolated to the natural counterpart. However,

the general principle studied can be applied to natural system with sufficient caution (Rice et al. 2006).

Two decades of research in sexual conflict using LH-system

The LH-population has a rich history of extremely sophisticated empirical studies on sexual selection and sexual conflict. Three approaches – experimental evolution, laboratory island analysis and hemiclonal analysis (discussed below) have produced several high quality results – a testimony to the suitability of the system. Here, I will briefly summarise the most important insights gained over the past (almost) two decades of studies.

In 1996, W. R. Rice adopted an ingenious empirical approach of male-limited (ML) evolution (also discussed in Chapter 1). Essentially, males in replicate populations (derived from LH) were allowed to evolve against a non-evolving target female phenotype. He made use of a number of special genetic constructs and the fact that recombination does not take place in *D. melanogaster* males in order to achieve this (Rice 1996). ML-males were found to evolve increased harming ability, coupled with increase in other components of their fitness, such as, ability to induce re-mating, sperm defence and offence (Rice 1996, 1998). On the one hand, these landmark studies showed the existence of sexually antagonistic (SA) alleles and ability of sexual antagonism to shift the sexes from their sex specific fitness optima (see gender load in Rice et al. 2006). On the other hand a model of Y-chromosome evolution was proposed, where a non-recombining Y-chromosome first evolves to accumulate SA-alleles favouring the heterogametic sex, but later due to the effects of interlocus conflict and genetic hitchhiking of deleterious mutations undergoes degeneration. Adopting the same approach, Prasad et al. (2007) showed in a separate study that ML-genome produced high-fitness males but low-fitness females. Hence, ML-genome evolved to accumulate male-benefiting SA-

allele – a direct evidence of intra-locus sexual conflict (Prasad et al. 2007). Later experiments suggested that ML-genome produced males with more efficient courtship (Bedhomme et al. 2008), however both harming ability and sperm competitive ability of such males was not different (Jiang et al. 2011). Jiang et al. (2011) argued that the difference in their results and the previously reported results from the similar study by Rice (1998) was possibly due to the long term laboratory adaptation of the base population. Nonetheless, another form of gender limited selection approach – gender limited middle class neighbourhood design was adopted by Morrow et al. (2008) to show increase in fitness of the selected sex along with the decrease in fitness of the non-selected sex. Another experimental evolution approach was adopted by Holland and Rice (1999) where replicate populations were derived from LH base population and subjected to experimentally enforced monogamy and promiscuity (control). As mentioned in the previous chapter as well, the authors observed monogamous males to become more benign and monogamous females to become more susceptible to male induced harm. Bijork et al. (2007) attempted to select for increased sperm defence and offence. Sperm defence in this system is quantified as P1, proportion of progeny sired by a male when it is the first one of the two mates of a given female. Sperm offence, similarly, is quantified as P2, proportion of progeny sired by a male when it is the second of the two mates of a given female. Bijork et al. (2007) selected a set of populations for increased P1 (defence lines) and another set for increased P2 (offence lines) with their matched controls. Essentially, the authors allowed test females to mate with males in specific order and quantified their P1 (for the defence lines) and P2 (for offence lines) for each of the males used. Then they picked males with the top 15 scores and allowed them to mate with females and ultimately produce the next generation. Even after 15 generations of such rigorous selection, the authors detected no significant increase in either components of sperm competitive ability of males (Bijork et

al. 2007). This lack of response was attributed to the complex ejaculate×ejaculate and ejaculate×female interactions besides low additive genetic variation for the relevant traits.

Although Rice (1996, 1998) and subsequent experimental evolution studies provided ample evidence of SA co-evolution in LH-system, the nature of sexual conflict and sexual selection in this system was established through a number of laboratory island analyses. The sexy-son theory (another name for Fisher's theory of evolution of exaggerated male traits through sexual selection, discussed in Chapter 1) received two important blows when Orteiza et al. (2005) and later Pischedda and Chippindale (2006) independently showed that the direct cost suffered by females severely out-weighs the indirect benefit of producing "sexy" sons that until then was hitherto assumed. While the later study made use of an approach that I will discuss shortly, Orteiza et al. (2005) directly quantified (a) the direct cost suffered by females due to male interactions and (b) a conservative estimate of the indirect benefit of producing sons through multiple mating (in effect the sexy son advantage). The results showed that while a meagre indirect benefit was indeed present in females that re-mated, however the direct cost was almost three times stronger. Kuijper et al. (2006) further quantified this female cost and showed that the cost increased non-linearly with number of mating. They also concluded that a substantial proportion of the cost was probably due to the non-mating effect of courtship, as opposed to the ejaculate toxicity previously reported in *D. melanogaster* (Chapman et al. 1995). Kuijper et al. (2006) also showed that SA male effects can be quantified in terms of life-time progeny production (or life-time fitness) of the females in this system, in addition to the long term survival cost. An interesting off-shoot of these studies was provided by Byrne and Rice in 2006 when they showed LH-males ability to show adaptive mate choice in favour of larger females, particularly under sperm limited condition. Long et al. (2009) synthesized this new finding with the existing knowledge of sexual

conflict using empirical dissection and proposed that a combination of SA-male interactions and male mate choice for high fitness females can potentially preserve variation in female fitness.

A third approach was an extraordinarily innovative way of cytogenetically cloning haploid sets of genomes from the base population and expressing them in random genetic back ground. The fitness variation observed among different haploid genomes was thought to represent the fitness variation among nuclear genomes. This method of comparing between different hemiclones (individuals sharing haploid sets) was termed 'hemiclonal analysis' (Pischedda and Chippindale 2006, Long and Rice 2007). Linder and Rice (2005) performed a hemiclonal analysis of 35 genomes for female resistance to mate-harm and showed the existence of a substantial genetic variation in female resistance. Similar analysis also showed low but substantial genetic variation in all components of sperm defence and offence (Friberg et al. 2005). Using this method, Chippindale et al. (2001) provided the first evidence of an 'ontogenetic conflict'. Haploid set of genomes were cytogenetically sampled and cloned from LH-base population and each of the sampled genomes was expressed in random genetic background (representing LH-gene pool only) as males and females. Intersexual correlation of juvenile fitness was found to be positive but that of the adult fitness was significantly negative, whereas there was no intersexual correlation of the total fitness (a measure representing both juvenile and adult components of fitness). The authors also found several instances where genomes that produced high fitness males also produced low fitness females and vice versa. Pischedda and Chippindale (2006) applied the same hemiclonal analysis to produce high and low fitness individuals of both the sexes. Then they allowed mating among these individuals in all possible combinations and quantified the reproductive success of the progeny. They found that paternal fitness had no significant correlation with the fitness of the

sons produced, while maternal fitness was positively correlated to the fitness of daughters but negative correlation to the fitness of the sons. This conclusively showed that, at least in this model system, there was no sexy-son advantage of female mate choice and the fitness (reproductive success) inheritance was consistent with the X-linked SA model. Another hemiclonal analysis in this system showed that locomotory activity, which was hitherto thought to be under the influence of sexual antagonism, is indeed under intralocus conflict (Long and Rice 2007). The authors found a significant positive intersexual genetic correlation of the trait, in spite of its role in intrasexual conflict.

In summary, the LH-system is evidently an ideal system to study intersexual conflict with abundant existing results. A combination of the well defined experimental framework and numerous evidence suggesting ample standing genetic variation, made this system suitable for the studies described in this thesis.

Sex Ratio Selection-line: experimental evolution of populations under altered operational sex ratio

Most of the work of this thesis was carried out on a set of *D. melanogaster* populations subjected to experimental evolution under different operational sex ratio (ratio of males to females available for reproduction). The aim was to look into the role of interlocus sexual conflict on the evolution of behavioural and life-history traits in a population. Different operational sex ratio is thought to generate different levels of male-male competition and interlocus sexual conflict in a population. Male biased sex ratio is expected to be a more competitive environment for the males, and together with increased opportunity of male interactions per female, is thought to generate 'high' conflict condition. Equal sex ratio is the standard ancestral condition. Females biased sex ratio on the other end is thought to relax intersexual conflict and male-male competition. This method of varying the level of

interlocus conflict was adopted in previous studies as well but with several differences (Wigby and Chapman 2004, 2006, Linklater et al. 2007).

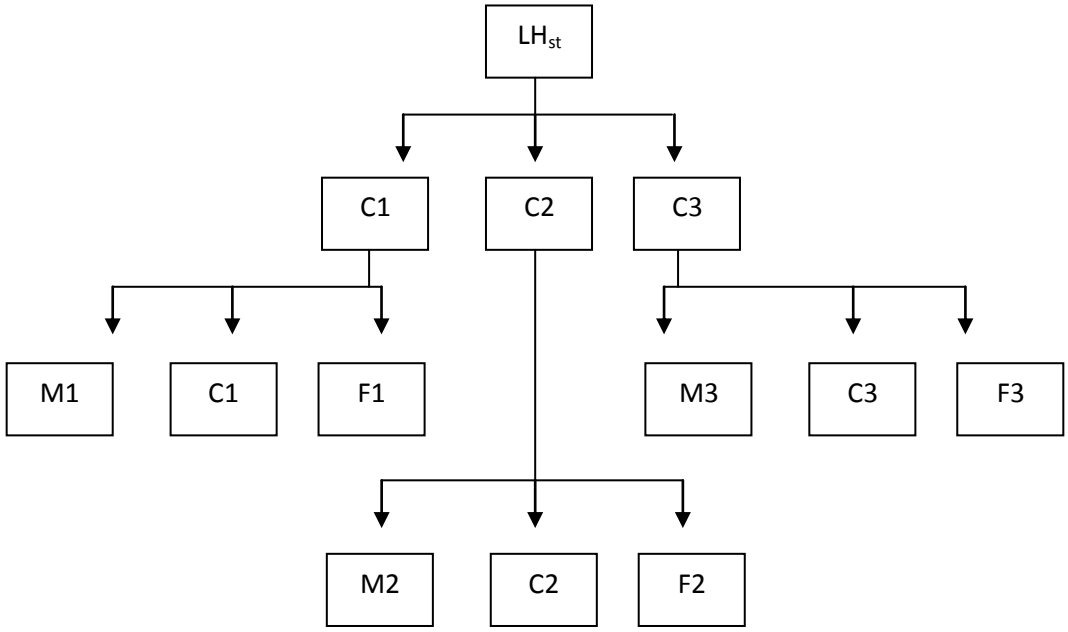

Figure 2.3: The 9 selected populations were created ultimately from LH_{st} baseline. LH_{st} was first used to create three replicate populations - C1, C2 and C3. These three populations were maintained for 5 generations and then each of them were split into three sex ratio regimes - male biased (M), female biased (F) and the ancestral equal sex ratio (C). Populations bearing same numeric subscript share common ancestry. For example, M1, F1 and C1 share common ancestor (i.e. C1).

Derivation and maintenance:

I derived three replicate populations, names C1-3, from the LH_{st} base population and maintained them for five generations under equal sex ratio (and other conditions described later). Then each replicate was used to derive two additional regimes – male biased (M1-3) and female biased (F1-3). Thus after six generations of splitting from the base populations, I established nine populations – three sex ratio regime, each with three replicates. Thus, populations bearing the same numerical subscript share a common ancestry (Figure 2.3) and are more closely related to each other compared to populations bearing different numerical subscripts. For example, M1 is more closely related to C1 and F1 than to M2. Additionally, during regular maintenance, replicate populations bearing the same numerical subscript are always handled together. Hence replicates bearing the same numerical subscripts are treated as statistical 'Blocks' in the analysis. The whole experiment consists of three statistical blocks (Blocks 1, 2 and 3). All aspect of the maintenance regime was kept equal across the regimes except the adult sex ratio. All the populations are maintained as 14-day discrete generation cycle (Figure 2.4), under 25^{o}C temperature, 60-80% relative humidity and 12-hours light / 12-hours dark. The maintenance regime of the populations is described in Figure 2.4. Every generation eggs are cultured in food vials under moderate density (140-160 eggs / 8-10ml of food in 8-dram vials). The flies take about 10 days to complete the preadult development. On 10th day adult flies star eclosing out of pupae. Just prior eclosion the pupae become dark in appearance indicating the eclosion phase. The adult flies are collected as very young (< 6 hours post eclosion) virgins and held in single sex vials (pre-reproductive vials). The adult flies take around 8-10 hours to become reproductively mature.

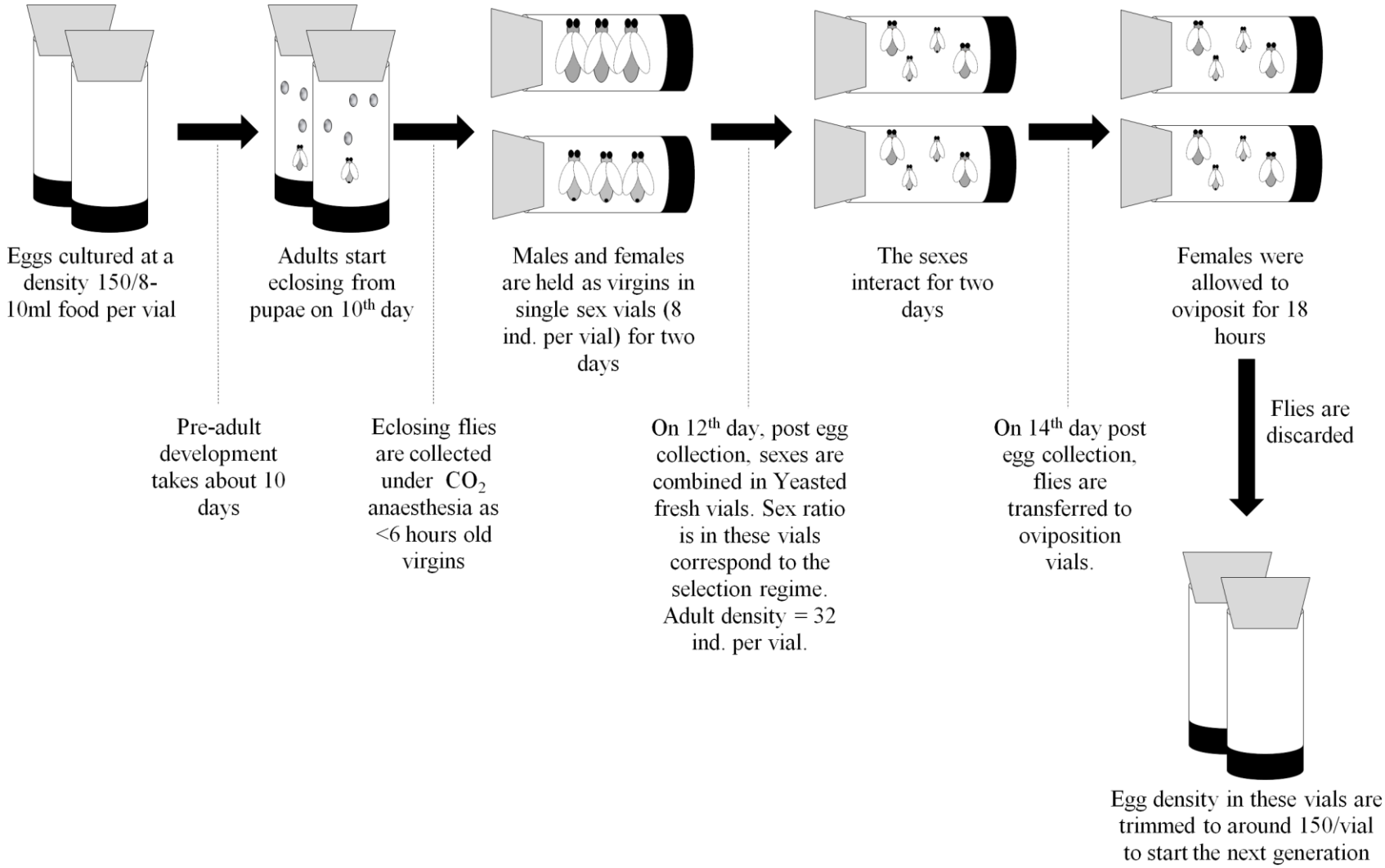

Figure 2.4: General maintenance protocol for the selected populations (M1-3, C1-3, and F1-3)

Therefore by this method of collecting the adult flies soon after eclosion ensures their virgin status. Virgin flies are held at a density of 8 flies per vial in the pre-reproductive vials. After two days, on 12th day post egg collection, the sexes are combined in food vials provided with measured amount of live Yeast (adult competition vials). The sex ratio in the adult competition vials were maintained according to the selection regime – male biased (24 males : 8 females) for M-populations, equal sex ratio (16 males : 16 females) for C-populations and female biased (8 males : 24 females) for F-populations. The amount of Yeast available to each female is controlled at 0.467mg. In the adult competition vials, the flies are allowed to interact for two days before transferring them to oviposition vials. Oviposition vials are provisioned with 8-10ml of food. Flies are given a window of 18 hours to lay eggs. After this 18 hour gap, flies are discarded and the egg density in the each vial is trimmed to around 150 per vial to start the next generation. The effective population size was controlled at around 450 for each these 9 populations. Calculation of N_e was done following Crow & Kimura (1970), $N_e = 4N_mN_f/(N_m+N_f)$. I discuss the N_e and possible effect of genetic drift in these populations in the following section.

Effective population size and effect of genetic drift in M, C and F regimes:

The method of calculation of N_e given by Crow and Kimura (1970) mentioned in the previous section assumes random mating in the population and therefore have limited application to the populations under study. For a population with female multiple mating and sperm precedence, the effective population size (N_e) is given by:

$$N_e = \frac{4N_F N_F}{N_F\left(P_1^2+P_2^2+P_3^2\right)+N_F}$$ (Rice and Holland 2005)

For M-populations:

$$N_e = \frac{4\times152\times152}{152(0.036^2+0.154^2+0.81^2)+152} = 361.67$$

(Assuming each female mates with 3 males and P1=0.036, P2=0.154 and P3=0.81. These values were used by Rice and Holland (2005) and comes from the LH-base population)

For C-populations:

$N_e = \frac{4\times224\times224}{224(0.19^2+0.81^2)+224}$ = 529.49

(Assuming each female mates with two males. This seems to be a reasonable assumption since in previous studies (Stewart et al. 2005), it has been shown that most of the females of this population mate twice. P1=0.19, P2=0.89 (Rice and Holland 2005))

For F-populations:

$N_e = \frac{4\times456\times456}{456(0.19^2+0.81^2)+456}$ = 1077.89

(Assuming each female mates with two different males)

$N_e = \frac{4\times456\times152}{(456+152)}$ = 456

(Assuming each female mates with only one male, there is no sperm competition, thus I stick to the original definition of N_e)

The actual Ne of the F populations is likely to be in between the above two values.Harmful mutation with a selection coefficient – s can only accumulate in a population if $|s| < 1/N_e$ (Kimura 1983).

For M-populations: mutations with $|s| < 0.0028$ can accumulate.

For C-populations: mutations with $|s| < 0.0019$ can accumulate.

For F-populations: mutations with $|s| < 0.0009$ can accumulate (assuming $N_e = 1077.89$).

These values give the upper bound of the magnitude of the mutations that can accumulate in the populations. This upper bound in M-population relative to that of the C and F-populations are 0.0009 (0.0028-0.0019) and 0.0019 (0.0028-0.0009). These values are quite small and indicate that virtually all mutations that are expected to accumulate in the M-populations are also likely to accumulate in the C and F-populations (See Rice and Holland 2005).Additionally, time to fixation for a neutral or mildly deleterious allele is $4N_e$ (Kimura 1983). A conservative measure of change in the frequency of such an allele in 60 generations (i.e., within the time frame of the present experiment) is given by:

$$\Delta p = \frac{60}{4N_e}$$

Following the above mentioned relation, Δp for M, C and F (assuming N_e of F-populations to be 456) populations would be 0.042, 0.028 and 0.033 respectively. These values are sufficiently small and comparable across the three regimes. Hence, these values reassure that the effect of differential genetic drift across the different populations in the present study is very likely to be negligible.

Generation of experimental flies: Standardization:

The three regimes differ in the adult environment in terms of different operational sex ratios. Although, all other conditions were kept constant across all the regimes, it is likely that the selection (different operational sex ratio) itself affected the adult condition in one way or the other. Thus it is possible to have some immediate parental effect in the progeny. In the assays described in the following chapters, the aim was to look at evolved genetic differences rather than the immediate parental effect. Hence it was important to equalize the parental effects across different regimes while running the assays. This was done by passing all the populations through one generation of standard maintenance, a process known as standardization (Rose 1984).

During standardization, eggs were collected from the selected populations at the density of 140-160 per 8-10ml of food in each vial. Flies were allowed to grow till adult stage for 12 days under the standard laboratory conditions, 25^{o}C temperature, 60-80% relative humidity, 12 hours light / 12 hours dark. No virgin collection was done. On 12th day post egg collection, flies are handled in one of the following two ways:

(a) Flies from different vials were mixed within each population and redistribute in fresh food vials (10-15 vial) containing food and limiting amount (but not measured) of live Yeast. They were allowed to remain in these vials for 2 days, following which they were transferred to oviposition vial having 8-10ml food. Flies were allowed to

oviposit for 17-18 hours before discarding them. Egg density in these vials was carefully trimmed to very close to 150 per vial. These vials were incubated under standard maintenance conditions and adults were collected for experiment.

(b) Flies of each of the populations were transferred to one fly cage (19cm×14cm×24cm) with a petri plate (90mm diameter) having food. The food was smeared with a paste of live Yeast (with water). They were allowed to remain in the cage undisturbed for the following two days before collecting eggs from them. To collect eggs, they were given a fresh food plate and allowed a window of 6 hours for oviposition. Females lay eggs on the surface of the food. Pieces of food having the desired number eggs (Usually 150±10) were transferred to food vials having 8-10ml of food. A variant of this method of collecting eggs was used while collecting eggs of 'exact' density. This is mentioned in Chapter 6.

Composition of Corn-meal food:

Sl. No.	Ingredient	Amount (per litre of food)
1.	Water (ml)	1000
2.	Agar powder (gm)	14.8
3.	Molasses (ml)	100
4.	Corn meal (gm)	100
5.	Baker's Yeast (gm)	41.2
6.	Propionic acid (ml)	8
7.	p-Hydroxymethyl benzoate (gm)	2.25
8.	Ethanol (ml)	22.5

Preparation: All the ingredients (except 6-8) are boiled in water to make a thick suspension. The suspension is cooled a bit from the boiling state before adding the preservatives – propionic acid (6) and a solution of p-Hydroxymethyl benzoate (7) in Ethanol (8). The hot food is then poured in vials and used after it has cooled down.

Chapter 3

Rapid evolution of adult competitive ability and components of reproductive behaviour

INTRODUCTION:

Unless there is a complete genetic monogamy, the two sexes rarely overlap in their evolutionary interests – leading to intersexual conflict (Chapman et al. 2003, Rice et al. 2006). As the two sexes invest differently in sexual reproduction, they differ in the strategies that maximise their fitness. While mating and/or fertilization success is the major determinant of male fitness, female fitness is usually limited by the number of gametes a given female can produce and how long she can live. While the males are benefitted by increasing the mating rate, females are not (Bateman 1948). This dichotomy between the evolutionary interests coupled with the fact that the two sexes share a common gene pool lays the foundation for intersexual conflict (Rice et al. 2006). One form of intersexual conflict, interlocus conflict, can potentially lead to a co-evolutionary arms' race wherein the sexes antagonistically influence the fitness of each other (Rice 2000, Arnqvist and Rowe 2005). Another form of intersexual conflict, intralocus conflict, arises from the expression of sexually antagonistic (SA) traits in both sexes leading to a genetic 'tug-of-war' (Prasad et al. 2007) between sexes over the expression of SA genes.

In intralocus conflict, optimum trait value of a trait (that is expressed in both sexes) differs between sexes. Genes that are beneficial to males are selected for in males, whereas they are selected against in the females due to some negative impact related to its expression in females and vice-versa. For example, in fruit flies, locomotor activity levels are positively

correlated between the sexes. However, 'high' activity is favoured in males (it possibly increases male mating success) whereas 'low' activity is favoured in females (possibly conserves energy and increases efficiency of favouring) (Long and Rice 2007). Hence, neither of the sexes can attain its optimum trait value, leading to a decline in fitness - termed as 'gender load'. In interlocus conflict, fitness of the two sexes is affected due to direct antagonistic interactions. During such conflict, traits that increase male fitness reduce female fitness as a by-product. Females in turn evolve resistance to such harming effects of the males, setting the stage for a co-evolutionary process. Thus, both the sexes prevent each other from optimizing their fitness, creating a gender load.

The role of sexual conflict in creating gender load was underlined by a number of experimental evolution studies. 'Male-limited evolution' allowed males in a population to evolve against a fixed target female phenotype thus effectively relaxing sexual antagonism (Rice 1996, 1998, Prasad et al. 2007). Such male limited evolution led to the increase in male fitness, indicating the role of sexual conflict in creating the gender load (Rice 1996, 1998, Prasad et al. 2007). Using a different approach of gender limited evolution (middle class neighbourhood design), Morrow et al. (2005) showed increase in fitness of the selected sex while decline in fitness of the opposite sex (unselected). A number of studies have documented evolution of fitness components in both sexes upon removal of sexual conflict by experimentally enforced monogamy (Holland and Rice 1999, Martin and Hosken 2003, Crudgington et al. 2005). However, it is important to know whether manipulation of the level of sexual conflict, as opposed to the complete removal of it, can affect fitness components of the two sexes. Previous studies have addressed this issue by looking at the response of populations to the experimental manipulation of operational sex ratio. Following this method of experimental evolution, Wigby and Chapman (2003) observed evolution of female fitness

component (viz. female resistance to male induced harm) in replicate populations of *Drosophila melanogaster* but the male fitness components remained unresponsive to such selection in this study. Michalczyk et al. (2010) observed the evolution of fitness components in both the sexes in response to the experimental manipulation of operational sex ratio in *Tribolium castaneum*.

Here I asked two questions of important consequences – (a) does alteration of the level of intersexual conflict result in the evolution of the competitive ability of adult males and females? (b) How do components of reproductive behaviour evolve in response to altered operational sex ratio? I addressed these issues by studying a set of laboratory adapted populations of *Drosophila melanogaster* subjected to altered levels of intersexual conflict. Intersexual conflict in this system is well studied and is discussed in Chapter 2 in detail. The level of intersexual conflict was manipulated in these populations by altering the operational sex ratio. The results suggest a rapid evolution of reproductive behaviour and adult fitness measures of the flies after just 8-12 generations of selection.

METHODS:

The detailed history and description of the three selection regimes (9 populations) are discussed in Chapter 2 (Experimental system), along with the details of the base populations used in this assay. The present set of assays was done after 8-12 generations of selection, followed by one generation of standardization.

Generation of experimental flies:

After one generation of standardization (see Chapter 2), the flies from all selected populations (M1-3, C1-3 and F1-3) were grown under controlled larval density (140-160 per 8-10ml food

in each vial). Adult flies were collected as virgins on 10^{th} day (post egg collection) and held in single sex vials at a density of 5 individuals per vial (for fitness assay) and 10 individuals per vial (for reproductive behaviour assay).

LH and LH_{st} flies were grown and collected under similar conditions as mentioned above and their ages were matched with the selected flies used in the assay. Virgin LH males and females were held in single sex vials as 10 per vial. Virgin LH_{st} males and females were held in single sex vials at a density of 15 per vial. For the reproductive behavious assay, LH males and females were generated and collected in the same way but they were held at a density of 10 per vial.

Male-fitness assay:

Competitive fitness of the selected males was assayed by allowing them to compete with ancestral males held along with ancestral females and then quantifying the proportion of the progeny sired by the selected males. Five 2-day old (post eclosion) virgin males (recessive scarlet eyed) from the selected populations (M1-2/C1-2/F1-2) were combined with 10 LH males (wild type red eyed) and 15 LH_{st} females (recessive scarlet eyed) in each 8-dram vial provisioned with food and 7mg live Yeast. 8-10 such vials were set up for each population. These vials were left undisturbed for the next two days. Following this, flies were sorted under light CO_2-anaesthesia and females were transferred into oviposition test tubes (dimension: 12mm×75mm) provisioned with food. Females were allowed to oviposit for 18 hours and then discarded. These test tubes were then incubated for the next 12 days under 25^oC, 60-80% relative humidity and 12 hours light /12 hours dark. The emerging adult flies from these test tubes were counted and scored based on their eye colour. As the scarlet-eye gene is an autosomal marker, progeny sired by the selected males were all scarlet eyed. Proportion of scarlet progeny averaged across all 15 females in a vial was taken as the

measure of mean competitive fitness of selection regime males in that vial. These vial means were taken as the unit of analysis.

Female-fitness assay:

Female fitness was quantified following a similar method as the one followed in the male-fitness assay. Five 2-days old virgin females belonging to one of the selected populations (M1-2/C1-2/F1-2) were combined with 10 virgin LH-females of the same age along with fifteen LH_{st}-males in an 8-dram vial containing food and 7mg live Yeast. 10 such vials were set up for each population. Female fitness critically depended upon their ability to forage and utilize this limiting quantity of Yeast. This quantity of Yeast matched the per female Yeast availability under the normal maintenance of the selected females. These vials were left undisturbed for the next two days. Following this the flies were sorted using light CO_2-anaesthesia and the selection regime females were transferred individually to oviposition test tubes (dimension: 12mm×75mm) provisioned with food and allowed a window of 18 hours for oviposition. This entire protocol of fitness closely matched the normal maintenance condition of the populations. Hence, the progeny produced by the selection regime females was taken as the absolute measure of their fitness under competitive condition. The number of progeny produced by all the five selection regime females in a vial was used to derive a vial mean. These vial means were taken as the unit of analysis.

Measures of mating behaviour of selected males and females:

Ten 2-days old (post eclosion) virgin males from one of the selected populations (M1-3/C1-3/F1-3) were combined with 10 virgin LH females of the same age in 8-dram observation vials in presence of food and 4.67mg live Yeast. Males and females were combined without the use of anaesthesia. Immediately following combination, these vials were manually

observed and number of copulating pairs was noted down over time. 10 such vials were set up for each population. The observation yielded the start and end time of the copulations. Using this raw data, average mating latency (time taken to start mating) and copulation duration (time for which the flies remained in copula) were calculated for each vial. Vial means were used as the unit of analysis. This way of observing mating latency and copulation duration is a standard method in this system (Byrne and Rice 2006, Nandy et al. 2012).

Selected females were assayed in the same way. Ten 2-day old virgin females belonging to one of the selected population were combined with 10 virgin LH-males in observation vials provisioned with food and 4.67mg live Yeast. The Yeast availability per female was same as that during the normal maintenance of the selected females. These vials were then observed for mating latency and copulation duration in the same way described above. 10 such vials for each of the 9 population were observed. Mean mating latency and copulation duration were calculated for each vial following the method described above. These vial means were used as the unit of analysis.

Courtship frequency:

I quantified the frequency of courtship behaviour (courtship frequency) performed by the selected males towards ancestral females. Five 2-days old virgin males from one of the selected populations (M1-3/C1-3/F1-3) were combined with five virgin LH-females of the same age in observation vial provisioned with food and 2.33mg live Yeast. For each of the nine populations, 10 replicate vials were observed. Courtship frequency was observed on 3rd and 4th day of adult life. During each of the observation days, 4 rounds of observations were taken. Each vial was manually observed for 30 seconds during one observation and during that period the total number of courtship events was recorded. Observations were done during the light phase of the Light/Dark cycle in a well lit place without the slightest of disturbances

and under standard laboratory conditions, i.e., 25^{o}C temperature, 60-80% relative humidity. Mean number of courtship events averaged across all 8 observations was calculated for each vial. These vial means were used as the unit of analysis.

A similar courtship frequency assay was done to quantify frequency of courtship by ancestral LH-males directed towards the selection regime females. For this purpose, five selection regime females were combined with five LH-males under similar conditions as mentioned above. 10 replicate vials were observed for each of the 9 populations. Courtship frequency was observed and analysed in the similar way as well.

Data analyses:

All the analyses were done using two-factor mixed model ANOVA with selection regime as fixed factor crossed with random blocks. STATISTICA (Statsoft, version 10) for windows was used for these analyses.

RESULTS:

Analyses of the male competitive fitness data revealed a significant main effect of selection regime (Table 3.1, Figure 3.1). Multiple comparisons using Tukey's HSD suggested a significant difference between the competitive fitness of M and F-males, with that of C-males being intermediate. Fitness of C-males was not significantly different from that of the other two regimes. Analysis of the female competitive fitness data revealed a significant interaction between selection regime and block in addition to the significant effect of the selection regime (Table 3.1). Due to the significant interaction between blocks, female fitness data from each of the two blocks were analysed separately using one-way ANOVA. In block-1, a significant effect of selection was found (SS=281.78, df=2, F=7.04, p=0.0036) with fitness of

M-females being significantly higher than that of both F and C-females (Tukey's HSD, Figure 3.2a). Fitness of F and C-females in block 1 was not significantly different (Tukey's HSD, Figure 3.2a). In block-2 I found a significant effect of selection regime (SS=591.83, df=2, F=11.84, p=0.0003) with fitness of F-females being significantly lower compared to that of the M and C-females (Tukey's HSD, Figure 3.2b). Fitness of the M-females was not different from that of C-females (Tukey's HSD, Figure 3.2b).

When selected males were combined with LH-females, no significant effect of selection regime on mating latency was observed (Table 3.2, minutes ±SE, F: 4.7±0.2, C: 5.15±0.2, M: 4.9±0.19), however copulation duration was significantly affected by selection regime of the males (p=0.01, Table 3.2, Figure 3.3). Tukey's HSD revealed that F-males mated significantly longer than both C and M-males, while there was no difference between copulation duration of the M and C-males (Figure 3.3).

When selected females were combined with ancestral LH-males, there was a significant effect of selection regime on mating latency (Table 3.2, Figure 3.4). M-females had significantly higher mating latency compared to both C and F-females (Tukey's HSD, Figure 3.4), whereas there was no significant difference between that of F and C-females. There was no significant effect of selection regime on copulation duration of the mating between selected females and LH-males (Table 3.2, minutes ±SE, F: 21.5±0.32, C: 21.4±0.43, M: 21.2±0.39).

Courtship frequency of selection regime males towards LH-females was significantly affected by selection regime (Table 3.2, Figure 3.5). Tukey's HSD showed that M-males had significantly higher courtship frequency relative to C-males. F-males were found to have

intermediate courtship frequency. Courtship frequency of LH-males towards selection regime females was found to have no significant affect of selection regime (Table 3.2, mean number of courtship events per observation ±SE, F: 3.34±0.29, C: 4.2±0.29, M: 4.12±0.29).

Trait	Effect	SS	MS Num	DF	F	p>F
(a) Male fitness	Selection regime	0.086	0.043	2	4.587	0.015*
	Block & random	0.001	0.001	1	0.076	0.784
	Block × Selection regime	0.014	0.007	2	0.729	0.487
(b) Female fitness	Selection regime	536.990	268.495	2	12.017	<0.001*
	Block & random	396.135	396.135	1	17.730	<0.001*
	Block × Selection regime	367.551	183.775	2	8.225	<0.001*

Table 3.1: Summary of the results of two-factor ANOVAs using selection regime as fixed factor crossed with random blocks on (a) male competitive fitness and (b) female fitness under competitive condition. For males, the proportion of progeny sired by each selection regime male in a vial was calculated. These vial means were then used as the unit of analysis. For females, mean number of progeny produced by each selection regime female in a vial was calculated. These vial means were used as the unit of analysis. p-values marked with * indicate significant effect.

Trait	Effect	SS	MS Num	DF Num	F	p>F
A. Reproductive behaviour of selected males						
Courtship frequency	Selection regime	23.36	11.68	2	3.27	0.043*
	Block&Random	46.56	23.28	2	6.51	0.002*
	Selection regime × Block	3.03	0.76	4	0.21	0.931
Mating latency	Selection regime	2.55	1.28	2	1.24	0.295
	Block&Random	1.92	0.96	2	0.93	0.398
	Selection regime × Block	5.88	1.47	4	1.43	0.233
Copulation duration	Selection regime	35.89	17.95	2	4.84	0.011*
	Block&Random	28.01	14.00	2	3.77	0.028*
	Selection regime × Block	3.91	0.98	4	0.26	0.900
B. Reproductive behaviour of selected females						
Courtship frequency	Selection regime	5.72	2.86	2	1.10	0.338
	Block&Random	12.84	6.42	2	2.46	0.091
	Selection regime × Block	2.58	0.64	4	0.25	0.910
Mating latency	Selection regime	8.85	4.42	2	7.42	0.001*
	Block&Random	2.58	1.29	2	2.16	0.122
	Selection regime × Block	2.32	0.58	4	0.97	0.428
Copulation duration	Selection regime	0.84	0.42	2	0.09	0.912
	Block&Random	16.55	8.28	2	1.83	0.168
	Selection regime × Block	7.64	1.91	4	0.42	0.792

Table 3.2: Summary of separate two-factor ANOVAs using selection regime as fixed factor crossed with random blocks on (A) courtship frequency, mating latency and copulation duration of selection regime males (assayed against LH-females), (B) courtship frequency of LH-males towards selection regime females, mating latency and copulation duration of selection regime females (assayed against LH-males). For all the analyses vial means (see methods) were taken as the unit of analysis. p-values marked with * indicate significant effect.

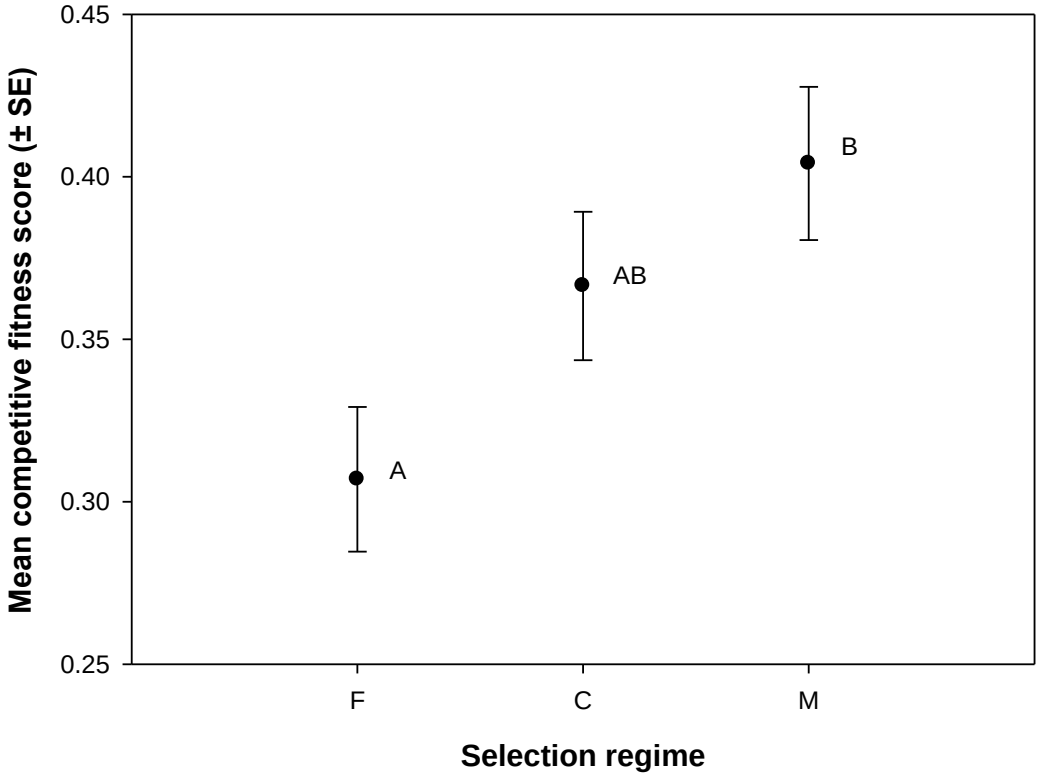

Figure 3.1: Competitive fitness of the selection regime males. Mean proportion of progeny sired by each selection line males in each vial was calculated using the raw progeny count data. These vial means were then used as the unit of analysis. Points not sharing common letters are significantly different (determined using Tukey's HSD).

