Role of sexual selection and conflict in the evolution of reproductive traits: A study using populations of *Drosophila melanogaster* evolving under different operational sex ratios.

A thesis submitted for the degree
Doctor of Philosophy

By

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Dedicated to my family and friends

CONTENTS

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Synopsis

Expanding on Fisher's idea of sexual selection, Rice and Holland (1998) proposed a 'Chase-away' model based on antagonistic coevolution between the sexes. This model suggested that pre-existing sensory bias in females induces a selection pressure on males to evolve an initial elaborate trait to increase its attractiveness to the females. These elaborate traits can be harmful to females. As a counter-response, females also evolve resistance against the males' elaborate characteristics, leading to a decrease in males' reproductive success. This cycle of adaptation and counter adaptation results in a scenario where the evolutionary interests of males and females are in direct conflict with respect to each other. This form of sexual conflict is referred to as interlocus sexual conflict, and the co-evolutionary 'arms race' that ensues is known as sexually antagonistic coevolution.

Many evolutionary studies have tested the predictions of the chase-away model. Consistent with the model's predictions, studies have shown the evolution of male fitness-related traits such as reproductive success, sperm competitive ability, sperm morphology etc., in response to the co-evolutionary arms race between the males and females. Additionally, a few studies have documented the correlated evolution of mate harming ability of males and mate harm resistance in the females. However, apart from the direct predictions about mate harm and mate harm resistance; the theory lends itself to nuanced and extended predictions. Many of these predictions have not been tested yet. In this thesis, using laboratory experimental evolution on populations of *Drosophila melanogaster,* I tried to test some extensions of sexual selection and conflict theories on the evolution of reproductive traits.

Some of the important predictions which I addressed are as follows:

1) Can evolution under different levels of sexual selection lead to the evolution of plasticity in male reproductive behavior in response to variable socio-sexual environments?

2) Can males evolving under divergent levels of sexual selection evolve to have different courtship learning abilities?
3) Does evolving under differential levels of sexual selection affect the relationship between reproductive effort and immune response in both males and females?
4) Does evolving under differential levels of sexual selection and conflict lead to evolution of maternal effects?

Laboratory studies investigating sexual conflict mainly use two methods to manipulate sexual selection and conflict intensity -a) by enforcing monogamy (b) biasing the sex ratios. The method which I used is sex ratio bias. For most of the experiments, I used two selected regimes– male-biased (M) regime with the operational sex ratio (male: female) as 3:1, and the female-biased (F) regime with a male: female sex ratio of 1:3. For each of the two regimes, there were three independent replicate populations. These populations were established by Dr Bodhisatta Nandy and are explained in detail in Nandy et al. (2013b). Males of the M regime are under higher sexual conflict and intrasexual selection compared to males from the F regime. Males of the M regime and F regime have diverged in terms of their life history and reproductive traits. For example, M males have evolved to have increased ejaculate investment, increased courtship activity and locomotor activity, and increased mate harm compared to F regime males. In response, M regime females (compared to F regime females) have evolved to become mate harm resistant and their basal level fecundity has decreased (Nandy et al. 2014).

In my first set of studies, I addressed the evolution of sperm competitive ability in the M and F regime males. Since M and F regime males are evolving under different levels of sexual conflict, these males can be assumed to be evolving under different intensities of sperm

competition for many generations. Indeed, M regime males are known to have greater sperm competitive ability than the F regime males. Sperm competition theory also suggests that mating expenditure of males should enhance with increasing risk (the possibility of a female being mated or mating again) whereas decrease with increasing intensity (the number of ejaculates struggling to gain paternity over a given set of eggs) of sperm competition Howbeit, production of reproductive resources is energetically very expensive. Hence males are expected to adopt different strategies in response to varying degrees of sperm competition. Studies have shown that males of diverse insect species show plastic behavioural strategies by altering their morphology, physiology or behaviour in response to rival males. These studies indicated that alteration of the density of rival males competing for fertilization of eggs could modify both the risk and intensity parameters of sperm competition.

Till present, studies that have demonstrated the plasticity in male's reproductive traits in response to perceived sperm competition are mostly single generation phenotypic manipulation studies. However, given that acquiring plasticity comes with a fitness advantage for males, it is still not well understood if plastic responses in male reproductive behaviour can evolve in response to specific selection pressures. This speculation built the framework for the 1st study of my thesis. To investigate if there is any influence of sexual selection on the evolution of plasticity in male reproductive behaviour, I subjected males from both male-biased (M) and female-biased (F) regime to treatments with the different number of competitors (1, 8, 32) in their early life. After this, these males were retained in these treatments for a conditioning period of 2 days before the mating trials. Results showed that males under male-biased and female-biased regimes evolved different plastic responses in their reproductive investment (measured as copulation duration and sperm competitive ability) in response to varying density of rival males. M males initially increased their

reproductive investment as the number of competitors increased from 1 to 7, but afterwards, on exposure to a higher number of competitors (31), these males showed a decrease in their reproductive investment. On the other hand, the F regime males were found to continually increase their reproductive investment with the increasing number of competitors. I speculated the most possible causal factor for the observed trend of results to be the differential intensity of sexual selection acting on M and F males. M males are under intense sexual selection pressure compared to F males as there is strong male-male competition in the M regime. It can be assumed that to cope with sexual selection pressure, M males have evolved to be more sensitive and strategic in response to any change in their socio-sexual environment. Also, it is possible that the potential level of sperm competition is perceived differently by M and F males due to their different evolutionary trajectories.

Further, in a -up experiment, M and F males were housed with ancestral (LH) males rather than the males from their own respective regimes (as that of the previous experiment) for two days. The different numbers of competitor LH males used for keeping with each of M and F regime males were 1, 7 and 31. In this case, I observed that M males' reproductive investment pattern changed compared to when they were housed with males of their own kind. It indicates that the evolutionary history of rival males also matters while conducting such experimental assay. Then, I further examined the effect of increasing the time between the perception of competitive cues and the time of mating assays. Interestingly, I observed that as the time from removal of competitive cues increased, the memory of early life experience wore off at different rates between the males evolving under male-biased and female-biased regimes. This study provides a new insight into review of sexual selection in the evolution of phenotypic plasticity in male reproductive behaviour in response to variable socio-sexual environment.