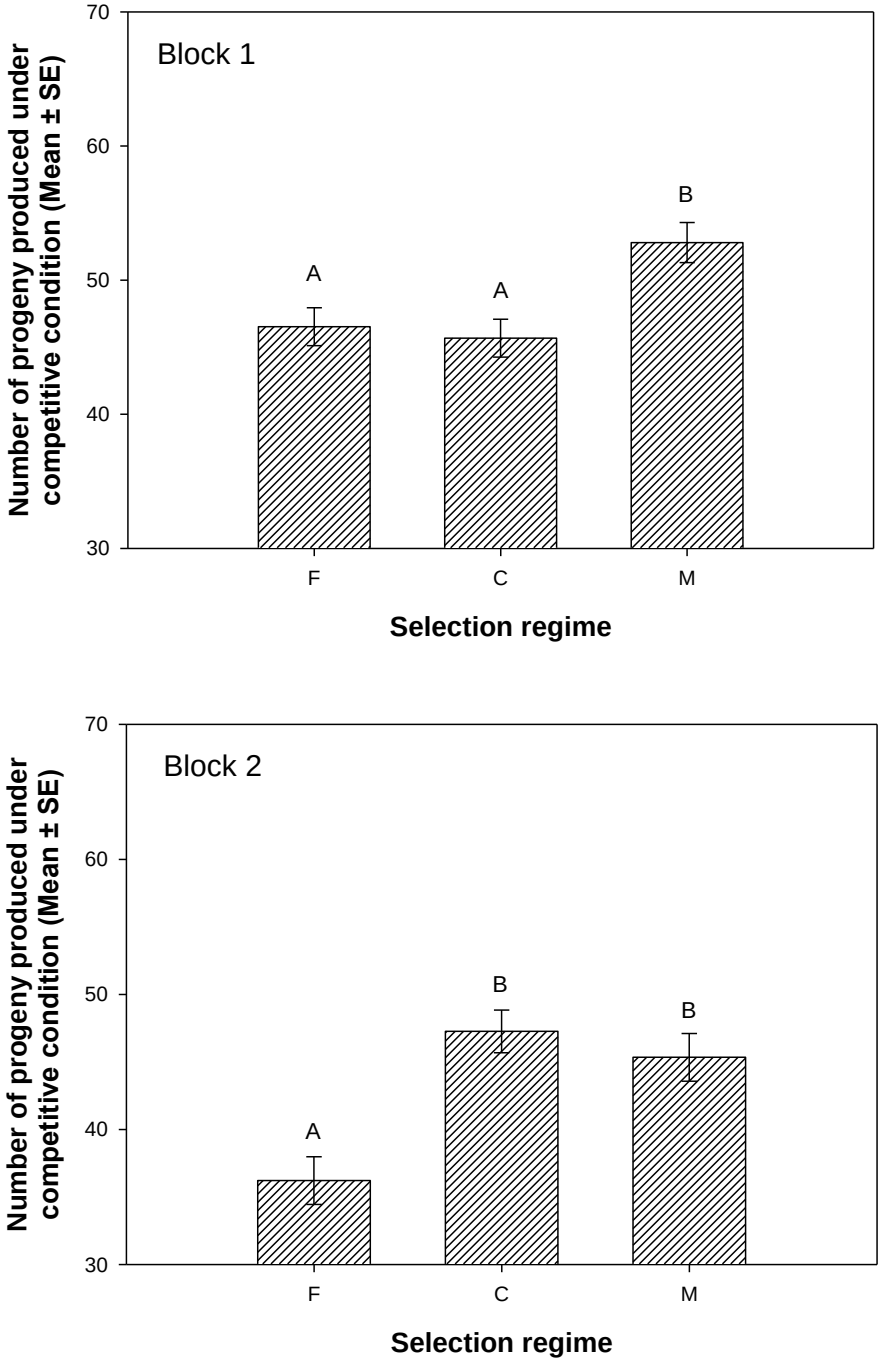

Figure 3.2: Fitness (progeny count) of the selection regime females under competitive condition. Mean number of progeny produced by each selection regime female was calculated. These vial means were then used as the unit of analysis. Points not sharing common letters are significantly different (determined using Tukey's HSD).

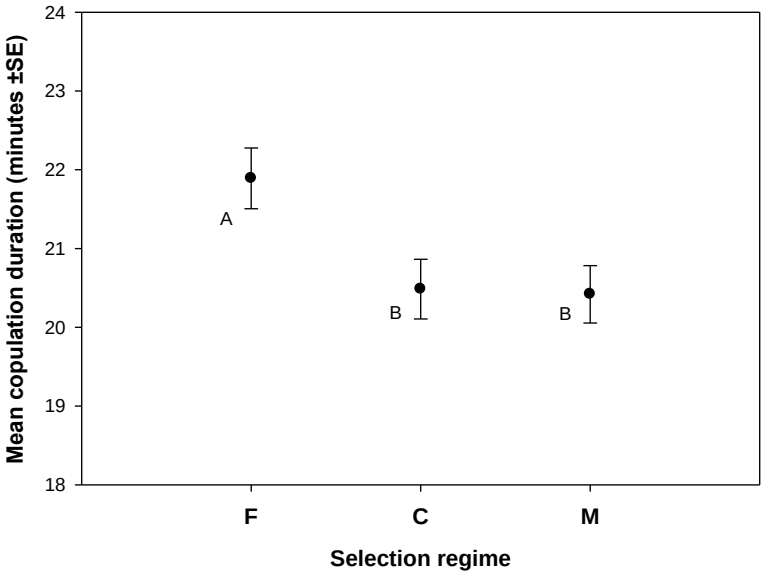

Figure 3.3: Copulation duration of the selection regime males assayed against LH-females. Mean copulation duration of each vial was calculated using the raw data. These vial means were then used as the unit of analysis. Points not sharing common letters are significantly different (determined using Tukey's HSD).

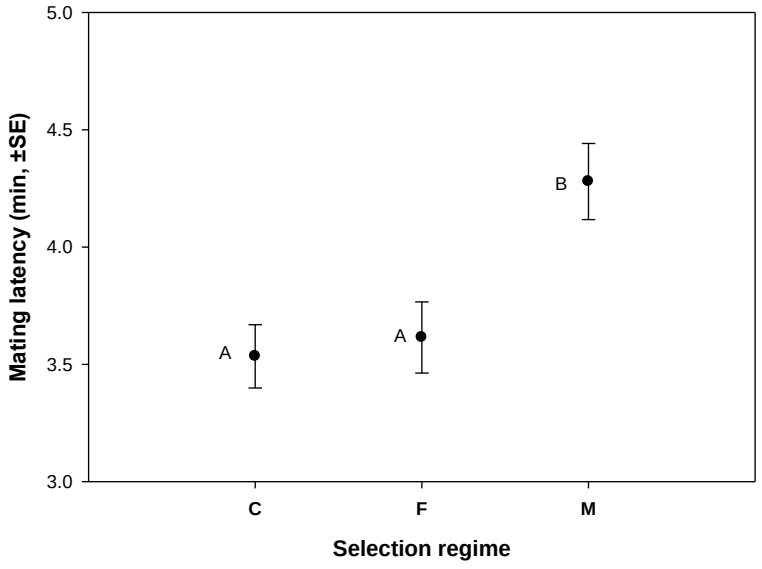

Figure 3.4: Mating latency of selection regime females assayed against LH-males. Mean mating latency was calculated for each vial using the raw data. These vial means were then used as the unit of analysis. Points not sharing common letters are significantly different (determined using Tukey's HSD).

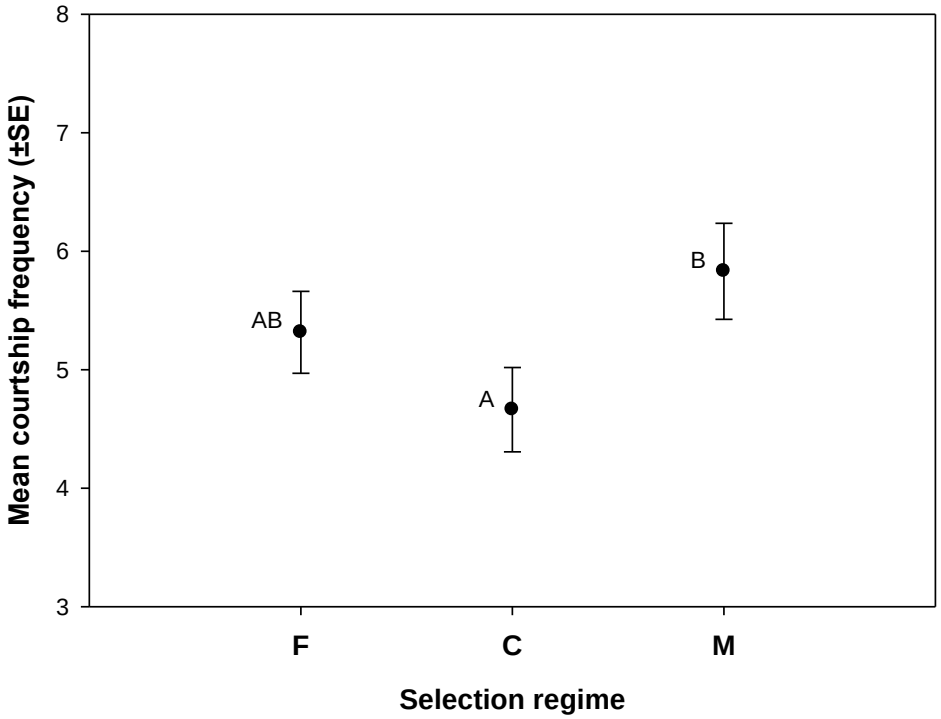

Figure 3.5: Courtship frequency of selection regime males towards LH-females. Mean courtship frequency per observation was calculated for each vial. These means were then used as the unit of analysis. Points not sharing common letters are significantly different (determined using Tukey's HSD).

DISCUSSION:

The present study demonstrates the evolution of male and female adult competitive fitness for the first time. In addition it shows the evolution of components of reproductive behaviour under the influence of altered operational sex ratio. Competitive ability in males evolved rapidly under both male biased (M) and female biased (F) operational sex ratio. Male fitness was found to be higher in M-regime compared to that in the F-regime, with C-males having intermediate competitive fitness. Female fitness under competitive condition was found to have diverged between M and F-regimes, with M-females having higher fitness compared to F-females. Significant changes in the components of reproductive behaviour of males were also observed. M-males were found to court more often and F-males were found to mate for longer duration. Although female competitive ability showed significant divergence between M and F-regimes, the trait value for C-regime relative to those of the other two regimes was not consistent across blocks. However, M-females of all three blocks showed significantly higher mating latency. Other components of reproductive behaviour in both sexes did not differ significantly across different selection regimes.

Response of male competitive fitness and components of reproductive behaviour:

In my laboratory adapted population, the flies have a short generation time of 14 days, of which 4-5 days consist the adult phase. Hence, selection acts on males within these ~4 days of reproductive life. Previous studies, using the same population have reported evolution of male competitive fitness and/or fitness components under (a) experimentally enforced monogamy (Holland and Rice 1999) and (b) male-limited evolution (Rice 1996, 1998, Prasad et al. 2007). The selection regime in my study altered the level of male-male competition in the populations and intersexual conflict by varying the operational sex ratio, with male biased operational sex ratio representing a three-fold increase compared to the ancestral equal sex

ratio condition and the female biased operational sex ratio representing a decline of similar magnitude. Thus one would predict the M-males to evolve increased adult competitive ability and hence have higher competitive fitness. Competitive fitness of F-males on the other hand is expected to decrease in response to the selection, given that under F-regime selection on male competitive ability is relaxed many fold. In line with such prediction, I found increase in male competitive fitness of M-males and decrease in the same in F-males. My study is one of the very few evidences showing evolution of male competitive fitness in response to alteration in operational sex ratio in fruit flies. Previous studies, using a similar selection protocol (but different base population of *D. melanogaster*) did not find any response in components of male fitness (Wigby and Chapman 2003). However, such response of male fitness was observed in flour beetles (*Tribolium castaneum*), where populations subjected to male biased and female biased operational sex ratio significantly diverged with respect to the male adult competitive ability after 20 generations of selection (Michalczyk et al. 2010). The males from the male biased operational sex ratio were found to have higher competitive ability compared to those from the female biased operational sex ratio.

Previous studies reported evolution of competitive fitness or other components of male fitness under a range of experimental evolution approach. Under male-limited evolution, while Rice (1996, 1998) observed increase in male fitness associated with an increase in sperm competitive ability and ability to cause mate-harm, Prasad et al. (2007) did find increase in male fitness but neither was it associated with any measurable change in sperm competitive ability nor with any change in mate-harm (Jiang et al. 2011). Populations described by Prasad et al. (2007) evolved increase in male fitness possibly by evolving more efficient courtship (Bedhomme et al. 2008). Holland and Rice (1999) observed males under enforced monogamy to have evolved reduced courtship activity, however, competitive fitness of such males were not measured. Flour beetles males, in the study reported by Michalczyk et

al. (2010), evolved increased competitive fitness under male biased regime possibly by becoming more efficient at inducing mating and by investing more per copulation. However, they did not observe any change in the males' sperm-offence and defence ability (Mychalczyk et al. 2010).

I recorded two important trends in the male reproductive behaviour. First, M-males were found to court the ancestral LH-females at a higher rate (see courtship frequency result). Given that courtship vigour is positively correlated with male mating success (Markow 1987), M-males' increased competitive success can at least in part be attributed to such behavioural difference. Under my assay condition, it is however difficult to distinguish between mating success and fertilization success. Secondly, copulation between F-males and ancestral LH-females were found to last for significantly longer duration, probably indicating increased ejaculate (sperm and/or seminal proteins) investment by F-males while mating with virgin females. Theories of sperm competition predict that under last male sperm precedence (as in my system), males should invest more in sperm defence, especially when risk of sperm competition (measured as female re-mating rate, number of competitors present, sex ratio) is not very high (Parker 1990, Enqvist and Reinhold 2006). The F-regime represents a significantly reduced risk of sperm competition and hence the observed result is possibly what one might predict based on the theories of sperm competition. However, the theoretical models mentioned above consider variation in sperm number only, while the duration of copulation represents the quantity of sperms and/or other components of ejaculate. Previous studies (Gilchrist and Partridge 2000) have shown that only a small part of the copulation represents 'sperm transfer', the rest representing transfer of ejaculate minus sperm (i.e., seminal proteins). Thus the increased copulation duration might represent (a) increased sperm transfer, (b) increased transfer of seminal proteins or (c) both. The present results cannot be

used to differentiate between these alternatives. I did not observe any significant change in the mating latency of the selected males, indicating that the efficiency of courtship of the selected males possibly did not evolve.

One important consequence of evolution in male fitness is the possibility of changes in their ability to inflict mate-harm on females. This was observed in the male-limited evolution studies by Rice (1996, 1998). However, Jiang et al. (2011), working with the same base population and a very similar selection design, could not reproduce the same results. I address this issue in Chapter-4a with the results from assays done after >45 generations. In addition, another important component of male fitness, sperm competitive ability, is likely to be affected by the selection. I address this in Chapter-4b.

Response of female competitive fitness and components of reproductive behaviour

I found the M-females to have significantly higher fitness relative to F-females. The possible explanation for such observation is discussed below.

Fitness of a female is thought to depend on the number of eggs she can produce, how long she can live and the quality of males she mates with (Bateman 1948). In *Drosophila*, mating has been shown to have a negative impact on female fitness in terms of survivorship (Partridge et al. 1987, Fowler and Partridge 1989, Chapman et al. 1993, 1995) and fecundity (Chapman et al. 1995, Holland and Rice 1999, Prout and Clark 2000, Pitnick and Garcia-Gonzalez 2002, Friberg and Arnqvist 2003, Linder and Rice 2005, Kuijper et al. 2006,). Hence, any form of resistance to such male-inflicted harm can be expected to bring large fitness advantage to the females.

In this system, female fitness is also determined by their access to live Yeast (Stewart et al. 2005, Nandy et al. 2012). As Yeast is given in limiting quantity, female's efficiency to forage for Yeast, especially under competitive condition, is expected to have large fitness consequence.

In the selection imposed during the present experiment, M-females can increase their competitive fitness either by evolving better resistance to mate-harm or by increasing their Yeast foraging efficiency. The females in the male biased (M) regime experienced nine times more male interactions and hence equally higher mate-harm compared to the female biased (F) regime. Therefore selection on female for resistance to mate-harm can be expected to be intense under M-regime and much weaker in F-regime. Since competitive fitness of the selected females was assayed against the mate-harm imposed by ancestral males, it is possible that the fitness difference observed in the results represents the difference in ability of females of F and M-regime to resist mate-harm.

Alternatively, the results might represent differential abilities of M and F-females to forage for Yeast. Under male biased operational sex ratio, M-females are expected to experience higher amount of mate-harm relative to the F-females. This condition is likely to select females for investing more in mate-harm resistance related activities, which are likely to be energetically costly. Under such conditions, M-females can potentially evolve increased resource acquisition or more efficient resource utilization or both. If this is true then such a female trait is likely to influence the fitness estimates, since the fitness assay was done under a controlled Yeast availability. However, further experiments are required to test this hypothesis.

Behavioural observations showed that virgin M-females took significantly longer time (~21% longer compared to C-females and ~18% longer compared to F-females) to start mating with LH-males (Figure 3.4). This possibly indicates M-females increased resistance to the mating attempts by LH-males, corroborating well with the previously mentioned explanation of the fitness results. I did not find any other significant behavioural differences between the females from different selection regimes.

At least two previous studies have reported evolution of resistance to mate-harm in response to alterations in operational sex ratio. Michalczyk et al. (2010) worked on *Triboleum* to show that upon 20 generations of selection females from the female biased operational sex ratio suffered a cumulative cost of re-mating while females from the male biased operational sex ratio were virtually unaffected by re-mating. In *Drosophila*, females evolved under female biased operational sex ratio were found to evolve increased susceptibility to mate-harm (in terms of mortality) after around 50 generations (Wigby and Chapman 2004). However, none of these studies measured female fitness under competitive condition. Hence, my study is the first evidence of its kind showing that the advantage/disadvantage of increased/decreased resistance to mate-harm is also expressed under competitive condition – a situation females are more likely to encounter naturally and therefore more relevant evolutionarily.

CONCLUSION:

I showed that competitive fitness of both males and females evolves rapidly as a response to experimentally imposed alterations in the level of sexual conflict in the population of fruit flies – a novel finding consistent with the theories of sexual conflict. Competitive fitness was

found to be higher in both sexes evolved under male biased operational sex ratio (representing increased sexual conflict). While increase in male fitness under M-regime was associated with increased courtship activity, response of female fitness was likely to represent evolution of females' ability to resist mate harm.

Chapter 4

Evolution of male traits

Chapter 4a

Evolution of mate-harm, longevity and behaviour in males

INTRODUCTION:

In most sexually reproducing species, males compete for access to females. This competition amongst males can potentially result in male specific adaptations including the ability to manipulate their mates – either physically or physiologically. As a by-product of such manipulation, males often end up causing fitness depression in females (Chapman et al. 2003). Such effect of males on female fitness is generally called mate-harm (Jiang et al. 2011). Mate-harm in turn selects for increased resistance to male-induced harm in females. This dynamic conflict between the two sexes, commonly known as interlocus sexual conflict, can potentially lead to open ended cycles of adaptation and counter adaptation – reminiscent of the "Red queen" between prey-predator or host-parasite (Van Valen 1973, Ridley 1993).

This form of conflict has been predicted to be one of the most important forces of evolution, potentially affecting evolution of life-history (Wedell 2005) and aging (Promislow 2003, Bonduriansky 2008) as well as promote speciation (Rice 1997, Gavrilets & Hayashi 2005, Martin and Hosken 2003a, Rice et al. 2005).

There are two aspects of interlocus conflict – (a) evolution of male traits related to "mate-harm ability" and (b) evolution of traits related to "resistance" in females. Here, I focus on the male part of the conflict. In addition to the evolution of mate-harming ability in males, I also explore the evolution life-history traits in males.

The mechanisms of mate-harm vary across species (reviewed in Koena 2012, more details see Arnqvist & Rowe 2005). It can range from purely physiological (mediated through chemical transferred to females during mating) to mechanical (injuries caused during mating). For example, in fruit flies (*Drosophila melanogaster*) mate harm is caused both by physical coercion during courtship (Fowler & Partridge 1989, Kuijper et al. 2006, Rice et al. 2006) and by physiological manipulation mediated through Accessory Gland Proteins (Chapman et al. 1995, Wolfner 1997). Due to these, females suffer mating costs both in terms of fecundity as well as longevity (Fowler & Partridge 1989, Kuijper et al. 2006, Rice et al. 2006). In water striders (Rowe et al. 1994) and bean weevil (Crudgington and Siva-Jothy 2000) on the other hand, mate-harm happens principally through mechanical route, leading to enhanced mortality in females.

A number of experimental evolution approaches have addressed the interesting dynamics related to the evolution of mate-harm and other components of male reproductive behaviour. However, there is still substantial discord across different studies. In one approach, populations were either released from sexual antagonism (by artificially enforcing life-long monogamy) or were subjected to sexual antagonism (by maintaining the normal polygamous mating system). Following several generations of selection, monogamous males were found to be more benign compared to polygamous males (Rice and Holland 1999, Pitnick et al. 2001, Martin and Hosken 2003, Crudgington et al. 2005). Removal of sexual conflict by experimentally enforced monogamy has been shown to select for reduced investment in sperm production (i.e., decrease in testes size) relative to the polygamous condition (Hosken et al. 2001, Pitnick et al. 2001, Simmons and Garcia-Gonzalez 2008). These studies indicated the maintenance cost of the relevant male traits. In another approach, populations were subjected to male-limited evolution, wherein males were allowed to evolve with respect to a

fixed target female phenotype (Rice 1996, 1998, Prasad et al 2007). Both the studies reported increase in male fitness in absence of the gender load. Rice (1996) found that males from the male-limited populations were more harming to their mates. However, in other studies using the same approach (and closely related base populations) males from the male-limited populations had higher fitness than the males from the control populations but the two types of males did not differ in their mate-harming ability (Prasad et al 2007, Jiang et al 2011). Yet another approach has been to experimentally evolve populations under different levels of sexual antagonism generated by varying the operational sex ratio of the populations (Wigby and Chapman 2004, 2006, Crudgington et al. 2005, Linklater et al. 2007, Michalczyk et al. 2010). In all these studies, females' ability to resist mate-harm has been found to evolve in response to such selection. Crudgington et al. (2005) found males evolved under male biased operational sex ratio to be more harming to their mates relative to males evolved under enforced monogamy. However, Wigby and Chapman (2004) found males' harming ability to be unresponsive to the selection (alteration of operational sex ratio). Thus from the multifarious results observed in a range of experimental evolution studies, it appears that the issue of evolution of mate-harm and male reproductive behaviour and/or physiology under intersexual conflict is far from being settled.

Theories suggest that males under stronger sexual conflict should evolve increased investment in sexual reproduction and related traits at the cost of faster aging and shorter life-span (Promislow 2003, Bonduriansky 2008). However, only few studies (Maklakov et al. 2007, Maklakov et al. 2009, Maklakov and Fricke 2009) have so far addressed the correlation between sexual conflict and evolution of life-span and aging. Maklakov et al. (2007) and Maklakov and Fricke (2009) did not find any effect of artificially imposed monogamy or polyandry on the life-span and rate of aging of the males of their study populations. However,

Maklakov et al. (2007) observed female life-span and aging rate to evolve in response to the changes in mating system in the population. Thus it is important to test whether evolution under different levels of sexual conflict leads to the predicted (Promislow 2003) changes in life-span and rate of aging.

Here I ask the following questions: (a) Does the ability of males to cause mate-harm evolve under different levels of sexual conflict? (b) If mate-harm evolves under such condition, how do males become more harming? Do their behavioural traits, such as courtship frequency and spontaneous locomotor activity, respond to such selection? (c) Is there a longevity cost to adaptation to varying levels of sexual conflict?

I present the results of an experimental evolution study addressing interlocus conflict. Three replicate populations of *Drosophila melanogaster* were subjected to three different levels of sexual antagonism by manipulating the operational sex ratio – male biased (M), equal sex ratio (C) and female biased (F). Intensity of interlocus conflict is expected to be high under male biased condition, moderate under equal sex ratio and low under female biased regime. I then quantified (a) harming ability of the males (in terms of both mortality and fecundity), (b) courtship frequency, (c) spontaneous locomotor activity and (d) longevity and rates of aging under both mated and virgin conditions.

METHODS:

The experiments were done on the males belonging to the nine populations of *Drosophila melanogaster* – M1-3, C1-3 and F1-3 representing male biased, equal and female biased operational sex ratio respectively described in Chapter 2. After standardization (see Chapter 2 for details), eggs were collected for the generation of the experimental flies at a density of

130-150 per vial (8-10ml of cornmeal food). On 10^{th} day after egg collection, males were collected as virgins during the peak of their eclosion and held as single sex vials at an adult density of 10 per vial (for mate-harm assay), 8 per vial (for longevity-assay) and 5 per vial (for courtship frequency assay and locomotor activity assay).

LH females used in this experiment were raised in similar conditions and collected as virgins during the peak of eclosion. Virgin LH-females were held as single sex vials in groups of 8 per vial (for mate-harm assay and longevity assay) and 5 per vial (for courtship and locomotor activity assay). Eggs for LH flies were collected on the same day as that of the selection lines. Thus for all populations the age of the experimental flies were same during the experiment.

A. Dry body weight of selection line males

This was done after 45-47 generations of selection. Freshly eclosed males were flash-frozen. The frozen flies were dried at 60^{o}C for 48 hours and weighed in a high precision electronic balance (Sartorius CPA225D) to the nearest 0.01mg. A total of 50 males per population were measured for body weight distributed in ten groups of 5 each. Mean body weight of each group was calculated and taken as the unit of analysis.

B. Mate-harm assay: Fitness of females exposed to selected males

This assay was also done after 45-47 generations of selection. Fitness (progeny produced) of LH females exposed to the selection line males were assayed under two conditions – singly mated (SM) and continuously exposed (CE). In case of SM, 8 virgin females (2-day old) were transferred into fresh mating vial (seeded with 3.736mg live Yeast) along with 10 virgin males (2 day old) from one of the nine populations (M1-3, C1-3, F1-3). Combinations were

done without anaesthesia. For each population, 20 such vials were set up. Of these, 10 were randomly assigned to SM and 10 to CE. In the SM set, males and females were allowed to interact for one hour. In our flies, this period is just enough to complete a single mating. After one hour, males and females from the SM set were separated under light CO_2 anaesthesia, males were discarded and the females returned to the same vials. They were then held for two days before oviposition. In the CE set, males and females were allowed to interact continuously for two days. After the two day period, females from the SM and CE sets were transferred to oviposition bottles (Laxbro, FLBT 20, 60mm diameter × 140mm height) with ample amount of food (8 females per bottle). Females were allowed a window of 18 hours for oviposition, after which they were discarded. The eggs were incubated at 25^{o}C for 12 days and frozen at -20^{o}C upon complete eclosion of the progeny. The bottles were checked for any sign of crowding. The progeny count was taken as measure of female fitness which can be compared across the different selection regimes. The total number of progeny produced in each vial (progeny count of a bottle) was taken as the unit of analysis.

C. Longevity assay

The longevity assay was done after 50 generations of selection. Longevity of selected males was measured under two conditions – (a) mated and (b) virgin. For the "mated" set, 8 virgin females (2day old) from the LH population were combined with 8 males (2day old) from one of the nine populations (M1-3, C1-3, F1-3) in a vial seeded with 3.74mg live Yeast. 10 such vials were set up for each of the nine populations. For the "virgin" set, 8 virgin males (2 day old) were transferred to Yeasted vials (3.74mg) without females. For this set as well, 8-10 vials were set up per population. Flies were transferred to fresh food every alternate day without anaesthesia. Dead flies were sexed and counted during every transfer. For mated set, sex ratio was maintained at 1:1 by introducing LH-female(s) (in case of a LH female death)

or removal of female(s) (in case of a male death) into a vial. Extra LH females were maintained as a separate set under similar conditions (uncrowded, equal sex ratio, ample food) with LH males. On day 49, I ran out of replacement females (either they were dead or in very bad condition) and thus the replacement had to be stopped. I calculated mean longevity of the selection regime males for each vial using the mortality data. During the analysis of mean longevity, these vial means were taken as the unit of analysis. During the entire assay period I also recorded number of death of LH-female in each vial in addition to keeping track of the mortality of selection line males. I used data of the death of ancestral females as an indicator of the mate-harm of the males that they were housed with. I used two measures of mate harm (a) total number of ancestral females that died in a given vial (analysed at the level of vials) and (b) the mortality rate of females. To calculate female mortality rate, the cumulative weekly female mortality was regressed against time (weeks) and the best fit slope was taken as the mortality rate. This was done for each population. Since female deaths observed during the entire course of the longevity experiment was used to calculate this 'rate', it is essentially the per week mortality rate of females averaged across the entire life-span of the selection regime males. Mortality rates were analysed using the population estimates.

I analysed the rate of aging of the selection line males in the following two ways:

(a) Gompertz model: age dependent and age independent rate of aging

The raw mortality data was used to derive instantaneous mortality at time t. This mortality was affected by three factors - mating status [i = 1 (mated), 2 (virgin)], selection regime [j = 1 (C), 2 (F), 3 (M)] and block [random, k = 1, 2 and 3]. The mortality data was fitted in the Gompertz model using R (version 2.14.1), nlme-R package. The three above mentioned

factors were built in to the model. Mortality between time t-2 and t (μ_{ijkt}) was modeled in the following way:

$\mu_{ijkt} = f_{ijkt} + €_{ijkt}$

where, $€_{ijk}$ is random error and $f_{ijkt} = 1 - \frac{p_{t-2}}{p_t}$

here p_t and p_{t-2} are defined as

$p_t = \frac{a_0}{a_1}\left(e^{1-e^{a_1 t}}\right)$, and $p_{t-2} = \frac{ao}{a1}\left(1 - e^{1-e^{a_1(t-2)}}\right)$

Hence, the function, f represents the Gompertz model (Jafari et al. 2007).

a_o and a_1 are commonly referred to as "age independent" and "age dependent" rates of aging respectively. These can be broken down into linear expressions:

$$a_{0ijk} = \alpha_0 + \delta_i\beta_0 + \delta_j\gamma_{0j} + \delta_i\delta_j\varphi_{0ij} + b_{0k}$$

$$a_{1ijk} = \alpha_1 + \delta_i\beta_1 + \delta_j\gamma_{1j} + \delta_i\delta_j\varphi_{1ij} + b_{1k}$$

Where, α, β, γ, φ are coefficients and b is the random error within populations, derived by fitting experimental data into Gompertz model. δ_s is the Kronecker delta, i.e., $\delta_s = 0$, when s =1, otherwise $\delta_s = 1$. b_k is a random factor, reflecting population effect and was found to be very small in my experiment (of the order of 10^{-9}) and hence, ignored. The model also gave the standard errors associated with these parameters and the correlation coefficients between these parameters.

(b) Age specific survivorship rates:

To see whether survival rates (mirror image of mortality rate) at different ages have changed across different populations, I did age specific survival rate analysis. Due to the heterogeneity of female life span across different mating status, fixed age windows could not be analysed. Instead, in each of the four type of analyses (mated/virgin and male/female) the entire life span of all flies was divided into four windows – Phase 1 (day-1 to 50% of the maximum

lifespan), Phase 2 (50-67.67% of the maximum lifespan), Phase 3 (67.67-83.33%) and Phase 4 (83.33% to maximum lifespan). In each of these windows, mean survival rate was analysed. Mean survival rate for a given vial was calculated as the proportion of individuals surviving at the end of the window divided by the proportion of individuals surviving at the start of the window in that vial. These vial means were then taken as the unit of analysis. Similar method of analyzing age specific survival rate was adopted by Adler and Bonduriansky (2011). Survival rate for Phase 4 is by definition, either zero or indeterminate. Therefore three age classes were analysed for survival rate.

LH-female mortality:

The design of the experiment allowed us to measure and compare selected males influence on females' mortality. Throughout the longevity assay, I recorded the deaths of LH-females in the 'mated'-longevity vials. I analysed the total number of female deaths across the three regimes. For this, total number of female deaths were calculated for each vial and these vial estimates were then used as the unit of analysis. I also analysed rate of mortality. Rate of mortality was derived by regressing cumulative week-wise mortality of ancestral females across all vials against time (in weeks) for each of the nine populations (M1-3, C1-3, F1-3) separately. The least square fit slope was taken as the LH-female mortality rate (mortality per week). This mortality rate could only be calculated for each population. Therefore, the mortality rate of each population was taken as the unit of analysis.

D. <u>Courtship frequency assay</u>

Courtship frequency of the selection line males was measured after 51-55 generations of selection. On 2nd day after eclosion, virgin selection line males (from one of the 9 populations) were combined with virgin LH-females (5 males: 5 females) in vials with

standard food and supplemented with live yeast (2.33mg per vial). 10 such vials were set up per population. The vials were then returned to the incubator where they were maintained for a day at standard conditions. Courtship frequency was assayed on the two subsequent days during the light phase of the 12:12 LD cycle. On each day of observation, the vials were placed under uniform over-head lighting at 25^{o}C. Observations started 3 hours after lights-on. Each vial was observed four times a day (total of eight observations over two days). Observations were spaced 1 hour apart. During each observation, a vial was observed for 30 seconds and total number of courtship events (chase, wing-flap, mounting-attempt etc.) was recorded. All vials were numerically coded to ensure a complete blindness of the observer to the identity of the males under observation. I calculated the mean number of courtship per vial per observation from the raw data and this was used as the unit of analysis.

E. Spontaneous locomotor activity

After 51-55 generations of selection, spontaneous locomotor activity of the selection line males was assayed. Activity of the males was measured using focal sampling method under a set up identical to that described in the previous section. On 2nd day post eclosion, virgin selection line males (from one of the 9 populations) were combined with virgin LH-females (5 males: 5 females) in observation vials with standard food supplemented with live yeast (2.33mg per vial). 10 such vials were set up per population. Activity was observed on 3rd and 4th day (post eclosion). The observation vials were divided into four equal sized regions by marking the surface of the vials with a marker. Each region was then numbered for identification. During each observation, a region of a given vial was selected with the help of a random number generator. The individual present in the chosen region was observed. Occasionally more than one individual was present in the selected region and in such situation one out of them was observed. If the chosen region did not have any individual,

another random number was generated and the process repeated until a target individual was spotted. Each observation consisted of watching a focal individual for two successive 4-second intervals. If the focal individual showed any displacement within this interval, it was scored as being active. Each vial was observed thrice in a given round of observation. There were four rounds of observation each on days 3 and 4 post eclosion. The observations started 3 hours post lights-on in their 12:12 LD cycle. Each round of observation was spaced one hour apart. Observer bias was controlled by randomization of the method of selecting the focal individual and by making the assay double blind. An "activity score" was calculated on the basis of the raw data. Mean number of times a given vial was scored as "active" during each observation was calculated. This was averaged across all eight observations for a given vial to derive the "activity score" for that vial. These activity scores (vial values) were taken as the unit of analysis.

F. Data analyses and experimental replication:

Mean longevity (8-10 replicate per treatment) was analysed using two factor mixed model ANOVA with selection regime as fixed and block as random factor. The two mating status (mated and virgin) were analysed separately. Mean survival rate for each age window (Phase 1/2/3) was analysed using two factor mixed model ANOVA, with selection regime as fixed and block as random factor. Total number of deaths of the ancestral females and ancestral (LH) female mortality rate were analysed using two-factor mixed model ANOVA with selection regime as fixed factor and block as random factor. In mortality rate analysis, multiple comparisons were done using paired t-test (paired with respect to blocks) with Dunn-Sidak correction (Sokal & Rahlf 1995). For the Gompertz analyses, the two mating status, virgin and mated, were analysed separately. To test whether a_0 and a_1 of any two regimes differed significantly, I calculated the difference in a_0 and a_1 using the Gompertz

estimates. Then I created a 99% confidence interval around this measure of difference using the model derived standard errors and correlation coefficients. If the 99% confidence interval included zero, I concluded that the 'difference' is not significantly different from zero and hence the two regimes did not have different a_0/a_1.

Dry body weight was measured in 9-10 replicates per population (section D). Thereafter the body weight data was analysed using two-factor, mixed model ANOVA with selection regime as fixed factor crossed with random blocks. For the fitness assays (section E), 8-10 replicate vials were set up for both "single mating" and "continuous exposure" sets. Courtship frequency (section G) and locomotor activity (section H) of selected males were assayed using 10 replicate vials for each population. Analyses of the three mentioned traits were done using vial means. Fitness data were analysed using three factor, mixed model ANOVA with selection regime and exposure status (single mating/continuous exposure) as fixed factors and block as random factor. All multiple comparisons were done using Tukey's HSD. All the analyses were done at α=0.05 level of significant using Statistica (for Windows).

RESULTS:

A. Dry Body weight:

Analysis of dry body weight revealed a significant effect of selection regime ($p<0.0001$, Table 4a.1, Figure 4a.1). Multiple comparisons using Tukey's HSD showed that F-males were significantly bigger compared to the males from other two regimes.

B. Fitness of the ancestral females exposed to selection line males:

Analysis of the fitness data suggested a significant effect of selection regime and mating status on LH-females fitness (Table 4a.2, Figure 4a.2). Multiple comparison using Tukey's HSD indicated that females mated to F-males produced significantly more progeny compared to females mated to the males of other two regimes. None of the interactions were statistically significant. Tukey's HSD suggested that females mated singly to the F-males produced more progeny compared to females mated to M and C-males, however, the differences were not significant. But females held with F-males continuously for two days produced significantly greater number of progeny relative to when they were exposed to M and C-males (Figure 4a.2).

C. Longevity assay:

1. MEAN LONGEVITY:

Under both virgin and mated conditions, selection regime had significant effects on the mean longevity of selection line males (Table 4a.3, Figure 4a.3a and 4a.3b). Multiple comparison (Tukey's HSD) showed that virgin F-males had significantly higher longevity compared to both C and M-males under same condition. Virgin M-males had lower mean longevity compared to virgin C-males but the difference was not significant. There was no interaction between block and selection regime (Table 4a.3). Analysis of the data from mated males showed a nearly significant interaction between selection regime and block ($p=0.057$). A closer look at the analysis suggested that the longevity of C relative to M and F was different in block 1 and thus the interaction. An analysis excluding C regime showed a highly significant ($p=0.01$, Table 4a.3) difference between M and F-mated males with a non-significant interaction term ($p=0.86$).

2. MEASURES OF RATE OF AGING:

Rate of aging was measured in two approaches – using Gompertz model and by analysing age specific rate of survival. The results are mentioned below:

Gompertz Model:

Table 4a.4 gives the details of all the estimated Gompertz parameters. Further analyses revealed that none of two aging parameters (a_1 and a_0) showed any significant difference in any of the comparisons (Table 4a.5).

Age specific rate survival:

I analysed age specific survival rate following an approach similar to what Adler and Bonduriansky (2011) followed to estimate early and late life mortality rate. Except phase-1, both phase-2 and 3 survival rate of virgin males had significant main effect of selection regime (Figure 4a.4a, Table 4a.4). In phase-2, M-virgin males had significantly lower survival rate compared to that of C-virgin males, which in turn had significantly lower survival rate compared to F-virgin males ($p<0.0001$, multiple comparison using Tukey's HSD). During phase-3, M-virgin males still had lower survival rate compared to the other two regimes, however, survival rates of F and C-virgin males were not significantly different ($p<0.0001$, multiple comparison using Tukey's HSD). Under mated condition, there was no difference in survival rate across the three selection regimes during phase-1. However, in phase-2, survival rate was found to be significantly affected by selection regime ($p=0.036$). M-males showed significantly lower survival rate compared to C and F-males (Tukey's HSD, Figure 4a.4b, Table 4a.4). There was no difference in survival rate during the third phase.

3. MORTALITY OF ANCESTRAL FEMALES:

Although more of females died in M-male vials, the total number of deaths recorded across all the populations were not significantly different across selection regime (Two factor mixed model ANOVA, p=0.137, Table 4a.6). Mortality rate of the ancestral females, i.e., number of female deaths observed per week averaged across the male life-span, was found to have significant effect of selection regime of the males (p=0.02, Table 4a.6, Figure 4a.5). Multiple comparisons using paired t-test (paired by blocks) revealed that LH-females died faster when exposed to M-males compared when they were exposed to C-males. Female mortality rates with F and C-males, and with F and M-males were not significantly different (Table 4a.7).

D. Courtship frequency of selected males:

Analysis of the courtship frequency data suggested a significant effect of selection regime (p=0.024, Table 4a.8a, Figure 4a.6a). Tukey's HSD indicated that M-males had significantly higher courtship frequency compared to that of C-males (Figure 4a.6a). However, courtship frequency of F-males was not different from that of C-males (Figure 4a.6a).

E. Spontaneous locomotor activity:

I found significant effect of my selection on the spontaneous locomotor activity of the selection line males (p<0.0001, Table 4a.8b, Figure 4a.6b). Through multiple comparisons using Tukey's HSD, M-males were found to have significantly higher activity score compared to both F and C-males (Figure 4a.6b). Though F-males were less active compared to C-males, the difference was not significant (Figure 4a.6b).

Effect	SS	MS Num	DF Num	F	p
Selection regime	0.0031	0.0015	2	11.25	<0.001*
Block & Random	0.0012	0.0006	2	4.39	0.016*
Selection regime × Block	0.0007	0.0002	4	1.23	0.304

Table 4a.1: Summary of the results of two-factor ANOVA on dry body weight data treating selection regime as the fixed factor crossed with random blocks. p-values marked with * are statistically significant.

Source	SS	MS Num	DF Num	F	p
Selection regime	534.71	267.35	2	5.77	0.004*
mating status	581.43	581.43	1	12.56	<0.001*
Selection regime×mating status	92.6	46.3	2	1	0.37
Block & Random	175.1	87.55	2	1.89	0.15
Selection regime×Block	87.62	21.91	4	0.47	0.76
Mating status×Block	171.47	85.73	2	1.85	0.16
Selection regime*mating stat*Block	199.96	49.99	4	1.08	0.37

Table 4a.2: Summary of results of three-factor ANOVA using selection regime and mating status as fixed factor crossed with random blocks on the progeny production data. Vial means were taken as the unit of analysis. p-values marked with * are statistically significant.

Analysis	Effect	SS	MS Num	df Num	F	p
Virgin male	Selection regime	745.96	372.98	2	9.49	<0.001
	Block & Random	163.93	81.96	2	2.09	0.131
	Selection regime × Block	301.17	75.29	4	1.92	0.116
Mated male	Selection regime	117.82	58.91	2	3.23	0.045
	Block & Random	115.23	57.62	2	3.16	0.048
	Selection regime × Block	177.10	44.27	4	2.43	0.055
Mated male (Excluding C)	Selection regime	95.13	95.13	1	6.03	0.018
	Block & Random	263.86	131.93	2	8.36	0.001
	Selection regime × Block	4.54	2.27	2	0.14	0.866

Table 4a.3: Summary of the result of two-factor ANOVA with selection regime as fixed factor crossed with random block on mean longevity. Vial means were taken as the unit of analysis. p-values marked with * are statistically significant.

Parameter	Value	Std.Error	DF	t-value	p-value
Age independent Gompertz parameter, a_0					
α_0	0.01053	0.004367	607	2.411302	0.0162
γ_{02}	-0.00155	0.005778	607	-0.26825	0.7886
γ_{03}	0.001731	0.006787	607	0.254969	0.7988
β_0	-0.00538	0.00535	607	-1.00648	0.3146
φ_{02}	0.000615	0.007059	607	0.087101	0.9306
φ_{03}	0.000704	0.00849	607	0.082966	0.9339
Age dependent Gompertz parameter, a_1					
α_1	0.013004	0.007542	607	1.724086	0.0852
γ_{12}	0.003209	0.010555	607	0.304034	0.7612
γ_{13}	-0.00407	0.011058	607	-0.36768	0.7132
β_1	0.00077	0.010948	607	0.07029	0.944
φ_{12}	0.0003	0.015424	607	0.019425	0.9845
φ_{13}	-0.00203	0.015583	607	-0.13047	0.8962

Table 4a.4: Summary of the Gompertz parameters estimated by fitting the raw mortality data in the Gompertz model using R (version 2.14.1), nlme-R package.

Comparison	Difference	Variance	SE	99% C.I.
Mated a_0				
M-C	0.00173051	4.61×10^{-5}	0.006787	±0.017538
F-C	-0.001549991	3.34×10^{-5}	0.005778	±0.01493
M-F	0.003280501	7.95×10^{-5}	0.008914	±0.023033
Virgin a_0				
M-C	0.002434884	2.61×10^{-5}	0.005105	±0.013192
M-F	0.003370055	6.16×10^{-5}	0.007847	±0.020277
F-C	-0.000935171	1.64×10^{-5}	0.00405	±0.010466
Mated a_1				
M-C	0.00173051	1.22×10^{-4}	0.011058	±0.028574
F-C	-0.001549991	1.11×10^{-4}	0.010555	±0.027273
M-F	-0.007274795	2.34×10^{-4}	0.015287	±0.039501
Virgin a_1				
M-C	-0.006098944	1.2×10^{-4}	0.010974	±0.028356
M-F	-0.009607528	3.73×10^{-4}	0.019308	±0.049893
F-C	0.003508584	1.27×10^{-4}	0.011251	±0.029074

Table 4a.5: Summary of the analyses of Gompertz parameters. Data from virgin and mated set were analysed separately. The difference between a pair of regimes were derived by using the Gompertz model estimates and then a 99% confidence interval was created around this difference by using model derived standard errors. In none of the comparison, the difference was significantly different from 0, indicating that there was no significant difference in the Gompertz parameters across the different regimes.

Treatment	Stage	Effect	SS	MS Num.	DF Num.	F	p > F
Virgin male	Phase 1	Selection regime	0.014	0.007	2	0.660	0.520
		Block & Random	0.009	0.005	2	0.441	0.645
		Selection regime×Block	0.023	0.006	4	0.541	0.706
	Phase 2	Selection regime	1.032	0.516	2	10.160	<0.001*
		Block & Random	0.538	0.269	2	5.302	0.007*
		Selection regime×Block	0.374	0.094	4	1.841	0.129
	Phase 3	Selection regime	0.337	0.169	2	4.852	0.01*
		Block & Random	0.082	0.041	2	1.187	0.311
		Selection regime×Block	0.250	0.062	4	1.796	0.138
Mated male	Phase 1	Selection regime	0.014	0.007	2	0.805	0.451
		Block & Random	0.078	0.039	2	4.482	0.014*
		Selection regime×Block	0.026	0.006	4	0.734	0.572
	Phase 2	Selection regime	0.343	0.172	2	3.470	0.036*
		Block & Random	0.049	0.024	2	0.494	0.612
		Selection regime×Block	0.194	0.049	4	0.980	0.423
	Phase 3	Selection regime	0.170	0.085	2	1.238	0.29
		Block & Random	0.706	0.353	2	5.129	0.008*
		Selection regime×Block	0.227	0.057	4	0.824	0.514

Table 4a.6: Summary of separate two-factor ANOVAs on survival rate at each of the age classes (phases) for females under both virgin and mated conditions. Selection regime was taken as fixed factor crossed with random blocks. Vial means were taken as the unit of analysis. p-values marked with * are statistically significant.

Trait	Effect	SS	DF	MS	F	p > F
Female mortality	Selection regime	10.69	5.34	2	1.14	0.33
	Block & random	9.62	4.81	2	1.02	0.36
	Selection regime×Block	4.64	1.16	4	0.25	0.91
Female mortality rate	Selection regime	5.19	2	2.60	10.57	0.03*
	Block & random	4.23	2	2.12	8.62	0.04*

Table 4a.7: Summary of the results of the analyses of LH-female (a) mortality and (b) mortality rate using two-factor ANOVA using selection regime as fixed factor and block as random factor. Vial means were taken as the unit of analysis for female mortality. Mortality rate of each population was calculated and these population level estimates were used as the unit of analysis. p-values marked with * are statistically significant.

Comparison	t-value	DF	p > |t|
M Vs. C	-7.5	2	0.017*
M Vs. F	4.4	2	0.048
F Vs. C	0.21	2	0.85

Table 4a.8: Summary of multiple comparisons of female mortality rate data using paired t-test. The p-value with * is significant after Dunn-Sidak correction.

Trait	Effect	SS	MS Num	DF Num	F	p>F
(a) Courtship frequency	Selection regime	31.15	15.57	2	3.90	0.024*
	Block&Random	71.67	35.84	2	8.97	<0.001*
	Selection regime*Block	4.70	1.18	4	0.29	0.881
(b) Locomotor activity	Selection regime	0.29	0.15	2	18.50	<0.001*
	Block & Random	0.03	0.02	2	2.17	0.122
	Selection regime*Block	0.04	0.01	4	1.37	0.253

Table 4a.9: Summary of the results of two-factor ANOVA using selection regime as fixed factor crossed with random blocks on (a) Courtship frequency and (b) Locomotor activity (activity score) of the selection line males. p-values marked with * are statistically significant.

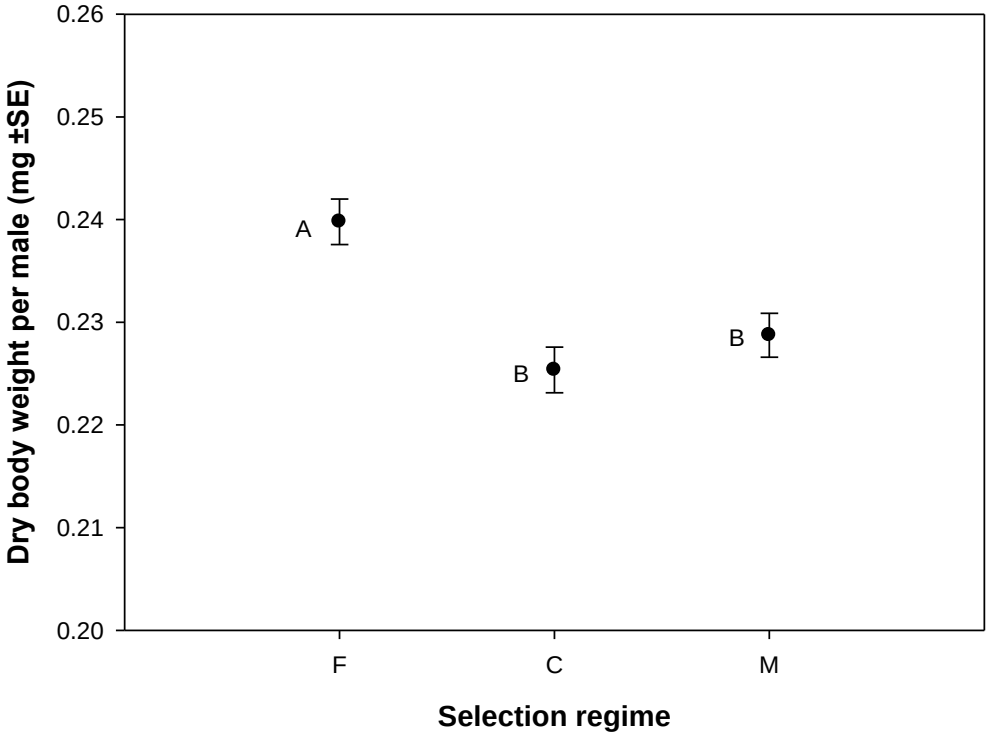

Figure 4a.1: Dry weight at eclosion of males from the three selection regimes. Individuals were weighed in groups of five. An average body weight was calculated using the weight of the five flies. These mean values were taken as the unit of analysis. Points not sharing common letters are significantly different (determined using Tukey's HSD).

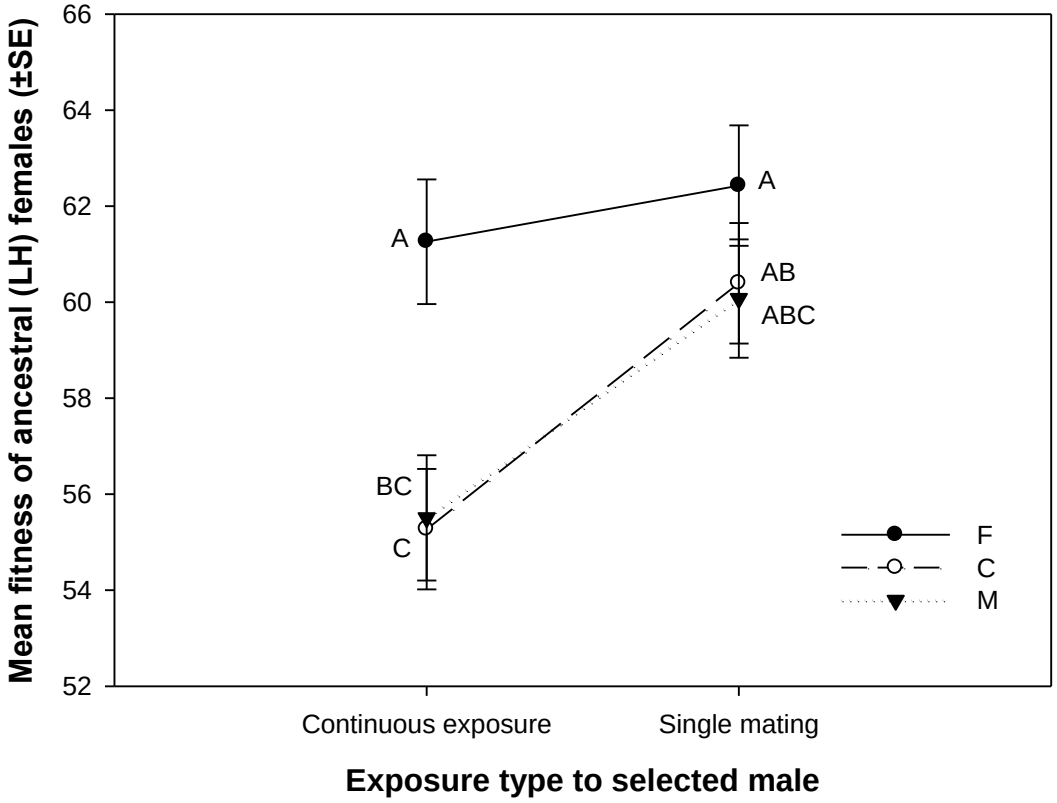

Figure 4a.2: Mean fitness of LH-females under the experimental conditions: after an exposure to the selection line males for one hour (single mating, SM) and two days (continuously exposure, CE). Total number of progeny produced by all the females in a vial was counted and a mean is calculated using this data. The vial means are then used as the unit of analysis. Points not sharing common letters are significantly different (determined using Tukey's HSD).

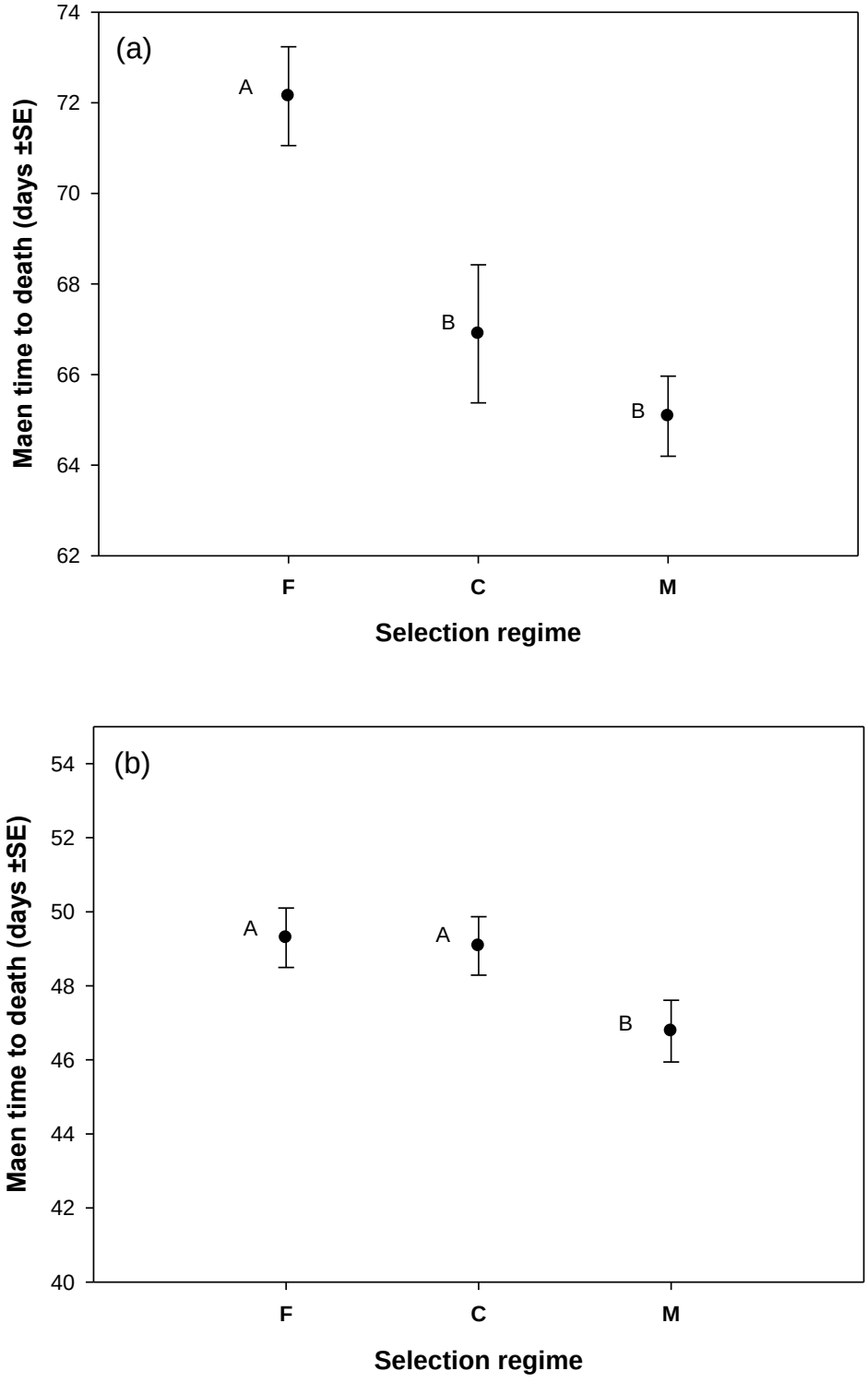

Figure 4a.3: Mean longevity of selected males under (a) virgin and (b) mated conditions (continuously held with LH-females). A mean longevity was calculated for each vial. These vial means are used as the unit of analysis. Points not sharing common letter are significantly different (determined using Tukey's HSD).

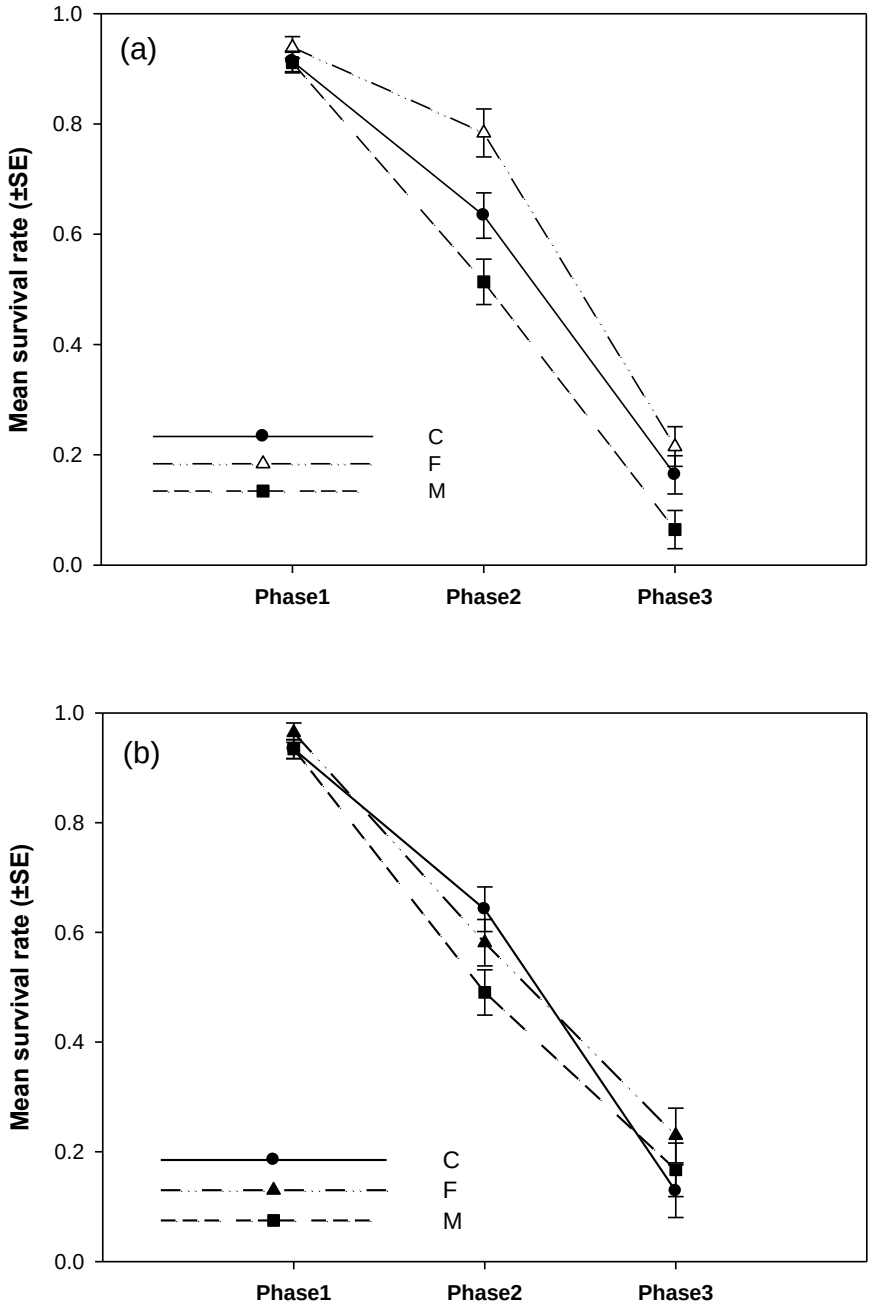

Figure 4a.4: Age specific survival rates of selected males under (a) virgin and (b) mated conditions. Phases were defined by systematically dividing the life-span of the females (see methods section for detail). Survival rate was calculated for each vial by dividing proportion of males surviving in that vial at the end of a phase by proportion of males surviving at the beginning of the phase. These vial estimates were then used as the unit of analysis. Each age class (phase) was analysed separately.

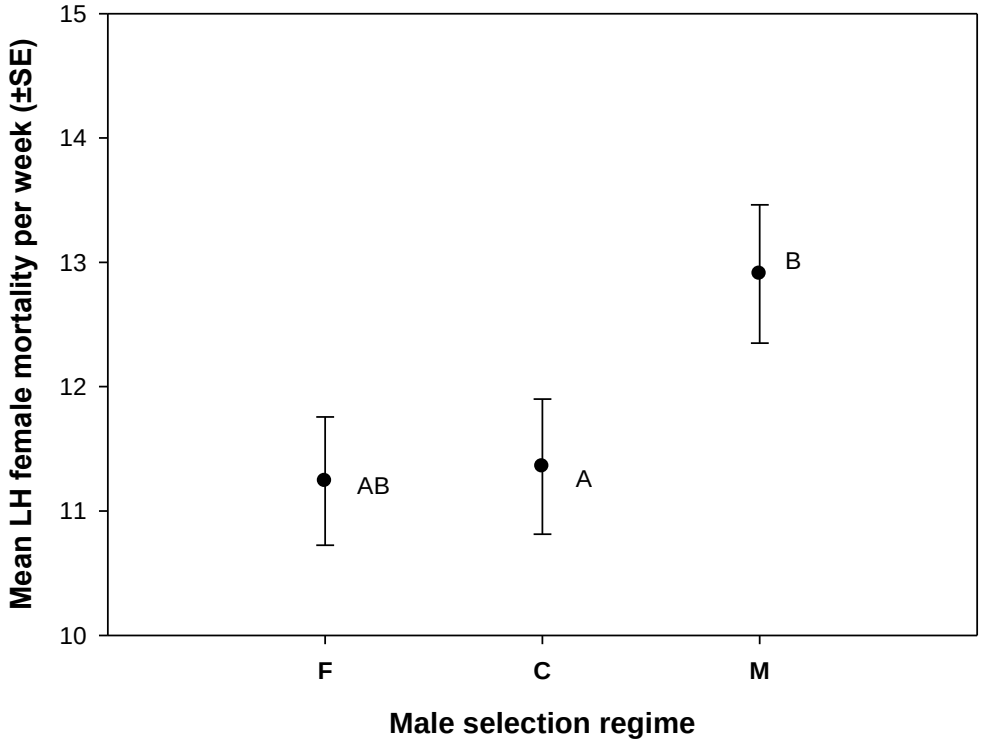

Figure 4a.5: LH-female mortality when exposed to selection line males: mean female mortality per week. Mortality rate was calculated for each of the nine populations by regressing cumulative weekly mortality across all the replicate vials against time (in weeks). These population level measures of mortality rates were then taken as the unit of analysis. Points not sharing common letter are significantly different (determined using Tukey's HSD).

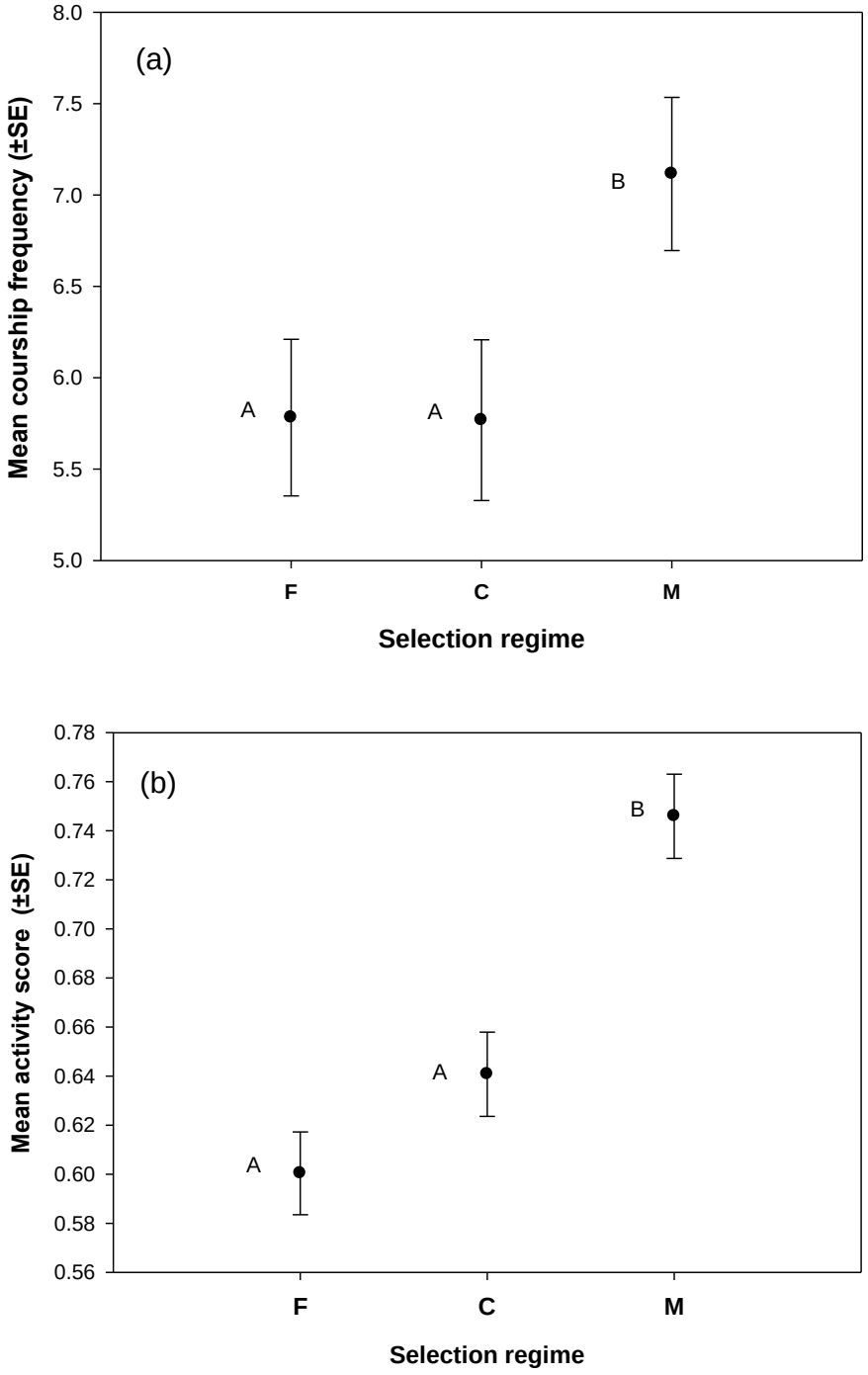

Figure 4a.6: (a) Courtship frequency and (b) Mean activity score of selection regime males when held with LH-females. Mean activity score (see methods section) and mean courtship frequency were calculated for each vial using the raw data and these were then used as the unit of analysis. Points not sharing common letter are significantly different (determined using Tukey's HSD).

DISCUSSION:

In my study, males from populations with increased level of sexual conflict (i.e., male biased operational sex ratio) evolved to be more harming. The males from the male biased selection regime caused more mortality in ancestral females while they did not cause greater fecundity depression in the ancestral females. These males were also found to have increased courtship frequency and locomotor activity. The overall increase in reproduction related activities evolved at the cost of decrease in mean longevity and slightly faster aging. Under decreased intensity of sexual conflict (i.e., female biased operational sex ratio), males evolved roughly in the opposite direction. These males evolved to be relatively benign towards females, at least when mate-harm was measured in terms of fecundity depression in females, in spite of evolving larger body size. I did not find them to be less active or less eager in courtship (compared to C-males). However, the baseline longevity of these males was found to have significantly increased, possibly due to their increased body size (resulting in more resources to start out with) or decreased investment in reproduction related physiology or both. The increase in body size of F-males is a likely consequence of relaxed male specific selection in F-regime resulting in the evolution of body size towards the female optima. Such mode of body size evolution has been reported previously in this system (Prasad et al. 2007). I now discuss each of these findings in detail.

Evolution of Courtship frequency:

I observed an increase in courtship frequency in M-males even when they were held under equal sex ratio. Under male biased operational sex ratio (M-regime), male-male competition is likely to be high and opportunities of mating are likely to be low. Given the positive correlation between male courtship frequency and competitive mating success (Markow 1987), males are likely to be selected for increased courtship activity under increased

competitive conditions. *D. pseudoobscura* populations subjected to increased male-biased operational sex ratio for more than 50 generations have been shown to evolve increased courtship frequency (Crudgington et al. 2009). Thus my results are in agreement with those of Crudgington et al (2009). Holland and Rice (1999) found courtship frequency to decrease under experimental removal of sexual selection through enforced monogamy. However, Crudgington et al. (2009) did not observe any such decline in courtship frequency in populations of *D. pseudoobscura* evolved under monogamous mating system. As female biased regime is expected to cause a general relaxation of the degree of male-male competition, males from F-regime could in principle evolve decreased courtship frequency. But I did not find any such evidence. One possible reason might be the assay environment, which had equal sex ratio. For F-males, equal sex ratio is a three-fold more male biased condition relative to their normal selection condition. Males in this system are known to show plasticity in the components of their reproductive behaviour in response to varying numbers of competitors (Bretman et al. 2009, 2010, Nandy and Prasad 2011). Hence, the difference in the selection versus assay condition can potentially explain the observed results.

Evolution of locomotor activity of males:

In a beautifully designed and executed study, Long & Rice (2007) showed that 'adult locomotory activity' is positively correlated with male fitness. The study also showed that the mentioned trait has antagonistic fitness consequences in the two sexes, i.e., it is involved in intra-locus conflict (Long & Rice 2007). Under intense competitive condition in M-populations, one would expect a heavy premium on male-fitness related traits, such as, locomotor activity. The results confirm this prediction and show a significant increase in spontaneous locomotor activity in M-males compared to C-males. However, I did not observe decline in locomotor activity in F-males relative to C-males, possibly indicating a basal level

of selection pressure maintaining male-fitness related traits. Additionally, as discussed in the previous section, *D. melanogaster* males are capable of showing plasticity in their reproductive behaviour based on the number of competitors (Bretman et al. 2009, 2010, Nandy and Prasad 2011). Since the assay was done under equal sex ratio, which is a relatively more male biased condition than the F-males' usual maintenance regime, plasticity in male behaviour can contribute to the observed results.

Evolution of harming ability in males:

Mate-harm is necessarily a by-product of a suit of reproductive success enhancing traits in males (Wedell 2005, Rice et al. 2006, Koene 2012). Populations, as simple as laboratory island populations (Rice et al. 2006), have been found to harbour significant amount of genetic variation with respect to male's ability to cause such mate-harm (Sawby & Hughes 2001, Civetta & Clark 2000). A number of previous studies have reported the selection response of mate-harm (Holland and Rice 1999, Rice 1996, Martin and Hosken 2003b, Crudgington et al. 2005, 2009). Enforced monogamy resulted in males becoming increasingly benign compared to the males under control or polyandrous mating system (Holland and Rice 1999, Martin and Hosken 2003, Crudgington et al. 2005, 2009). One of the previous studies (Martin and Hosken 2003b) recorded less mortality of females kept with monogamous males, however, their fecundity was not affected. Holland and Rice (1999) and Crudgington et al. (2005, 2009) on the other hand, reported evolution of harming ability of the males measured in terms of life-time progeny production of females but the mortality of females mated to different kinds of males was not significantly different (Crudgington et al. 2009). Rice (1996) adopted the ingenious approach of "male-limited evolution" where males were allowed to evolve against a fixed target female phenotype, with no opportunity in the females for counter evolution. After 36 generations, the males were found to have become more

competitive and they also caused more female mortality compared to control males (Rice 1996).

However, other studies failed to find any evidence of evolution of mate-harm. Manipulation of intensity of sexual conflict by altering the sex ratio did not cause any evolution in male's ability to cause harming effects in females (Wigby & Chapman 2004). Though Rice (1996) observed evolution of mate-harm using male-limited evolution, a more recent study using the same approach did not see such evolutionary response (Jiang et al. 2011).

My results clearly demonstrate the evolution of mate-harm under altered levels of sexual conflict. Under increased intensity of conflict, i.e., under male biased selection regime, males were found to have become more harming. While males have been shown to harm females in terms of both mortality and life-time fitness (Kuijper et al. 2006, Fowler & Partridge 1989), the natural question my observation raises is – why did my M-males evolve to be harming only in terms of mortality and not in terms of progeny production? The mechanism by which males cause increased mate-harm could be chemical (more toxic ejaculate) or physical (higher amount of courtship). At this point it is difficult to predict whether increase in mate-harming abilities of M-males is due to the evolution of behavioural components or due to the evolution of the ejaculate. Increase in courtship frequency (discussed above) in M-males is an indication of changes in the physical component of mate-harm (Fowler & Partridge 1989, Kuijper et al. 2006). However, since the change is small, the effect of this was probably only experimentally resolvable under long term exposure rather than short term (2 days) exposure as was done in the mate-harm assay (see Methods, section D).

Under F-condition, males evolved to be relatively benign to females. Females continuously exposed to F-males produced significantly greater number of progeny compared to the females exposed to the males of the other two populations. Even, females which were allowed a single mating with F-males produced more number of progeny compared to those mated to the males of the other two regimes, though this difference was not significant. This indicates that the F-males are benign to their mates, at least in terms of affecting progeny production. Since the benign nature of F-males was expressed even after a single round of mating, it is possible that the ejaculate quality and/or quantity of these males have evolved. However, I did not find any measurable difference in the mortality rate of females held continuously with F-males or C-males. While mate-harm has been shown to affect both female fecundity and longevity (Fowler and Partridge 1989, Kuijper et al. 2006, Rice et al. 2006), my finding was not unexpected given that females mated to F-males also produced significantly more number of progeny. Additionally, previous studies about evolution of mate-harm have produced mixed results. While some studies (Rice 1996, Martin and Hosken 2003b) have shown evolution in males' ability to cause mortality in their mates without affecting their fecundity, others have not seen any measurable change in mate harming ability of males (Wigby and Chapman 2004). Thus it is possible that evolution of mate harm ability in males in terms of fecundity and survivorship are, at least, to some extent independent of each other.

My results are different from those of Wigby and Chapman (2004) even though the same approach was used to alter the level of sexual conflict. Wigby and Chapman (2004) did not find any effect of selection on the mate harm ability in males from the male biased regime, whereas my results suggest evolution in this trait under both male biased and female biased regimes. This difference in results can possibly be attributed to one major difference in the

selection design – collection of virgin flies prior to the setup of adult competition vials and sex ratio treatments. As Wigby & Chapman (2004) did not collect virgin flies all the populations experienced similar sex ratio during the first (or more) mating. As a result, in their selection design strength of selection on males is expected to depend on the frequency of mating after the sex ratio regimes are set up. Additionally, some progeny can always be expected to be sired by the males that mated before the sex ratios were set up, diluting the effect of the selection.

My finding of evolution of increased male mate harm is in contrast to that of Jiang et al. (2011) who found no evidence of evolution of increased mate-harm (in the form of decreased longevity of females exposed to such males) in the males from "male-limited" (ML) populations even though the ML males evolved higher fitness (Prasad et al. 2007). It is particularly surprising because populations used in my study and that of Jiang et al. (2011) share a common ancestry. Jiang et al. (2011) cited the possible lack of sufficient additive genetic variation with respect to mate harm as one of the explanations for their result. However, as is evident from my finding, this is not the case. I argue that their finding only suggests that "ML" (male limited) males evolved to reduce the gender load by some mechanism which did not interfere with interlocus conflict. Bedhomme et al. (2008), working on the same populations, observed increased efficiency but decreased frequency of courtship activity in males expressing the ML genome compared those expressing C (control) genome. In addition, male limited evolution was associated with decrease in body size (dry weight) (Prasad et al. 2007). This indicates a decrease in at least the physical component of mate harm. Whereas male-limited evolution "masculinised" the genome without making it more harming, my selection regime directly selected for components of interlocus conflict and, led to the evolution of males that were either more or less harming.

Evolution of life-span and aging:

Sexual conflict has been implicated to have major consequences in the evolution of life-span and aging by affecting baseline investment in reproduction (Promislow 2003, Bonduriansky 2008). Under high level of male-male competition (increased conflict), males are expected to increase their investment in reproduction, thereby causing the evolution of faster aging and shorter life-span. When sexual conflict is absent or low, populations are thus expected to evolve slower rate of aging and longer life-span. Previous studies have largely ignored the effect of sexually antagonistic adaptations on life-span and aging (Bonduriansky 2008). Wigby and Chapman (2003) looked at the effect of alteration in level of sexual conflict on measures of female longevity but did not address aging in males. In another study, Maklakov et al. (2007) observed selection response of seed beetle populations to experimentally enforced monogamy and polygamy and found no effect of selection on male life-span and aging rate.

In the present study, I did not observe any significant effect of selection regime on the rate of aging quantified by the Gompertz parameters (a_1 and a_0). However, I observed that under virgin condition F-males had higher mean longevity and mid-life (Phase-2) survival rate compared to those of the other two regimes. This indicates greater availability of resources for somatic maintenance in F-males, either because of their higher body size or because of the evolved decrease in base line investment in reproductive physiology or both. On the other hand, M-males did not show decreased mean longevity under virgin condition. However, age specific survivorship analysis showed significantly lower phase-2 and phase-3 survival rate in M-males (virgin). Hence, although not distinguishable in terms of mean longevity and Gompertz parameters, M-males probably evolved faster aging measured in terms of age

specific survival rate. Since this was observed even under virgin condition, the faster aging in M-males was likely to be caused by increased baseline investment in reproductive physiology rather than the increase in somatic wear and tear resulting from increased reproductive activities.

When held with females (ancestral), F-males' mean longevity and age specific rate of survival were not different from those of C-males. M-males, however, showed shorter mean longevity and lower Phase-2 survival rate, pointing to the greater amount of resources consumed in reproductive physiology and behaviour. This conclusion is supported by the courtship frequency and locomotor activity observations – M-males had higher courtship frequency compared to C-males and also were found to be significantly more active. The lack of difference between F and C-males might be attributed to the assay condition. In the assay, F-males were subjected to more male biased condition than what they are used to during their normal maintenance, possibly causing some behavioural change in them (see discussion of courtship frequency and locomotor activity as well). Such plasticity in male behaviour is known in fruit flies (Nandy and Prasad 2011, Bretman et al. 2009, 2010). This change in behaviour can potentially increase mortality rate in males. Thus, it is possible that even with lower baseline mortality, F-males were found to have survival rate and mean longevity similar to those of the C-males when held continuously with ancestral females at a 1:1 sex ratio.

My results add to our understanding of the effects of sexual conflict on aging (Promislow 2003, Bonduriansky 2008) in males. I found that males, under increased sexual conflict, evolved increased investment in reproductive behaviour and physiology at the cost of reduced survival rate. Males from the populations experiencing less intense conflict evolved higher

basal investment in somatic maintenance either due availability of greater amount of resources (higher body size) or because of decreased basal investment in reproductive physiology or both. However, in presence of females, under equal sex ratio, this advantage of higher survival rate was not visible.

CONCLUSION:

The present study shows the evolution of male traits under altered levels of sexual conflict. I have shown that male courtship frequency, locomotor activity, life-span and aging evolve in response to the prevailing level of intersexual conflict and male-male competition. I also provide direct evidence of the evolution of mate-harm under the influence of altered level of sexual conflict. At least part of my observations can be explained in terms of the body size evolution. my study is the first comprehensive empirical demonstration of the evolutionary connection between intersexual conflict and evolution of life-span and aging in males. While evolution of males' ability to harm females is addressed here in detail, other components of male fitness (e.g., sperm defence/offence ability, ejaculate quality and quantity etc.) and their life-history consequences (e.g., stress resistance) remain important subjects for future consideration.

Note: The work reported in this Chapter is submitted for publication as

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Chapter 4b

Evolution of Sperm competitive ability

INTRODUCTION:

Females of several species of animals are found to mate more than once and store sperm from different males in their genital tract or some specialized storage organ (Lefevre & Jonsson 1962). This leads to "sperm competition", where sperm from different males compete in the female body for limited opportunity of fertilization (Parker 1970, Wedell et al. 2002). Sperm competitive ability is typically defined and quantified in terms of two components – defence and offence (Parker 1970, Boorman & Parker 1976, Clark et al. 2000, Friberg et al. 2005, Bjork et al. 2007). When a given male is the first mate of a female, the proportion of progeny sired by him gives a measure of sperm defence (P1) and the probability that the female will undergo a second mating is termed fidelity. On the other hand, if a male mates with an already mated female, the proportion of progeny fathered by him is termed P2. Together with the probability of mating with an already mated female, P2 is the measure of sperm offence. Males can potentially maximise their sperm competitive ability either by manipulating sperm/ejaculate physiology or by changing female behaviour and/or physiology (Snook, R. 2005). Since most of the sexually reproducing species are promiscuous, sperm competition is expected to be widespread (Birkhead and Moller 1998, Birkhead and Pizzari 2002). Apart from its wide-spread occurrence, sperm competition is predicted to be an important factor in the process of speciation (Parker and Partridge 1998, Simmons, L. 2001) due to its role in driving inter sexual conflict (Stockey 1997, Civetta and Clark 2000, Rice 2000, Friberg et al. 2005). Sperm competition and the resulting post-copulatory sexual selection also have the potential to significantly alter male reproductive behaviour (Gage and Barnard 1996, Wedell and Cook 1999, Bretman et al. 2009, 2010, Nandy and Prasad 2011) and physiology

(Wolfner 1997) thereby playing important role in the evolution of male reproductive and life-history traits.

Empirical studies using model organisms have been a key source of my understanding of the process of evolution of sperm competitive ability. Several studies have documented genetic and phenotypic variation in this trait. Most quantitative genetic studies have shown substantial genetic variation in all components of defence and offence abilities in fruit flies (Clark et al. 1995, Hughes 1997, Civetta and Clark 2000, Friberg et al. 2005, Hughes and Leips 2006). However, while Hughes (1997) found this variance to be mainly non-additive, Friberg et al. (2005) reported significant amount of additive genetic variance within the population of *D. melanogaster*. In addition, significant male×male (Clark et al. 2000) and male×female (Clark and Begun 1998, Clark et al. 1999, Miller and Pitnick 2002) interactions were found to influence the outcome of sperm competition.

Despite the existence of strong fitness consequences, attempts to observe evolution of sperm competitive ability have produced inconsistent results across different studies. When males were allowed to evolve against a fixed target female phenotype (male limited evolution), Rice (1996, 1998) observed increase in offence and defence ability in males. However, using the same ancestral populations, two different studies failed to find any such evolution. Bjork et al. (2007) directly selected for both defence and offence but could not find the predicted response. Another study using the similar "male limited evolution" approach could not reproduce Rice's results (Rice 1996, 1998), in spite of observing increase in male fitness (Jiang et al. 2011). Alteration in the levels of sexual selection by changing the operational sex ratio was found to have no significant effect on male reproductive traits (Wigby and Chapman 2004) and sperm competitive ability (Michalczyk et al. 2010). However, removal of sexual selection through experimentally enforced monogamy led to the evolution of male reproductive traits and sperm competitive ability (Hosken et al. 2001, Simmons and Garcia-

Gonzalez 2008). In a relatively recent study, decrease in sperm competitive ability was also observed in mice populations subjected to enforced monogamy (Firman and Simmons 2011).