An exciting inference of the above study was that M males retained the memory of early life experience for a slightly longer time relative to F males. The reproductive investment pattern in the case of CD shown by M males was found to be almost the same after 3 days of excluding the cues, whereas F males did not show a similar pattern even after 3 days of excluding the cues. This observation took my attention and led to the next question of my thesis to check for the evolution of different learning abilities in M and F males in terms of courtship learning. Courtship conditioning or learning is a phenomenon where males are trained to avoid courting non-receptive mated females by holding males with these females for a short duration. From the last two decades, courtship conditioning or learning has been demonstrated as a well-established phenomenon in *Drosophila*. It is considered to play a significant role in determining the mating success of males, hence affecting their fitness and depicting the adaptive significance of courtship learning. Sexual conflict can drive the evolution of exaggerated male courtship displays as these exaggerated traits can be useful for gaining mates. However, the role of sexual conflict in the evolution of courtship learning (which also may have adaptive significance) has not been explored yet. Therefore, I asked if males evolving under differential levels of sexual conflict can evolve differential levels of courtship learning to discriminate between receptive and unreceptive females. It has been shown that M regime males have evolved to have greater courtship activity than F regime males. So, we hypothesised that as M regime males court more and courtship provides an opportunity to gather information about the mating target, M males may have evolved to retain and use this information to increase their reproductive success. To test this, we subjected both M and F regime males to a conditioning phase by holding them with unreceptive females. Then we tested if these males learned to avoid the unreceptive females and direct courtship towards the receptive female. M regime males were better at recognising the receptive female in both the cases when they were conditioned or not conditioned relative

to F regime males. It indicates that M males have evolved to become inherently better at distinguishing between receptive and unreceptive females. However, I did not find a clear difference in courtship learning ability between the M and F regime males. Courtship latency was also recorded to check if courtship suppressed as a result of conditioning in males as suggested by various experimental pieces of evidence. Also, as in the test phase, there was one receptive female with unreceptive females, mating latency was noted to see which males are faster at recognising the receptive female between M and F regime males. Both mating latency and courtship latency were not affected by the selection but responded to the treatments as conditioned males showed lower mating latency and higher courtship latency relative to males that were not subjected to conditioning. This study highlights the importance of sexual conflict and selection in driving the innate abilities to recognise the appropriate mating partners, which is an important parameter that can influence the reproductive success of males.

Sexual selection theory indicates that reproductive traits and immune traits are correlated, and experimental evolutionary evidence from many taxa documented that phenotypic tradeoffs exist between reproductive effort and immune response. There is ample evidence of experimental evolutionary studies that have shown that reproductive output and performance deteriorate as a result of activation of the immune response in response to a pathogen. Sexual conflict theory suggests that males should invest in traits that increase the reproductive fitness of males, even at a cost to survival. Thus, it is possible that sexual selection and conflict can affect the relationship between reproductive performance and immune function. Therefore, in this study, I investigated the effect of heat-killed bacterial challenge on the reproductive performance of males and females evolving under differential levels of sexual conflict. In the experimental setup, there were three kinds of treatments- I (infected with heat-killed bacteria), U (control, uninfected), S (pricked with a needle dipped in 10mM $MgSO_4$ slurry,

as a control for the pricking). For males, assayed reproductive traits include mating latency, copulation duration, and competitive fertilization success. In females, fecundity was quantified as a measure of reproductive fitness. Males subjected to infected treatment from each M and F regime took longer to initiate the mating. However, there was no significant difference between M and F males for mating latency. In M males, for copulation duration and competitive fertilization success, I observed no significant difference among infected, sham and unhandled treatments. In F regime males, males from infected treatment were found to decrease their reproductive performance (CD and P1) compared with sham and unhandled treatments. In females from both M and F regimes, a significant decrease in the number of laid eggs was observed following exposure to heat-killed bacteria. However, the rate of decline in fecundity was almost similar in both kinds of females. Therefore, my study reports that sexual conflict affected the type of relationship between the reproductive effort and immune function in males evolving under altered operational sex ratios.

Sexual conflict over mating frequency is ubiquitous in nature due to the divergent evolutionary interests of males and females. In the M regime, females are exposed to high mating rates every generation, whereas F regime females are not much exposed to multiple mating throughout their evolutionary history. The increased mating rate has been shown to have detrimental effects on females at physiological and morphological levels. Various adaptive explanations have been suggested for polyandry's existence despite the harmful effects of this phenomenon on females. These explanations suggest that costs of polyandry can be compensated either through direct benefits to the females by increasing her own fitness (i.e., survival and/or reproductive success) or through indirect benefits, where a female is benefited indirectly through the elevated fitness of her offspring. In the past decade, diverse theoretical and empirical studies have provided evidence supporting the adaptive value of multi-male mating based on indirect benefits. These indirect benefits can also be mediated

through maternal effects wherein the environment experienced by the mother can affect offspring fitness. Given these maternal effects can lead to an increase in offspring fitness; it is still not well documented if these maternal effects can evolve in response to selection imposed on the mothers. Therefore, through this study, I tested if such transgenerational effects can involve in selected regimes where females are exposed to different kinds of sexual environments every generation. To investigate this, I subjected both M and F regime females to multiple mating for four days and tested the effects on their offspring fitness. M and F regime females were also subjected to single mating, which served as the controls. There was no difference between the reproductive fitness of daughters (measured as fecundity) and sons (measured as ML, CD and P1) sired by both multiply mated and singly mated M mothers. Conversely, daughters sired by multiply mated F regime mothers suffered a decline in their fecundity comparative to daughters sired by singly mated F mothers. Also, I observed no effect of multiple mating by F mothers on their sons' fitness. Hence my results from this study did not support the idea of adaptiveness of multiple mating measured in terms of indirect benefits. However, I show that differential evolutionary trajectories of mothers driven by sexual selection and conflict can lead to the evolution of maternal effects. To my knowledge, this is the 1st empirical evidence to show the evolution of such transgenerational effects in response to sexual selection and conflict.

To sum up, in this thesis, I highlight some extended questions of sexual selection and conflict theories on the evolution of reproductive traits. The main findings of my thesis are that evolution under different levels of sexual selection and conflict leads to the evolution of-

1) divergent responses in the reproductive behaviour of males when subjected to variable socio-sexual environments.
2) inherent ability in males to assess the mating status of the females.

3) different kinds of association between reproduction and immunity in males but not in females.
4) trans-generational maternal effects.

Chapter -1

Introduction

It's not the strongest of the species nor the most intelligent who survives but the most responsive to change.

- Charles Darwin, *On the origin of species*

In the book *On The origin of species* (1859), Darwin proposed the concept of one of the most influential evolutionary forces, 'Sexual selection'. However, the mechanism of sexual selection was developed further by Darwin in his book *The Descent of Man and Selection in Relation to Sex* in 1871. Definition of Sexual Selection proposed by Darwin is (Darwin 1871a) (Part I, pp 254–255): -

"*We are, however, here concerned only with that kind of selection, which I have called sexual selection. This depends on the advantage which certain individuals have over other individuals of the same sex and species, in exclusive relation to reproduction."*

Theory of sexual selection proposed by Darwin was not entirely accepted by evolutionary biologists and faced considerable confusion and disagreement at that time due to some of its controversial ideas. For instance, Darwin suggested that sexual selection is not a subcategory of natural selection, and this process occurs through differences in mating success. On the other hand, natural selection arises due to differences in all other fitness components. Also, apparently, Darwin considered natural selection to be stronger than sexual selection, which may not always be the case as suggested by a few evidences (Kirkpatrick 1982; Svensson et al. 2006). Further, while defining sexual selection, Darwin focussed only on the pre-copulatory mechanisms of sexual selection and paid no attention to post-copulatory scenario of sexual selection. However, the conceptual framework for the idea of sexual selection was laid by Darwin and his contribution in explaining the mechanism for the evolution of unusual

traits having no fitness-related benefits such as bright colouration, costly courtship, horns and antlers etc. cannot be neglected.