Here I report the results from an experimental evolution study using a long term laboratory adapted population of *Drosophila melanogaster*. I held populations at different operational sex ratios and then assayed the evolutionary response of these populations in terms of different components of sperm competitive ability of the males – defence and offence. Alteration in operational sex ratio is predicted to generate a range of intensities of male-male competition – high (male biased), medium (equal sex ratio) and low (female biased). After 55-60 generations of selection, I quantified males' sperm defence and offence abilities (P1 and P2 respectively) under single pair and group conditions. I also observed different components of male behaviour.

METHODS:

The experiments were done on males belonging to the populations of *Drosophila melanogaster* subjected to different operational sex ratios described in detail in Chapter 2. Prior to the experiment, all the selected populations were passed through one generation of standardization (Chapter 2). During standardization, all the populations were grown at the usual larval density (140-150 per 10ml of cornmeal food per vial). Instead of collecting them as virgins, on 12^{th} day post egg collection, flies of each of the nine populations were transferred into fly-cages (dimension: 19cm×14cm×24cm) provisioned with food and live Yeast. Close to 2,250 adult flies (at approximately equal sex ratio) were transferred into each cage. Two days later, the flies were allowed to oviposit on fresh food plates and the eggs were collected for the experiment. Females for the assay came from the ancestral population - LH_{st}. Competitor males were taken from LH (Chippindale et al. 2001) population, which is

the ancestor of LH_{st}. The LH_{st} populations are regularly back crossed into LH background to ensure that the two populations have identical genetic backgrounds and differ only with respect to the eye colour marker (detail mentioned in Chapter 2). Importantly, LH is wild type red eyed and LH_{st} and the selected populations are scarlet eyed (autosomal recessive st marked). The assays were carried out following two designs – group and single pair.

Generation of experimental flies:

All the experimental flies were generated under controlled larval density and standard culture conditions ($25^{o}C$, 60-80% RH, 12hours-12hours light/dark cycle). For each of the nine selected populations, 150 eggs were cultured in 8-10ml of cornmeal-molasses food per vial. On 10^{th} day post egg collection, adult flies started emerging. Males were collected as very young ($\leq$ 6 hours post eclosion) virgins during the peak of their eclosion rhythm under light CO_2 anaesthesia (< 3 minutes exposure). Males were held in single sex vials, at the density of 10 per vial (for Group design) and 1 per vial (for Single pair design) till the mating trials. Two day old virgin flies were used to set up the mating trials.

The LH and LH_{st} flies were generated under similar conditions – controlled larval density (140-160 per 10 ml cornmeal molasses food per vial). LH_{st} females were collected as virgins in the same way described above and held at a density of 8 per vial (for Group design) and 1 per vial (for Single pair design) in single sex-vials for two days before the mating trial. LH-males were collected in the same way and held in single sex vials at a density of 10 per vial (for Group design) and 1 per vial (for Single sex design). LH males and LH_{st} females were also held for two days before the mating trials.

Group design:

For sperm defence (P1) assay, selection regime males (from one of the nine populations), in groups of 10, were combined with eight virgin LH_{st} females in a mating vial. 9-10 such replicate vials were set up for each of the nine populations (M1-3, C1-3, F1-3). The males were discarded after one round of mating and females were held back in the same vial. Single mating was ensured by direct manual observation and the limited time of exposure (<60-75minutes). This limited exposure is sufficient for only a single round of mating per female and has been successfully adopted previously (Nandy et al. 2012). Females were separated from the males under light CO_2-anaesthesia and were allowed half an hour to recover from the effect. Following this the red eyed (LH) competitor males were introduced into the vials in groups of 10. These vials were left undisturbed for the next 18-20 hours. After this exposure time, males were discarded and females were transferred individually to oviposition test tubes (dimension: 12mm×75mm) provisioned with food under anaesthesia. A window of 18 hours was allowed for oviposition, following which the females were discarded and the test tubes were incubated under standard conditions (25^{o}C, 12hours-12hours light/dark cycle, 60% Relative Humidity) for 12 days before freezing them. The progeny was scored for eye colour and proportion of scarlet eyed progeny was taken as a measure of P1 of the selection regime males. Each female in a vial was scored for P1. Females which produced only scarlet eyed progeny (i.e., P1=1) very likely failed to remate and therefore were removed from the analyses. Finally, a vial mean was calculated for each replicate vial using P1 values from all the females (excluding females with P1=1) in that vial. Vial means were used as the unit of analysis.

For sperm offence (P2) assay, the design was identical to that of the P1-assay described earlier, except that the first males were taken as LH-males and the second males were

selection regime males. LH_{st} females were first allowed a single mating (manually observed) with LH-males (8 females and 10 males per vial), following which the males were separated and discarded. The second male (i.e., males from one of the 9 populations) were introduced after allowing the females to recover from the effect of anaesthesia for half an hour to 8-10 vials per population were set up for this part of the experiment. The second males interacted with the females for 18-20 hours, after which they were separated. Females were then transferred to the oviposition test tubes as mentioned in the previous section and allowed to oviposit for 18 hours. Similar to the P1-assay, the proportion of scarlet eyed progeny was taken as the measure of P2 of the selection regime males. Each female in a vial was scored for P2. Females which produced only red eyed progeny (i.e., P2=0) were removed from the analyses as they very likely failed to re-mate with the selection line males. Similar to the P1-assay, vial means were calculated for each vial, using P2 values from all the females in that vial (excluding females with P2=0). Vial means were used as the unit of analysis. In each vial, proportion of females with P2>0 was taken as a measure of re-mating success (proportion of males that successfully re-mated with the test-female). Re-mating success was analysed using vial means.

Single-pair design: sperm defence and related behaviours

One virgin LH_{st} female was combined with a single selection regime male in an 8-dram vial provisioned with food. The cotton plug of the vial was push deep into the vial to adjust the space available to the flies to roughly 30mm×30mm. The pair was observed till they finished mating and mating latency (time taken by a pair to start mating after being put together in mating vial) and copulation duration (duration for which the pair remained in copula) were recorded. After the first mating, female was quickly sorted using CO_2-anaesthesia and the male was discarded. The female was held back into the vial, allowed a recovery time of half

an hour before introducing the second male (red eyed LH). Following this the vial is left undisturbed for 44 hours during which competitor LH-male mated with the female. This 44-hour exposure closely matched the normal male-female interaction time for the selected populations. After this exposure window, the male was discarded and the female was transferred to test tube provisioned with food and was allowed an oviposition window of 18 hours. The progeny produced during this window was allowed to emerge and then they were scored for their eye colour marker. The proportion of scarlet progeny was taken as an estimate of P1 of the selection regime male. 39-44 males from each of the population were assayed for P1. Vials in which females failed to re-mate (P1=1) were excluded for the P1-analysis. Final sample size for P1 analysis was n=35-43 for each population. Since individual females were the unit of independent replication within each population in this assay, mating latency, copulation duration and P1 values from individual females were used as the unit of analysis. Proportion of females that did not re-mate with the LH-males (i.e., produced only scarlet-eyed progeny, P1=1) was noted and taken as a measure of re-mating fidelity of the selection regime males. Re-mating fidelity was analysed using population means.

Data analyses:

P1 (group and single pair design), P2, copulation duration, mating latency and re-mating success were analysed using two-factor mixed model ANOVA with selection regime as fixed factor crossed with random blocks. I calculated mean re-mating fidelity for each of the populations and analysed the data using a two-factor ANOVA with selection regime as fixed factor and block as random factor. All multiple comparisons were done using Tukey's HSD. All the analyses were done using STATISTICA for Windows, Version 10 (StatSoft). Level of significance (α) was taken as 0.05 for all the tests done.

RESULTS:

Group Design:

In the group design, I found a significant effect of selection regime on sperm defence score (P1) as well as sperm offence score (P2) of the selected males (Table 4b.1). Both analyses were consistent across blocks. Multiple comparison using Tukey's HSD showed that M-males had the highest P1, which was significantly different from that of F-males. C-males had intermediate P1, not significantly different from either F or M-males (Figure 4b.1a). F-males were found to have significantly lower P2 compared to both C and M-males (Figure 4b.1b). There was no significant effect of selection regime on the re-mating success (Table 4b.1).

Single pair design:

In the single pair design assay, selection regime was found to have a significant effect on copulation duration (CD) and P1 (Table 4b.2). Tukey's HSD suggested that M-males mated for significantly longer duration compared to both F and C-males (Figure 4b.2a). CD for F-males was longer than C-males but the difference was not significant (Figure 4b.2a). Multiple comparisons on the P1 data revealed a significant difference between M and F-males with M-males having the highest P1 and F-males having the least P1 (Figure 4b.2b). C-males were again found to have intermediate P1 (Figure 4b.2b). Neither M nor F-males had P1 significantly different from that of C-males. P1 results were thus consistent across the group and single pair design assays (see Figure 4b.1a and 4b.2b).

No significant effect (Table 4b.2) of selection regime on mating latency (mean ±SE, F: 0.04 ±0.01; C: 0.09 ±0.01; M: 0.05 ±0.01), re-mating fidelity (mean ±SE, F: 0.04 ±0.01; C: 0.09 ±0.01; M: 0.05 ±0.01) was found.

Trait	Source	SS	MS Num	DF Num	F Ratio	Prob>F
P1	Selection regime	0.099	0.049	2	4.437	0.015*
	Block & Random	0.017	0.009	2	0.781	0.462
	Selection regime×Block	0.025	0.006	4	0.561	0.692
P2	Selection regime	0.057	0.028	2	4.117	0.020*
	Block & Random	0.016	0.008	2	1.169	0.316
	Selection regime×Block	0.004	0.001	4	0.157	0.959
Re-mating success	Selection regime	0.012	0.006	2	0.399	0.672
	Block & Random	0.058	0.029	2	1.938	0.150
	Selection regime×Block	0.014	0.003	4	0.229	0.921

Table 4b.1: Summary of the two-factor mixed model ANOVA on P1, P2 and Re-mating success data from the group design assays treating selection regime as fixed factor crossed with random blocks. Vial means were treated as units of analyses. p-values marked with * indicate significant effects.

Trait	Source	SS	MS Num	DF Num	F Ratio	Prob>F
Copulation duration	Selection regime	387.548	193.774	2	32.163	<0.001*
	Block & Random	76.024	38.012	2	6.309	0.002*
	Selection regime×Block	48.798	12.199	4	2.025	0.090
P1	Selection regime	0.137	0.068	2	3.225	0.041*
	Block & Random	0.028	0.014	2	0.668	0.513
	Selection regime×Block	0.022	0.005	4	0.255	0.907
Mating latency	Selection regime	28.461	14.230	2	0.979	0.377
	Block & Random	92.527	46.264	2	3.181	0.043*
	Selection regime×Block	33.666	8.417	4	0.579	0.678
Re-mating fidelity	Selection regime	0.003	0.002	2	3.284	0.143
	Block & Random	0.001	0.0003	2	0.552	0.614

Table 4b.2: Summary of two-factor ANOVA on the copulation duration, P1, mating latency and Re-mating fidelity data from single pair design assays treating selection regime as fixed factor crossed with random blocks. Except for re-mating fidelity, data from each individual (replicate) was treated as the unit of analysis. Mean re-mating fidelity of each population was used as unit of analysis. p-values marked with * indicate significant effects.

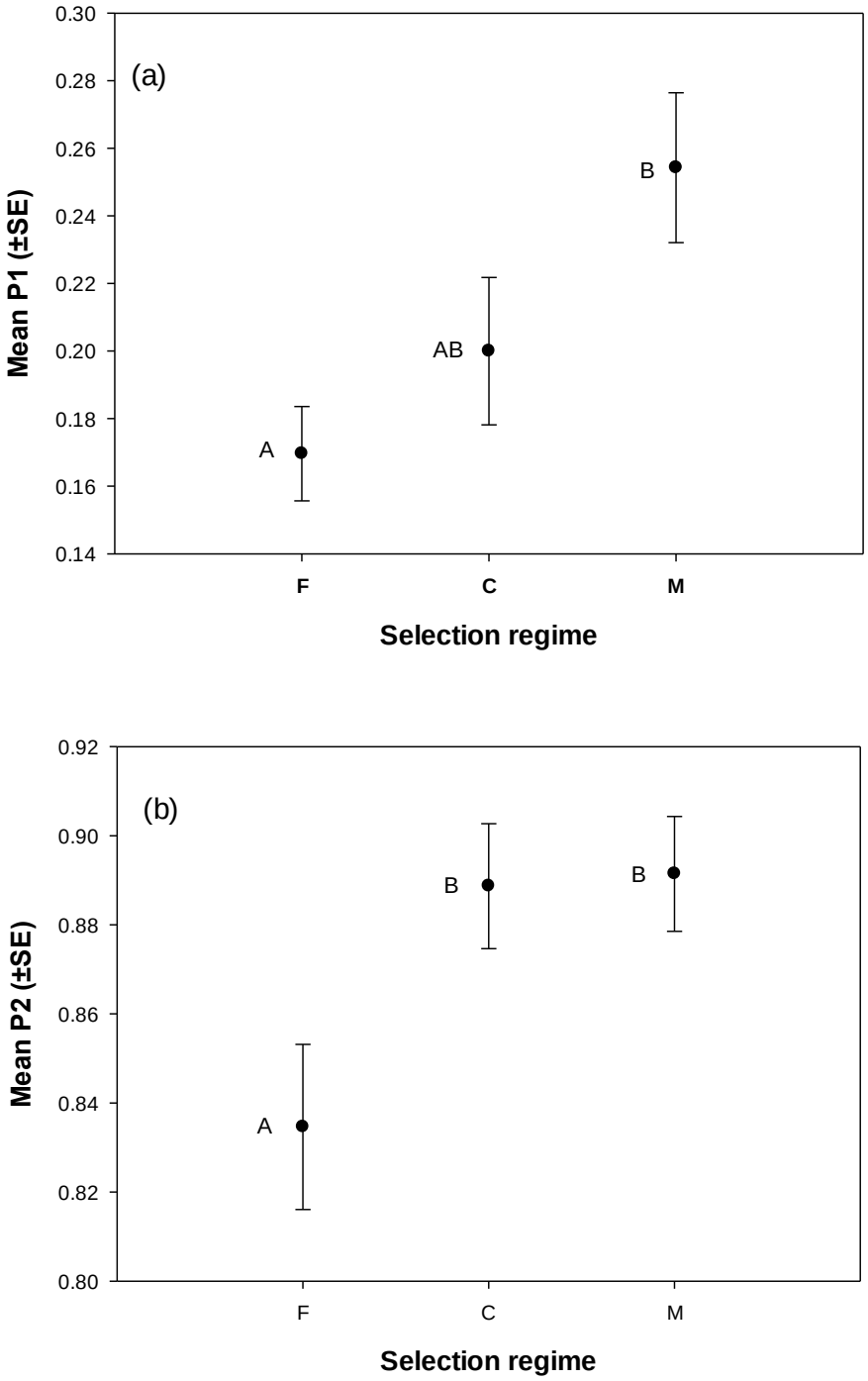

Figure 4b.1: Effect of selection regime on (a) P1 and (b) P2 of the selected males competed against ancestral males (LH) as detected in the group design assay. For each vial, a mean was calculated using the raw data. These vial means were then taken as the unit of analysis. Points not sharing common letters are significantly different (determined using Tukey's HSD).

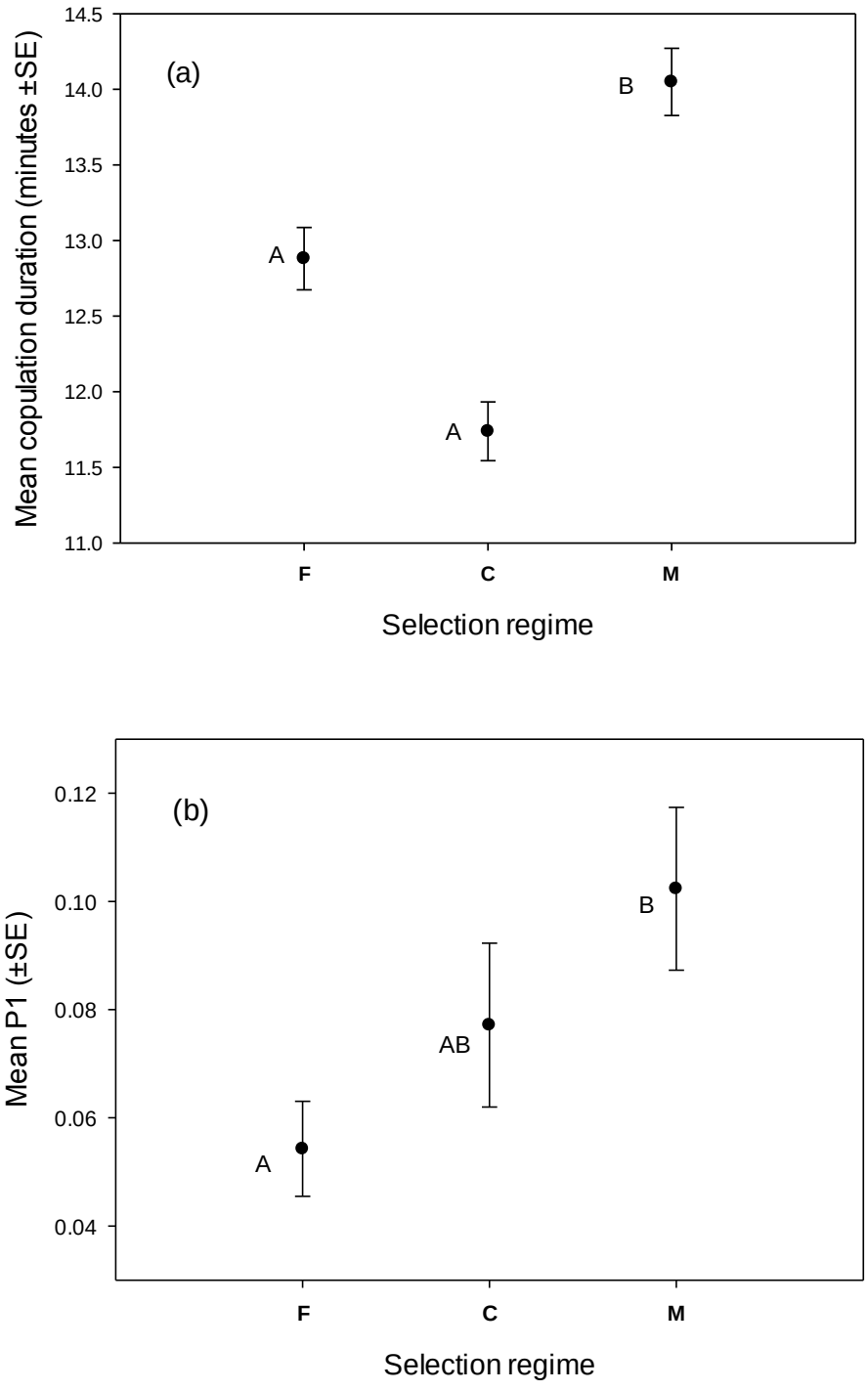

Figure 4b.2: Results of single pair trials: effect of selection regime on (a) copulation duration and (b) P1 of the selected males. Data was analysed using the copulation duration and P1 observed in individual females as the unit of analysis. Points not sharing common letters are significantly different (determined using Tukey's HSD).

DISCUSSION:

Increased male-male competition (male biased sex ratio in my regime), is likely to select for higher sperm competitive ability. Under relaxation of such competition (female biased sex ratio in my regime), the traits relevant to sperm competitive ability are expected to degenerate if there is cost of bearing them. Theories of sperm competition predict the evolution of several male traits under the influence of such selection (Parker 1970, Simmons 2001). Empirical studies in a range of taxa also suggest that male behaviour and/or physiology can potentially evolve in response to sperm competition experienced by the males (Birkhead and Moller 1998, Simmons 2001). Male reproductive behaviour and/or physiology, along with sperm competitive ability have been shown to evolve under various conditions using a number of species (Rice 1996, 1998, Pitnick et al. 2001, Hosken and Ward 2001, Hosken et al. 2001, Simmons and Garcia-Gonzalez 2008, Firman and Simmons 2011). Here I show the evolution of sperm competitive ability of males under the altered operational sex ratio.

Pre-adult survivorship differences of the progeny of males across the three selection regimes can potentially lead to differences in the P1 and P2 measures in my assay (Gilchrist and Partridge 1997, Garcia-Gonzalez 2008). I found no significant difference in the pre-adult survivorship (egg to adult survival rate) of the M, C and F flies in a separate experiment (proportion survived, mean ±SE, M: 0.90 ±0.009, C: 0.90 ±0.009, F: 0.87 ±0.009, see Chapter 6 for details). In addition, LH_{st} is an outbred base population with pre-adult survivorship of about 90%. Hence, the progeny of M, C, F males with females of the LH_{st} base population are very unlikely to have differential pre-adult survivorship. Therefore the observed differences in P1 and P2 of the three selection regimes are representation of their sperm competitive ability. My results suggest that males from populations with female biased operational sex ratio (F) evolved reduced sperm competitive ability in terms of both sperm

defence and offence. These differences can be caused either by qualitative change in the male ejaculate (Ravi Ram and Wolfner 2007, Sirot et al. 2011) or by changes in different components of male mating behaviour, such as quantity of ejaculate invested per copulation (Bretman et al. 2009, Nandy and Prasad 2011). However I did not observe any significant decline in copulation duration, ability to induce fidelity and re-mating success of F-males. In a separate assay, I also observed F-males to be equally active in courting the females (see Chapter 4a). The lack of these behavioural changes indicates likely qualitative changes (discussed further later) in the ejaculate of F-males. In populations with male biased sex ratio (M), males evolved increased P1 without inducing higher fidelity. M-males did not have increased offence ability.

Largely, my results are in line with the predictions of theories of sperm competition (Parker 1970). Under F-regime, due to the abundance of mating opportunities, intensity of competition between males, including sperm competition (risk and intensity) can be expected to be low. Such relaxation of selection is likely to cause the loss (or deterioration) of the costly traits which are otherwise advantageous in male-male competition. Male ejaculate contains accessory gland proteins or Acps (Wolfner 2002) which have substantial manufacturing cost (Chapman and Edwards 2011). Most of these proteins have been shown to have post copulatory effects on females and their evolution is believed to be affected by intersexual conflict and sperm competition (Civetta and Clark 2000, Wolfner 2009). It is possible that, due to the relaxation of the intensity of sperm competition, F-males have evolved Acps that are qualitatively and/or quantitatively different. Such changes are likely to make their ejaculate less competitive, causing the decline in P1 and P2 of F males, as observed in my experiment. However, since I did not quantify the ejaculate components in my assay, at this point I cannot confirm this hypothesis. However, it is important to note here

that I found no obvious decline in behaviours associated with sperm competitive ability in the F males (see above).

Alternatively, the observed decline in the sperm competitive ability in F-males could possibly be due to differential genetic drift in the F-populations during the course of selection leading to inbreeding-like effects. I attempted to equalize N_e across the three regimes to avoid differential inbreeding. However, the very nature of the mating system and sexual selection in the system (multiple mating by females, last male sperm precedence etc.) can potentially complicate this equalization method. Alternative methods of calculating N_e (accounting for multiple mating, sperm precedence) has been suggested by Rice and Holland (2005). Following this method, even after considering sperm precedence and female multiple mating, the N_e in all the regimes was greater than 350 (see supplementary information). Previous studies have shown that laboratory selection experiments, such as ours, where $N_e > 100$ are very unlikely to suffer effects of inbreeding and drift within the time scales of this study, i.e., 50-60 generations (Rice and Holland 2005, Snook et al. 2009, also see Chapter 2). Additionally, the regime that experienced smallest N_e (see Chapter 2) in my experiment was M and not F. However, M-populations showed increase in components of fitness, thereby indicating the absence of any confounding effect of inbreeding in the experiment.

Males of M-regime were found to have evolved increased P1 (defence) even though they did not induce higher fidelity to their mates. However,I I found M-males to copulate with virgin females for significantly longer duration. Previous studies using the same (Nandy & Prasad 2011) as well as different (Bretman et al. 2009, 2010) populations of *D. melanogaster* have shown a positive correlation between copulation duration and P1. In absence of further

quantitative data on sperm and/or Acps, it is, however, not clear whether this is caused by increased transfer of sperm or other components of ejaculate or both (Gilchrist & Partridge 2000, Sirot et al. 2011).

I did not find any change in P2 of the M-males. While it is difficult to predict the reason for this, I cite few possibilities. First, even in my baseline population, according to previous studies (Boorman & Parker 1976, Friberg et al. 2005, Bjork et al. 2007), P2 is usually very high – close to 0.8 (i.e., 80% of the progeny is sired by the second male). Further increase in P2 might be too costly for males to evolve even under M-regime. Secondly, under severely high risk and intensity of sperm competition (which can be expected to represent my M-regime) it might not be worth investing more in P2 (Enqvist & Reinhold 2006).

A number of studies have reported the evolution of decreased sperm competitive ability and testes size in response to complete removal of sexual selection by experimentally enforced monogamy (relative to polygamy) in a variety of model organisms (Pitnick et al. 2001, Hosken et al. 2001, Simmons and Garcia-Gonzalez 2008, Firman and Simmons 2011). However, relaxation of sexual selection (as opposed to complete removal) by having a female biased operational sex ratio did not produce similar response in at least two previous studies (Wigby and Chapman 2004, Michalczyk et al. 2010). While Rice (1996, 1998) observed evolution of sperm competitive ability in response to "male limited selection", Jiang et al. (2011) did not find any such evidence following the same approach of experimental evolution. Direct selection of sperm competitive ability by Bjork et al. (2007) did not cause any change in the offence and defence ability. However, Friberg et al. (2005) detected small but measurable additive variation with respect to all components of offence and defence.

Bjork et al. (2007) suggested that complex interactions with rival males and females could possibly explain their failure to find response to the selection. Jiang et al. (2011) suggested yet another novel explanation that included a possible loophole in the experimental evolution studies using long term laboratory adapted populations. Long term laboratory domestication under a controlled condition (specifically for LH-population) leading to strong directional selection on the relevant traits can potentially erode additive genetic variation (Jiang et al. 2011). my results at least to some extent contradict these two studies. I did find significant effect of selection regime on sperm competitive abilities of males after 60 generations of selection, indicating the presence of substantial additive genetic variation in the ancestral population. There are several differences between my study and the two (Bjork et al 2007, Jiang et al. 2011) mentioned earlier, potentially explaining such different results. (a) Given that the outcome of sperm competition depends on the complex male-male and male-female interactions (Clark 2002, Miller and Pitnick 2002), direct selection on only males either for P1/P2 or fitness might not lead to the expected selection response (Bjork et al. 2007). my selection, on the other hand, was of a more multifarious type, selecting for a suite of traits in both the sexes. (b) Earlier studies (Bjork et al. 2007, Jiang et al. 2011) have looked only at increasing defence and offence abilities. my F-regime (female biased sex ratio) relaxed the selection of male-male competition and thus the population could evolve in the direction (i.e., decrease of defence and offence) not studied in these previous experiments. As discussed earlier, it is possible that evolution in one direction (i.e., increased P2) is too costly to evolve, while evolution in the other direction is possible.

Copulation duration of selection regime males after 8 and 60 generations of selection:

Copulation duration was found to be significantly higher in M-males in the present assay. However, as mentioned in Chapter 3, I found this trait in F-males to be higher after 8

generations of selection while that of M-males was unresponsive till then. Though this change in the response of this trait to the selection is difficult to explain, I mention few relevant issues here. First, it should be noted here that even in the present assay F-males did show higher copulation duration relative to C-males, however the difference was statistically non-significant. It is possible that M-populations started responding to selection somewhere between 8^{th} and 60^{th} generation. Additionally, previous theoretical works predict an increased investment in first mating following relaxation of the risk of sperm competition in species with high last male sperm precedence (Parker 1990, Enqvist and Reinhold 2006). Thus the early response observed in my experimental evolution study is consistent with the existing theoretical work. However, the theoretical predictions are relevant on the number of sperm invested per mating and not the ejaculate per se. Gilchrist and Partridge (2000) have shown that changes in copulation duration, particularly in fruit flies, might indicate changes in the amount of non-sperm components of ejaculate transferred. Additionally, recent empirical studies have also found copulation duration to increase under male-biased operational sex ratio (Michalczyk et al. 2010). Thus the predictions related to the evolution of copulation duration are fairly ambiguous. Here, M-males were found to have both high copulation duration as well as higher P1. Given that empirically these two traits have been found to have positive correlation in multiple independent studies (Bretman et al. 2009, 2010, Nandy and Prasad 2011), it is quite possible that evolutionary response of these two traits in M-males was also correlated.

CONCLUSION:

In summary, I have shown in this study that sperm competitive ability (both defence and offence), at least in fruit flies, can potentially undergo adaptive evolution in response to the changes in the operational sex ratio. Due to the cost of maintenance, traits related to sperm

competitive ability undergo degeneration upon relaxation of competition among males. I have also shown increase in sperm defence ability under male biased selection condition that represents increased intensity of competition among males.

Note: The work reported in this chapter has been accepted in Evolution for publication:

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Chapter 5

Evolution of female traits

INTRODUCTION:

Promiscuity leads to a situation wherein male-fitness is limited by number of mates whereas female-fitness depends upon the number of offspring (Bateman 1948, Trivers 1972, Wedell et al. 2006, Chapman et al. 2003). In such mating systems, males are expected to be under selection for traits that increase their mating success, sperm competitive ability and their ability to ensure their mate's fidelity (Parker 1970, Trivers 1972, Simmons 2001, Snook 2005). Accordingly, several studies have documented the evolution of male specific adaptations, such as, elaborate and persistent courtship, forceful mating, traumatic insemination, mating plug, accessory gland proteins etc (Crudgington and Siva-Jothi 2000, Rowe et al. 1994, Chapman et al. 1995, Arnqvist and Rowe 2005, Rice 2000, Koene 2012). Such male specific adaptations often reduce female fitness as a by-product (commonly referred to as mate-harm), leading to selection on females to evolve "resistance" to male induced harm (Stockley 1997). This evolutionary conflict is referred to as "Interlocus Sexual Conflict" (Parker 1979, Arnqvist & Rowe 2005, Chapman et al. 2003, Koene 2012). It has the potential to maintain an open ended co-evolutionary arms race between the sexes wherein male adaptations to male-male competition drive evolution of female traits and counter evolution in females drives further evolution in male traits (Parker 1979, Rice 1984, Rice 2000, Arnqvist & Rowe 2005, Rice et al. 2006).

Here I focus on the outcome of Intersexual conflict on female traits. The conflict has been proposed to affect a wide range of female traits, including female behaviour and life-history (Rice 2000, Promislow 2003, Wedell et al. 2006, Bonduriansky et al. 2008). In populations

with higher levels of conflict, male adaptations (such as persistent courtship, toxic ejaculate, spiky genitalia or other forms of traumatic inseminations, that harm female) are predicted to increase extrinsic mortality rates of females, which can lead to the evolution of faster intrinsic rates of aging (Promislow 2003, Maklakov et al. 2005, 2006, 2007, Bonduriansky et al. 2008) through 'mutation accumulation' (Medawar 1952) and/or 'antagonistic pleiotropy' (Williams 1957). In addition, females are selected for resistance which can delay the effect of such mate-harm to later in their life (Bonduriansky et al. 2008). Such resistance traits have been documented in a number of species and often take the form of changes in behaviour and/or morphology and/or physiology (Birkhead et al. 1988, Rowe et al. 1994, Arnqvist and Rowe 1995, Bonduriansky 2003, Anderson et al. 2004, Snook and Hosken 2004). The resistance traits are expected to be costly to the females. For example, females in water striders have been shown to bear ecological cost of resistance, in the form of increased risk of predation (Rowe 1994) and physiological cost of resistance, in the form of increased energy expenditure (Watson et al. 1998). In fruit flies (*Drosophila melanogaster*), specifically in the laboratory populations, female resistance is expressed in the form of intense pre-mating struggle between the sexes (Rice et al. 2006), including such female behaviour as kicking, flicking and extrusion of genitalia (Connolly and Cook 1973). In addition, Linder and Rice (2005) found substantial standing genetic variation with reference to female resistance to mate harm in the laboratory population of *D. melanogaster* using the ingenious method of hemiclonal analysis.

Given that organisms are limited by the availability of resources, evolution of resistance to mate harm is predicted to come at the cost of other life-history traits, such as aging and life-span, fecundity etc. (Promislow 2003). While a number of empirical studies have addressed evolution of female resistance to mate harm (Holland and Rice 1999, Martin and Hosken 2003, Wigby and Chapman 2004, Crudgington et al. 2005, Michalczyk et al. 2010), few have

tried to test the predictions on the life-history consequences of such female adaptations (Maklakov et al. 2007).

Three lines of experimental evolution studies have addressed this issue. In one approach, populations of fruit flies, dung flies and seed beetles were subjected to enforced monogamy and polygamy (Holland and Rice 1999, Martin and Hosken 2003, Crudgington et al. 2005, Maklakov et al. 2007). Complete lifelong monogamy (as was experimentally imposed in the mentioned studies) is thought to remove interlocus conflict from a population (Rice 2000, Promislow 2003, Wedell et al. 2006). Thus the comparison between monogamy and polygamy is really a comparison between "conflict" and "no-conflict" conditions. Compared to individuals from polygamous populations, monogamous females evolved to be less resistant to male harassment. Polygamous-female seed beetles evolved faster rate of aging and shorter life-span under unmated condition, where as there was no response in lifespan of polygamous and monogamous males (Maklakov et al. 2007). The second approach altered operational sex ratio in replicate populations of *Drosophila melanogaster* (Wigby and Chapman 2004) and *Tribolium castaneum* (Michalczyk et al. 2010), thereby altering the levels of sexual conflict in these populations. Male biased operational sex ratio is expected to increase the degree of interlocus sexual conflict, whereas female biased sex ratio is expected to relax interlocus sexual conflict. Compared to females from the female-biased regime, those from male biased regimes evolved higher resistance to mate harm in terms of increased survivorship upon continuous male interaction and/or increased number of copulations (Wigby and Chapman 2004, Michalczyk et al. 2010). Crudgington et al. (2005) also found the females subjected to male biased operational sex ratio (for >50 generations) to be more resistant to mate-harm relative to the females evolved under complete monogamy. However, these studies did not address the life-history consequence of such female adaptation, specifically aging and life-span of the females.

Maklakov et al. (2005, 2006) adopted the completely opposite approach to relate intersexual conflict and evolution of life-span and aging. While males selected for late reproduction were shown to be less harming early in life (Maklakov et al. 2005), females selected for late reproduction were had increased resistance to mate harm late in life relative to early reproducing females (Maklakov et al. 2006).

Here I report results from a long term experimental evolution study using laboratory adapted populations of *Drosophila melanogaster*, where intensity of interlocus sexual conflict was varied by manipulating adult sex ratio. Upon 45 generations of selection, I recorded female fitness (progeny produced) under two different conditions – single mating and continuous exposure to males. After 50 generations of selection, I measured adult longevity of flies from the experimental populations under both mated and virgin conditions. I also recorded the mortality rates of males from the base population when combined with mates from the three sex-ratio regimes. Finally, after 52 generations, I assayed (a) female (spontaneous) locomotor activity when held with ancestral males and (b) frequency of courtship activity of ancestral males directed towards females from the three selection regimes.

METHODS:

The detail of the experimental evolution protocol followed is mentioned in Chapter 2 (Experimental system). Here I used females from M1-3, C1-3 and F1-3 populations and males from the LH base population (see Chapter 2). The selection regime females were generated after one generation of standardization (described in Chapter 2). All the flies were grown under standard larval density (150±10 per 8-10ml food in each vial). More detail of the method of generation of the flies for the assays is mentioned in Chapter 2.

A. Measurement of dry body weight (Generation-45):

After 45-47 generations of selection, I measured the dry body weight of the selected females after standardization for one generation. Flies were grown at their usual larval density (150 per 8-10ml of food) and collected soon after eclosion (<6hours). These freshly eclosed females were flash frozen in groups of five. Later they were dried at 60^{o}C for 48 hours and weighed in a high precision electronic balance (Sartorius CPA225D) to the nearest 0.01mg in groups of five (previously formed). A total of 50 females per population were weighed.

B. Fitness (progeny produced) of selected females:

This assay was done after 45-47 generations of selection. Fitness of the selected females was measured under two conditions – (a) Single-Mating (SM) and (b) Continuously-Exposed (CE). Selection regime females and LH-males were grown under standard conditions and were collected as virgins (<6 hour post eclosion) using light CO_2-anaesthesia. Virgin flies were held in single sex vials (selection regime females: 8 per vial, LH-males: 10 per vial). 2days old (post eclosion) virgin females of each population were combined with virgin LH-males of the same age. This was done by combining one vial of selected females (=8 females) with one vial of LH-males (=10 males) into a fresh food vial (seeded with 3.73mg live Yeast) without anaesthesia. 20 such vials were set up for each population, out of which 10 were randomly assigned as SM-vials and rest 10 as CE-vials. The SM-vials were sorted (using light CO_2 anaesthesia) after 1 hour - males were discarded and females were kept in the same vial. Two days later these females were transferred (using light CO_2 anaesthesia) to bottles provisioned with ample amount of food and were left undisturbed for 18 hours during which they oviposited. The CE-vials were left undisturbed after the initial combination for two days. Following the two days of continuous exposure to males, females from these vials were sorted (using light CO_2 anaesthesia) and transferred to bottles for oviposition (similar to SM-

vials). Males were discarded. After the 18-hours oviposition window females from both types (SM & CE) of treatments were discarded and the bottles were preserved for progeny count. 12-days later the bottles were frozen at -20^{0}C and the progeny was counted. Progeny count was taken as the measure of fitness of females from the three different selection regimes under two conditions – CE and SM, when exposed to a common back ground (LH) male-phenotype (=mate-harm). The bottles, in which the progeny grew, did not show any sign of crowding and hence my measure of fitness was not confounded by the progeny growth environment. The progeny count of each bottle was used to calculate the mean production of each female in that vial:

$$\text{Mean progeny production} = \frac{Total\ progeny\ count\ of\ one\ bottle}{number\ of\ females\ (usually\ 8)}$$

These vial means were used as the unit of analysis.

C. Longevity experiment:

This assay was done following 50 generations of selection. After one generation of standardization, selected females were assayed for their longevity under both virgin and mated condition. Selected females were collected as virgins on 10th days post egg-collection and held in single sex vials. 8 flies were held in each vial. LH males were collected on the same day as virgins and held under similar condition. On 12th day, selected flies were randomly divided in to two groups – virgin and mated (10 replicates for each population).). For mated set, 8 selected females were combined with 8 LH-males in a food vial containing yeast supplement on top (amount of Yeast per female matching the selection protocol). Ten such vials were set up per population. For the virgin set, 8 selected females were introduced into a food vial containing yeast supplement on top (amount of Yeast per female matching the selection protocol). 8-10 such vials were set up per population. Other than the first day

day of setting up the experiment, the experimental vials were never supplied with Yeast. Flies were transferred into fresh food vials and dead flies were counted every alternate day. For mated set, sex ratio within a vial was readjusted on alternate days by introducing a replacement LH-male (if LH male died) or removal of LH-males (if selected female died). These replacement males were held under similar condition in bottles, under equal sex ratio (with LH-females). I calculated mean longevity values for each vial from the mortality data. These vial means were used as the units of analysis.

Measures of rate of aging:

(c) Gompertz model: age dependent and age independent rate of aging

The Gompertz parameters were determined for the selection line females under two above mentioned conditions (virgin and mated) following an identical method described in Chapter 4a.

(d) Age specific survivorship rates

I analysed aging rate by looking at age specific survival rate across the different selection regimes under both mated and virgin status. Such method is a crude way of looking at aging rate and has been previously used by Adler and Bonduriansky (2011) as an alternate measure of aging rate.

The entire life-span of the flies were divided into four phases - Phase 1 (day-1 to 50% of the maximum lifespan), Phase 2 (50-67.67% of the maximum lifespan), Phase 3 (67.67-83.33%) and Phase 4 (83.33% to maximum lifespan). Mean survival rate in each of these phases was defined in the following way:

$$Mean\ survival\ rate\ in\ Phase_n = \frac{proportion\ surviving\ at\ the\ end\ of\ Phase_n}{proportion\ surviving\ at\ the\ start\ of\ Phase_n}$$

Mean survivorship rate of each phase was analysed. Phase 4 – survival rates were by definition either zero or indeterminate and hence the remaining three phases were analysed. In addition, mated females died much faster and hence there was no meaningful measure of survival rate beyond Phase 2. The two mating status (mated and virgin) were analysed separately.

Mortality of LH-males:

I recorded the deaths of LH-males in the mated longevity vials. The total number male deaths recorded in each vial throughout the experiment was used as a unit of analysis.

D. Courtship frequency of ancestral males directed towards selected females:

For courtship observations selected females were separately kept with the LH-males in standard 8-dram vials with food. For the ease of observation the adult density was kept at 5 pairs (5 males, 5 females) per observation vial. All flies were grown under standard condition after one generation of standardization and collected as virgins during the peak of their eclosion. Virgin females from each of the 9-populations were held for two days in single-sex vials (5 per vial). Following this, on 12^{th} day post egg collection (i.e., 2^{nd} day post eclosion), selected females were combined with LH-males into observation vials containing food provisioned with 2.33mg live Yeast (i.e., 0.47mg per female). 10 observation vials were set up per population. The vials were observed over the next two days with the observers being blind to the identity of the females in a given vial. Each vial was observed four times a day. All observations were done during the "light"-phase of the 12:12 LD cycle, with ample ambient light and 25^{o}C temperature. Each observation was spaced by 1 hour from another. During the entire period of observation vials were kept absolutely undisturbed. Each observation lasted for 30 seconds, during which the total number of courtship events (directed

to females) was recorded. Using the data from all the observations, I calculated the mean number of courtship events per vial per observation. This vial mean was then used as the unit of analysis.

E. Spontaneous locomotor activity:

The set up for the locomotor activity observation was identical to that of the courtship frequency observation, except that the observation, instead of being a "scan" (as the courtship observation), was of a "focal sampling" type. Activity of the selected females was observed. The vials were observed on 3rd and 4th day (post eclosion), during the light phase, under ample ambient light and standard laboratory conditions (25^{o}C and 60-80% relative humidity). During each observation one individual (selected female) was chosen randomly as the focal animal. Potential observer bias was avoided by careful randomization of the selection of individuals and by the fact that observers were blind to the identity of the females in a given vial. The observation vials were divided into four regions (roughly equal in size) and numbered. During each reading the region was selected with the help of a random number generator. The individual present in the chosen region was observed. Occasionally more than one individual was present in the selected region and in such situation one out of them was observed. If the chosen region did not have any individual, another random number was generated and the process is repeated until a target individual was spotted. Once the focal animal was spotted, it was observed for 4 seconds. During this time if the animal showed any displacement it was scored as 'active', otherwise 'inactive'. Immediately following this, the same individual was observed for another 4 seconds and scored before moving to the next vial. For each vial, this process was repeated thrice within a span of 1 hour. This whole 1 hour long observation was repeated 4 times in each of the observation day. All the observations were taken during the light phase of the 12 hours light/12 hours dark cycle,

starting from at least after 3 hours after lights-on. Finally, the average number of times a vial was scored 'active' (defined as 'activity score') throughout the two observation days was taken as the unit of analysis.

F. Data analyses:

Mean longevity (8-10 replicate per treatment) was analysed using two factor mixed model ANOVA with selection regime as fixed and block as random factor using vial means as the unit of analysis. The two mating status (mated and virgin) were analysed separately. Gompertz parameters were analysed using identical method followed in Chapter 4a. The difference in a_0 and a_1 was calculated using the Gompertz estimates. Then a 99% confidence interval around this measure of difference was created using the model derived standard errors and correlation coefficients. If the 99% confidence interval included zero, it was concluded that the 'difference' is not significantly different from zero and hence the two populations did not differ in terms of their a_0/a_1. Mean survival rate (vials means) for each age window (Phase 1/2/3) was analysed using two factor mixed model ANOVA, with selection regime as fixed and block as random factors. Total number of deaths of the ancestral males in each vial was analysed using two factor mixed model ANOVA with selection regime as fixed and block as random factor (vial values = unit of analysis). The ancestral (LH) male-mortality was analysed using two-factor mixed model ANOVA with selection regime as fixed factor and block as random factor taking population estimates as the unit of analysis.

Dry body weight was measured in 9-10 replicates (groups of 5 females) for each population (section D). Thereafter the body weight data was analysed using two factor,

mixed model ANOVA with selection regime as fixed factor crossed with random blocks taking the body weight of each group as the unit of analysis.

For the progeny production assays (section E), 8-10 replicate vials were set up for both "single mating" and "continuous exposure" sets. Courtship frequency (section G) and activity score (section H) of selected females were assayed using 8-10 vials for each population. Analyses of the three mentioned traits were done using vial means. Progeny production data was analysed using three factor, mixed model ANOVA with selection regime and exposure status (single mating/continuous exposure) as fixed factors and block as random factor taking vial means as the unit of analysis. The data from the two mating status were also analysed separately using two factor mixed model ANOVA with selection regime as fixed factor crossed with random blocks. In addition, females' resistance to mate-harm was also analysed by using a "cost score" (ω), which is defined as the following:

$$\omega = \frac{Mean\ prog.count\ of\ the\ treatment\ as\ SM\ - Mean\ prog.count\ of\ a\ vial\ under\ CE}{Mean\ prog.count\ of\ the\ treatment\ as\ SM}$$

Assuming the progeny production by a female under SM condition to represent the fitness of an unharmed female, a higher ω indicates a greater reduction in progeny production in females upon the CE treatment. ω was calculated for each CE-vial for all the populations. These values were then analysed using two-factor ANOVA with selection regime as fixed factor crossed with random blocks.

All multiple comparisons (except in the analysis of LH-male mortality rate) were done using Tukey's HSD. All the analyses were done at α=0.05 level of significant using Statistica (for Windows, version 10).

RESULTS:

A. Dry body weight of selected females:

Selection regime was found to have significant effect on the dry body weight of the selection line females ($p=0.016$, Table 5.1). There was no interaction between block and selection regime ($p=0.26$, Table 5.1). Multiple comparison (Tukey's HSD) showed that F females were significantly heavier than C females while the difference between dry weight of C and M-females was not significant (Figure 5.1).

B. Number of progeny produced by selected females:

The analysis of fitness data using three factor ANOVA indicated a nearly significant effect of selection regime ($p=0.051$) and a significant effect of mating status ($p<0.0001$). There was a significant interaction between selection regime and mating status ($p<0.0001$). Block had significant effect but none of the other interactions, including the three-way interaction, were significant (Table 5.2). I found significant effect of selection regime on female fitness (progeny produced) under both "Continuous Exposure" (to LH-males) and "Singly Mated" (to LH-males) conditions (Table 5.2). Tukey's HSD showed that when held with LH-males for two days (i.e., the continuous exposure set) M-females had highest fitness followed by C-females, while F-females had the minimum fitness (Figure 5.2). Multiple comparison of the "singly mated" set suggested that F-females had significantly higher fitness compared to both M and C-females, while there was no difference between M and C-females' fitness (Figure 5.2). Analysis of ω revealed a significant effect of selection regime, consistent across the three blocks (Table 5.2, Figure 5.3). Multiple comparisons using Tukey's HSD revealed that F-females had significantly higher ω compared to C-females, which in turn had significantly higher ω compared to M-females (Figure 5.3). Thus M-females were found to be the least affected by the CE treatment, while F-females were found to be the worst sufferers.

C. Longevity of selected females:

i. Female mean longevity:

Selection regime has significant effects on mean female longevity under both virgin and mated conditions ($p<0.0001$ for virgin, Figure 5.4a; $p=0.04$ for mated, Figure 5.4b; Table 5.3). Tukey's HSD suggested that F-virgin females had significantly longer life span compared to both M and C virgin females (Figure 5.4a). Under mated condition, M-females had approximately significantly higher longevity compared to both F and C (Tukey's HSD, Figure 5.4b). There was a significant effect of block under virgin conditions, but none of the interactions were significant.

ii. Measures of rate of aging:

(a) Gompertz model:

The estimated Gompertz parameters are given in Table 5.4. None of the parameters differ significantly across the three regimes (Table 5.5).

(b) Age specific survival rates:

Survival rate of virgin females did not show any significant difference across selection regimes during Phase-1 and 3 (Table 5.6). However, there was significant effect of selection regime on Phase-2 survival rates ($p=0.031$, Figure 5.5a, Table 5.6). Multiple comparison (Tukey's HSD) suggested that F-virgin females had significantly higher survival rate than that of M and C females during this phase. The significant interaction term during the third phase can be attributed to the very low number of deaths during that phase resulting in across block heterogeneity.

In mated females, a significant effect of selection regime was observed ($p>0.0001$, Figure 5.5b, Table 6) during Phase-1. M-females showed a significantly higher survival

rate compared to both F and C-females (Tukey's HSD). However, there was no significant difference in the survival rate across populations during Phase-2 (Table 5.6).

iii. Ancestral (LH) male mortality when exposed to the selected females:

I found significant effect of selection regime of the females on the number of ancestral male deaths recorded (Table 5.7). Multiple comparisons using Tukey's HSD showed that when males were held with M-females, more male deaths were recorded compared to when the males were held with C or F-females (Figure 5.6). The male mortality recorded against C and F-females was not significantly different (Tukey's HSD, Figure 5.6).

D. Frequency of courtship directed to selection line females:

Courtship frequency of LH-males directed to the selected females were not different across selection regimes ($p=0.35$, Table 5.8a).

E. Spontaneous locomotor activity of selection line females:

Spontaneous locomotor activity significantly differed across selection regimes ($p=0.001$, Table 5.8b). Multiple comparisons suggested M-females were significantly more active compared to C and F-females (Figure 5.7). Even though, F-females were less active than C-females, this difference was not significant.

Effect	SS	MS Num	DF Num	F	P
Selection regime	0.001	0.001	2	4.303	0.017*
Block & Random	0.003	0.001	2	8.706	<0.001
Selection regime×Block	0.001	0.000	4	1.348	0.260

Table 5.1: Summary of two-factor ANOVA on dry body weight data treating selection regime as the fixed factor crossed with random blocks. p-values marked with * are statistically significant.

Analysis	Source	SS	MS Num	DF Num	F	p
(a) Both mating status combined	Selection regime	219.79	109.90	2	3.03	0.051*
	Mating status	1305.08	1305.08	1	35.93	<0.001*
	Selection regime×Mating status	1568.02	784.01	2	21.59	<0.001*
	Block & Random	539.63	269.82	2	7.43	0.001*
	Selection regime×Block	196.41	49.10	4	1.35	0.253
	Mating status×Block	4.87	2.43	2	0.07	0.935
	Selection regime×Mating status×Block	78.50	19.63	4	0.54	0.706
(b) Singly Mated	Selection regime	356.32	178.16	2	6.44	0.003*
	Block & Random	258.99	129.49	2	4.68	0.012*
	Selection regime×Block	56.89	14.22	4	0.51	0.726
(c) Continuous Exposure	Selection regime	1430.71	715.36	2	15.90	<0.001*
	Block & Random	285.35	142.67	2	3.17	0.048*
	Selection regime×Block	222.91	55.73	4	1.24	0.302
(d) Cost score	Selection regime	1.01	0.50	2	32.29	<0.001*
	Block & Random	0.004	0.002	2	0.13	0.875
	Selection regime×Block	0.06	0.01	4	0.94	0.445

Table 5.2: Summary of results of (a) three-factor ANOVA using selection regime and mating status as fixed factor crossed with random blocks on the progeny production data, (b) two-factor ANOVA using selection regime as fixed factor crossed with random blocks on the progeny production data from singly mated set, (c) two-factor ANOVA using selection regime as fixed factor crossed with random blocks on the progeny production data from continuously exposed set. Vial means were taken as the unit of analysis. p-values marked with * are statistically significant.

Mating status	Effect	SS	MS Num	df Num	F	p > F
Virgin	Selection regime	593.46	296.73	2	18.05	< 0.001*
	Block & Random	169.04	84.52	2	5.14	0.008*
	selection regime×Block	47.01	11.75	4	0.71	0.584
Mated	Selection regime	170.02	85.01	2	3.31	0.042*
	Block & Random	85.94	42.97	2	1.67	0.195
	selection regime×Block	4.62	1.16	4	0.04	0.996

Table 5.3: Summary of the results of two-factor ANOVA with selection regime as fixed factor crossed with random block on mean longevity. Vial means were taken as the unit of analysis. p-values marked with * are statistically significant.

Parameter	Value	SE	DF	t-value	p-value
Age independent Gompertz parameter, a_0					
α_0	0.03572	0.01259	473	2.83628	0.0048
γ_{02}	-0.0138	0.01543	473	-0.8966	0.3704
γ_{03}	-0.0108	0.01543	473	-0.7015	0.4833
β_0	-0.0275	0.01314	473	-2.0911	0.037
φ_{02}	0.012	0.01614	473	0.74361	0.4575
φ_{03}	0.00842	0.01613	473	0.52185	0.602
Age dependent Gompertz parameter, a_1					
α_1	-0.0115	0.00988	473	-1.165	0.2446
γ_{12}	0.01231	0.0145	473	0.84946	0.3961
γ_{13}	0.00826	0.01327	473	0.62234	0.534
β_1	0.02261	0.01232	473	1.83552	0.0671
φ_{12}	-0.0062	0.01774	473	-0.349	0.7272
φ_{13}	0.00054	0.01709	473	0.03185	0.9746

Table 5.4: Summary of the Gompertz parameters estimated by fitting the raw mortality data in the Gompertz model using R (version 2.14.1), nlme-R package.

Comparison	Difference	Variance	SE	99% C.I.
		Mated a_0		
M-C	-0.01082368	2.38×10^{-04}	0.015429	±0.0403622
F-C	-0.01383317	1.73×10^{-04}	0.0131356	±0.0343628
M-F	0.00300949	7.93×10^{-04}	0.0281633	±0.0736751
		Virgin a_0		
M-C	-0.00240498	2.24×10^{-05}	0.0047327	±0.0123808
F-C	-0.00182885	2.24×10^{-05}	0.0047359	±0.0123891
M-F	-0.00057613	7.28×10^{-05}	0.0085333	±0.0223231
		Mated a_1		
M-C	0.00826131	1.76×10^{-04}	0.0132747	±0.0347265
F-C	0.01231445	2.10×10^{-04}	0.0144968	±0.0379236
M-F	-0.00405314	5.82×10^{-04}	0.0241145	±0.0630835
		Virgin a_1		
M-C	0.00880558	1.44×10^{-02}	0.1201511	±0.3143152
F-C	0.00612304	4.80×10^{-05}	0.0069316	±0.0181329
M-F	0.00268254	8.10×10^{-04}	0.0284518	±0.0744299

Table 5.5: Summary of the analyses of Gompertz parameters. Data from virgin and mated set were analysed separately. The difference between a pair of regimes were derived by using the Gompertz model estimates and then a 99% confidence interval was created around this difference by using model derived standard errors. In none of the comparison, the difference was significantly different from 0, indicating that there was no significant difference in the Gompertz parameters across the different regimes.

Treatment	Stage	Effect	SS	MS Num	df Num	F-Ratio	p > F
Virgin	Phase 1	Selection regime	0.034	0.017	2	1.09	0.342
		Block & Random	0.008	0.004	2	0.239	0.788
		Selection regime×Block	0.025	0.006	4	0.393	0.813
	Phase 2	Selection regime	0.469	0.234	2	3.637	0.031*
		Block & Random	0.704	0.352	2	5.462	0.006*
		Selection regime×Block	0.201	0.05	4	0.781	0.541
	Phase 3	Selection regime	0.028	0.014	2	1.231	0.298
		Block & Random	0.055	0.027	2	2.41	0.097
		Selection regime×Block	0.141	0.035	4	3.098	0.021*
Mated	Phase 1	Selection regime	0.626	0.313	2	10.032	<0.001*
		Block & Random	0.282	0.141	2	4.515	0.014*
		Selection regime×Block	0.213	0.053	4	1.707	0.157
	Phase 2	Selection regime	0.06	0.03	2	0.624	0.539
		Block & Random	0.045	0.022	2	0.466	0.629
		Selection regime×Block	0.149	0.037	4	0.777	0.544

Table 5.6: Summary of separate two-factor ANOVAs on survival rate at each of the age classes for females under both virgin and mated conditions. Selection regime was taken as fixed factor crossed with random blocks. Vial means were taken as the unit of analysis. p-values marked with * are statistically significant.

Source	SS	df	MS	F	P
Selection regime	7.62	3.81	2	3.51	0.034*
Block&Random	4.82	2.41	2	2.22	0.115
Selection regime×Block	2.11	0.53	4	0.49	0.746

Table 5.7: Summary of results of two-factor ANOVA with selection regime as fixed factor crossed with random block on LH-male mortality data. Total mortality observed in each vial was taken as the unit of analysis. p-values marked with * are statistically significant.

Trait	Source	SS	MS	DF	F	p
(a) Courtship frequency	Selection regime	14.33	7.17	2	1.06	0.352
	Block & Random	324.88	162.44	2	23.96	<0.001*
	Selection regime×Block	13.65	3.41	4	0.5	0.733
(b) Spontaneous locomotor activity	Selection regime	0.09	0.04	2	7.35	0.001*
	Block & Random	0.20	0.10	2	16.86	<0.001*
	Selection regime×Block	0.01	0.00	4	0.57	0.684

Table 5.8: Summary of the results of two-factor ANOVA with selection regime as fixed factor crossed with random block on (a) Mean courtship frequency of LH-males directed towards selection regime females and (b) Mean spontaneous locomotor activity of selection regime females. Vial means were used as the unit of analysis. p-values marked with * are statistically significant.

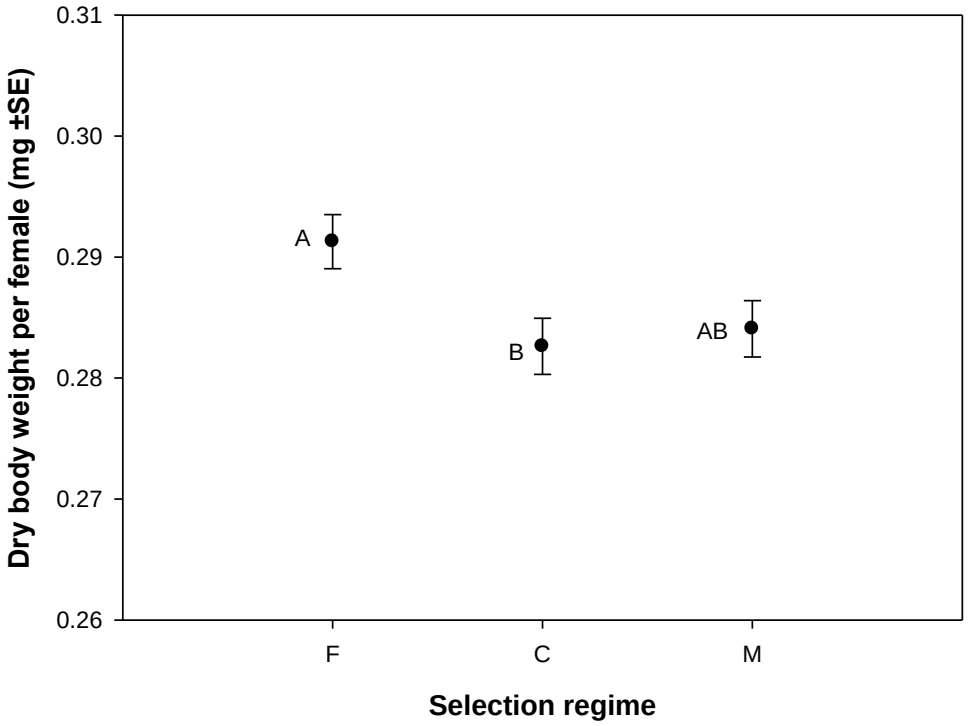

Figure 5.1: Dry weight at eclosion of females from the three selection regimes. Individuals were weighed in groups of five. An average body weight was calculated using the weight of the five flies. These mean values were taken as the unit of analysis. Points not sharing common letters are significantly different (determined using Tukey's HSD).

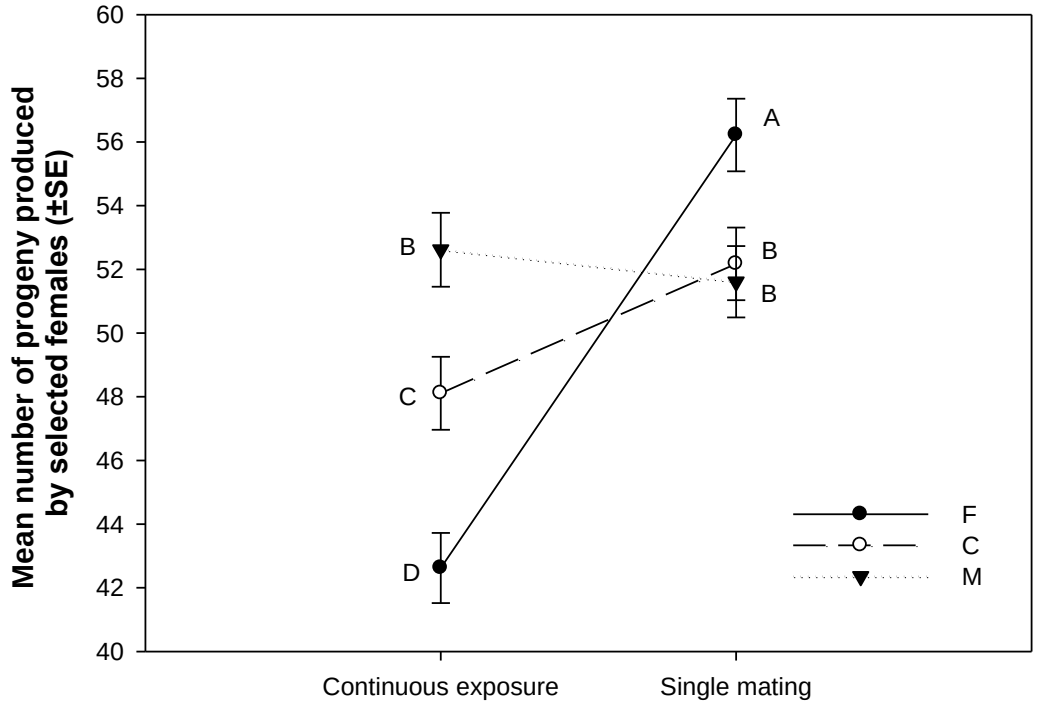

Figure 5.2: Number of progeny produced by the selection line females after continuous exposure to LH-males for two days and single mating with LH-males. Total number of progeny produced by all the females in a vial was counted and a mean is calculated using this data. The vial means are then used as the unit of analysis. Points not sharing common letters are significantly different (determined using Tukey's HSD).

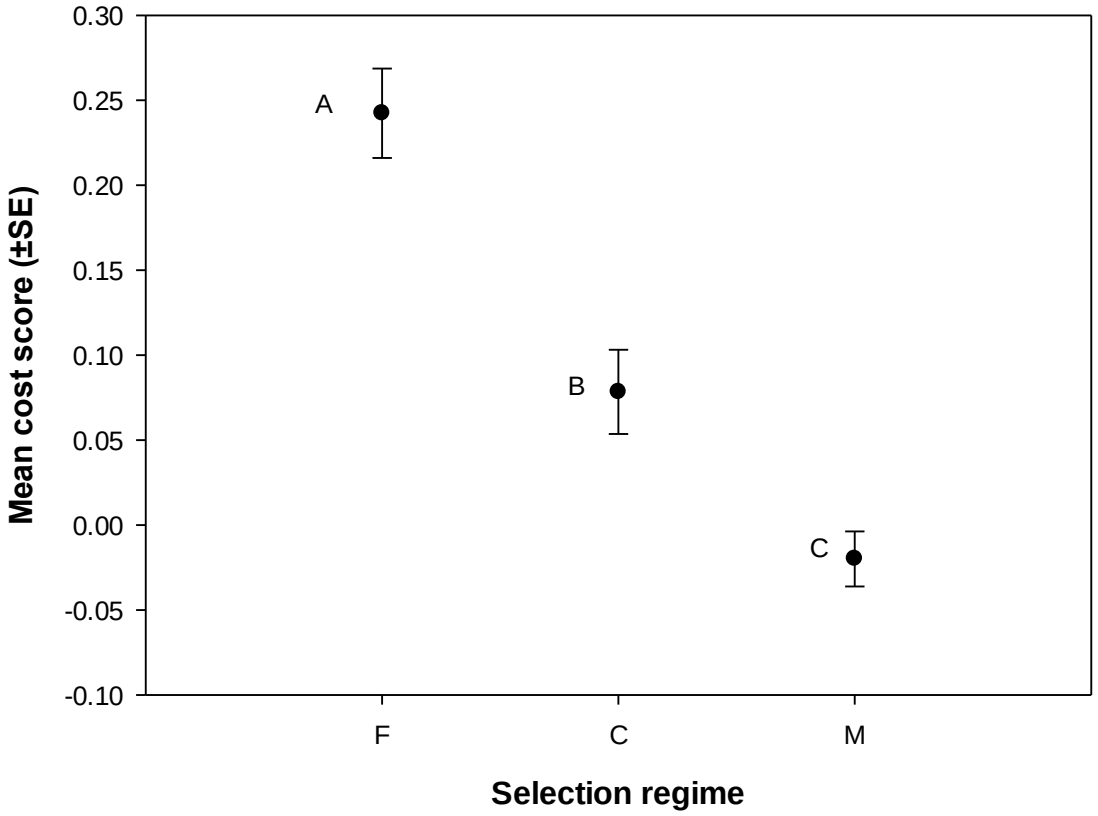

Figure 5.3: Mean cost scores (ω) for the selection regime females. ω was calculated for each CE-vial using the formula given in the methods section. ω represents the reduction in progeny production by selection regime females upon CE treatment (i.e., continuous exposure to LH-males relative to single mating). The ω calculated for each CE-vial was used as the unit of analysis. Points not sharing common letters are significantly different (determined using Tukey's HSD).

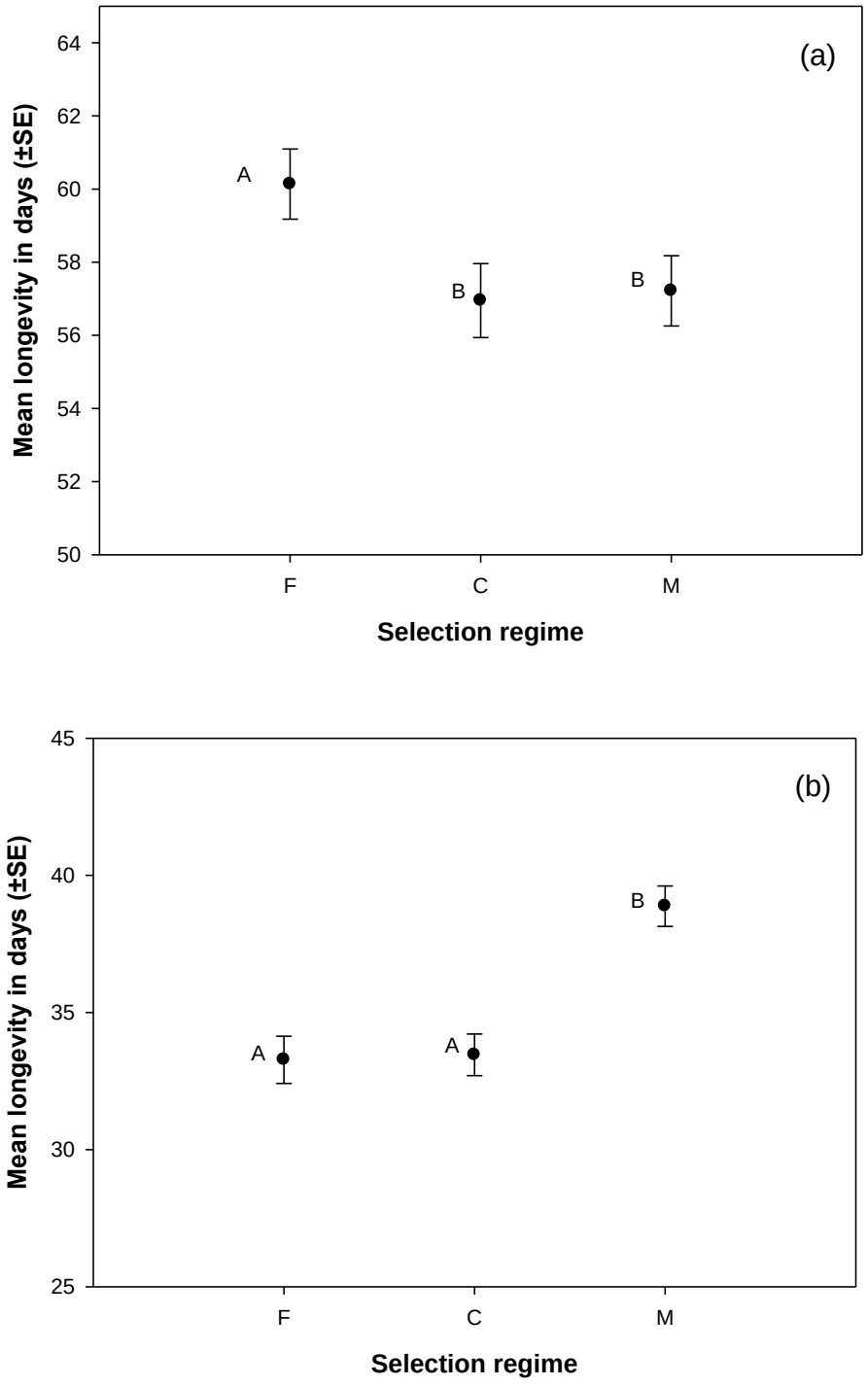

Figure 5.4: Effect of selection regime on mean longevity of (a) virgin female, (b) mated female (continuously held with LH-males throughout the life-span). A mean longevity was calculated for each vial. These vial means are used as the unit of analysis. Points not sharing common letter are significantly different (determined using Tukey's HSD).

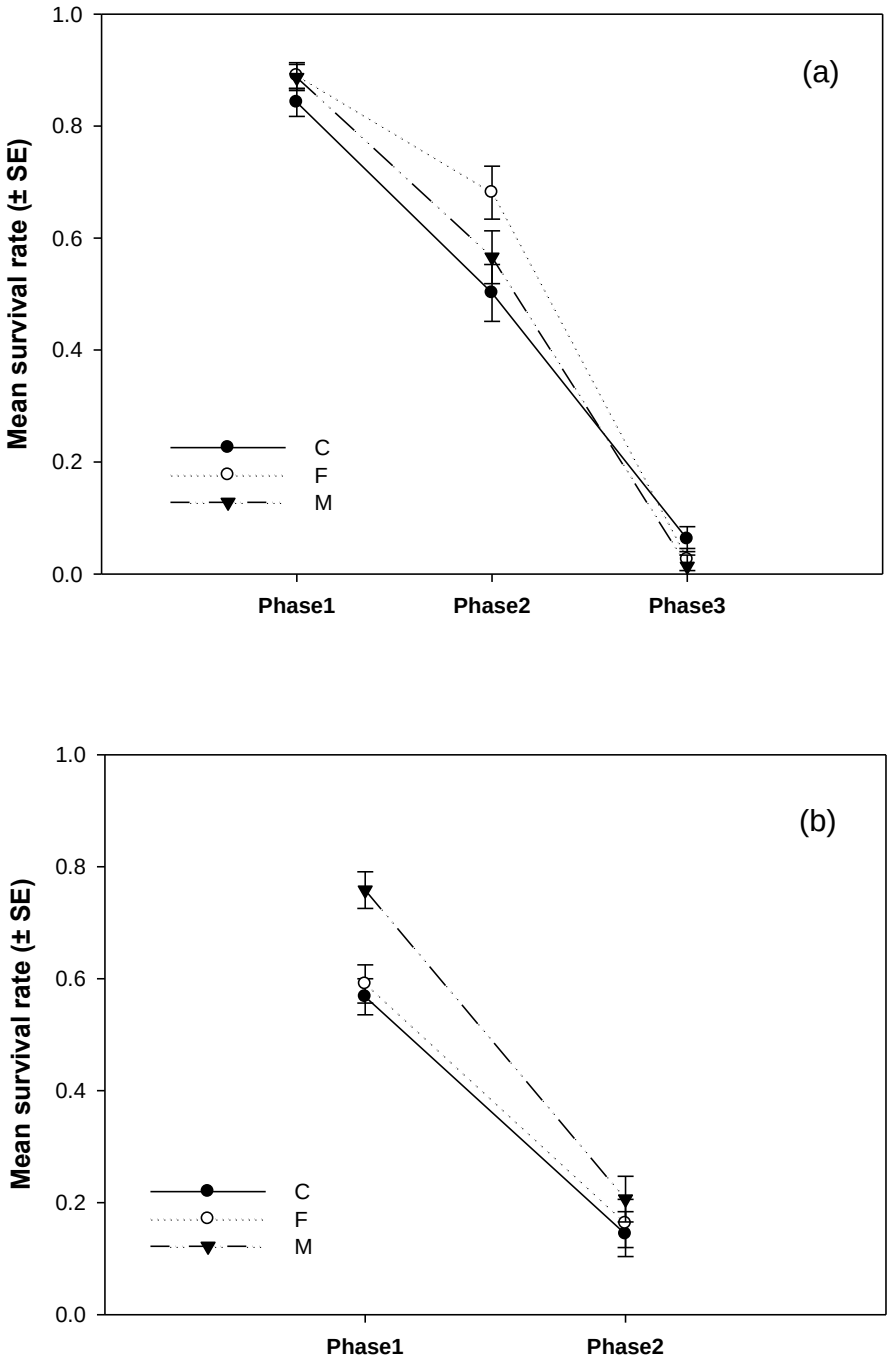

Figure 5.5: Age specific survival rates of selected females under (a) virgin and (b) mated conditions. Phases were defined by systematically dividing the life-span of the females (see methods section for detail). Survival rate was calculated for each vial by dividing proportion of females surviving in that vial at the end of a phase by proportion of females surviving at the beginning of the phase. These vial estimates were then used as the unit of analysis. Each age class (phase) was analysed separately.

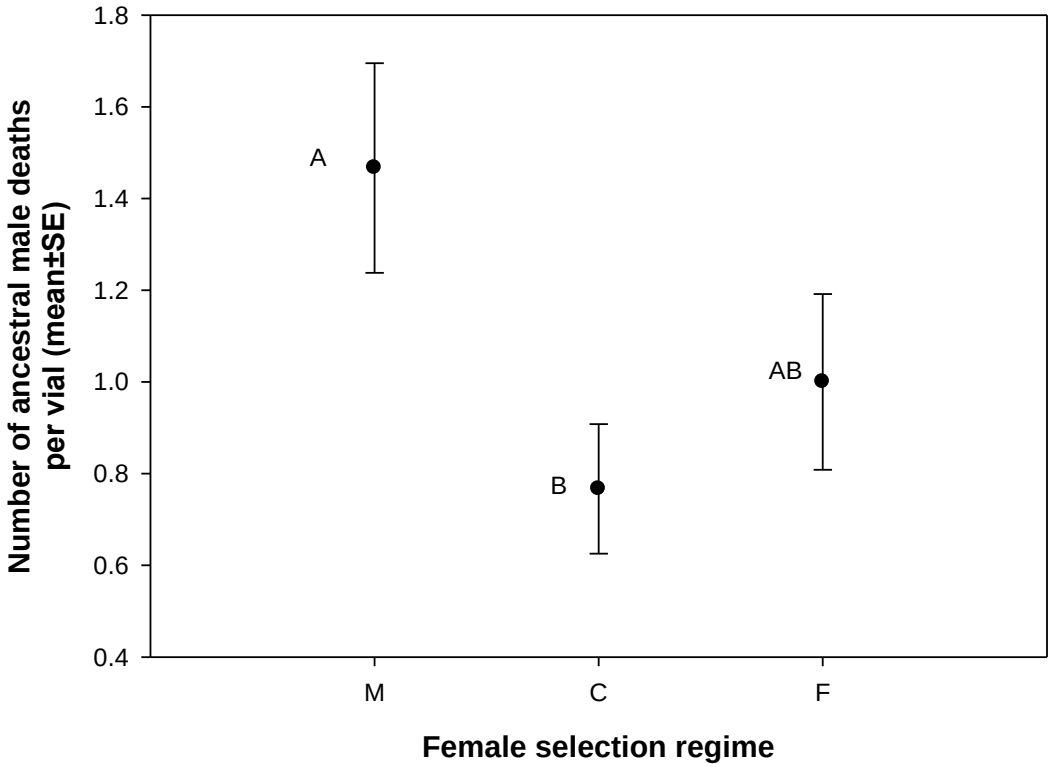

Figure 5.6: Number of deaths of ancestral (LH) males continuously held with selected females (M/C/F) per vial. Total number of male deaths observed in each vial throughout the longevity assay was taken as the unit of analysis. Points not sharing common letter are significantly different (determined using Tukey's HSD).

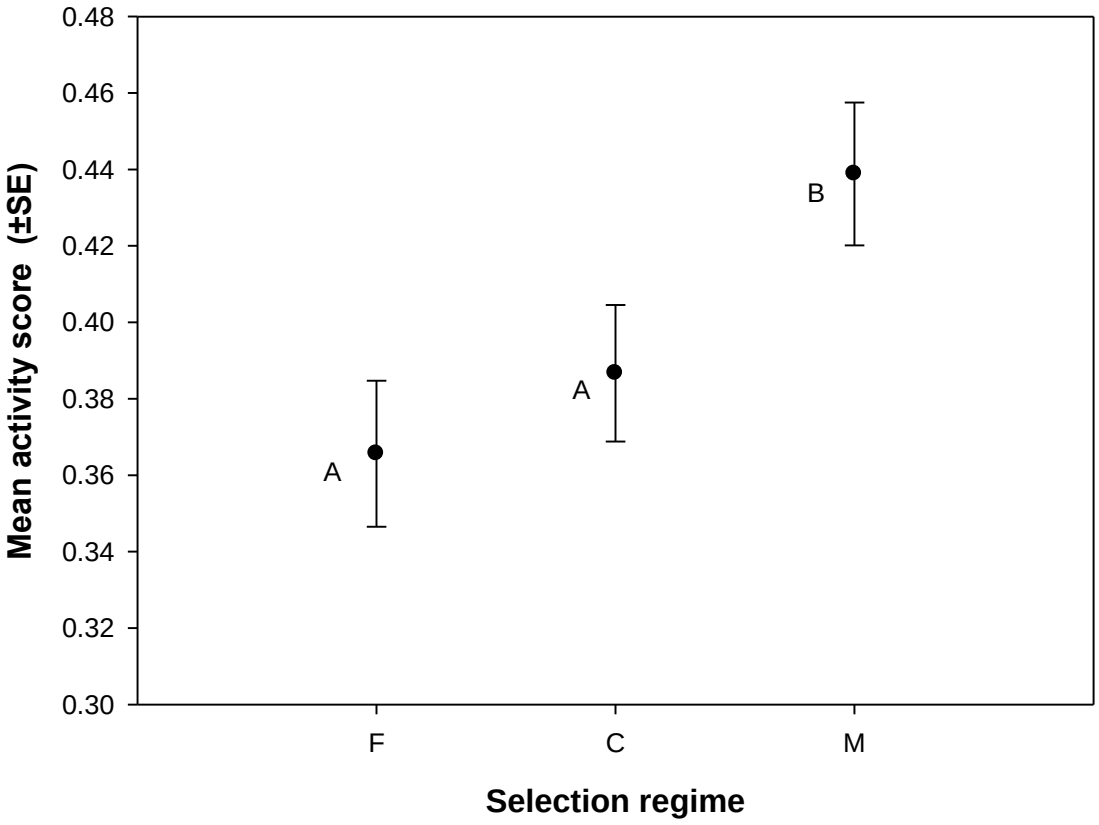

Figure 5.7: Spontaneous locomotor activity of the selection line females held with ancestral males. A mean activity score (see methods section) was calculated for each vial using the raw locomor activity data and this was used as the unit of analysis. Points not sharing common letter are significantly different (determined using Tukey's HSD).

DISCUSSION:

A number of experimental evolution studies have addressed the evolution of female resistance to male induced harm (Holand and Rice 1999, Martin and Hosken 2003, Wigby and Chapman 2004, Crudgington et al. 2005, Maklakov et al. 2007, Michalczyk et al. 2010). While removal of sexual conflict (by enforced life-long monogamy) and alteration of intensity of sexual conflict (by altering operational sex ratio) have been shown to result in the evolution in females ability to resist male induced harassment (or mate-harm), few studies have documented the consequences of such evolution to the rate of aging and life span. Also the mechanism by which females become more (or less) resistant is unknown. Results from my study clearly show that females from the male biased populations (M-females) evolved increased resistance to mate harm (in terms of number of progeny produced as well as longevity). I did not find any evidence of cost of maintaining the resistance physiology in terms of faster aging and/or reduced longevity of the M-females. Additionally I found that M-females evolved increased locomotor activity. I also found tentative evidence to suggest that as a by product of evolution of female resistance to mate-harm, mortality rate in males can increase. On the other hand, relaxation of the pressure of sexual conflict led to the increase in body size (body weight at eclosion) of females from the female biased selection regime (F-females). The F-females were also found to have increased baseline longevity and progeny production. However, when held with males (LH) F-females suffered a greater mate-harm in terms of severely curtailed progeny production. I now discuss each of these findings in detail.

Evolution of female resistance to mate-harm:

Females, in fruit flies, experience harming male effects (i.e., mate harm) both in terms of reduced longevity as well as fecundity (Fowler & Partridge 1989, Kuijper et al. 2006, Rice et al. 2006). In my selection regime M-females experienced three times more male interaction

compared to the C-females and thus potentially, three times more mate-harm. As one would predict, I observed adaptation of M-females to mate-harm. When held continuously with ancestral males, M-females produced almost 10% more progeny (thus has higher total fitness) and had significantly higher mean longevity compared to the C-females. This finding confirms the results of Wigby & Chapman (2004) where the authors found their females from male biased regime to have lower, albeit non-significant, survival risk ratio compared to that of the females from "Equal sex ratio" population.

During their normal maintenance regime, F-females experienced three times less (antagonistic) male interactions compared to C regime. Thus in this much benign condition, the selection pressure on females to maintain the resistance traits was relaxed, at least to some extent. Therefore one would predict these females to evolve lower resistance to mate harm if maintaining a resistance mechanism is costly. Here, I observed F-females to produce about 11% less number of progeny compared to C-females when held with ancestral males continuously (Figure 5.2), indicating their higher susceptibility to mate-harm. However, in the longevity assay, upon continuous exposure to ancestral males, F-females did not show any difference in mean longevity compared to C-females. Thus the F-females' increased susceptibility to mate-harm was only expressed in terms of decreased progeny production but not in terms of increased mortality.