Models of sexual selection

In the early 20th century, Darwin's intuitive concept of sexual selection was further developed by Ronald Fisher in 1930 in his book '*The Genetical Theory of Natural Selection'*. Fisher suggested that evolution of certain male traits was driven by a female preference for such traits. The evolution of these male sexually selected traits resulted in the evolution of the female preference itself, thereby generating a positive feedback loop and deriving the co-evolution of exaggerated male traits and increased female preference for such exaggeration. As long as trait value and mating/reproductive success are positively correlated, this process is obviously advantageous to males, whereas the usefulness of this process is not so apparent for females. Therefore, Fisher proposed that by preferring such traits, females gained an advantage by producing 'sexy' sons that will probably inherit their father's trait(s). Similarly, the female progeny also inherits their mother's preference trait(s), thereby attaining the 'Run-away'. In this way, female preference can evolve just because of its genetic correlation with genes for sexually selected male traits. However, this Run away model also has limitations in the form of viability selection, where its antagonistic effects on viability prevent immense exaggeration of traits. Contrary to this, the theory of direct benefits was proposed, which states that if there was an immediate fitness benefit to the females (such as easy access to resources in choosing males that defend larger territories, or provide nuptial gifts etc.) for showing preference, only then female preference could evolve..This theory proposed that 'sexy son' is not enough to completely explain the evolution of female preference and male traits (Kirkpatrick 1985).

There is ample evolutionary evidence to underpin both sets of theories and suggest that they're discordant to each other. Although, the mechanism of this runaway process remained

debated regarding the origin and spread of trait exaggeration in males and preference in females. This runaway model was further explained by Dawkins (1986) through the classic example of long-tailed widowbird. While males have long tails that are selected for by female preference, female choices in tail length are quite more profound with females being attracted to tails longer than those that exists naturally. Females that selected long-tailed males tend to have mothers that preferred long-tailed fathers. Consequently, they have both sets of genes in their bodies, i.e., genes for long tails and for choosing long tails become interlinked. Therefore, the choice for long tails and the tail length itself may become correlated, tending to increase together. The more tails length increases, the more long tails are preferred. Any minor preliminary imbalance between preferences and tails may set forth an explosion in tail lengths. After this, in the early ’90s an alternate coevolutionary model known as ‘sensory exploitation’ (Basolo 1990; Ryan 1990; Basolo and Endler 1995; Sinervo and Basolo 1996) was proposed, leading to similar kind of results. Sinervo and Basolo (1996) observed female choice to have evolved before the preferred male trait in a swordtail fish species (Xyphosura). It was hypothesized that female preference (or sensory bias for certain male features) is likely to be a by-product of viability selection on the sensory system of the females. Hence, males at this point can be believed to be selected for exploiting the pre-existing sensory bias in females in terms of inducing affinity in females for mating.

Rise of a new paradigm- Sexually antagonistic coevolution

In 1948, Bateman A.J. showed that there is greater variability in the reproductive success of males than the females in fruit flies. It can lead to different fitness optima of two sexes. Therefore, all else being equal, the sexes should evolve towards their own optimal phenotype. However, in traits such as mating rate, parental care etc. which requires participation of both the sexes, the males and females cannot evolve independently towards their optimum. Further, if the traits show a positive genetic correlation between the sexes, independent

evolution of the two sexes towards their optimum is again prevented. Thus, conflicts can arise between males and females over the level of expression of a trait. Such conflict is referred to as intersexual conflict. Parker (1979) formalized and re-expanded the theory of sexual selection and demonstrated how competition among males (i.e., intra-sexual selection) could cause adverse effects to their mates, leading to intersexual conflict. Later on, experimental work by some evolutionary biologists, such as those of Rice (1984, 1986, 1987, 1996), Arnqvist (1989a, b, 1992) and Arnqvist and Rowe (1995), established a new paradigm in our comprehension of male-female co-evolution – sexually antagonistic co-evolution (Rice 2000 ; Arnqvist and Rowe 2002). Rice and Holland contributed a thought-provoking model of 'chase-away selection' (Holland and Rice 1998) to this new paradigm. Chase-away model proposed that preexisting sensory bias of females imposes a selection pressure on males to evolve an initial, rudimentary display trait that increases their attractiveness to females, for instance, a moderately longer tail. These highly attractive males then induce females to mate beyond their optimum. This, in turn, imposes a selection pressure on females to counter evolve 'resistance' rather than 'preference' for the male display trait. Now, males are under higher selection pressure to evolve a more extreme display trait to overcome the resistance evolved by females. It leads to a cycle of adaptation and counter adaptation between the males and females, resulting in a sexually antagonistic coevolution process. According to this process, in contrast to 'preference', females are selected to evolve 'resistance' to male persistence. This female resistance is now well investigated in model organisms such as bed bugs, water strider and fruit flies (Arnqvist and Rowe 1995; Crudgington and Siva-Jothy 2000; Rice et al. 2005, 2006; Reinhardt et al. 2007; Nandy et al. 2013). The attraction of this hypothesis was that it involved no complex presumptions and assumed that any male feature that increases the frequency of mating in females is selected for in males. It can be any simple behavioural coercion or some cryptic form of modification (for example, sensory bias

suggested by Basolo 1990). The suboptimal mating rate is predicted to lower female fitness, thereby selecting females to evolve resistance to male stimulation and/or coercion. This is believed to initiate intersexual antagonistic co-evolution (Rice 2000). It is also known as interlocus sexual conflict as this process involves different loci from males and females, which are often sex-limited or biased in their expression. There is another form of sexual conflict that involves the same allele being expressed in both sexes. In this case, a genomic tug of war develops between the males and females as each sex have a different optimal trait value. This kind of conflict is intralocus sexual conflict (Rice and Chippindale 2001; Prasad et al. 2007).

Taken together, sexual conflict can occur either by direct male-female conflicting interactions (Inter-locus conflict) or through the non-sex-limited expression of the traits which have antagonistic fitness effects in males and females (Intra-locus conflict). It is clear from the above discussion that sexual conflict can shape the fitness of both the sexes by affecting life-history traits and reproductive traits. However, the evolutionary conclusion of the interlocus conflict, that how it can shape the strategies associated with reproductive behaviour of the males and females, is debated. Therefore, in this thesis, I tried to investigate the role of altered levels of sexual conflict in the evolution of reproductive traits of males and females in response to variable environments.

Sexual conflict and evolution of reproductive traits

Interlocus conflict is widespread and has been documented in several species across different taxa (Rice 2000; Arnqvist and Rowe 2005). In *D. melanogaster*, males' reproductive success primarily depends upon their ability to mate with the available females and their sperm competitive abilities. (Chapman et al. 1995; Rice et al. 2006). As a consequence, a range of relevant traits has evolved in males that may result in decline in their mates' fitness (Civetta and Clark 2000; Rice 2000). These traits can have effects at the precopulatory level (i.e.,

behavioural), such as persistent courtship (Fowler and Partridge 1989; Partridge and Fowler 1990; Kuijper et al. 2006), or at the post-copulatory level, stimulated by the physiological effects of seminal fluid (Chapman et al. 1995; Wolfner 1997). All these harmful effects of males on female fitness are jointly referred to as Mate-harm (Jiang et al., 2011). As explained above, since mate-harm decreases female fitness, natural selection is expected to act on females to evolve resistance to mate-harm. In *D. melanogaster*, evolving resistance to mate harm involves frequent mate-rejection, extrusion of genitalia and some unknown physiologic mechanisms (Cook and Connolly 1973; Rice et al. 2006; Wolfner 2009). Hence, interlocus sexual conflict can be stated as the co-evolution between mate-harm and resistance to mate-harm. Various studies have tried to investigate the process of evolution under both intralocus sexual conflict and interlocus sexual conflict using laboratory experimental evolution. For instance, Nandy (2013) showed an empirical evidence for the evolution of mate harm and mate harm resistance in *D. melanogaster* populations selected for different levels of interlocus sexual conflict for 40-50 generations (these are the same populations which I used. I will describe these populations later in chapter 2). To manipulate the levels of interlocus sexual conflict, Nandy altered the adult operational sex ratios in these populations. Changing the operational sex ratio in the population thereby changes the level of inter male competition and male-female encounter rate in the population. As a result of this skewed sex ratio change in the intensity of interlocus sexual conflict is expected. Male-biased sex ratio regime is expected to display high conflict, female-biased sex ratio –minimized conflict and equal sex ratio is the basic ancestral environment. Wigby and Chapman (2004) exposed replicate populations of *D. melanogaster* to such skewed operational sex ratio for 33 generations. The only significant response to selection was found in the female-biased regime in the form of significantly lessened resistance to mate-harm in females (Wigby and Chapman 2004). Although, after 60-67 generations of selection, males from the female-biased regime were

found to have evolved slower ejaculate depletion pattern (Linklater et al. 2007a). Also, one of the recent studies, changed the operational sex ratio in flour beetles – *Tribolium castaneum* for 20 generations and found that females from the female biased regime were sensitive to multi-male mating resulting in a decrease in fitness (mate-harm), whereas for females from the male-biased regime no such effect was observed (Michalczyk et al. 2011). Although they did not go for direct quantification of harming ability of males, they found that the competitive fitness of the males from the male-biased regime was significantly higher than those from the female-biased regime (Michalczyk et al. 2011). This study provided evidence for divergence between the populations experiencing male-biased and female-biased operational sex ratio.

Other than altering the operational sex ratios, levels of sexual selection or conflict can be manipulated by other means also. As an instance, a native method of 'male limited evolution' was used by Rice (1996, 1998) wherein a set of *D. melanogaster* populations, only males were allowed to evolve against a static female phenotype as females were not allowed to counter adapt. This led to the evolution of increased mate harming ability in males associated with an increase in male reproductive fitness along with sperm competitive ability (Rice 1996; Holland and Rice 1998). Conversely, using the same experimental approach, Jiang et al. (2011) reported no evidence of the evolution of mate-harm and sperm competitive ability in males, however, an increase in male fitness was observed as compared to the controls (Prasad et al. 2007). To note here, both of these studies used the same approach but gained contrasting results. For this observation, it was suggested that long term laboratory cultivation has the potential to dilute the additive genetic variation with respect to the pertinent traits through strong directional selection on the male fitness components. This observation was supported by 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) did not find any detectable response to the subjected selection pressure and speculated two possibilities for this observation– (a) lack of substantial additive genetic variation in the proper direction and (b) complex encounters between males, their mates and their competitors with respect to the outcome of sperm competition (Bjork et al. 2007). .

Another method for investigating sexual conflict using laboratory experimental evHowever, across a number of studies using different model systems, sperm competitive ability and related male reproductive traits were observed to be responsive to experimental relaxation of sexual selection through enforced monogamy (Hosken and Ward 2001a; Pitnick et al. 2001; Simmons and García-González 2008; Firman and Simmons 2012)olution was to subject populations to evolve populations under experimentally enforced monogamous (relaxing sexual conflict) and polyandrous/polygynous/promiscuous (retaining sexual conflict) mating system. *D. melanogaster* populations with imposed monogamy evolved males with less noxious seminal fluid and females with increased susceptibility to mate-harm (Holland and Rice 1999). In the same way, Dung fly (*Sypsis cynapsea*) males enforced to evolve under monogamous regimes for 29 generations were observed to be relatively benign while females from the same regime were found to be more susceptible to the mate-harm (Martin and Hosken 2003). Similar results were reported by Crudington et al. (2005, 2009) of the evolution of male and female-specific traits using *D.pseudoobscura*.

To summarise, studies using laboratory experimental system are have proved to be very useful in understanding the process of sexual selection. This research sector is very complex, and still, a lot needs to be done to completely interpret the consequences of this evolutionary process. Through this thesis, using laboratory experimental evolution, I tried to uncover some facts about the role of sexual selection and conflict in the evolution of reproductive traits.

Testing the extended predictions of 'Chase-away model of Sexual selection.'

Chase away model suggests that sexually antagonistic coevolution can lead to the evolution of a different suite of traits in both males and females under selection. Studies have shown the evolution of various life-history traits and reproductive traits in response to sexually antagonistic co-evolution between the sexes. However, the predictions of the chase away model extend far beyond what has been investigated till now, and there can be various indirect consequences of sexually antagonistic evolution. Therefore, in this thesis, I attempted to investigate the role of sexual selection in the evolution of such aspects of reproductive traits that have received relatively less attention. For example, phenotypic plasticity is an important phenomenon that can increase the fitness of organisms in the face of challenges concerning various ecological contexts.

Given that acquiring plastic responses in a trait can lead to an increase in an organisms' reproductive fitness, I predicted that such plastic responses could be expected to be favoured by sexual selection. Studies have reported that males can show plastic responses in their reproductive behaviour according to the perceived intensity of sperm competition through the rivals in their vicinity (Parker 1990c, a, 1993a; Parker et al. 1997a; Wedell et al. 2002a; Engqvist and Reinhold 2005a). In *D. melanogaster,* exposure to rivals leads to an increase in mating duration in males (Bretman et al. 2009b; Nandy and Prasad 2011; Dore et al. 2020), followed by a subsequent rise in paternity success (Bretman et al. 2009b; Nandy and Prasad 2011) and an adjustment in ejaculate composition as a response to conspecific potential rival males (Wigby et al. 2009; Garbaczewska et al. 2013; Moatt et al. 2014). Here, I asked if plastic responses in reproductive traits of males evolving under differential levels of sexual selection can themselves evolve. Males evolving under different levels of sexual selection experience different intensities of sperm competition every generation. Therefore, I predicted

that plastic responses might have evolved in response to the different intensities of intrasexual interactions in these regimes.