In my selection regime the amount of progeny produced by the females during the 18 hour window on 14^{th} day of the maintenance cycle represents their total life-time progeny production. The assay condition matched the selection condition very closely and the progeny production was measured at 14days (post egg collection) age during an 18 hour window. Thus, the progeny count represents the total life-time progeny production of the selected

females – the ultimate currency in which their fitness is measured. Previous studies (Kuijper et al. 2005, Rice et al. 2006) have shown that mate-harm in this system can be detected even by this measure of female fitness. In my assay, C-females produced about 8% less progeny under CE condition compared to the SM condition. This difference in progeny production between SM and CE conditions was not significant in M-females. F-females, on the other hand, produced significantly more (about 7.5% more relative to M and C-females) number of progeny under SM condition relative to M and C-females but under CE condition they produced significantly less number of progeny than either C or M-females. Interestingly, the progeny production of M-females was not significantly different in the SM and CE conditions, indicating that the two day interaction period with the males had virtually no negative effect on the fitness of M-females. It is important to point out here that the experiment closely resembled the regular maintenance schedule. Given that the mortality of females over the two day interaction period in the experiment and in the regular maintenance protocol is negligible, female fitness is dependent on progeny production post the interaction period. Thus my results indicate that the M females seem to have evolved absolute resistance to male-induced harm. The F-females produced 24% less progeny under CE condition relative to SM condition (Figure 5.2) – the heaviest drop in progeny production among the three types of females. Thus, F females seem to have the least resistance to male induced harm among all three different types of females. C-females suffered an intermediate decline of about 8% in their progeny production. The analysis using cost score (ϖ) also points to similar conclusion. While M-females were found to have the least ϖ, F-females showed highest ϖ values in all blocks. C-females were found to have intermediate ϖ. These collectively indicate that (a) females under M-regime evolve increased resistance to mate-harm and (b) ability to resist mate-harm degenerated in F-females. The increased progeny production of F females under SM indicates that there is a significant cost of maintaining a

resistance mechanism. Hence, under F selection regime, where there is relaxed selection on maintaining a resistance mechanism, resources are freed up for investment into progeny. Thus, when male harassment is absent, F females show the highest fitness. Alternatively, such increase in fecundity can also be attributed to the increased body size of the F-females. However, it is interesting to note that F females suffer greater decline in progeny production under CE condition, despite their increased body size. Thus, is very likely that the differences in the fitness of the three types of females under SM and CE conditions actually represent their altered patterns of investment of resources in resistance related traits.

Evolution of life-span and aging in females

In my analyses, I tested whether (a) increased sexual conflict leads to decreased baseline female life-span and survival rates (Promislow 2003) and (b) relaxing (as opposed to complete removal) the conflict increases base line lifespan and survival rates (Maklakov et al. 2007) by assaying female longevity under virgin conditions. When not exposed to males, M-females lived as long as C-females and there was no significant difference in age specific survivorship between the females of these two regimes. However, virgin F-females had significantly longer mean longevity and had higher survival rate during Phase-2. This indicates that while in M-regime baseline female mortality rates did not change, same evolved under F-regime. There are two possible explanations (non-mutually exclusive) for the observed decline in baseline mortality in F-females. (a) Under decreased sexual antagonism, F-females evolved decrease in baseline investment in mate-harm resistance related (i.e., conflict related) traits and thereby investing more in other life-history traits, such as, longevity. (b) The higher longevity of virgin F-females is mainly because of their increased body size.

Under mated condition, M-females lived longer on an average (see mean longevity data) presumably because of their increased resistance to mate-harm as discussed earlier. Rate of aging of mated females, measured in terms of Gompertz parameters, was not significantly affected by selection regime. If rate of aging is not different across the three regimes, one would expect similar pattern of survival rates across different age classes in all the populations. However, M-females survived significantly better during phase-1 while in phase-2 there was no difference across different regimes. This is a suggestive evidence of slightly slower aging in M-females under mated condition, not resolvable in terms of Gompertz parameters.

Simple Y-model of resource allocation predicts that increased investment in a resource consuming physiology, like resistance to mate-harm should decrease the amount of resource available to other physiological processes leading to trade-offs (Williams 1966, Van Noordwijk and de Jong 1986, Zera and Harshman 2001). The most common form of such trade-off is that between reproduction and longevity, i.e., fecundity in females and longevity and/or aging rate (Harshman and Zera 2007, Williams 2005, Flatt 2011). Later work on life-history evolution also predicted sexual conflict related traits (such as resistance to mate-harm) to trade-off with longevity and/or rate of aging (Promislow 2003, Bonduriansky et al. 2008). In my study, Compared to the C females, the F females had significantly lower progeny production when housed with males continuously (indicating decreased resistance to mate harm) coupled with significantly higher progeny production when exposed to males minimally, indicating a trade-off between resistance and progeny production. However, when compared to C females, the M females had significantly higher resistance to mate harm (in terms of increased progeny production and longevity when continuously exposed to males) but did not differ from C females in terms of progeny production when exposed to males

minimally or in virgin longevity. Thus, I did not find evidence of a trade-off in my M-females. There are at least two possible interpretations of such finding. (a) Trade-offs between life-history traits are often much more complicated than the simple Y-model, possibly involving more than two traits. This might give rise to a situation where two traits (e.g., resistance and longevity) can show positive or no correlation even though they share a common limiting resource (Zera and Harshman 2001). Hence it is possible that the predicted trade-off might exist between resistance and life-history traits other than longevity and aging (e.g., starvation and desiccation resistance). (b) Resource acquisition in females (e.g., live Yeast foraging) can increase under M-regime ameliorating the trade-off (Sterns 1989, Zera and Harshman 2001). Since at this point I do not have a measure of the resource acquisition it is difficult to account for this possibility. In addition, without the exact knowledge of the nature of physiology of the resistance, it is probably safe not to assume a trade-off (Zera and Harshman 2001, Flatt and Kawecki 2007).

Why did body size at eclosion evolve under F-regime?

Although I do not have any direct evidence explaining the evolution of increased body size (measured as dry body weight) in F-regime, I here provide a plausible hypothesis. Body size, in this system, has been shown to be influenced by sexual conflict. Previous studies using the same base population (LH) have shown that when female specific selection is relaxed, populations evolve decreased body size (Prasad et al. 2007). Prasad et al. (2007) suggested that this is because female specific selection favours larger body size and smaller body size is selected for by the male specific selection. My F-regime represents a condition where male specific selection is likely to be weak, a condition opposite to that of male-limited evolution (Prasad et al. 2007). Hence the observed change in body size possibly represents evolution under female specific selection.

Increased locomotor activity of M females

Long and Rice (2007) beautifully demonstrated locomotor activity to be under intralocus conflict. A significant negative correlation was found between locomotor activity and female fitness (Long and Rice 2007). This negative genetic correlation was believed to be caused by at least two factors – (a) active females attract more courtship from males, which has been shown to bring down female fitness and (b) at least in this laboratory system, higher activity is not beneficial to the females *Drosophila* whereas low activity might be beneficial by conserving energy and increasing the efficiency of live-Yeast foraging (Long and Rice 2007). However, in my M-regime I observed an increase in locomotor activity in M-females. This possibly represents a correlated response to the selection on males in this regime. In M-regime males are under strong selection for traits related to adult fitness (e.g., activity). Therefore it is possible that locomotor activity is strongly selected for in males and the population evolved towards male optima creating a gender load for females. My data also suggest increase in locomotor activity in M-males (see Chapter 4a), supporting the above mentioned argument to some extent. Alternatively, the observed result can be a direct response to the selection on females. One might speculate, that higher activity might allow females to escape from male coercion, thereby getting selected for in M-regime even in females. However, whether increased locomotor activity can provide any form of protection for females from male interactions is not clear.

D. melanogaster males have been shown to have mating and/or courtship preference for (a) larger females (Long et al. 2009) and (b) females with higher locomotor activity (Tompkins et al. 1982). In my assay I found significant difference in both body size and locomotor activity across females of different selection regimes. But I did not observe any difference in the frequency of courtship received by these females.

Male-cost of being with 'resistant' females?

During sexually antagonistic co-evolution female evolution is driven by the mating cost imposed by male interaction on females. Females are thus expected to adapt to such cost, thereby becoming resistant to mate-harm. This in turn is predicted to make mating cost higher for males (Bonduriansky 2008). However, none of the experimental evolution studies shed any light on this prediction. I found my M-females to be more resistant to mate-harm. Thus, one would predict males to invest more in courting these females and thereby pay higher cost of mating. I found tentative evidence in support of this prediction. I observed more ancestral-male deaths in M-female vials compared to that in C-female vials, while the mortality rate difference was marginally not significant. However, I did not find any difference in courtship frequency directed to M-females and the females of other types. However, there are a number of other unexplored ways (such as, cumulative amount of courtship over the entire life-time of the females, male-male aggression, ejaculate investment per female etc.) in which males can potentially invest more. Thus at this point, the cause of higher mortality in males exposed to M-females is not clear.

CONCLUSION:

My study clearly demonstrates the evolution of female resistance to male induced harm. I have shown that females under male biased regime evolved resistance to mate-harm that virtually ameliorated the cost of male interaction in terms of their life-time progeny production in addition to reducing the mortality cost. Resistance to mate-harm degenerated in the females under females biased regime and thereby made them highly susceptible to mate-harm measured in terms of life-time progeny production. I point out the evolutionary relationship between such female adaptation and evolution of life-span and aging in females. Most of my results are consistent with previous empirical and theoretical studies.

Chapter 6

Evolution of life-history traits

INTRODUCTION:

The scheduling of reproduction and mortality throughout the life-time is termed life-history of an organism (Sterns 1992). The study of life-history evolution involves the understanding of the genetic correlations between various traits that constitutes the life-history and how they give rise to a particular life-history under a given ecological condition (Prasad and Joshi 2003). While reproduction and mortality constitute the primary life-history traits, other traits such as pre-adult development time, body size, age at first reproduction, resistance to stress etc, are commonly referred to as life-history related traits. A large number of studies (especially using experimental evolution) have addressed the evolution of life history and related traits (for example, Rose 1984, Mueller 1987, Chippindale et al. 1996, Chippindale et al. 1998, Prasad et al. 2001, see Prasad and Joshi 2003 for review). Over the past couple of decades, with the emergence of sexual conflict theory, there is a growing body of evidence that suggests a role for sexual conflict in life-history evolution (Wedell et al. 2006, Bonduriansky et al. 2008).

Sexual conflict (see Chapter 1) has the potential to alter the pattern of resource allocation, resource acquisition and mortality rate in a sex specific manner, thereby affecting the evolution of life-history and related traits (Promislow and Pletcher 2002, Promislow 2003, Wedell et al. 2006, Bonduriansky et al. 2008). Previous studies, using *Drosophila melanogaster*, have shown that life-history traits, such as, starvation resistance and longevity of both males and females suffer due to the presence of their mates (Chippindale et al. 1997). Populations selected for slower senescence (and late life reproduction), evolved males which

were poor competitors early in life, being less efficient at inducing females to lay eggs (Service and Vossbrink 1996). Similar results were found by Maklakov et al. (2005), where males from populations selected for late reproduction (hence, elongated life-span) were found to be less harming to their mates relative to the males from populations selected for early reproduction (hence shorter life-span). Females from populations selected for elongated life-span were found to have decreased mating rate (Sgro et al. 2000). Similar results were reported by Maklakov et al. (2006). Females from populations selected for late reproduction were found to be more resistant to re-mating relatively late in life compared to females from populations selected for early reproduction (Maklakov et al. 2006). Even though there has been suggestive evidence linking sexual conflict with life-history evolution, few studies have directly attempted to study this inter-relationship. Specifically, studies addressing sexual conflict have rarely addressed life-history traits in detail (but see Maklakov et al 2007, Adler and Bonduriansky 2011). Those that do only limited themselves to the analyses concerning life-span and aging rate (Bonduriansky et al. 2008).

In Chapter 4a and Chapter 5, I have reported the evidence of evolution of life-span and aging rate in both sexes in response to different levels of sexual conflict in my study. Here, I ask the question – do life-history traits, such as, development time, pre-adult survivorship and starvation resistance evolve under different levels of sexual conflict? For this purpose, I used the nine populations (M1-3, C1-3 and F1-3) subjected to different operational sex ratios. After >45 generations of selection I quantified the evolutionary response of the above mentioned life-history related traits.

METHODS:

The details of the experimental evolution protocol adopted along with all the details of the populations used in this experiment are mentioned in Chapter 2. The experiments were done with the populations selected for different levels of sexual conflict (M1-3, C1-3, F1-3) after one generation of standardization. The method of standardization is also mentioned in Chapter 2.

Pre-adult development time and larval survival rate:

This was done after 52-55 generations of selection. For this part of the experiment standardization was done in cages. During standardization the flies from the selected populations were transferred into cages and provided with food and ample amount of live Yeast. After two days eggs were collected within a window of 1 hour. To collect eggs, each cage (i.e., each population) was first given a food plate (60mm diameter) and allowed to oviposit. An hour later another fresh food plate was introduced and allowed a window of 1 hour. The eggs for the assay were collected from the second plate. This method synchronised the larvae in terms of their developmental stage. The females in this system can store eggs and lay them when suitable medium is available. This behaviour of females can potentially introduce a lot of asynchrony among the eggs of a single clutch. The first food plate allowed females lay almost all the stored eggs. Eggs from the second plate were transferred to an Agar-gel (1% Agar-agar solution) strip with the help of a fine brush. The eggs were then counted on the Agar-gel surface and pieces of Agar-gel with exactly 60 eggs were transferred to 8-dram vials containing 8ml of corn meal food. Each vial was labelled individually and placed into the incubator with the standard laboratory conditions. Vials were randomly shuffled twice every day to equalize any positional effect.

The vials were monitored carefully for the sign of beginning of eclosion. Eclosing adults were counted and sexed every 4 hours after the start of eclosion till all the pupae eclosed. Time of each count was carefully noted down. The raw data was used to derive average measures of preadult development time per vial for each sex. The proportion of flies, starting from the 60 eggs cultured at the beginning of the assay, that finally eclosed from each vial by end of the assay (or 11^{th} day post egg-collection) was considered as the measure of pre-adult (egg to adult) survivorship.

Starvation resistance assay:

This was done after 45-47 generations of selection. Starvation resistance was assayed by quantifying longevity of selected flies under starved condition following the protocol similar to that followed by Chippindale et al. (1997). After 40-45 generations of selection, starvation resistance of the selected males and females were assayed under two conditions – freshly eclosed and 4days old, held with mates. Eggs were collected from standardized flies from each of the nine selected populations (M1-3, C1-3, F1-3) at a density of 150±10 per 8-10 ml food in each vial. They were allowed o grow under standard laboratory conditions – 25^{o}C temperature, 60-80% relative humidity and 12 hours light / 12 hours dark. On 10^{th} day post egg collection, adult flies were collected as very young virgins (<6 hours) and held in single sex vials at the density of 5 individuals / vial. For each population, 2 sets of flies were collected. One set (Virgin set) of 10 vials per population per sex was collected in 'starvation vials'. These starvation vials were standard 8-dram vials provisioned with non-nutritive agar gel (1% Agar-agar solution + 1% p-hydroxy benzoic acid solution) and plugged with cotton. The Agar-gel provided moisture for the flies and ensured that flies are only starved but not desiccated. Throughout the course of the starvation assay, there were no sign of the agar gels

becoming too dry. The other set of 7-10 vials was collected in food vials and assigned to the "Mated set" described later.

LH-flies were grown under similar conditions - 150±10 per vial larval density, 25^{o}C temperature, 60-80% relative humidity and 12 hours light / 12 hours dark. On 10th day post eclosion, the adult flies were collected as virgins (<6 hours old) and held in single sex vials (with food) at the density of 5 individuals per vial.

On 12th day post egg collection, the set (Mated set) of selected males from all the nine populations were combined with LH-females in mating vials provisioned with food and 2.33mg live Yeast. Each of these mating vials had 5 males belonging to one of the nine selected populations (M1-3/C1-3/F1-3) and 5 LH-females. Selected females were combined with LH males in the similar way to set up the mating vials. These mating vials were left undisturbed for the following two days.

On 14th day post egg collection, i.e., 4th day post eclosion, the selected males and females from the mating vials were transferred to starvation vials using light CO_2-anaeesthesia. The LH-males and the females were discarded. Mortality was recorded in the starvation vials (both virgin and mated set) every 6 hours. The time a given fly survived starvation, i.e., longevity under starvation was referred to as 'starvation time' and was considered as a measure of starvation resistance.

Along with the starvation resistance assay, I also measured dry body weight of the selected males and females. This was done for flies of two age class used in the starvation assay – freshly eclosed and 4-day old. Freshly eclosed flies were frozen in -20^{O}C. Subsets of 4-day

old flies from block 2 (M2, C2 and F2) and block 3 (M3, C3 and F3) were taken out of mating vials (Mated set, mentioned above) and flash frozen at -20^{O}C. Frozen flies were dried in 60^{O}C for 48 hours and weighed following the method described in Chapter 4a and 5.

Data analysis:

Pre-adult survivorship data was Arcsine-square root transformed. Both the transformed and untransformed data were analysed using two-factor mixed model ANOVA with selection regime as fixed factor crossed with random blocks. Mean pre-adult development time (vial means) was analysed using three-factor mixed model ANOVA, with selection regime and sex as fixed factor and block as random factor.

Data from the two sexes were analysed separately. In each sex, the data was first analysed using three-factor ANOVA with selection regime and age (0day and 4day) as fixed factors crossed with random blocks. However, both sexes revealed significant effects of 'block×selection regime' interaction along with significant effect of 'block×age' (for females) and 'block×selection regime×age' (for males) interactions (see result section for detail). Therefore, data from each sex and each block was analysed separately using two-factor ANOVA with selection regime and age as fixed factors. Additionally, the change in starvation resistance due to the 4days of adult life was analysed using the parameter – starvation cost score (δ), which was defined as follows:

$$\delta = \frac{Mean\ starvation\ time\ of\ the\ treatment\ at\ 0day - vial\ mean\ starvation\ time\ at\ 4day}{Mean\ starvation\ time\ of\ the\ treatment\ at\ 0day}$$

δ should be smaller in case the decline in starvation time is small (indicating less cost of reproduction) and larger in case the decline is large (indicating high cost of reproduction). δ

was calculated for each vial for all populations and sexes. δ for both the sexes were analysed separately using two-factor ANOVA with selection regime as fixed factor crossed with random blocks. The unit of analysis was vial values here.

Body weight data for each age class (0d and 4d) and each sex (male and female) was analysed separately using two-factor ANOVA with selection regime as fixed factor and block as random factor.

All multiple comparisons were done using Tukey's HSD. All analyses were done using STATISTICA (for windows, version 10).

RESULTS:

Pre-adult survivorship:

Selection regime had no significant effect on pre-adult survivorship (proportion survived, mean ±SE, M: 0.90 ±0.009, C: 0.90 ±0.009, F: 0.87 ±0.009, Table 6.1). Since transformation did not make a qualitative difference to the result of the analysis, I report only the untransformed data.

Pre-adult development time:

Selection regime, sex and block had significant effect on pre-adult development time (Table 6.2, Figure 6.1a, b). Males in all the populations had significantly higher development time compared to that of the females (Table 6.2) consistent with the well known sexual dimorphism in pre-adult development time. Development time in F-flies was found to be significantly higher relative to that of the other two regimes (Tukey's HSD, Figure 6.1a, b).

The interaction between selection regime and sex was not significant, indicating that the dimorphism in development time did not evolve in the present selection regime.

Starvation resistance:

In selection regime males, the three-way ANOVA revealed a significant effect of selection regime, age, block and selection regime×age interaction on mean starvation time (Table 6.3). However, the effect of selection regime×block interaction was nearly significant ($p=0.051$, Table 6.3). Therefore, each block was analysed separately. In each block, effects of selection regime and age on starvation time of males were significant (Table 6.4, Figure 6.2). In block 1, selection regime×age interaction was found to have a significant effect (Table 6.4). Analysis of δ for selected males revealed a significant effect of selection regime and selection regime×block interaction (Table 6.5). Due to the significant selection regime×block interaction, δ in each block was analysed separately. This separate analysis indicated significant effect of selection regime in block 1 and 2, however in block 3 the effect was marginally not significant (Table 6.6, Figure 6.3). Multiple comparisons using Tukey's HSD indicated that δ was significantly smaller in F-males relative to that in C-males in block 1 and 2. In block 3, δ was smaller in F-males compared to C-males however the difference was not significant. Further details of the results of multiple comparisons are summarised in Figure 6.3.

In selection regime females, the three-way ANOVA indicated a non-significant effect of selection regime but significant effects of age and block on starvation time (Table 6.3). The selection regime×age interaction had a significant effect of starvation time of the females (Table 6.3) and this was consistent across all the blocks indicated by the non-significant

effect of selection regime×age×block interaction (p=64, Table 6.3). There were significant effects of selection regime×block and age×block interactions (Table 6.3), which led me to analyse each block separately. The block-wise analysis revealed a significant effect of age and selection regime×age interaction in all three blocks (Table 6.4, Figure 6.4). The results of multiple comparisons using Tukey's HSD are represented in Figure 6.4. Selection regime was found to have a significant effect on δ for the selected females (Table 6.5, Figure 6.5). The selection regime×block interaction was not significant in this analysis indicating that the effect of selection regime on δ was consistent across blocks. Multiple comparisons using Tukey's HSD indicated that F-females had significantly higher values relative to M and C-females while there was no significant difference between M and C-females (Figure 6.5).

Dry body weight:

There was a significant effect of selection regime on the dry body weight of the freshly eclosed (0-day old) males and females (Table 6.7). Multiple comparisons using Tukey's HSD suggested that in both sexes, flies from the F-regime were significantly larger than flies from the other two regimes, whereas there was no significant difference between the dry body weight between C and M-flies. The effect of selection regime on dry weight of 4-day old males was significant (Table 6.7, Figure 6.6a) with F-males being significantly larger than M and C-males. In females however, there was no significant effect of selection regime of on dry body weight of the 4-day old flies (Table 6.7, Figure 6.6b).

Effect	SS	MS Num	DF Num	F	p>F
Selection regime	0.011	0.005	2	2.290	0.108
Block & Random	0.012	0.006	2	2.492	0.089
Selection regime × Block	0.012	0.003	4	1.286	0.283

Table 6.1: Summary of the results of two-factor ANOVA using selection regime as fixed factor crossed with random blocks on pre-adult survivorship. Mean survivorship was calculated for each replicate vials and these means were then used as the unit of analysis.

Effect	SS	MS Num	DF	F	p>F
Selection regime	196.06	98.03	2	10.75	<0.001*
Sex	709.30	709.30	1	77.77	<0.001*
Selection regime × Sex	7.54	3.77	2	0.41	0.662
Block & Random	123.39	61.69	2	6.76	0.002*
Selection regime × Block	46.67	11.67	4	1.28	0.281
Sex × Block	10.39	5.20	2	0.57	0.567
Selection regime × Block × Sex	18.80	4.70	4	0.52	0.725

Table 6.2: Summary of the results of three-factor ANOVA using selection regime and sex as fixed factors crossed with random blocks on pre-adult development time. Mean development time was calculated for each replicate vials (separately for the two sexes) and these means were then used as the unit of analysis. p-values marked with * are statistically significant.

Sex	Effect	SS	MS Num	df Num	F Ratio	p > F
Starvation resistance of selected males	**Selection regime**	21814.21	10907.11	2	16.02	<0.001*
	Block & random	6410.91	3205.45	2	4.71	0.010*
	Selection regime × Block	6593.94	1648.49	4	2.42	0.051
	Age	28909.98	28909.98	1	42.45	<0.001*
	Selection regime × Age	11666.93	5833.47	2	8.57	<0.001*
	Age × Block	507.99	254.00	2	0.37	0.689
	Selection regime × Age × Block	5864.69	1466.17	4	2.15	0.077
Starvation resistance of selected females	**Selection regime**	1390.50	695.25	2	1.37	0.258
	Age	235453.01	235453.01	1	462.73	<0.001*
	Selection regime × Age	13416.52	6708.26	2	13.18	<0.001*
	Block & random	21344.08	10672.04	2	20.97	<0.001*
	Selection regime × Block	5449.58	1362.39	4	2.68	0.034*
	Age × Block	35402.59	17701.30	2	34.79	<0.001*
	Selection regime × Age × Block	1273.58	318.39	4	0.63	0.645

Table 6.3: Summary of the results of three-factor ANOVA using selection regime and age as fixed factors crossed with random blocks on starvation resistance (longevity under starvation) of selection regime (a) males and (b) females. Mean starvation resistance was calculated for each replicate vials and these means were then used as the unit of analysis. p-values marked with * are statistically significant.

Sex	Block	Effect	DF	SS	F	p>F
Selection regime Male	1	**Selection regime**	2	11650.704	6.487709	0.003*
		Age	1	11103.041	12.36548	0.001*
		Selection regime × Age	2	14764.546	8.221656	0.001*
	2	**Selection regime**	2	11949.199	12.97246	<0.001*
		Age	1	12669.398	27.50867	<0.001*
		Selection regime × Age	2	1636.8867	1.777061	0.180
	3	**Selection regime**	2	4946.2109	3.713172	0.032*
		Age	1	6040.4576	9.069269	0.004*
		Selection regime × Age	2	2082.4792	1.563339	0.220
Selection regime Female	1	**Selection regime**	2	1337.419	1.237	0.299
		Age	1	29865.793	55.229	<0.001*
		Selection regime × Age	2	7153.899	6.615	0.003*
	2	**Selection regime**	2	1661.115	2.342	0.107
		Age	1	184088.556	518.995	<0.001*
		Selection regime × Age	2	3184.728	4.489	0.016*
	3	**Selection regime**	2	3978.881	3.140	0.052
		Age	1	56938.859	89.862	<0.001*
		Selection regime × Age	2	4365.320	3.445	0.040*

Table 6.4: Block-wise analysis of starvation resistance. Summary of the results of two-factor ANOVA using selection regime and age as fixed factors on starvation resistance of the two sexes. Mean starvation resistance was calculated for each replicate vials and these means were then used as the unit of analysis. p-values marked with * are statistically significant.

Sex	Effect	SS	MS Num	DF Num	F	p
Selected males	Selection regime	0.69	0.35	2	21.85	<0.001*
	Block & Random	0.02	0.01	2	0.64	0.5297
	Selection regime × Block	0.31	0.08	4	4.90	0.001*
Selected females	Selection regime	0.49	0.24	2	18.73	<0.001*
	Block & Random	1.27	0.63	2	48.75	<0.001*
	Selection regime × Block	0.06	0.02	4	1.24	0.301

Table 6.5: Summary of the results of two-factor ANOVA using selection regime as fixed factor crossed with random blocks on starvation cost score. Cost score was calculated for each replicate vials of 4-days age. These means were then used as the unit of analysis. p-values marked with * are statistically significant.

Block	SS	MS Num	DF Num	F	p
1	0.87	0.29	2	25.85	<0.001*
2	0.10	0.04	2	4.13	0.028*
3	0.10	0.10	2	2.78	0.086

Table 6.6: Summary of the results of three separate one-way ANOVA for each block testing for the effect of selection regime on starvation cost score of the selection regime males. p-values marked with * are statistically significant.

Sex	Age	Effect	SS	MS Num	DF	F	p>F
Female	0-day	Selection regime	0.0013	0.0007	2	6.30	0.004*
		Block & Random	5.8×10^{-5}	5.8×10^{-5}	1	0.55	0.461
		Selection regime × Block	0.0004	0.0002	2	2.14	0.128
	4-day	Selection regime	0.0017	0.0009	2	0.83	0.441
		Block & Random	0.0001	0.0001	1	0.10	0.749
		Selection regime × Block	0.0011	0.0005	2	0.51	0.605
Male	0d	Selection regime	0.0031	0.0015	2	11.25	<0.001*
		Block & Random	0.0012	0.0006	2	4.39	0.016*
		Selection regime × Block	0.0007	0.0002	4	1.23	0.304
	4d.	Selection regime	0.0011	0.0006	2	11.49	<0.001*
		Block & Random	0.0049	0.0024	2	50.87	<0.001*
		Selection regime × Block	0.0004	0.0001	4	1.83	0.132

Table 6.7: Summary of the results of two-factor ANOVA using selection regime as fixed factor crossed with random blocks on dry body weight of the selection regime males and females at two age classes (0day and 4day). Body weight was measured in groups of f5 flies. Mean body weight for each of these groups was calculated. These means were then used as the unit of analysis. p-values marked with * are statistically significant.

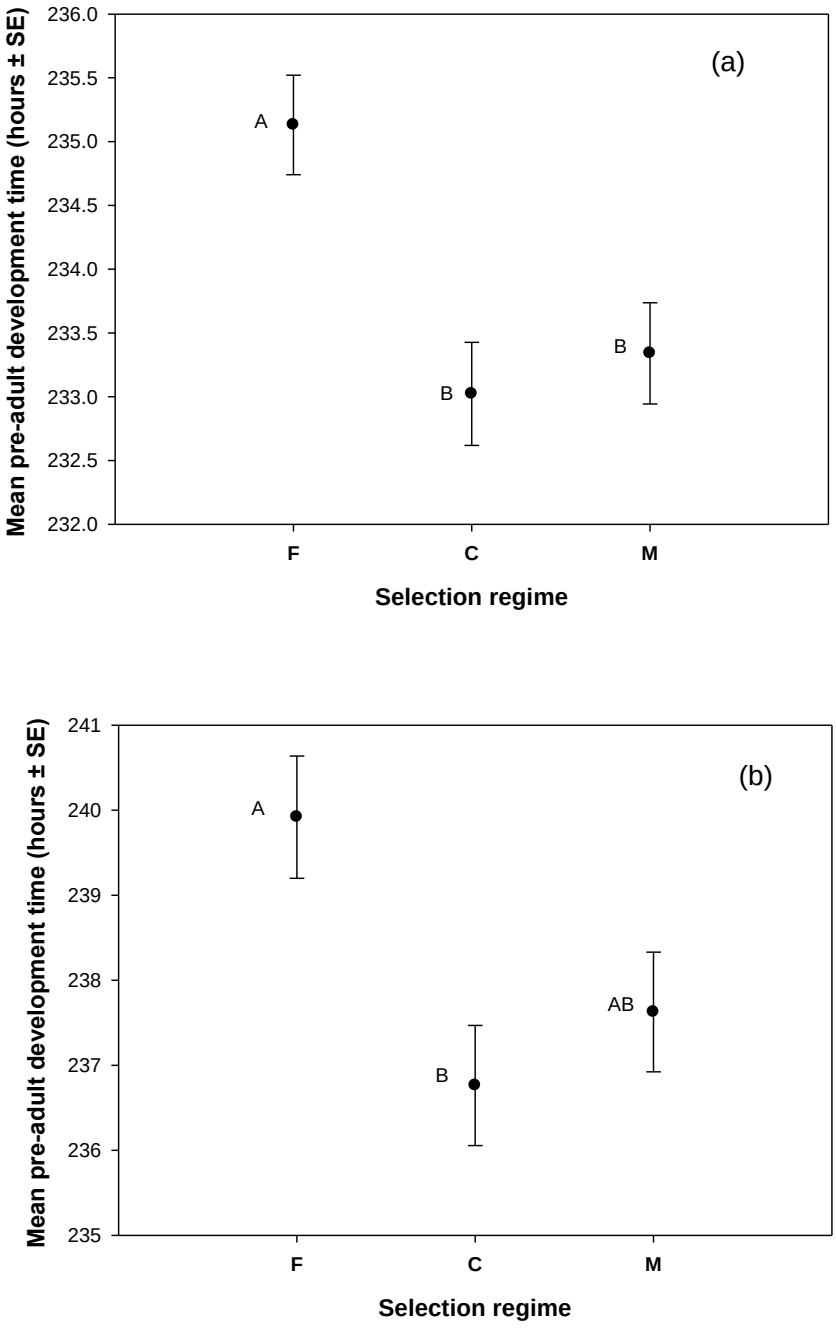

Figure 6.1: Pre-adult development time of selection regime (a) females and (b) males. Flies were grown at a density of 60 per 8-10ml of food in each vial. Mean development time for each vial was calculated (separately for males and females). These vial means were then taken as the unit of analysis. Points not sharing common letters are significantly different (determined using Tukey's HSD).

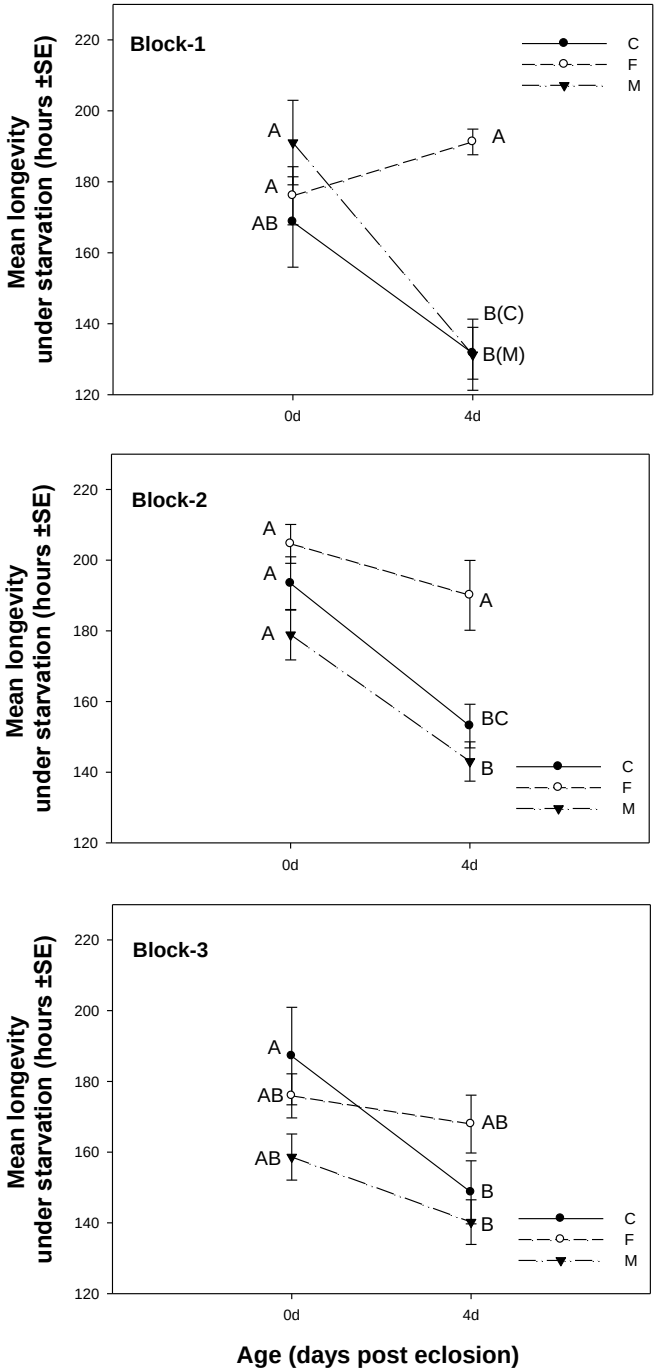

Figure 6.2: Longevity (in hours) under starvation (starvation resistance) of selection regime males in each block. Mean starvation resistance of each vial was calculated and these means were then used as the unit of analysis. Points not sharing common letters are significantly different (determined using Tukey's HSD).

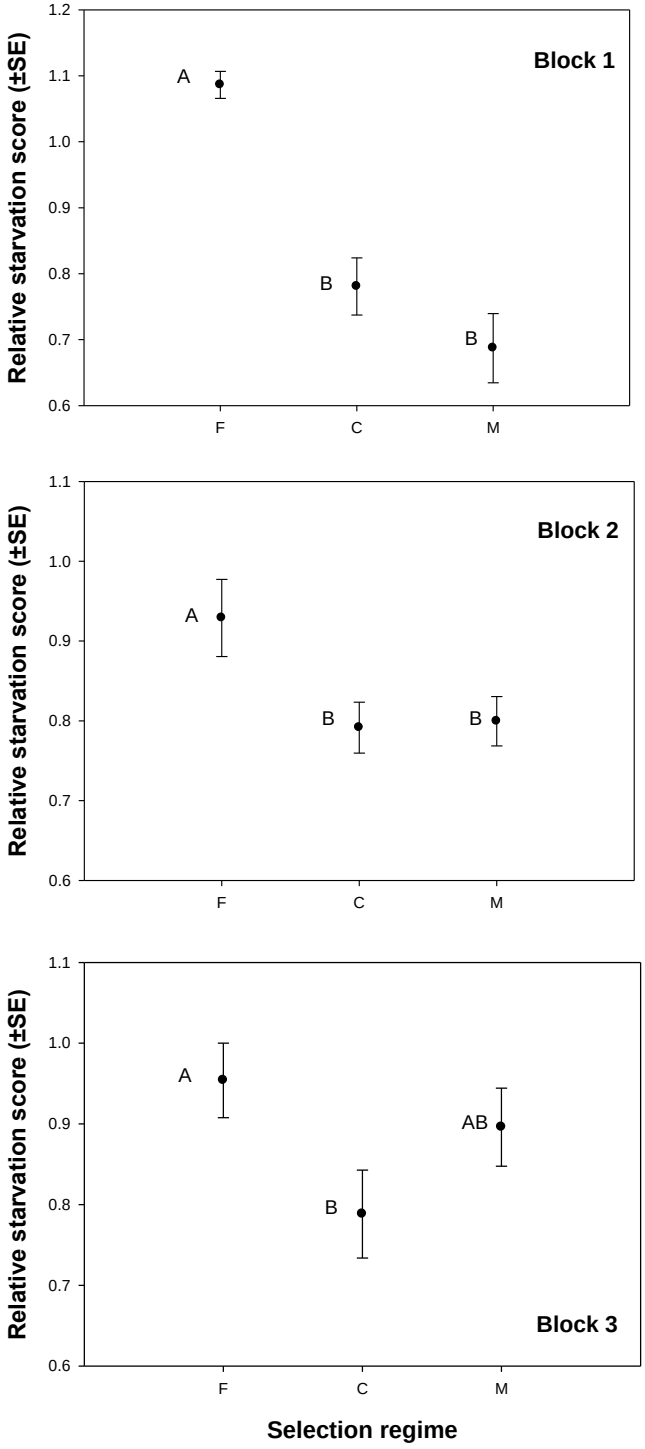

Figure 6.3: Starvation cost score (δ) of selection regime males in three blocks. δ was calculated for each vial (of 4-days age) using a formula mentioned in the methods section. δ measured the change in starvation resistance due to the four days of adult life. δ for each vial was taken as the unit of analysis. Points not sharing common letters are significantly different (determined using Tukey's HSD).

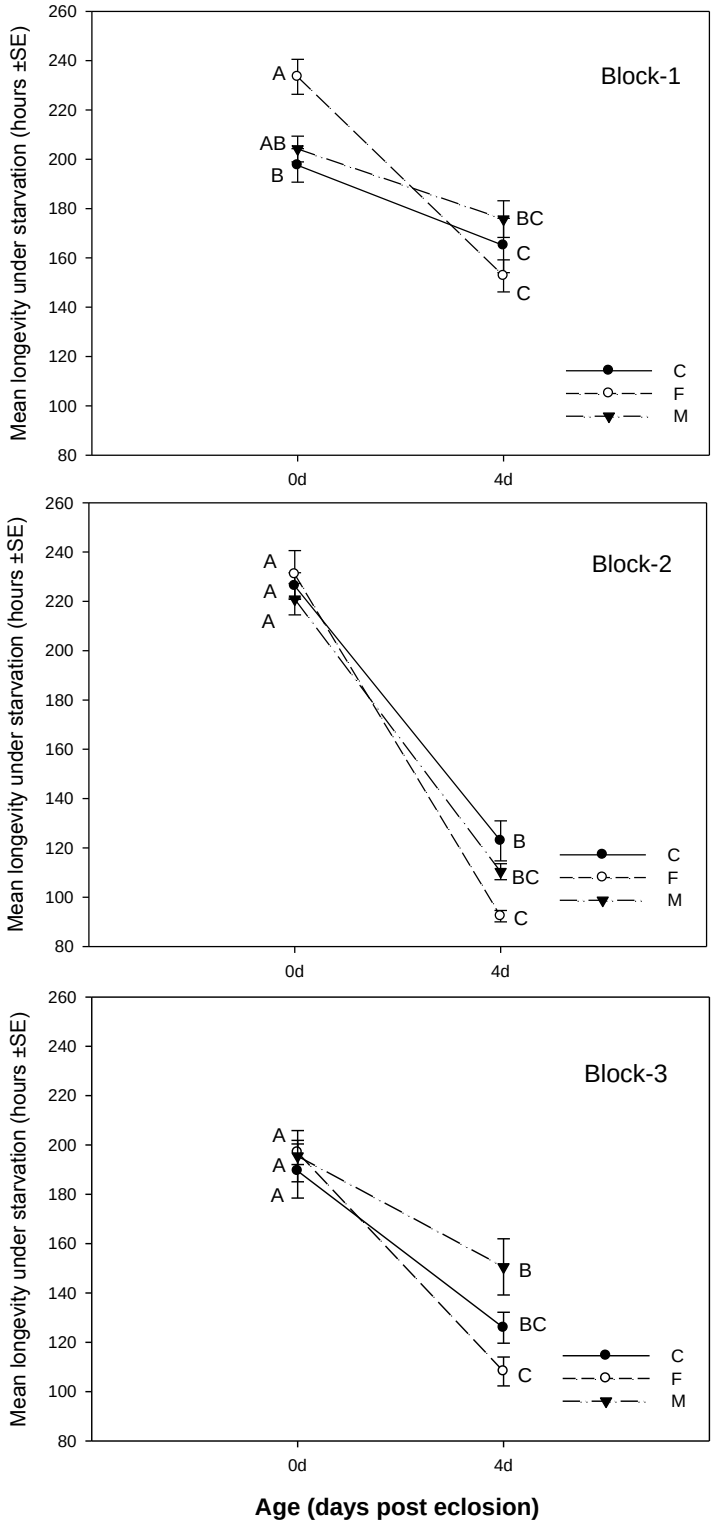

Figure6.4: Longevity (in hours) under starvation (starvation resistance) of selection regime females in each block. Mean starvation resistance of each vial was calculated and these means were then used as the unit of analysis. Points not sharing common letters are significantly different (determined using Tukey's HSD).