Courtship is an important component of male reproductive behaviour that determines the reproductive fitness of males. Chase away model suggests that conflict over mating rate may continually select males for the evolution of male courtship behaviour. Therefore, , any adaptation that increases the chances of successful courtship and therefore increased reproductive fitness should evolve. *Drosophila* males possess an innate behaviour of courting potential mates in their surroundings. But social experience with the conspecific or heterospecific individuals can alter their behavioural courtship strategies (Dukas 2005; Villella and Hall 2008). Courtship conditioning is an established paradigm in *D. melanogaster* males that enables males to refine their courtship behaviour according to the receptivity of females (Siegel and Hall 1979; Gailey et al. 1982). In populations with regimes under differential levels of sexual selection, males are exposed to females of different receptivity. Males evolving under higher sexual selection will have greater exposure to unreceptive females in their maintenance regime. Hence, males under higher sexual conflict regime evolve in a complex environment in terms of females with different receptivities than the males evolving under lower sexual selection regime. Therefore, I predicted that males under higher sexual selection might have evolved to assess the receptivity of females more accurately and faster than the males under lower sexual selection.

Next, the models of sexual selection predict the evolution of male display traits that increase the probability of mating for males. In other words, traits that increase the chances of matings or increased investment into reproduction will be favoured by sexual selection. In the populations used here, males from male-biased regimes (M) have evolved to invest more in reproduction compared to males from female-biased regime. Now, theory suggests that reproductive activity and immune system activation are costly to maintain for organisms.

Hence, reproductive effort and immune response activation are expected to trade off against each other. Various studies have shown the phenotypic trade-off relationship between the reproductive effort and immune response (Sheldon and Verhulst 1996; Zuk 1996; Moret and Schmid-Hempel 2000; Ahmed et al. 2002; Lazzaro et al. 2008; McKean et al. 2008; Bashir-Tanoli and Tinsley 2014; Howick and Lazzaro 2014). However, the relationship between the reproductive traits and immune traits in response to sexual selection is not well documented. Here, I tried to explore the effect of immune response activation on the reproductive performance of males and females evolving under different levels of sexual conflict. Theory suggests that males should invest more in reproduction to increase their fitness. Therefore, I asked if males selected for increased reproductive performance when forced to invest in immune system function, continue to channel their resources in reproductive activities or modify their reproductive behaviour following heat-killed bacterial challenge.

Finally, theory suggests that intersexual conflict over mating rate will continuously select males to evolve more exaggerated traits to make females mate beyond their optimum. The increased mating rate has been shown to affect females adversely in most of the species in nature (Arnqvist and Nilsson 2000; Crudgington and Siva-Jothy 2000; Gavrilets et al. 2001; Blanckenhorn et al. 2002). Recent studies suggest that an increased mating rate can have transgenerational effects with multiply mated females producing progeny with lower fitness (Brommer et al. 2012; Gasparini et al. 2012; Dowling et al. 2014). Such maternal effects are though not consistent. A few studies have shown multiply mated females producing progeny with higher fitness (Priest et al. 2008). In either case, maternal effects based on maternal mating status can have transgenerational fitness consequences. Therefore, given the suitable variation, such maternal effects can principally evolve. For example, if multiple mating has negative effects on progeny fitness than in populations where females are subjected to multiple mating every generation, we can expect the evolution of mechanisms which will

ameliorate such transgenerational fitness costs. In populations used by me, females experience differential rates of mating every generation. Females evolving with the males under higher sexual selection are subjected to elevated mating rate and hence higher intensity of mate harm every generation than the females evolving with the males under lower sexual selection. Therefore, as these females under different levels of sexual conflict are exposed to the variable sexual environment every generation, it is possible that maternal effects have evolved in these regimes. I tested this prediction by assaying the reproductive fitness of offspring produced by females evolving under different levels of sexual conflict.

I used *Drosophila melanogaster* as the model organism to address the above questions using experimental evolution. In the next chapter, I discuss experimental evolution and details of my particular experimental system.

Chapter -2

Experimental system

Slow though the process of selection may be, if feeble man can do much by his powers of artificial selection, I can see no limit to the amount of change, to the beauty and infinite complexity of the co-adaptations between all organic beings, one with another and with their physical conditions of life, which may be effected in the long course of time by nature's power of selection.

— Charles Darwin 1871

Since Darwin presented his seminal work on evolution by natural selection that operates on the diversity and selects the fittest individual (Darwin's 1859), there has been growing interest in understanding evolution and its elementary principles. Darwin's theory suggested that evolution is a too gradual process to be studied directly and that it could be possible only by indirect comparisons of living species and/or fossils. Such kind of comparative investigations are still of great significance today, specifically when looking at long time scales and when mediated by modern techniques and fossils to analyse and compare, for example, sequences of DNA or proteins (Gubry-Rangin et al. 2015; Gallone et al. 2016; Hug et al. 2016).

Experimental evolution and laboratory selection

Experimental evolution involves the review of evolutionary processes emerging in experimental populations in response to environmental conditions subjected by the researcher. This research paradigm is progressively used to study adaptation, determine evolutionary parameters, and analyse diverse evolutionary hypotheses. Experimental evolution may be visualised in the laboratory as individuals/populations adapt according to new environmental conditions through natural selection. Adaptation can originate in experimental evolution in 2 different ways – One method is via an individual organism acquiring a novel beneficial mutation. Alternatively, a change in allele frequency in standing genetic variation already present in a population of organisms can lead to adaptation (Long et al., 2015). Apart from this, other evolutionary forces such as genetic drift and gene flow can

also contribute into experimental evolutionary studies (Kawecki et al., 2012). Experimental evolution optimizes biological systems through adaptation; the adapted systems with their mutations present unique perturbed states of the systems that generate new and often unexpected output.

Experimental evolution can be largely described as research in which populations are examined across multiple generations under defined and reproducible environment (Garland and Rose 2009). A crucial component of experimental evolution is laboratory selection, where a researcher can set up isolated populations with sufficient additive genetic variation and can monitor the evolution of such populations under a well-defined force of selection. Laboratory selection studies have contributed assets of biological insights. They facilitate particular conditions to be imposed on replicate populations, allowing evolutionary modifications to occur in a confined or relatively unconstrained environment. As a result, we now have an ample amount of data demonstrating evolutionary responses in bacteria, yeast, nematodes, *Drosophila* and mice (Hoffmann and Parsons 1993; Travisano et al. 1995; Garland Jr et al. 2002; Bennett 2003; Kliman et al. 2003; Riehle et al. 2003). Further, in the past two decades, many evolutionary reviews have noticed some of the drawbacks concerned with selection studies (Gibbs 1999; Harshman and Hoffmann 2000). Examples include unintended selection criteria, inappropriate controls, variability in replicate populations, and disparate results under identical selection regimes, to name a few.