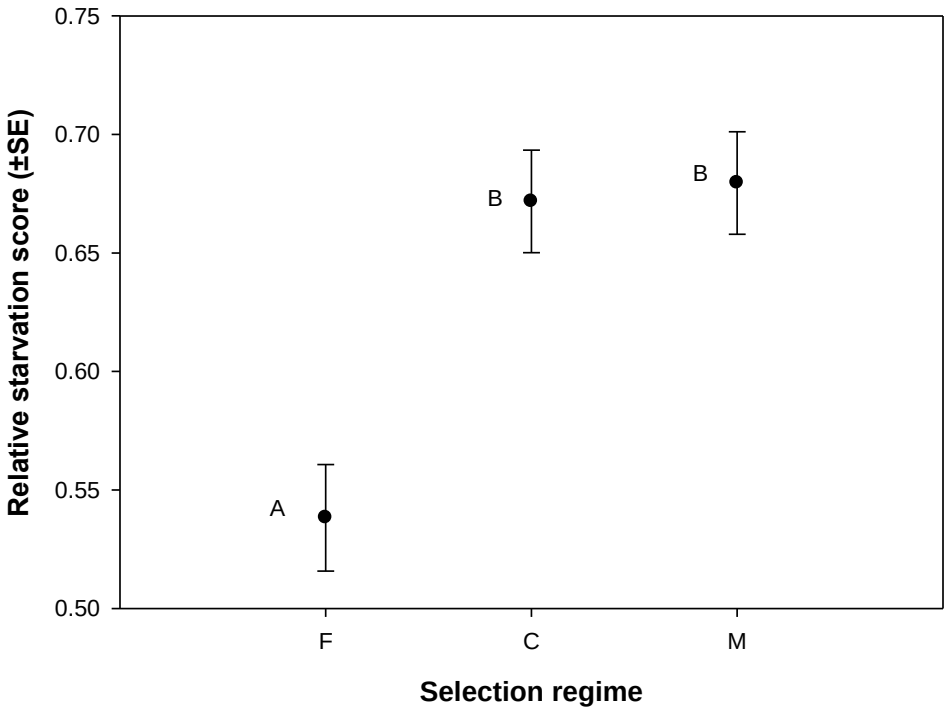

Figure 6.5: Starvation cost score (δ) of selection regime females. δ was calculated for each vial (of 4-days age) using a formula mentioned in the methods section. δ measured the change in starvation resistance due to the four days of adult life. δ for each vial was taken as the unit of analysis. Points not sharing common letters are significantly different (determined using Tukey's HSD).

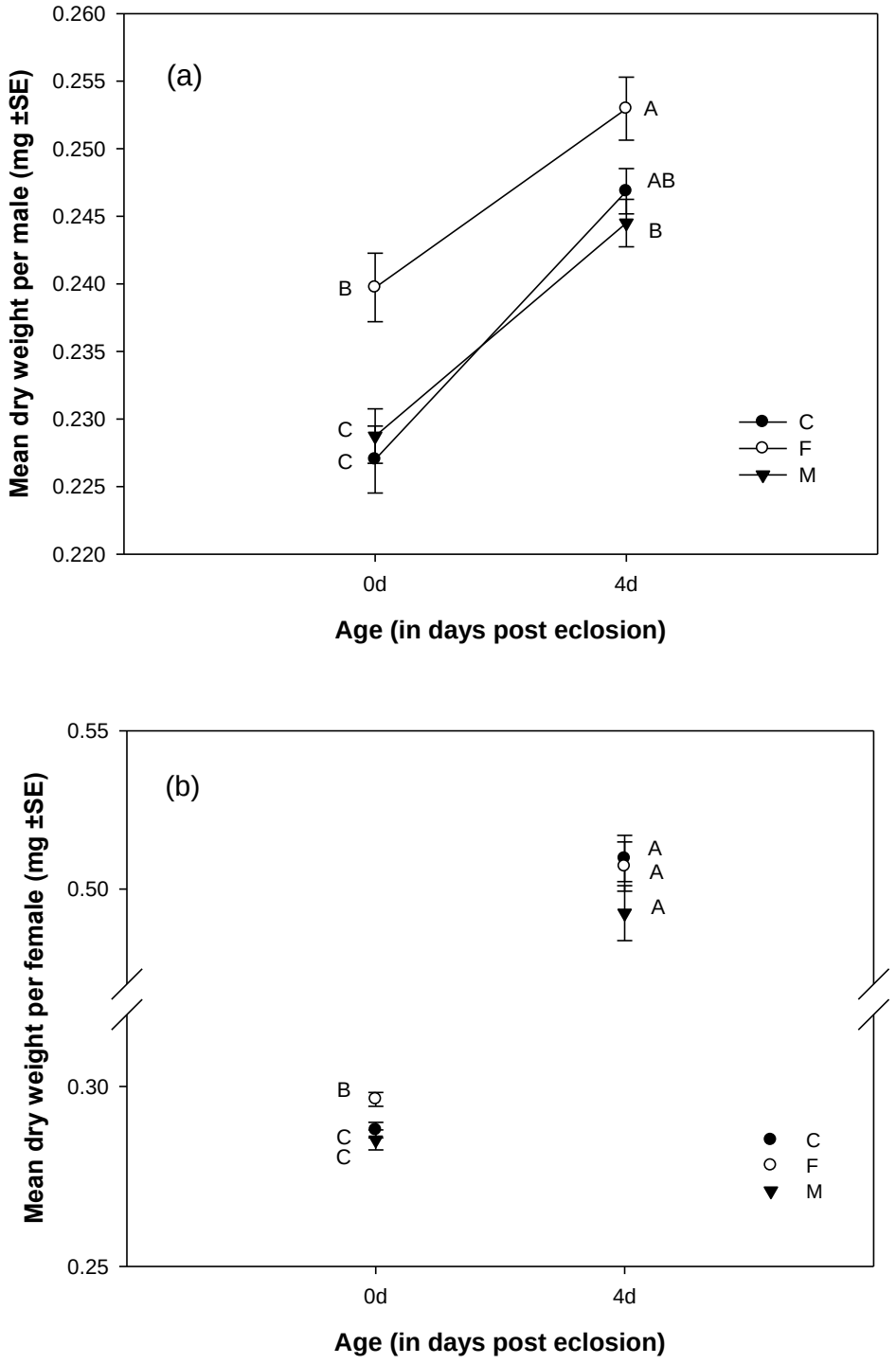

Figure 6.6: Dry body weight of selection regime (a) males and (b) females at two ages. Dry weight was measured for groups of 5 flies. A mean was calculated for each of these groups. These means were then used as the unit of analysis. Points not sharing common letters are significantly different (determined using Tukey's HSD).

DISCUSSION:

Although sexual conflict and life-history evolution are predicted to be correlated to each other, this idea is only rarely put to empirical assessment (Wedell et al. 2006, Bonduriansky et al. 2008). Here I have shown that key life-history traits such as pre-adult development time, body size and starvation resistance evolve under the laboratory selection for adaptation to different levels of sexual conflict. There was no direct, intentional selection imposed on these traits and hence the observed responses can be assigned to the correlated response due to the evolution in male and female traits described in previous chapters.

Body size at different age:

This was done after 45-47 generations of selection. As mentioned in Chapter 4a and 5 as well, I observed a significant increase in body size at eclosion (measured as dry body weight) in F-flies of both sexes. This possibly evolved due to the relaxation of male-specific selection under F-regime (see Chapter 4a and 5 for detailed discussion). Here I have observed that the initial body size difference was retained for males even after 4 days of adult life including two days of interaction with mates in mating vials in presence of Yeast. However, the body size difference was found to disappear in females after similar treatment (4-days of adult life, including two days of interaction with males). This can be attributed to female's ability to feed on live Yeast during this period of life (Simmons and Bradley 1997).

Pre-adult development time and pre-adult survivorship:

A positive correlation between pre-adult development time and adult body size is known in *Drosophila*. Previous studies showed that larger flies take longer to finish larval development (Robertson 1963). Additionally, selection for shorter pre-adult development time has been

shown to lead to the decrease in adult body size, indicating the genetic correlation of the two traits (Prasad et al. 2001). The opposite trend, i.e., increase in body size and slower pre-adult development time was also observed in *D, melanogaster* (Zwaan et al. 1995). Therefore it is likely that in the present experiment F-flies evolved increased body size by extending their pre-adult development. In essence, I observed the evolution of larval traits (pre-adult development time) in response to a selection that acted during the adult phase. To the best of my knowledge this is only the second such report, the only other one being that of McKean and Nunny (2005). However, my results are not in line with that of McKean and Nunny (2005). While an increase in development time was observed under increased sexual selection in the above mentioned study, I observed an increase in development time under female biased regime (i.e., under relaxation of the strength of sexual selection and conflict).

Starvation resistance of adult flies:

I found significant effect of selection regime on the pattern of starvation resistance (longevity under starvation) in both sexes. Previous studies have suggested that cost of reproduction can be quantified in terms of reduction in starvation resistance (Chippindale et al. 1997). I quantified starvation resistance of both sexes at two ages – freshly eclosed (i.e., 0day) and 4day post eclosion. The observed decline in starvation resistance over the four days of adult life in both sexes indicates the cost of reproduction (as quantified by Chippindale et al. 2007). Hereafter, I will refer to this decline in starvation resistance in the course of four days of adult life as 'starvation cost of reproduction'. I found an asymmetric effect of selection on the starvation cost across the two sexes. Starvation cost was found to have reduced in F-males, while it remained unchanged in M-males. This was also demonstrated by the significantly low starvation cost score (δ) of F-males. There was no significant difference between δ's of

the M and C-males. In females, on the other hand, the starvation cost was found to have increased in F-females and remained unresponsive in M-females.

Reduction in starvation cost in F-males can be explained in at least two different ways. The observed result might indicate the possible reduction in the reproductive investment (behaviour and/or physiology) in F-males. As reported in Chapter 4a and 4b, I did not find any obvious change in the reproductive behaviour of F-males. However, multiple evidences suggest that the reproductive physiology has probably significantly changed in F-males (see Chapter 4a for discussion in mate-harm and Chapter 4b for sperm competitive ability). Alternatively the reduction in starvation cost in F-males might also represent the effect of increased body size, therefore greater availability of resources in these males.

Increased starvation cost in F-females is, very likely, another expression of the increased susceptibility of F-females to mate-harm. It is important to note here that F-females did not show increased longevity-cost in previous assay (see longevity results in Chapter 5). However, under more stressful condition, such cost was detected. Martin and Hosken (2003) found evidences of sexually antagonistic co-evolution in an experimental evolution study using *Sepsis cynipsea*. Experimentally enforced monogamy was found to lead to more susceptible (to mate-harm) females (Martin and Hosken 2003). However the effect of increased susceptibility in such females was more easily detected under starvation compared to more benign, un-starved condition. It was argued that cost detection might have been facilitated under stressful condition (Martin and Hosken 2003). My results of starvation cost in females described here, along with the longevity results described in Chapter 5, are in line with this argument of more efficient cost detection under stressful condition.

CONCLUSION:

In general, my results indicate that relaxation of selection imposed by sexual conflict through experimentally manipulated female biased operational sex ratio leads to evolution of key life-history related traits (body size, development time, starvation resistance) in both males and females in *D. melanogaster*. Together with the body size data, increased development time indicate increased pre-adult resource acquisition in F-females. My study however, did not detect any response of these life-history related traits under male biased operational sex ratio (i.e., increased sexual conflict). To the best of my knowledge, this is the only study showing evolutionary trends in life-history related traits, such as starvation resistance and development time, in response to experimental manipulation of the levels of sexual conflict (generated by different operational sex ratio).

Chapter 7

Degree of adaptive male mate choice is positively correlated with female quality variance

INTRODUCTION:

Male mate choice is defined as differential male sexual response to different reproductively mature conspecific females (Bonduriansky 2001). In contrast with the age old perception (Trivers 1972), results from studies over the past few decades indicate that males pay a non-trivial cost related to sexual reproduction and consequently, male-mate choice, either in the form of mating decisions or post-copulatory events (differential ejaculate investment), may evolve as an adaptive strategy (Trivers 1972, Edward and Chapman 2011).

As males can derive at least some fitness from each additional mating (Bateman 1948), variance in the quality of females is thought to be one of the prerequisites for the evolution of male mate choice, ensuring fitness returns in spite of the cost associated with rejecting available mating opportunities (Bonduriansky and Brooks 1998). Fecundity is one of the most important components of fitness in females and thus mating efforts of males in most promiscuous species are both expected and observed to be sensitive to some indicators of female fecundity, e.g., body size, fatness, gravid or non-gravid condition etc. (Bonduriansky 2001, Bonduriansky and Brooks 1998, Pitafi et al. 1995, Gage and Barnard 1996, Wedell and Cook 1999, Lefranc and Bundgaard 2000, Katvala and Kaitala 2001, Byrne and Rice 2006). In general, factors having strong influence on, and perceivable phenotypic correlation with female fecundity are

expected to serve as honest indicators of female quality. In addition, non-trivial amount of resources utilized by the males for the production of ejaculate, courtship and other mating related activities provide a favourable condition for the evolution of male mate choice (Dewsbury 1982, Pitnick and Markow 1994b, Cordts and Partridge 1996).

Although both age and immediate nutritional status have been shown to affect female fecundity (Boorman and Parker 1976), there are very few studies looking at male mating behaviour towards females differing in these two factors. Here I report the effect of age and nutritional status on female fitness in an outbred population of *Drosophila melanogaster*. I then examine the behaviour of the males of this population when subjected to two-way choice conditions across a range of variance in female fitness (generated by altering the two above mentioned factors). I also report the male behaviour under no-choice conditions.

METHODS:

Experimental organism:

The experiments were done using a large (N≅9,000), outbred, laboratory population of *Drosophila melanogaster* – LH (see Chapter 2 for details of maintenance regime and history).

Experimental females and their dry body weight:

Four different types of experimental females, Young High Yeasted (Y/HY), Young Low Yeasted (Y/LY), Old High Yeasted (O/HY) and Old Low Yeasted (O/LY), were generated following a scheme described in Figure 4. To generate young experimental females (3day old), eggs were cultured at a density of approximately 150 per vial (8-

10ml of corn meal-molasses food) and were incubated at 25^0C under 12:12 LD condition similar to the standard culture condition of the LH population. 10 days later, when pupae started darkening the vials were observed every 3-4 hours. Adult virgin females were collected during the peak of their eclosion cycle using light CO_2 anaesthesia. Virginity was ensured by collecting flies within 6 hours of eclosion. Collected females were held in single sex groups at a density of 10 individuals per vial. One day later they were divided into two sets - "HighYeast" and "Low Yeast" and were transferred to food-vials with 15mg and 5mg of Yeast respectively and were left undisturbed for 2days. Experiments were done when they were 3day old. Old females were generated following a similar protocol but eggs were cultured 10days before culturing eggs for generation of the young females. Virgin old females were held in single sex vials till they were 11day old with food changes every alternate day and at the age of 11day they were given the similar Yeast-treatment as that given to young females. Experiments were done when they were 13day old (same day when young females were 3day old). Eggs were collected for the generation of medium aged - sperm limiter females 4day before collecting eggs for the generation of young females. Virgin females were collected from these vials in the similar way and were held as virgins till they were approximately 7day old before using them for sperm-limitation.

Just prior to conducting experiment 1, 45-50 experimental females of each type (O/HY, O/LY, Y/HY and Y/LY) were randomly chosen and flash frozen for measurement of body weights. These flies were then dispensed into clean dry vials in groups of 5, dried at 70^oC for 24 hours and weighed in a high precision electronic balance (Sartorius CPA225D) to the nearest 0.01mg.

Experimental males:

Males used in Experiment-1 and 2 (described below) were generated using a similar protocol as that followed while generating young females. They were collected as virgins and were held in single sex groups of 10 per vial. All the males used in this study were 3day old at the time of experiment. For Experiment 2, males were made sperm limited by keeping them with excess of 7day old virgin (sperm limiter, described earlier) females (male:female = 10:30 in each vial) for approximately 12 hours, just before the start of the experiment. Females and males were combined in food vials without using anaesthesia and after 12 hours, the males were separated from the females under light CO_2 anaesthesia. This treatment ensured multiple mating opportunities for each of the males and was sufficient to make them resource (sperm and other components of ejaculate) depleted (Markow et al. 1978, Byrne and Rice 2006). These males were kept undisturbed for 30 – 45 minutes to allow them to recover from the effect of CO_2 anaesthesia before starting the experiment.

Experiment 1: Effect of age and nutritional status on fitness (number of progeny produced) and reproductive behaviour of the females under no-choice condition

Fitness of the four different types of experimental females (O/HY, O/LY, Y/HY and Y/LY) was assayed using the following protocol. 10 females of a given type were combined with 10 males (3day old, virgin) without using anaesthesia and were allowed to interact for 1 hour in a food vial. For each type of experimental female, I set up 11-13 such vials. The females were then separated under light CO_2 anaesthesia One day later, they were transferred (under mild CO_2 anaesthesia) individually into test tubes (12mm×75mm) containing media and allowed to oviposit for 18 hours. Females were discarded and the test tubes were incubated at 25^0C. Emerging progeny were counted after 13 days by which time all progeny had completed development. The progeny count

was taken as the measure of their fitness. The one hour interaction time between the males and females ensures a single mating. This is because the mean copulation duration in my flies measured under similar laboratory conditions is typically 20-30 minutes. Additionally, there is a latency period preceding the mating and a refractory period succeeding the mating. I also ensured a single mating by observing the vials continuously.

Under similar conditions, I combined 10 females of a given type (O/HY or O/LY or Y/HY or Y/LY) with ten 3day old virgin males in a food-vial and their mating behaviour was continuously observed (manually) for 1 hour. 9-10 such vials were observed for each female type (O/HY, O/LY, Y/HY and Y/LY). I recorded the number of mating pairs over time which yielded start and end time for copulation. Using this data, I calculated average mating latency (time taken by a pair of virgin flies to initiate mating), copulation duration (time taken to complete mating) and mating success (proportion of females inseminated within 1 hour under no-choice condition) for each vial. In this experiment, in each vial males were exposed to only one type of experimental female (i.e., either O/HY or O/LY or Y/HY or Y/LY). Hence I term this as "no-choice" condition.

The entire protocol to measure fitness and reproductive behaviour of the four different types of experimental females was designed to closely mimic the natural maintenance protocol of this population. Hence experiment was done during the light phase of their 12:12 LD cycle at 25^0C temperature and uniform overhead laboratory lighting.

Experiment 2: Assay of precopulatory male mate choice

During the choice experiments, different types of females were identified by feeding them yeast suspension with non-toxic food colours (commercially available, green and red). Females were allowed to feed on Yeast-colour suspension for half an hour prior to the choice trial, upon which the abdomen of the females were coloured. To control for the effect of the abdomen-colours on mate choice, I replicated the experiments with reciprocal colouring of females. All combinations of female types (Table3) were used in the experiment – O/HY vs. O/LY (C1), Y/HY vs. O/HY (C2), Y/LY vs. O/HY (C3), Y/HY vs. O/LY (C4), Y/LY vs. O/LY (C5), Y/HY vs. Y/LY (C6). Mating vials were set up by combining two types of females (10 individuals of each type) and the sperm limited 3 day old males (10 individuals) without anaesthesia in vials containing food. As mentioned before, the two types of females in a given vial were identified by red and green colouration of their abdomen. 6 such vials for each combination type and each colour-code were setup (see following section: Experimental replications and data analyses). The mating vials were left undisturbed for 30 minutes. Mating was stopped by mechanically disturbing the vials and then females were immediately sorted on the basis of their abdomen-colour using light CO_2 – anaesthesia and transferred singly into test tubes (12mm × 75mm) containing food. Females were allowed to lay eggs for 48 hrs, after which they were discarded and the test tubes were incubated at 25^0C. After two days, the test tubes were observed for the presence of larvae. Females in the test tubes with live larvae were scored as "mated" and those with none as "unmated".

My own observations with these flies suggest that usually virgin pairs take 3-9 minutes to start mating and then if not disturbed they mate for 20-30 minutes. Thus 30 minute exposure is sufficient to ensure single mating per male. More exposure time might have allowed at least some males to start second round of mating as excess virgin females were present in the mating vials. Another study using a derivative of the LH population

(Byrne and Rice 2006) also followed the same protocol to successfully to ensure single mating per male.

The experiment was done during the light phase of the 12:12 LD cycle of the flies at 25^0C temperature and uniform overhead laboratory lighting

Experimental Replications and Data Analysis:

Fitness (11-13 replicate vials per female type), mating latency (9-10 replicate vials per female type), copulation duration (9-10 replicate vials per female type) and dry body weight (9-10 replicate vials per female type) data were analysed using two factor ANOVA with age and nutritional status as fixed factors. Mating success (9-10 replicate vials per female type) data were not normally distributed, so they were analysed using Kruskal-Wallis tests. Total of six tests were done, corresponding to all possible comparisons between the four types of females used in the experiment. A sequential Bonferroni test was used in these pair wise comparisons. As there were a total of six comparisons (Table 2), for each of them the level of significance was revised to 0.008 following Bonferroni method (Sokal and Rohlf 1995). Experiment 2 had 12 replicate vials for each combination type. Of these, 6 vials had a particular abdominal colour code (for identifying the two different types of females) while the other six vials had the reciprocal colour code. The raw choice data (proportion of each type of female inseminated during the choice trial) were converted into *choice score (CS)* following the equation given below:

$$CS = \frac{P}{P+Q}$$

Where P is the proportion of higher-fitness females fertilized and Q is the proportion of lower-fitness females fertilized. This was possible because, for every combination, the

two kinds of females could be categorised as higher fitness and lower fitness, based on earlier fitness measurement results (Table 3). In the absence of any mating bias, this score is expected to be equal to 0.5, assuming no difference in intrinsic mating success of the females. If there is a bias towards the females of higher fitness, CS should range between 0.5 and 1, with higher values indicating stronger bias. If the bias is towards lower fitness females, CS should range between 0.5 and 0, with lower values indicating stronger bias. Effects of food colour and combination type on CS were analysed by modelling food colour and combination type as a fixed factors in a two factor ANOVA. Since, food colour had no significant main effect or interaction ($p>0.4$ for both), choice scores from reciprocal colour combinations were pooled for the rest of the analyses. For each combination, choice scores were analysed using one sample t-test (two tail) with hypothesised mean as 0.5. For all the statistical tests (ANOVA and t-tests) data were tested for normality using Shapiro-Wilk W tests, and, unless otherwise mentioned, the data were found to not be significantly different from normality. Level of significance (α) was taken as 0.05 in all the tests done.

RESULTS:

Fitness (number of progeny produced) of the experimental females:

The experiment was done with four kinds of females differing in two factors, viz. age and nutritional status - Young High Yeasted (Y/HY), Young Low Yeasted (Y/LY), Old High Yeasted (O/HY) and Old Low Yeasted (O/LY). These four kinds of females are hereafter referred to as "Experimental Females". Young females were 3day old post-eclosion and old females were 13day old post eclosion. High and Low Yeasting status were created by supplying 5mg and 15mg of live Yeast per 10 females respectively. Fitness of the above mentioned females was measured by combining 10 females of a

given type (Y/HY or Y/LY or O/HY or O/LY) with ten 3day old virgin males in a food vial. For each type of experimental female 11-13 such vials were set up. The experimental females were allowed to mate and interact with males for an hour and then held in single sex groups for one day following which they were allowed to oviposit individually in test tubes. The progeny count was taken as fitness of the females (for details see Methods section). I found significant main effects of age and nutritional status of the females on their fitness, with old females having 32.6% lesser fitness compared to young females and females from the low nutritional regime having 21% lower fitness than females from the high nutritional regime (Table **7.**1a, Figure **7.**1a). However, young females were relatively less affected by the difference in the nutritional status while the level of nutrition strongly affected the fitness of old females, leading to a significant interaction between the two factors (Table **7.**1a, Figure **7.**1a).

Dry body weight of experimental females:

Both age and nutritional status had significant effect on dry body weight (see Methods for measurement of dry weight) of the females along with a significant interaction between the two factors (Table **7.**1b, Figure **7.**1b). However, multiple comparisons using Tukey's HSD suggested that only dry body weight of O/LY – female was significantly less than that of the other three types.

Components of reproductive behaviour under no-choice condition:

Ten Experimental females of a given type were combined with ten virgin males that were 3 day old in a food vial. This vial was then observed for components of reproductive behaviour for one hour (see Methods section for details). Neither age nor nutritional status had any significant effect on mating latency (time taken to initiate

mating) and copulation duration (time for which the copulation lasted) (Table **7.**1c,d; Figure **7.**2a,b). Analyses of the intrinsic mating success (proportion of females successfully mating within one hour after being combined with young, virgin males, Table 2, Figure **7.**3) suggested that both young and old females were not significantly affected by their nutritional status (see comparisons - O/HY vs. O/LY and Y/HY vs. Y/LY, Table **7.**2). I did four additional pair-wise comparisons of mating success (Table **7.**2, Figure **7.**3) thus completing all possible comparisons between the four types of females mentioned. Only Y/LY had significantly higher mating success than O/LY. The comparison - Y/HY vs. O/LY was marginally not significant, whereas rest of the comparisons were not statistically significant (see Methods section for statistical details).

Two way choice experiment:

Six different combinations (C1-6, Table **7.**3, see Methods for details) of females were assayed in the choice experiment. Each combination was assayed using a two-way choice design. I combined 10 sperm depleted, 3day old males with 20 experimental females of which 10 females were of one type while 10 others were of a different type (see Table **7.**3) in a standard vial (95 mm height × 25 mm diameter) with food. Ten such vials were set up for each combination. I then counted the number of females of each type mated within each vial within half an hour of combining with the males. From each of the experimental vials, the raw mating data was used to generate a "Choice Score" (CS) – a measurement of the intensity of mating bias ($1 \geq CS \geq 0$, see Methods for calculation and details). A CS=0.5 indicates no-choice, $0.5 > CS \geq 0$ indicates bias for lower-fitness females and $1 \geq CS > 0.5$ indicates bias for higher-fitness females. Choice scores were tested using a one sample t-test (two-tailed) with a null

hypothesis of mean-CS=0.5, i.e., no-choice. Apart from C6, choice scores from all other combinations were significantly greater than 0.5 ($p \leq 0.04$ for all combination types) indicating a bias in favour of higher-fitness females (Figure 4a). The choice score of C6 (=0.54) was marginally not significantly different ($p=0.053$) from the expected CS of 0.5.

The expected choice score of 0.5 assumes equal intrinsic mating successes of the two types of females under choice assay. Due to the difference in intrinsic mating success between Y/LY and O/LY-females (see mating success results earlier), the expected mean CS for C5 was revised considering the experimentally derived intrinsic mating successes of these experimental females using the formula:

$$\text{Expected CS} = \frac{\text{Intrinsic Mating success of female of higher fitness}}{\text{Summation of Intrinsic Mating successes of both types of females}}$$

The revised expected choice score (indicating absence of mate choice) for C5 was 0.523. The observed CS of C5 was then analysed using one sample t-test (two tail), this time considering the revised hypothesised mean (=0.523). The analysis revealed a significant ($p=0.0011$) difference, indicating mating bias towards Y/LY females.

I then analysed the choice score to see whether it is different across different choice combinations (C1-6). A one factor ANOVA was done with combination type as fixed factor. Choice score was significantly affected by combination type ($df=5$, $SS=0.69$, $F=6.82$, $p<0.0001$). Multiple comparisons using Tukey's HSD showed that C4 and C5 were significantly different from C1, C3 and C6, whereas C2 had intermediate value and was not significantly different from any of the combinations (Figure **7.**3a).

Choice score and variance in female fitness:

A linear regression of mean choice scores from each of the combination on the difference in fitness of the females corresponding to the combination yielded a significant positive slope (Slope = 0.012, $r^2 = 0.73$, p = 0.03, Figure **7.**3b).

Trait	Effect	Df Num.	Df Den.	SS	F	p
(a) Fitness	Age	1	44	3476.33	110.77	<0.001*
	Nutritional status	1	44	1348.46	42.97	<0.001*
	Age×Nutritional status	1	44	143.51	4.57	0.04*
(b) Dry Body Weight	Age	1	34	0.02	37.55	<0.001*
	Nutritional status	1	34	0.004	8.04	0.008*
	Age×Nutritional status	1	34	0.007	13.09	0.001*
(c) Mating Latency	Age	1	32	3.54	2.11	0.16
	Nutritional status	1	32	0.07	0.04	0.84
	Age×Nutritional status	1	32	0.21	0.12	0.73
(d) Copulation Duration	Age	1	32	1.70	0.32	0.57
	Nutritional status	1	32	2.60	0.50	0.48
	Age×Nutritional status	1	32	2.31	0.44	0.50

Table 7.1: Summary of two factor ANOVA for (a) Fitness, (b) Dry body weight, (c) Mating latency and (d) Copulation duration of experimental females with age and nutritional status as fixed factors. p-value marked with * indicates statistically significant value.

Comparison	χ^2-square	Numertor df	Denominator df	p
O/HY vs. O/LY	0.31	1	17	0.58
O/HY vs. Y/HY	3.21	1	17	0.07
O/HY vs. Y/LY	4.76	1	17	0.03
O/LY vs. Y/HY	6.78	1	17	0.009
O/LY vs. Y/LY	8.10	1	17	0.004*
Y/HY vs. Y/LY	0.90	1	17	0.34

Table 7.2: Summary of Kruskal-Wallis analyses of all six comparisons between the mating successes of the four types of females. P-value marked with * indicates statistically significant value after sequential Bonferroni correction.

Combination type	Females involved	Higher-fitness female	Lower-fitness female
C1	O/HY vs. O/LY	O/HY	O/LY
C2	O/HY vs. Y/HY	Y/HY	O/HY
C3	O/HY vs. Y/LY	Y/LY	O/HY
C4	Y/HY vs. O/LY	Y/HY	O/LY
C5	O/LY vs. Y/LY	Y/LY	O/LY
C6	Y/HY vs. Y/LY	Y/HY	Y/LY

Table 7.3: Choice combinations: C1-6 are combinations assayed during the two-way choice experiment. Each combination had two types of females differing with respect to their age (young/old) and/or nutritional status (high/low Yeasted): Young High Yeasted (Y/HY), Young Low Yeasted (Y/LY), Old High Yeasted (O/HY) and Old Low Yeasted (O/LY). Based on fitness measurement these females are categorized as higher-fitness female and lower-fitness female in each combination type.

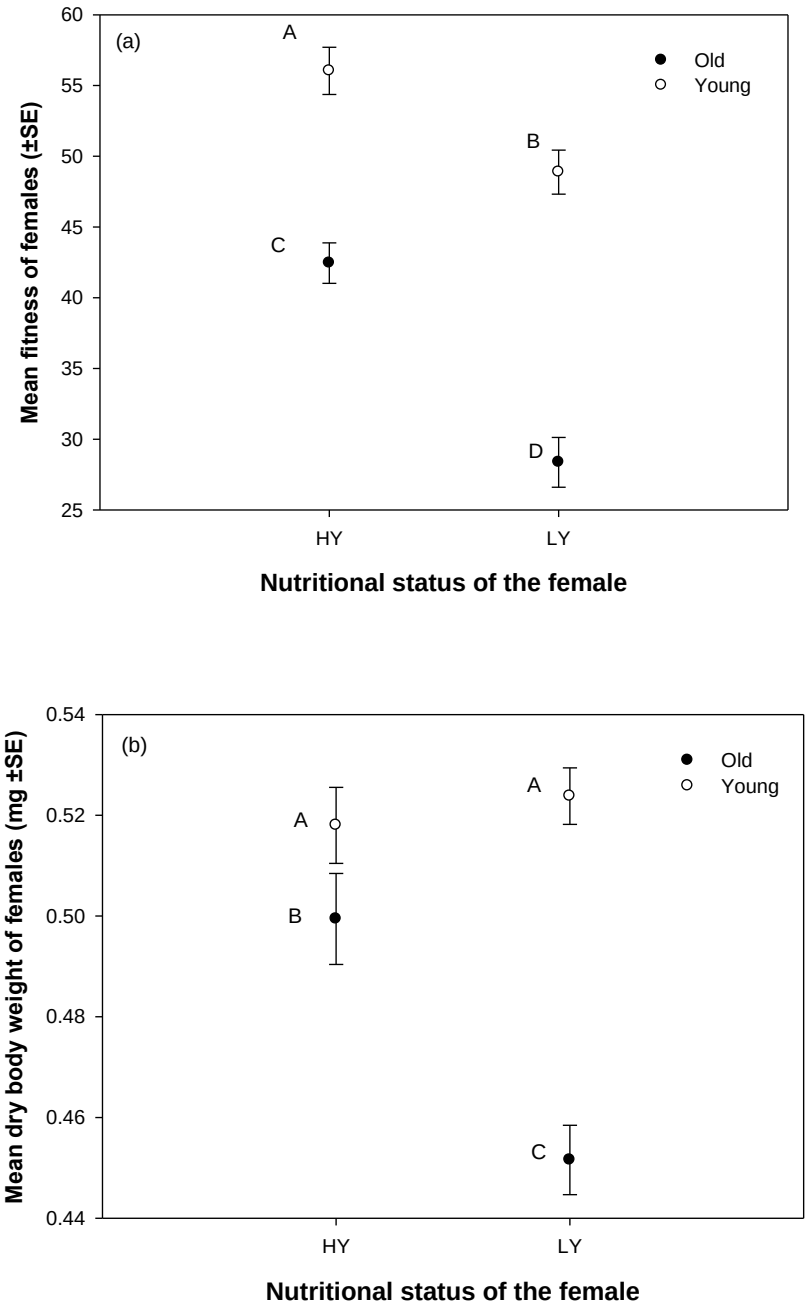

Figure 7.1: Effect of Nutritional status and Age of the females on: (a) Fitness (number of progeny produced) of the females; (b) Dry Body Weight. Points not connected by common letters are significantly different. Open circles (◦): Young females; Closed circles (•): Old females.

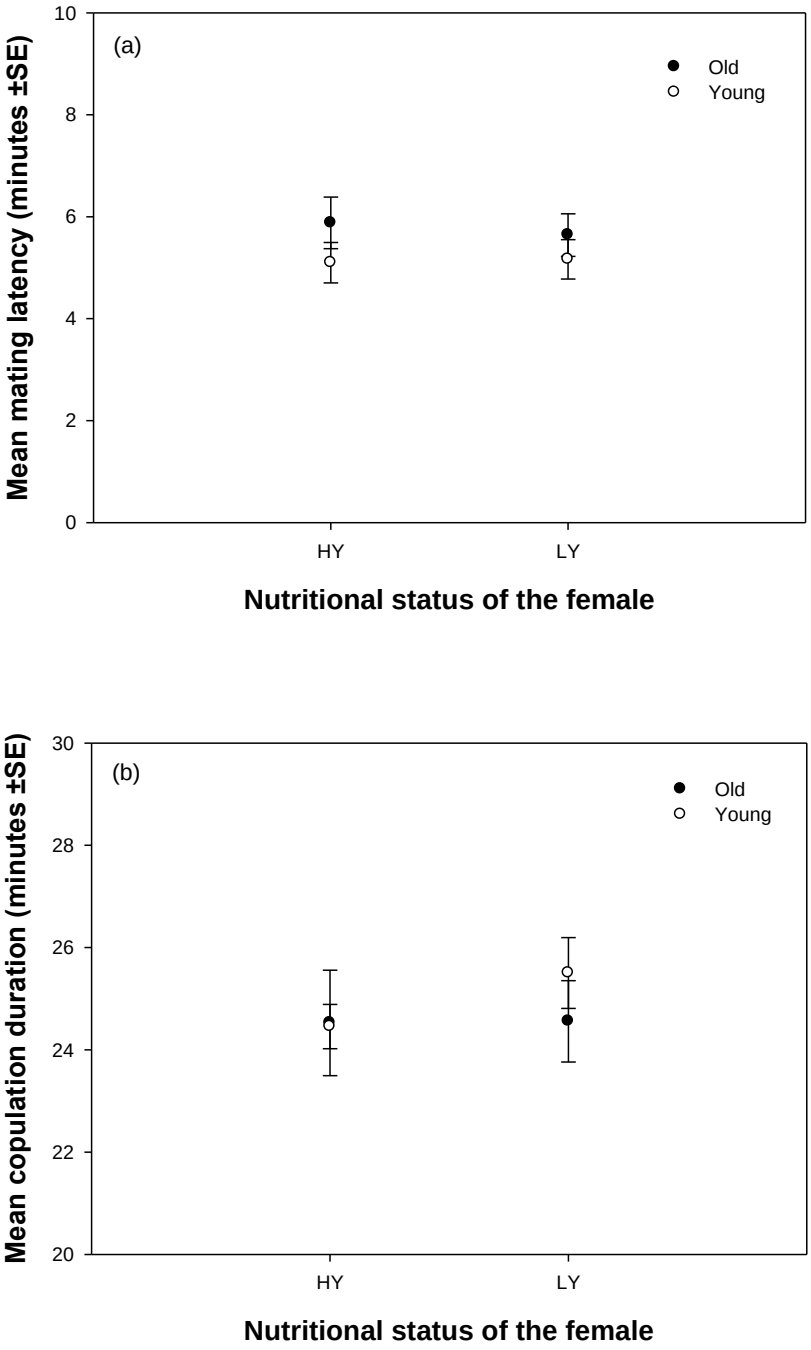

Figure 7.2: Effect of Nutritional status and Age of the female on the components of reproductive behaviour: (a) Mating latency; (b) Copulation duration. None of the points are significantly different from each other.

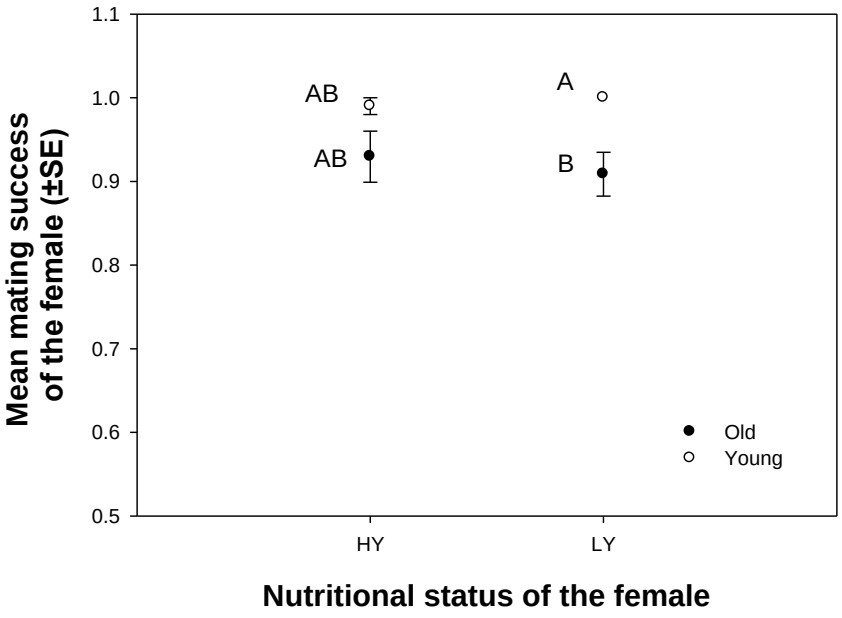

Figure 7.3: Effect of Nutritional status and Age of the females on Mating Success (proportion of virgin females inseminated within 1hour under no-choice condition) of the females. Points not connected by common letters are significantly different. Open circles (◦): Young females; Closed circles (•): Old females.

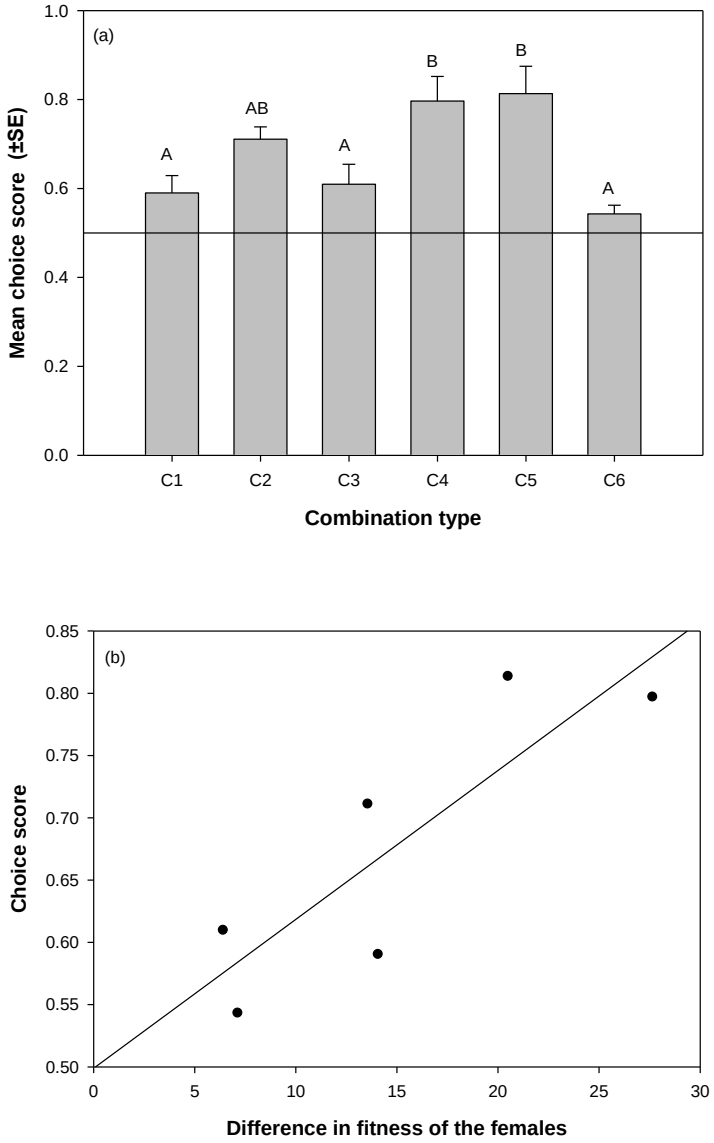

Figure 7.4: Choice scores and variance in female quality: (a) Mean Choice Scores of all the combinations (C1: O/HY vs. O/LY, C2: Y/HY vs. O/HY, C3: Y/LY vs. O/HY, C4: Y/HY vs. O/LY, C5: Y/LY vs. O/LY, C6: Y/HY vs. Y/LY). Bars not sharing common letters are significantly different. The horizontal line indicates expected choice score if there is no mating bias (CS=0.5). Except C6, in all combinations Choice Score was significantly greater than this expected value of 0.5 ($p<0.04$ for all combination). Choice score in C6 was marginally not-significantly different from 0.5 ($p=0.053$); (b) Regression between Choice Score and difference in fitness of the females. Slope = 0.012, $r^2 = 0.73$, $p = 0.03$.

DISCUSSION:

Theory predicts that under certain conditions, viz. considerable variation in female quality and sufficiently high cost of mating to the males, it will be advantageous for the males to bias their reproductive effort (Bonduriansky 2001). In my experiment, I manipulated both these factors. First, I generated female quality variation by manipulating the age and nutritional status which are known to have major effects on female fitness in *D. melanogaster* (Boorman and Parker 1976, Stewart et al. 2005). Second, I assessed mate choice in sperm depleted males, which are expected to have greater propensity to show mating bias (Byrne and Rice 2006). My results indicate that males tend to mate with females of higher potential reproductive fitness with the intensity of mating bias being positively correlated with the variance in the female fitness. Age as well as nutritional status of the females were found to be important determinants of male mating preference.

Apart from male mate choice, there are at least two alternative explanations for the observed pattern of mating bias in males. First, since mating decision depends on both males and females (Moehring and Mackay 2004), the bias in mating pattern observed in my experiment can potentially be explained in terms of higher receptivity of the young females compared to the old females. However, I found no significant difference in the receptivity, measured in terms of mating latency of the four different classes of females. However, young females were found to have a higher mating success under no-choice condition. The data suggests that O/LY-females, with an intrinsic mating success of 0.93, were significantly less successful in mating compared to Y/LY-females. However, the choice score in C5, where males were allowed to choose between O/LY and Y/LY females, was 0.81. This score was significantly different from the expected choice score

(even after considering the difference in mating success of the females), indicating mating bias in favour of Y/LY (see revised choice score in Results section).

Second, males can learn (Reif et al. 2002) to mate with the type of females they were exposed to during sperm depletion treatment. However, I rule this possibility out as the females used during sperm depletion came from a different age class (7day post eclosion) compared to the two age classes (old = 13day and young = 3day post eclosion) used in the experiment.

Thus it seems very likely that the observed bias in mating can be attributed to male mate choice rather than female receptivity or male learning. To summarise, I found no difference in mating latency and insufficient difference in the mating success under no-choice situation. However, a mating bias towards one type of females was evident under choice conditions. Thus I conclude that the observed pattern of results offer strong evidence for male mate choice. I did not find any evidence of post-copulatory male mate choice in the form of variation in copulation duration, which is often used as an indicator of male ejaculate investment in females and has been found to vary in a number of situations (Friberg 2006, Nandy and Prasad 2011). However, a recent study by Lupold et al. (2011) using *D. melanogaster* suggests that males might vary their ejaculate investment, especially sperms, without varying the copulation duration (Lüpold et al. 2011). This study showed that males invested more sperm in young and larger females. Thus at present I cannot completely rule out the possibility of post copulatory, cryptic male mate choice within my experimental regime.

In all the six choice combinations tested, fitness of the preferred females was significantly higher. Hence, at least in my experimental system, male mate choice is adaptive. More interestingly, the difference in the fitness of the two types of females within each of the six mate-choice combinations varied greatly and so did the degree of mating bias exhibited by the males (i.e., there was a significant effect of mating combination on choice score). A regression fit of choice scores on the difference in fitness of the experimental females resulted in a significantly positive slope indicating a positive correlation between variance in female fitness and degree of mating bias in line with the theories of adaptive male mate choice. Although theoretically indicated, effect of variance in female quality on male mating decision has rarely been addressed. Males in a bush cricket species were shown to be more likely to reject mates when perceived mate quality variance and mate encounter rate were both high (Kvarnemo and Simmons 1999). However, the organism studied has reversed sex roles, at least under nutrition deprived condition, and thus males are expected to be choosy (Kvarnemo and Simmons 1999). Here, I have shown that males of *D. melanogaster*, a species with conventional sex roles, adaptively vary their degree of mating bias according to the prevailing mate quality variance. My finding is the only the second empirical evidence of its kind, supporting, with even greater strength, theories of evolution of male mate choice. In addition, the pattern of choice scores in my experiment suggests males' ability to respond to both age as well as nutritional status of the females.

Although one previous study (Cook and Cook 1975) suggested that young and well fed *Drosophila* females receive higher amount of courtship, the study lacked any evidence regarding the actual mating success and fitness of the females and could only resolve the extreme difference. Thus my study is the first unambiguous evidence of adaptive

precopulatory male mate choice based on female age and nutritional status in *Drosophila* and one of the very few evidences for adaptive precopulatory male mate choice in general. It should also be noted that the population used in this study was maintained under optimum laboratory conditions for a large number of generations and it is likely that the mate quality variation (at least with respect to the two factors of age and nutritional status addressed here) experienced by the males is relatively low. The fact that I could show adaptive male mate choice based on experimentally generated mate quality variation even in this population points to mechanisms ingrained over the course of evolutionary history, prior to their laboratory adaptation and/or sufficient variation in mate quality even under laboratory conditions.

There can be several potential mechanisms by which males can differentiate between the two types of females. Theoretically, male mate choice is expected to depend on male's ability to assess the quality of females based on certain signals as well as how honest the signals are in indicating female quality (Bonduriansky 2001). In the present experiment, there are two factors affecting female quality- age and nutritional status. There are potentially, multiple signals that can be associated with ageing. Specifically, the cuticular hydrocarbon profile of a female is known to change with age (Ferveur 2005) and males of *Drosophila* can differentiate females based on cuticular hydrocarbon profiles (Friberg 2006). In an elegant study, Byrne and Rice (2006) showed that males of *D. melanogaster* that were resource limited preferentially mate with females of a larger body size (Byrne and Rice 2006). The body size variation among the females was generated by altering larval density. In my study, I varied the amount of yeast supplement available to the adults and found that nutritional manipulation significantly affected dry body weight of the females, specifically that of the old flies. Since all the

adults in my experiment came from standard density cultures, body size, measured as thorax length, is not expected to change due to nutritional manipulation. Therefore, body weight, but not body size, is one factor that can potentially explain the mechanism of the observed choice in the experiment. However, as suggested by the multiple comparison (Tukey's HSD), only O/LY females were significantly lighter than all the other type of females and thus only combinations (C1, C4 and C5) which had this type of females can be explained. Abdominal distension, although I did not quantify it, can be a potent cue for the nutritional status.

My finding is important in the understanding of the mechanism of maintenance of fitness variation in females of a population. As shown by Long et al. (2009), in an organism, like *D. melanogaster*, that experience sexual selection and sexual conflict simultaneously, the distribution of female fitness in a population is determined by both the distribution of the intrinsic fitness of the females and the distribution of the amount of fitness depressing male interactions (Long et al. 2009). The latter factor is a direct outcome of male mate choice. Preferred females are expected to attract more male attention and thus higher mate-harm. If males can vary the degree of mate choice depending upon female fitness variance, it will scale the intensity of the effect proposed by Long et al. (2009). Greater fecundity variance will lead to stronger male mate choice and thus greater reduction in the fecundity of preferred females (due to sexually antagonistic effects). On the other hand lesser fitness variance will lead to weaker male mate choice, consequently less fitness depression of the preferred females. Hence, all else being equal, the positive correlation between intensity of adaptive male mate choice and variance in female fitness together with sexually antagonistic interactions can potentially maintain variation in female fecundity and other fitness related traits much

more effectively (compared to a situation where adaptive male mate choice is not plastic).

My results have important consequences for the evolution of senescence. Empirical and theoretical studies suggest that female mate choice for older males (Beck and Powell 2000) has the potential to significantly lower the mortality rate in a population (Beck et al. 2002). I propose that male mate choice based on female age is very likely to have major consequences for the evolution of mortality rates. However, this has not received sufficient attention either theoretically or empirically. It is interesting to note that my study raises the possibility that the preferences of males and females with respect to the age of their mates might be in the opposite directions. However, the relative importance of male-mate choice will depend on two factors. First, if mate choice is exhibited mostly by males that are subjected to ejaculate depletion, then, this may not be common in the wild since ejaculate depletion is unlikely to be common. However, resource limitation, in terms of nutritional limitations, can affect males' ability to produce sperm (or ejaculate as a whole), given that they are energetically costly and such situation of resource limitation is expected to be common. Secondly, since intensity of male mate choice scales with the variance in the female quality, its relative importance is also expected to depend on factors affecting female quality (e.g., availability and distribution of food, age structure of the natural populations etc.). When variance in female quality is high male mate choice is expected to be very important compared to when it is low.

CONCLUSION:

When the cost of reproduction for males and variance in female quality are high, males are predicted to show adaptive mate choice. Using *Drosophila melanogaster*, I test this

prediction and show that sperm limited males preferentially mated with young and/or well fed females. The preferred females had higher reproductive output – direct evidence of adaptive precopulatory male mate choice. My most striking finding is the strong positive correlation between the degree of mating bias showed by the males and the variance in the fitness of the females. I proposed that such choice has important consequences with respect to the existing understanding of the mating system and the evolution of aging.

Note: The work reported in this chapter was published as

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Chapter 8

Effect of number of male co-inhabitants early in life on male reproductive behaviour and fitness component

INTRODUCTION:

In sexually reproducing species with little or no parental care, male fitness depends largely on the number of matings and the average number of progeny sired from each mating (Bateman 1948). Mating success of males in many species is largely dependent on the ability of the males to perform a set of complex behaviors, which together are termed mating behavior. Fruit flies are one of the best model systems to study male reproductive behavior because of a promiscuous mating system with considerable genetic variation for an elaborate male reproductive behavioral repertoire (Moehring and Mackay 2004).

Previous studies have documented the 'plasticity' of male mating behavior in various species of *Drosophila*. Immature *Drosophila* males that elicited courtship from mature males had significantly lower mating latency (time taken by a virgin pair to start mating, ML) as adults compared to males that did not elicit homosexual courtship (McRobert and Tompkins 1988). Flies housed in groups had lower mating frequencies and higher ML compared to flies housed singly, and males preferred females housed singly to those housed in groups (Ellis and Kessler 1975). Flies housed under light-dark cycles had greater mating success than flies housed in constant darkness (Hirsch et al. 1995). Fruit flies (*Drosophila* sp.) maintained in an enriched environment (presence of combination of complex inanimate and social stimulation

during housing) during early adult life had higher mating success than flies maintained in standard environments (Dukas and Moores 2003). Thus, there is a growing body of evidence that early life experience affects male mating behavior and mating success to a great degree in *Drosophila*. Some of these findings are relevant to the theories of sperm competition (Parker 1993, Engqvist and Reinhold 2005), wherein sperm from different males compete with each other with in the female genital tract (Parker 1970). Sperm competition is considered a potent driving force for the evolution of several morphological, behavioral and physiological traits (Snook 2005). Models of sperm competition recognize two different parameters – *risk* and *intensity* (Parker 1990, 1996, Engqvist and Reinhold 2005). It is predicted that males should evolve (a) mechanisms to gauge levels of sperm competition and (b) prudent ejaculate investment strategies based on varying levels of these two parameters (Engqvist and Reinhold 2005, 2006). Williams et al. (2005) provide an alternative model of sperm competition, where degree of sperm competition is coupled with sperm allocation. They show that factors, such as cost of mating, total resource availability and degree of sperm precedence (rather than degree of sperm competition per se), can drive the evolution of sperm allocation strategy.

Empirical evidence in support of the ability of males to gauge levels of sperm competition and invest accordingly comes from diverse species of insects, including crickets, butterflies and fruit flies (Gage and Barnard 1996, Wedell and Cook 1999, Friberg 2006, Bretman et al. 2009). In *Drosophila,* males can use female mating status and the number of potential competitors to gauge levels of sperm competition. Males were found to mate longer with females that are perceived as previously mated compared to females perceived as virgins (Friberg 2006). Two recent studies have shown that males held in groups during early adult life mated longer than males held singly (Bretman et al. 2009, 2010). Thus, at least in

Drosophila, copulation duration is a potential measure of male investment in response to perceived levels of sperm competition. Although copulation duration is considered an indicator of male ejaculate investment, results from some of the recent studies indicate that the variation in copulation duration can be attributed to variation in amount of accessory factors transferred (Friberg 2006, Bretman 2009, 2010).

In the present study, I addressed the following questions: (a) Is male mating behavior in *D. melanogaster* affected by the number of male co-inhabitants experienced early in adult life in the way predicted by the theory of sperm competition? (b) Do changes in mating behavior affect male competitive fitness? I exposed male *D. melanogaster* to different numbers of male co-inhabitants very early in their adult life and then assayed their mating latency and copulation duration. Sperm defence ability (the ability to resist displacement by sperm from other males) of the males from different treatments, was quantified as a measure of the fitness consequence of the behavior.

METHODS:

The experiment was done using the LH_{st} (Prasad et al. 2007) and LH (Chippindale and Rice 2001) populations of *D. melanogaster* (see Chapter 2 for details of the mentioned populations). While LH is the wild type (red-eyed) base population, LHst is a derivative of LH having autosomal recessive scarlet eye marker. Both populations were maintained on a 14 day discrete generation cycle at 25^{o}C and 12 hours light: 12 hours dark, on standard cornmeal-molasses food. Further details about the two populations are provided in Chapter 2.

For the present experiment, eggs were collected from adult flies and dispensed into 8 dram vials containing cornmeal-molasses food at a density of 150 eggs/vial. During peak eclosion, males were collected as very young virgins (<4 hrs post eclosion) and randomly assigned to one of five different treatments, which differed in the number of males (1, 8, 16, 24 or 32 males per vial) that were held together for a period of two days post eclosion. Space within the vial was adjusted to keep the space available per individual constant across the treatments. This was done by pushing the cotton plug to different depths. I allowed a space of about 3ml per individual between food and cotton plug. During the experiment, a single male from each treatment group was paired with a single 3-day old, virgin LH_{st} female. Each pair was observed individually to get the ML and CD. All experimental males were successful in mating. After about an hour, by which time almost all flies had completed mating, the flies were separated using light CO_2 anaesthesia. After half an hour, females were combined with control, red eyed males and allowed to interact for 20 - 22 hours, after which the males were discarded and females were put into individual test tubes (12 mm × 75 mm) with medium and allowed to oviposit for 18 hrs. Twelve days later, the progeny were scored on the basis of their eye colour. The proportion of scarlet-eyed flies gave the sperm defence (P1: proportion of progeny sired by the first male when the female has mated with two males sequentially) value of the experimental males. The fraction of the females that did not re-mate yielded a value of fidelity.

The entire experiment was done in three separate blocks, which were run on three successive days, with 15 replicates of each treatment in each block. Block means were used as the units of analysis. For sperm defence, analyses were done on both raw and arcsine square-root

transformed data. Data for each of the traits measured were analysed using a two-way mixed model analysis of variance (ANOVA) with treatment as the fixed factor crossed with randomised block. Multiple comparisons were implemented using Tukey's HSD. All these analyses were done using STATISTICA for Windows.

RESULTS:

The results of the ANOVAs are summarised in Table 8.1. There was no significant effect of treatment on mating latency (Figure 8.1). Copulation duration varied significantly across the treatments (Figure 8.1). Copulation duration increased till the 16-male treatment and then decreased till the 32-male treatment. Multiple comparisons indicated that the single male treatment was significantly different from all the other treatments. Additionally, the 16-male treatment was significantly different from the 32-male treatment.

Treatment had a significant effect on sperm defence (Table 8.1). The P1 values showed a distribution similar to that of the Copulation duration (Fig 8.1). P1 increased from single to 16-male treatment and thereafter declined till the 32-male treatment. Multiple comparisons indicated that the single male treatment was significantly different from the 16-male treatment. A linear regression of mean P1 values on mean CD yielded a significant positive slope (slope = 0.015, $r^2 = 0.27$, $p = 0.049$). No significant effect of the treatment on mating fidelity was observed.

Trait	df	MS	*F*	*p*
Mating Latency	4	0.43	2.35	0.142
Copulation Duration	4	10.76	25.32	< 0.001
Sperm defence	4	0.01	4.50	0.034

Table 8.1: Summary of results from three separate two-way mixed model ANOVA on ML, CD and P1 data, with treatment as the fixed factor crossed with random blocks.

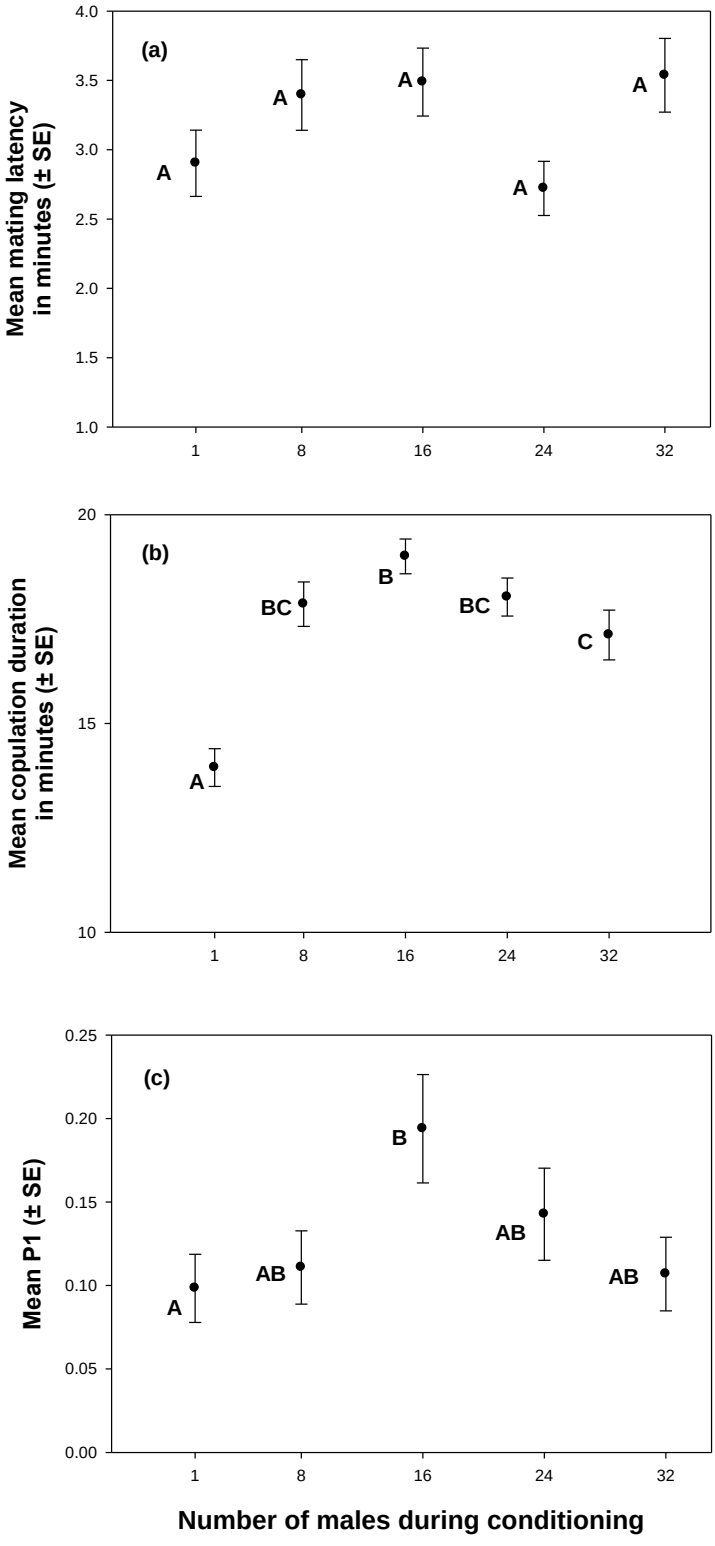

Figure 8.1: Effect of number of co-inhabitants experienced early in life on (a) Mating Latency, (b) Copulation Duration and (c) Sperm Defence ability (P1). Points not sharing at least one common letter are significantly different.

DISCUSSION:

In my study, males were exposed to increasing numbers of co-inhabitants during early adult life and then components of their reproductive behaviour and fitness were measured. The results show that copulation duration, an important component of the reproductive behaviour, is plastic, with the males confined with either high or low numbers of co-inhabitants showing lower copulation duration compared to males confined with intermediate numbers of co-inhabitants. Moreover, the variation in copulation duration was positively correlated with an important component of male fitness, namely sperm defence ability. Males from the 16-male treatment had higher P1 values compared to either males from the single or 32-male treatment. This fitness difference among the males is not attributable to the differences in their ability to inhibit further mating. Thus, my results indicate that the number of early life co-inhabitants faced by males may affect their later life fitness by altering components of reproductive behaviour.

There are several potential explanations for the observed change in copulation duration with the number of co-inhabitants. Male density prior to assay can have major effects on male courtship. *Drosophila* males held at high density tend to have lesser courtship intensity compared to males held isolated (Noor 1997). However, I rule out such density effects in my experiment. I ensured that the males had the same per capita space across treatments by varying the total available volume within the container. I also rule out density effects being mediated through competition for food. This is because (a) the food provided in the vial is enough to support a large number of flies and (b) males do not feed much compared to the females (Stewart et al. 2005).

Increasing the number of co-inhabitants increases the chances of interactions, which has the potential to affect male reproductive behaviour, quite independent of space- and food-limitation related effects. In *Drosophila*, the reproductive behaviour of a male can be affected by its interactions with other sexually mature males during its early, immature stages (Gailey et al. 1982, McRobert and Tompkins 1988). However, in my experiment, all individuals were of the same age; consequently, the differences in the reproductive behaviour of males from various treatments cannot be attributed to the interaction between mature and immature males. Additionally, while increased male-male interaction has considerable fitness cost (Gaskin et al. 2002), it is unlikely to be a major factor in my experiment, as males of the population I used commonly show very little male-male courtship. But lack of direct observation during the experiment prevents us from confirming this prediction.

Alternatively, the observed responses may reflect the adaptive, plastic ejaculate investments by males based on their perception of the level of sperm competition. In promiscuous species like *Drosophila*, females may mate multiply and store sperm from multiple males leading to sperm competition (Snook 2005). Increasing the number of co-inhabitants might alter the male's perception of sperm competition intensity or sperm competition risk. Theory predicts that male investment should vary based on both sperm competition intensity and sperm competition risk (Engqvist and Reinhold 2005). Additionally, theories distinguish between "average" (long- term average in a population) and "immediate" (in a given round of mating) levels of intensity and risk. Male investment is predicted to increase with increasing average levels of both intensity and risk. However, risk models predict increasing investment with increased immediate levels of risk, whereas intensity models predict decreasing investment with increased immediate levels of intensity (Parker et al. 1997, Engqvist and Reinhold 2005). In a recent study, Bretman et al. (2009) altered both average and immediate levels of

sperm competition by varying the number of competitors that males are housed with prior to and during the assay respectively and found that males adaptively varied their investment. Male investment increased with average levels of sperm competition but decreased with increasing immediate levels of sperm competition, an observation largely consistent with the predictions of intensity models of sperm competition. In my experiments, males were confined with cohabitants for two days and then assayed in the absence of a competitor. Hence in my experiments, average sperm competition intensity/risk levels were varied but immediate sperm competition intensity/risk levels were constant and zero. Sperm competition theory (Engqvist and Reinhold 2006) predicts increased investment with increased average levels of sperm competition intensity/risk. My results agree partly with the predictions of sperm competition theory and the results of Bretman *et al*. (2009) in that the copulation duration increases as the male number increases from one to 16. The observed decline in copulation duration as male numbers increase from 16 to 32 is not in agreement with the predictions of sperm competition theory. While at present I do not have a mechanism to explain the observed decline in copulation duration at higher male numbers, I make three observations- (a) Group sizes in my study are larger than those of Bretman et al. (2009, 2010). The sizes of the largest groups in the study of Bretman et al. were 4 (Bretman et al 2009) and 16 (Bretman et al 2010), whereas it was 32 in my study. While theories suggest increased investment with increased average levels of sperm competition, it is very likely that there exists a certain limit beyond which it might not be biologically feasible for an organism to invest in larger ejaculates and/or the costs of investing in such ejaculates might be very high. In fact, alternative treatments of sperm competition (Williams et al 2005) suggest that Evolutionarily Stable Strategy (ESS) level of sperm allocation decreases with increasing mating cost and strong last male precedence. However, I are not aware of any efforts to extend these alternative treatments of sperm competition to plastic ejaculate investments by

the males based on perceived levels of sperm competition. (b) It is quite possible that housing males with other males for a period of time might alter their perception of both average and immediate intensity/risk, thereby making comparisons with predictions from sperm competition theory more difficult. (c) It is important to note that the theoretical predictions (Engqvist and Reinhold 2006) assume numerical competition between sperm and concern investment of sperm in different matings by the males. Ejaculate (sperm along with the seminal proteins) investment pattern might be much more complicated than what is predicted. For example, in species like *Drosophila melanogaster*, with high last male sperm precedence and moderate level of remating frequency, theory predicts very little change in sperm investment with changing risk of sperm competition when mating with virgin females (Engqvist and Reinhold 2006). However, with increasing risk of sperm competition, males might still be selected for injecting more of the Accessory Gland Proteins (Acps) even to virgin females which might give them higher ability to defend against possible sperm displacement, which would mean an increase in the copulation duration. Hence, given that variation in copulation duration in *Drosophila* is likely to represent a variation in Acps rather than a variation in sperm numbers and that sperm competition is affected by Acps, the theoretical predictions of the pattern of variation in copulation duration with changing levels of sperm competition are not clear.

CONCLUSION:

In conclusion, my study clearly shows that (a) reproductive behaviour in male *D. melanogaster* can be non-linearly affected by the number of male co-inhabitants experienced early in adult life, and these changes in behaviour are partly consistent with the predictions

from theories of sperm competition and (b) these changes in behaviour directly affect at least one component of male fitness, sperm defence ability.

Note: The work reported in this chapter was published as

Nandy, B. and Prasad, N.G., Reproductive behaviour and fitness components in male *Drosophila* are non-linearly affected by the number of male co-inhabitants early in adult life. *J. Insect Sci.* 11, 67 (2011), available online: insectscience.org/11.67.

Chapter 9

Conclusion

Holland and Rice (1999) proposed the model of intersexual co-evolution through sexual conflict – chase-away selection. The model received a wide empirical support from a variety of species (Rice 2000, Arnqvist and Rowe 2005, Koene 2012). Particularly, the interlocus form of the intersexual conflict has received a lot of attention, presumably because of its role in initiating and driving the open ended arms race between the sexes and its predicted implications in the process of speciation (Rice 1996, Parker and Partridge 1998, Arnqvist et al. 2000, Gavrilets 2000, Martin and Hosken 2003a, Gavrilets and Hayashi 2005). In this thesis I have empirically tested the model of interlocus sexual conflict using laboratory experimental evolution. While my results generally support the model of co-evolution of mate-harm and resistance to mate-harm, one of the first comprehensive studies to do so, I have also found new evidence suggesting life-history consequences of intersexual conflict, which until recently received very little attention of the empiricists. I have also discovered two novel outcomes of intrasexual selection on males in the form of plasticity in their pre-copulatory mate choice based on mate-quality variance and gametic strategy based on early life experience. In this chapter I will summarize these novel findings and emphasize their importance in the context of my understanding of sexual selection.

Sexually antagonistic co-evolution – a comprehensive empirical assessment

Theories of sexual conflict predict that the fitness correlation between a male and its mate will rarely be positive under even low levels of promiscuity (Parker 1979, Rice 2000, Wedell et al. 2006). This, along with intense intrasexual selection on males, leads to intersexual conflict. Here I will first briefly outline the model of sexual conflict (Rice 2000, Rice et al.

2006) mediated by direct antagonistic interactions between the sexes. I will then show how the results presented in this thesis substantiate this model.

Males in most sexual species experience intense intrasexual selection (male-male competition). Such competition can take place prior to mating when males compete for access to mates (Bateman 1948) or even post-mating through sperm competition (Parker 1979, Simmons 2001, Snook et al. 2003). While males across several species have been known to compete vigorously for mating success investing heavily in courtship (see Trivers 1972), both male behaviour and ejaculate composition have been predicted to evolve in response to sperm competition (Birkhead and Moller 1998, Simmons 2001). Many of the male adaptations to such competition have been shown to affect females adversely – causing increased mortality (Fowler and Partridge 1989, Chapman et al. 1995) and decreased life-time progeny production (Linder and Rice 2005, Kuijper et al. 2006). These female harming effects from males do not benefit males directly but they are very likely to be by-products of male-benefiting functions of the traits (Civetta and Clark 2000, Rice 2000, Morrow et al. 2003). For example, components of ejaculate in *Drosophila* have been shown to reduce female remating propensity and increase short-term fecundity (Civetta and Clark 2000, Wolfner 2002). Given that male fitness can be expected to be diluted if his mate copulates again with a different male, these are beneficial for the males. Due to this male induced harm, females are selected to evolve resistance. Such female counter adaptation might render the male adaptations useless, thereby selecting males to further increase their investment in reproductive activities or evolve different mechanism to maximize fitness. This model is summarised in Figure 9.1.

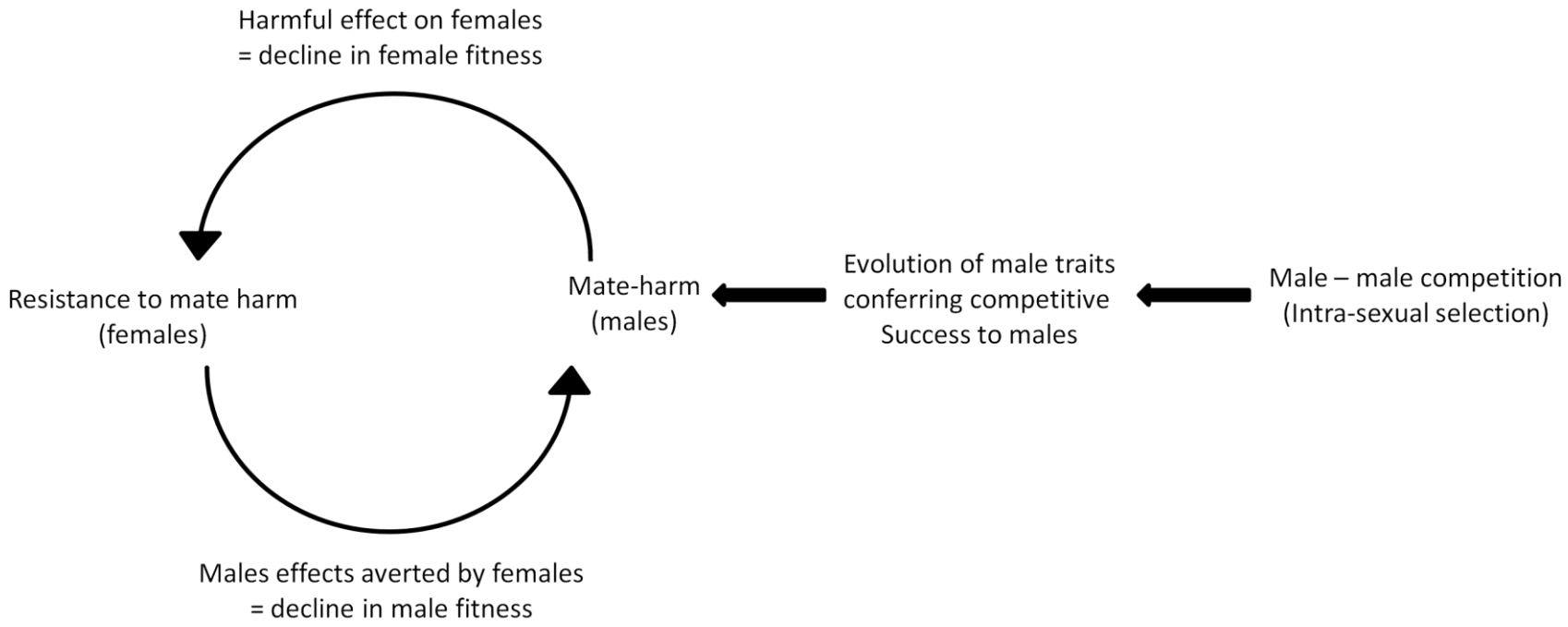

Figure 9.1: A simplified model of *Intersexual conflict* showing the male-female co-evolutionary dynamics

Implicit in this model is the assumption that intersexual conflict is initiated by male-male competition and can thus be manipulated by varying the degree of male-male competition, as opposed to the complete removal of it, within a population. However, this has rarely been tested empirically in a comprehensive manner.

The experimental evolution presented in this thesis tested this by varying the operational sex ratio within populations thereby altering the level of male-male competition. This alteration in operational sex ratio also altered the level of intersexual interaction, further influencing the intersexual conflict within the populations.

My results suggested that selection regime had significant effects on fitness components of the males. Males in the regime which experienced high male-male competition (i.e., the M-regime), evolved increased competitive ability and better sperm defence ability. The males from the low-competition regime (F-regime) evolved reduced competitive fitness and reduced sperm competitive ability (defence and offence). This, at least to my knowledge, is the first empirical evidence of its sort and underlines the importance of further investigations addressing different components of male fitness in detail. The finding substantiates the most basic component of the model depicted above, namely, evolution of male fitness components under different levels of intrasexual selection.

The obvious next question is that, do these male adaptations to such selection lead to evolution in their mate-harming ability? I observed that males from the M-regime caused increased mortality in females, indicating increased cost of cohabitation for females. The males from the F-regime were found to be significantly benign to female fitness. These results, again, unambiguously supported the model.

Along with the evolution in male traits, I also observed evolution of female resistance to mate-harm as predicted by the model. Females from the M-regime were found to be highly resistant to the male-induced fitness reducing effects while females from the F-regime were found to be significantly more susceptible. Although previous studies adopted similar selection design to observe evolution of components of female resistance, the results described in this thesis is of high importance as the resistance measured here were measures of total life-time fecundity of the females under both competitive condition (Chapter 3) and non-competitive condition (Chapter 5).

Thus, by and large my experimental results conform with the theories of intersexual conflict. The results indeed suggest that the fitness correlation between males and their mates in a population can respond to laboratory selection of altered operational sex ratio in the manner predicted by sexually antagonistic co-evolution. These results also implicitly indicate that investment in reproduction related (or sexual conflict related) traits can evolve under such co-evolution. Given that organisms seldom, if ever, have access to limitless resources, such change in investment should have important life-history consequences.

Life-history consequences of sexually antagonistic co-evolution

> "..expenditures on reproductive processes must be in functional harmony with each other and worth the costs, in relation to the long-range reproductive interest; and the use of resources for somatic processes is favoured to the extent that somatic survival, and perhaps growth, are important for future reproduction"
>
> - G.C.Williams, 1966.

Theories of life-history evolution even in their infancy predicted a physiological trade-off between reproduction and other life-history traits, such as longevity and stress resistance (Fisher 1930, Williams 1966). Since, inherently the amount of resources available to the organisms are limited, allocation of resources to one physiological process can be expected to limit its availability to some other resource demanding process (Van Noordwijk 1986, Zera and Harshman 2001). The simplest of such trade-off approximates to the so called "Y-model", with the base representing the available resources and the two arms representing the two alternate resource consuming process (Zera and Harshman 2001 and the references therein). Trade-offs surrounding reproduction related activities have been discussed in several occasions (see Flatt 2011 and citations therein for trade-offs between reproduction and longevity) and have frequently been referred to as cost of reproduction (for example Chippindale et al. 1997, see Zera and Harshman 2001 for definition). However, whether such cost of reproduction can evolve or not, specifically under intersexual conflict is largely unexplored (see Bonduriansky et al. 2008). Relatively recently, theories have been put forward to link intersexual conflict and life-history traits, such as life-span and aging rate (Promislow and Pletcher 2002, Promislow 2003, Bonduriansky et al. 2008). However, to the best of my knowledge, the experimental evolution study by Maklakov et al. (2007) remains the only direct test of such theories.

In this thesis I have reported the first comprehensive study of the life history consequences of evolution under sexual conflict. Previous studies have documented cost of reproduction in the currency of reduced longevity and increased susceptibility to stress (e.g., Starvation) in both sexes (Bell and Koufopanou 1986, Wedell et al. 2006, Chippindale et al. 1997). I found this cost of reproduction to evolve in my experimental evolution study. A decline in the cost of

reproduction in F-males in terms of reduced susceptibility to starvation stress (hereafter referred to as 'starvation-cost') was observed, while their longevity under mated condition (hereafter referred to as 'longevity-cost') was unresponsive. M-males on the other hand showed increased longevity-cost but their starvation-cost remained unresponsive to selection.. Asymmetry in the cost of reproduction measured in terms of longevity and starvation resistance is not unheard of (Chippindale et al. 1993, 1997) and can be attributed to the way they are usually measured. Measurement of starvation-cost allowed a two-day interaction period (male-female interaction), immediately following which the starvation susceptibility was measured. Hence, starvation-cost represents short-term effects of reproductive activities. Longevity-cost was measured under life-long cohabitation with females. Hence, it is likely to be sensitive to the cumulative long term effects of reproductive activities.

I also found the M-females to be significantly long lived under mated condition compared to the females of the other two regimes under similar condition, possibly indicating their evolved resistance to the male induced harm. F-females on the other hand showed increased cost of reproduction and/or male encounter in terms of their susceptibility to starvation stress. As mentioned above, there was substantial evidence to suggest the degeneration of resistance (to mate-harm) related traits in F-females. According to the theories of life-history evolution, the reduction in the investment in resistance related traits can potentially free up resources available for other life-history traits in F-females. Two observations in F-females are of interest here - (a) increase in longevity under virgin condition and (b) increase in fecundity when allowed a single round of mating. While these increases might represent the hitherto predicted trade-off between life-history traits and resistance, the possible role of body size increase (observed in the F-females) cannot be ruled out. Curiously, I did not find any life-

history cost of evolution of resistance to mate-harm in M-females. There are at least two possible explanations for this observation. (a) The trade-off assumes a fixed resource acquisition. However, resource acquisition itself can evolve in the M-females through the evolution in the Yeast-foraging behaviour, a hypothesis open to future studies. A similar situation can potentially be created if the available resources, which are assumed to be limiting, are in fact sufficiently non-limiting to allow some degree of variation in resource allocation pattern across different physiological processes. (b) Trade-offs between life-history traits are often much more complicated than the simple Y-model (Zera and Harshman 2001). Even though I found no evidence of trade-offs between resistance to mate-harm and life-history traits (such as longevity and starvation resistance) in M-females, the predicted trade-off might exist between some other life-history traits not studied in this thesis.

Resistant females make mating more costly for males

Theories of sexual conflict predict females to evolve adaptations to male induced harassments – resistance to mate harm. Such resistance mechanism is often associated with female reluctance to mating, physical struggle during/before mating, female aggressive behaviour towards potential mate and other such behaviours/physiologies (Arnqvist and Rowe 2005, Koena 2012). These adaptations are expected to increase extrinsic mortality rate in males, selecting males to invest more resources in reproduction to enable males (a) reproduce as much as possible during the short life time and/or (b) overcome female resistance by even more persistent mating attempts. In Chapter 4, I have tested this hypothesis and provided the first ever evidence from an experimental evolution study. M-females in my study evolved to be more resistant (as discussed above and in Chapter 4). Males co-inhabited with these females suffered greater mortality. This finding is sufficient to encourage future investigators

to direct some attention to this curious phenomena, which has received no attention whatsoever in all the previous experimental evolution studies.

Reproduction is a costly venture even for males: pre and post-copulatory male strategies

In Chapter 7 and 8, I have shown that males in *D. melanogaster* are capable of adaptive mating strategies in the form of plasticity in their behaviour, both in the pre-copulatory and the post-copulatory stage. Males were shown to preferentially mate with young and well fed females and copulate longer when they have experienced more competitors. Theories show that if only male cost to sexual reproduction is nontrivial (as opposed to the traditional wisdom of low male mating cost) such behaviour can evolve in males. I also found that such behaviours are adaptive. Copulation duration had a positive correlation with at least one component of male fitness and preferred females (young and/or well fed females) always produced more progeny. There are at least two important questions for future consideration – (a) Do populations harbour genetic variation for such plasticity? (b) Under what condition can such behaviour evolve?

Plasticity in male mate choice

Theoretically, the two prerequisites for the evolution of male mate choice are (a) cost of mating for males and (b) variance in female quality (Bonduriansky 2001 and the citations therein). While the former has been shown to affect male mate choice in at least one empirical study (Byrne and Rice 2006), the later has never been addressed experimentally. In this thesis, I have reported the first evidence suggesting the effect of mate quality variance on male mate choice. Using sperm-depleted males, I found a significant positive correlation between the degree of pre-copulatory male mate choice and the prevailing variance in mate

quality. The same study also showed male's sensitivity to female age (young and old) and nutritional status (well fed or less fed on live Yeast). These results have important consequence in term of the theories of aging and our understanding of how fitness variation is maintained in females, specifically in a species that experience sexual conflict and male mate choice.

Conclusions

To conclude, in this thesis, I have shown that sexual conflict related traits can evolve depending upon the intensity of sexual conflict within the population. Due to the very nature of this conflict, none of the sexes gain absolute advantage over the other. Rather any adaptation in one sex (with respect to the intersexual conflict) drives counter-evolution in the opposite sex. I found that males can show plasticity in their reproductive behaviour to cope with the intense intrasexual selection. I also found that males can also exercise mate choice, provided there is sufficient variation in female quality. More interestingly, life-history traits, which are important determinants of organisms' fitness (for example, life-span and aging, stress resistance etc.), trade-off with the sexual conflict related traits. While sexual conflict is a relatively new theory with potentially widespread importance, perhaps it now needs to be viewed in the grander context of life-history evolution. I hope that my thesis will generate fresh interest towards this view of intersexual conflict.

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