However, it can be argued that the speculation that laboratory selection leads to complexity and diversity of responses interprets an important envision into the processes and mechanisms of evolution (Garland Jr 2003; Folk and Bradley 2005). We are moving out of a period in which we felt that strict control of the environment during selection could result in canalized responses. In fact, the variability of responses under conditions where the

researchers have done their utmost to control the conditions of selection tells a great deal about the power and influence of chance and genetic diversity on evolutionary outcomes. Despite all these drawbacks, laboratory selection techniques offers an advantage of repeatability, control and statistical power over the selection environment and sometimes a direct cause-effect relationship comparative to, for example, field studies and phylogenetic studies. While this paradigm is more suitable for micro-evolutionary studies, it is helpful for macro-evolutionary investigations as well. Despite its great success, experimental evolution has some limitations also. For example, phenomena like the 'Cheshire Cat syndrome' (Rose et al. 1996) or the effects of inbreeding have the potential to affect the experimental evolution studies. Specific outcomes to selection can sometimes be exclusive to a particular study population among many, even within the same species and significant effects of gene × environment interaction can conceal inferences especially if assay conditions vary from population maintenance conditions. Thus, though experimental evolution is a potent system to study evolutionary processes, an experimenter should be attentive to the possible pitfalls.

Drosophila melanogaster

(Phylum: Arthropoda, Class: Insecta, Order: Diptera, Family: Drosophilidae)

Drosophila melanogaster is a fly species generally known as the fruit fly or vinegar fly. Charles W. Woodworth's proposed the use of this species as a model organism. *D. melanogaster* is widely used for biological research in genetics, physiology, microbial pathogenesis, and life history evolution. *Drosophila melanogaster* is a holometabolous insect with a life cycle consisting of four distinct phases: egg, larva, pupa and adult. Populations of *Drosophila melanogaster* used for this study were maintained at 25^{o}C temperature, 60-90% relative humidity (which is the standard laboratory environment for the populations I have used), and their life cycle follows the same route described below

every generation. After female oviposit, those eggs hatch into larva and go through three instars. After 4-5 days (and upon reaching a "critical mass"), the late third instar larva withdraws feeding, moves out of the food and develops into pupa, secreting a chitinous covering at an appropriate location (usually on the walls or cotton plugs of rearing vials). A further 4-5 days later, the adult fly makes an appearance from the pupal shell – a process commonly known as 'eclosion'. In particular, the adult males usually take ~8 hours to become reproductively mature and start the mating activity. The significance of this time window lies in the fact that in this time period, flies can be separated and held in single-sex groups as virgins. After attaining sexual maturity, mated females can start laying eggs. Female fecundity primarily relies upon their ability to gain access to some protein source, e.g., yeast, which is commonly used as the protein source in laboratory cultures (Prasad and Joshi 2003; Stewart et al. 2005; Nandy et al. 2012). Females can mate with multiple males and store sperms in seminal receptacle and spermatheca for short-term storage, respectively, and use them to fertilize their eggs (Lefevre Jr and Jonsson 1962; Manier et al. 2010).

Baseline and selected laboratory-adapted population: LH and derivatives

For all the experiments presented in this thesis, baseline population LH, LHst and their derivatives are used. LH population was established by Lawrence Harshman with 400 wild caught *Drosophila melanogaster* females from central California, USA in 1991 (Rice and Chippindale 2001). A 14-day discrete generation cycle is followed for the population at standard laboratory condition, i.e. 25^0C, 60-80% relative humidity and 12/12 light/day cycle, on standard cornmeal-yeast-molasses fly food (Table 2.1). The flies of this population are maintained in 60 vials with the dimensions as 25mm diameter × 90mm height. Larvae are cultured at the density of ~150 eggs per 8-10ml of food in these vials every generation. On the 12th day after egg collection, when all flies have eclosed, adult flies across different vials are mixed and reshuffled (16 males and 16 females per vial) under light CO_2 anaesthesia in

fresh vials containing food supplemented with a fixed amount of live yeast. These vials, referred to as ‘adult competition vials’, are then kept undisturbed for two days. On the 14th day flies in these vials are moved to ‘oviposition vials’ containing 8-10ml of fresh food, where the females oviposit for 18 hours. After this, all adults are disposed of, and the egg density is controlled at a density of 150 eggs in each of the vials by scooping the extra eggs with the help of a spatula. These vials now become the rearing vials for the next generation.

LHst was derived from LH base population by inserting the autosomal- recessive trait scarlet-eye coloured marker (‘st’) by repeated back cross (Prasad et al. 2007). The maintenance of LHst is similar to that of LH, except that the population's (LHst) effective size is made up of 30 vials. Also, LHst is regularly backcrossed with LH to maintain the genetic uniformity across the two populations. Considering that these populations have been maintained under constant laboratory conditions for more than 500 generations now, they are assumed to have adapted to the laboratory regime. The 2-week discrete generation cycle and 18-hour oviposition window aid us to define time windows that are important in determining the fitness of the individuals in the population. For instance, selection acts most strongly on adult traits during the three-day period when flies are present in competition vials and oviposition vials. During this period, females compete with each other to get access to the limited amount of live yeast and resist male coercion, while males are exposed to intense pre-and post-copulatory sexual selection. 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.

The LH experimental system has been used to study sexual selection for almost three decades now – ranging from male limited evolution (Rice 1996; Holland and Rice 1999 ; Prasad et al. 2007), the study of diversity in sexually antagonistic and sexually selected traits, and, ontogenetic conflict using the ingenious hemiclonal analysis (Chippindale et al. 2001; Friberg

2006a; Pischedda and Chippindale 2006), intralocus sexual conflict (Pischedda and Chippindale 2006), and, most recently sexually antagonistic coevolution through alteration of sex ratio . Hence the LH system is a remarkable model system to study intersexual conflict.

Selected populations: Derived through manipulation of operational sex ratio

All of the Experiments described in this thesis are carried out on a set of *D. melanogaster* populations subjected to experimental evolution under varying operational sex ratio (ratio of males to females available for reproduction). Bodhisatta Nandy established the lines in 2009 from the LHst base population. The complete information about this population's derivation and evolutionary ancestry has been described in detail in his doctoral thesis (Nandy et al. 2013b). Hence, I will be presenting a brief introduction about this population which is as follows

Maintenance:

This population consists of nine subpopulations – three sex ratio regimes, each with three replicates– male-biased (M1-3), equal sex ratio (C1-3) and female-biased (F1-3) regime. Populations with the same numerical subscript (replicate number) share a common ancestry and are more closely linked to each other compared to populations with dissimilar subscripts, i.e., M1 is more closely linked to C1 and F1 than to M2 or M3. Further, subpopulations carrying the same numerical subscript are handled together during stock maintenance and experimentation. Therefore, M1, C1 and F1 subpopulations together constitute the 'Block-1', M2, F2 and C2 make 'Block-2', and similarly, M3, F3 and C3 constitute 'Block-3'. Hence replicates bearing similar numerical subscripts are treated as statistical 'blocks' in the analysis. All aspects of the maintenance regime were kept the same across the regimes except the adult operational sex ratio. The populations are maintained in 2-week discrete generation cycles (Figure 2.4), under 25^{o}C temperature, 60-80% relative humidity and 12-hours light / 12-hours dark. Eggs are cultured in food vials containing cornmeal molasses agar food at a

density of 140-160 eggs / 8-10ml of food in 8-dram vials. It takes about ten days for the flies to complete the preadult development. On the 9th -10th-day post egg collection, adult flies start emerging out of pupae. The adult flies are collected as very young (< 6hours post eclosion) virgins and kept in single-sex vials at a density of 8 flies per vial. After 12 days, post egg collection, the sexes are combined in food vials provided with a fixed amount (0.467mg/female) of live yeast smeared on the food. The sex ratio in these adult competition vials was 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. After this, the rest of the maintenance is the same as that of the LH population. The effective population size was maintained at around 450 for each of these nine populations. The effective population size in these populations was counted to be considerably high even by conservative standards (with M having the lowest Ne of 361.67) such that any possibility of the effect of drift was ruled out (Nandy et al. 2013).

Excluding parental effects: Standardisation

While conducting any experimental assay, it is necessary to neutralize the parental effects across different regimes to differentiate genetic changes due to selection from non-genetic parental effects. This was achieved by imposing all the populations to pass through one generation of maintenance under standard conditions, a process known as standardization (modified from 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 develop till the adult stage for 12 days under the standard laboratory environment instead of a virgin collection.

On 12th-day post egg collection, flies of all the populations were shifted to one fly cage

(19cm×14cm×24cm) with a petri plate (90mm diameter) poured with traditional food with a paste of live yeast (with water) smeared on it. A fresh food plate was kept in the fly cage for egg collection, and a window of 6 hours was provided for oviposition. After females laid eggs on the surface of the food, accurately 150 eggs were transferred into fresh food vials containing 8-10 ml of food to generate experimental flies.

Table 2.1 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	22.5

Recipe of Fly media: All the ingredients (except 6-8) according to given quantities are mixed boiled in water to make a thick suspension. The suspension is cooled a little 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 media is then poured in vials and used after it has cooled down to 25▫C.

What has been done till now on these populations?

Earlier experimental studies on these populations have contributed considerably to answering important questions regarding sexual selection and conflict. Some of the important results from these populations are summarised below-

1) Competitive fitness of males from the male-biased and female-biased regime has evolved to diverge significantly. Males from the M regime have evolved to be more competitive, compared to F regime males. Also, Females from the M regime had evolved to be less susceptible to mate harm, thus having evolved higher fitness than females from the other two regimes (Nandy et al. 2013b).

2) Sperm competitive ability (sperm defense - P1, offence - P2) have been found to evolve with M regime males having increased P1 relative to that of males of the F regime. Increase in P1 was correlated with increased copulation duration, possibly suggesting greater ejaculate investment by these males (Nandy et al. 2013a). However, this was not reflected in terms of evolutionary changes in either testis and accessory gland size or their depletion patterns (Chechi et al. 2017).

3) Higher locomotor activity and courtship frequency have evolved in M regime males which came at the expense of increased rates of ageing and a decrease in mean lifespan. It was the first empirical evidence, clearly documenting the evolution of male reproductive traits under intersexual conflict and the related life-history trade-offs (Nandy et al. 2013b).

4) Increased mate harm resistance has evolved in M regime females quantified in terms of both longevity and fitness, which again traded off with an increased rate of ageing. Further, F regime females were found to have higher reproductive success upon single mating (minimum mate harm sustained for progeny production) and significantly greater average lifespan in the absence of reproductive activity - suggesting a trade-off between life-history traits (such as longevity and fecundity) and resistance-related traits (Nandy et al. 2014).

5) Increased levels of sexually antagonistic coevolution resulted in the evolution of early stages of reproductive isolation at (a) premating and (b) postmating prezygotic stages in these populations of *Drosophila melanogaster.* When presented with the conspecific sympatric and allopatric males, in populations under high sexual conflict (M), females showed assortative mating, indicating the evolution of reproductive isolation. However, no such trend was displayed in F regime females. This study showed that sexual selection could serve as a mediator in the process of speciation (Syed et al. 2017).

6) Evolution of senescence in components of competitive fitness, secondary sexual traits and correlated mate harming ability in male *D. melanogaster* (Syed et al. 2017).

7) No evidence of trade–off between reproduction and immune components was observed between the males evolving under differential levels of sexual selection (Syed et al.2020).

8) Evolution of female influence on male competitive fertilization in response to the sexual conflict (Syed et al. 2018).

This divergence of M and F populations in terms of reproductive and life-history traits can be attributed to the varying pressure of sexual selection and sexual conflict acting on them. Given this substantial information, I have tried to answer some relevant consequences of sexual selection pressure on the evolution of reproductive traits.

Chapter -3

Evolution of divergent responses in males evolving under altered operational sex ratios in response to different socio-sexual environment

Introduction

Sexual selection is a major evolutionary force that favours any trait that can enhance the reproductive success of an individual as one sex competes for access to mating opportunities (Darwin 1871; Andersson 1994)**.** In most of the sexually propagating species, females can store sperm from multiple males simultaneously. As a result, the opportunity for post-copulatory sexual selection (PSS) arises in the female reproductive tract (FRT) through competition for fertilization between sperm from different males (sperm competition) as well as sperm choice mediated by the FRT (Parker 1970; Thornhill 1983; Eberhard 1996). Therefore, sperm competition may play a vital role in determining the reproductive fitness of males. Empirical evidence suggest that in promiscuous species where fertilization occurs internally, sperm competition can influence male morphology, anatomy and physiology (Møller 1998; Parker 1998; Simmons 2001). However, the production of reproductive resources has been reported to be very costly in males (Cordts and Partridge 1996; Martin and Hosken 2004; Perez-Staples and Aluja 2006). Hence, males are expected to evolve reproductive strategies in response to varying levels of sperm competition. There are several pieces of evidences of males acquiring phenotypic plasticity in their reproductive traits in response to perceived sperm competition. Males of diverse insect species, including crickets, butterflies and beetles, display plastic behavioural strategies by altering their morphology, physiology or behaviour in response to rival males (Parker et al. 1996; Wedell and Cook 1999; Friberg 2006; Sakaluk and Müller 2008; Bretman et al. 2009). Additionally, males can differentially allocate their reproductive resources between pre and post-copulatory mating behaviour in accordance with the perceived intensity of competitive reproductive cues (Wedell et al. 2002; Bretman et al. 2009, 2011a).

One way to alter the level of sperm competition is by changing the number of rival males around a focal male before or during the mating. Studies have reported that modifying the

number of rival males competing for the fertilization of eggs can change both the risk and intensity parameters of sperm competition (Parker 1970; Parker 1982, 1990b; Parker et al. 1996; Wedell et al. 2002; Bretman et al. 2011a).

According to sperm competition theory (Parker 1970), when the intensity of male-male competition is high, selection favours the males with increased reproductive investment (defined as the amount of ejaculate spent by the males) (Parker 1970; Gage 1991; Wedell et al. 2002). Theory also predicts that mating expenditure of males should increase with increasing risk (the possibility of a female being mated or mating again) and decrease with increasing intensity (the number of ejaculates struggling to gain paternity over a given set of eggs) of sperm competition (Parker 1990b,, 1993; Parker et al. 1997; Wedell et al. 2002; Engqvist and Reinhold 2005). An empirical investigation of the "intensity model" shows different patterns of plastic ejaculate investment in different systems. For example, in crickets (Simmons and Kvarnemo 1997; Schaus and Sakaluk 2001), red jungle fowl (Pizzari et al. 2003), and several species of fish (Candolin and Reynolds 2002; Pilastro et al. 2002; Smith et al. 2003), males show a reduction in ejaculate expenditure in response to the increasing intensity of sperm competition, while males of field crickets *Gryllodessigillatus* and *Acheta domesticus* (Gage and Barnard 1996) and butterfly *Pieris rapae* (Wedell and Cook 1999) showed an increase in the amount of ejaculate released in response to increasing sperm competition intensity. In *D. melanogaster,* exposure to rivals resulted in an increase in copulation duration in males (Bretman et al. 2009; Nandy and Prasad 2011; Dore et al. 2021), followed by a subsequent rise in paternity success (Bretman et al. 2009; Nandy and Prasad 2011) and modification in ejaculate composition as a response to conspecific potential rival males (Wigby et al. 2009; Garbaczewska et al. 2013; Moatt et al. 2014). Most of the studies mentioned above are single-generation phenotypic manipulation studies that have investigated reproductive trait expression under altered socio-sexual environments. However,

the plastic responses exhibited by males are expected to be adaptive in nature (Bretman et al. 2011a) and therefore, there are several areas of reproductive plasticity yet to be fully explored. Given that plasticity is adaptive and can contribute to fitness of males, it can be assumed that plasticity can itself evolve in response to different selection pressures. In support of this idea, there are several theoretical models as well as empirical studies which show that phenotypic plasticity can evolve (Via and Lande 1985; Stearns and Koella 1986; Van Tienderen 1991; Gomulkiewicz and Kirkpatrick 1992; Houston and McNamara 1992; Kawecki and Stearns 1993a; Sasaki and de Jong 1999).

Evolution of plasticity in reproductive traits in males has been explored by a few studies using *D. melanogaster* populations evolving under different operational sex ratios. For example, *D. melanogaster* males evolving under male-biased (MB) and female-biased (FB) regimes expressed higher mating duration as well as competitive reproductive success following exposure to rivals in comparison to wild-type males (not exposed to rivals). However, this increase was considerably larger (though this trend was statistically non-significant) in the case of MB males (Edward et al. 2010). Further, a recent study by Dore et al. (2021) reported that in *D. melanogaster*, an encounter with the rivals resulted in longer mating latencies, increased mating duration and decreased courtship delivery in males evolving under male-biased (MB) sex ratio compared to males evolving under equal sex ratio (EQ) or female-biased (FB) sex ratios. This study also reported no significant effect of changing the evolutionary history of rival males on the reproductive behaviour of focal males (Dore et al. 2021). Thus, there exists considerable evidence of the evolution of plastic reproductive strategies in males in response to the presence versus absence of rivals in populations evolving under different operational sex ratios.

Though the studies mentioned above (Kawecki and Stearns 1993b; Hosken and Ward 2001; Edward et al. 2010; Dore et al. 2021) have documented the role of sexual selection in the

evolution of reproductive plasticity, the role of the density of competitors is still underexplored in the evolution of reproductive plastic responses. According to sperm competition theory, the different densities of competitors can signal varying intensity of sperm competition (Parker 1998). In addition to the numbers, the quality of the competitors can also potentially affect sperm competition. Studies suggest that the intensity of male-male competition is affected not only by the presence of rivals but also by examining the condition of those rivals (Parker 1982; Parker et al. 1996, 1997; Wedell et al. 2002; Bretman et al. 2011b). Given that males can assess the condition of rivals, the quality/ identity of competitors might also be an essential factor that can influence the perceived level of sperm competition.

Therefore, in the present study using experimental evolution, I specifically investigated the modification of reproductive investment by focal males in response to the attributes that have gained comparatively less attention- social group size/density of rivals and quality/evolutionary history of rivals. For this, I used replicate laboratory populations of *D. melanogaster* evolving under Male-biased (M) (1 female: 3 male) or Female-biased (F) (3 female: 1 male) adult sex ratios for more than 150 generations. Earlier studies on these populations have shown that M males have evolved increased sperm competitive ability, courtship frequency, locomotor activity and mate harming ability than F regime males (Nandy et al. 2013a,b). Furthermore, the return on ejaculate expenditure in terms of fitness is expected to be low in the case of M males due to intense male-male competition. Therefore, for increasing their reproductive success, M males might have evolved to become more strategic in terms of reproductive/ejaculate investment than F males.

Further, it has been documented that to adjust according to fluctuating environmental conditions; an individual must be able to receive, process, learn, and/or memorize multiple sensory cue components accurately (Bretman et al. 2011b; Mohorianu et al. 2017; Rouse et

al. 2018). Since it has been suggested that the intensity of sexual selection can significantly affect the evolution of cognitive abilities in males (Hollis and Kawecki 2014), it is logical to expect that M and F regime males might be processing the information (received from competitive cues) in different ways. Also, it has been observed in *D. melanogaster* that with an increase in the time lag between the removal of competitive cues and reproductive trait expression, the influence of experienced cues (such as increased mating duration) fades off (Rouse and Bretman 2016). Thus, I addressed the question of whether there are evolved differences between M and F males in their 'memory retention' of competitive cues experienced in early life.

To put it briefly, in this study, I used *D. melanogaster* populations evolving under different levels of sexual selection and conflict to address the following questions:

1) How does changing the number of rivals affect reproductive traits - mating latency, copulation duration and sperm defense ability in populations evolving under altered operational sex ratios?

2) Does changing the evolutionary history of rivals affect the pattern of plastic reproductive investment?

3) How long-lasting are these plastic responses?

Materials and methods

Standardization and generation of experimental flies

For generating experimental flies, one generation of standardization of populations (modified from Rose 1984) was followed to eliminate any potential non-genetic parental effects between the two regimes. In the process of standardization, populations were maintained in the same conditions as that of the ancestral LHst population, i.e. flies were not collected as virgins and were maintained under an equal sex ratio.

Egg collection was done on the same day for test males from M and F regimes, for females from LHst and the second male for the sperm defense assay from LH population. Eggs were cultured at a moderate density of 150 eggs/vial in 8–10 mL of cornmeal-molasses-yeast food for each of the populations. On the 9-10th day after egg collection, males from M and F regimes were collected as virgins by isolating them within 6 hours of eclosion, using light CO_2 anaesthesia. Soon after this, focal males from both regimes were subjected to experimental treatments. LHst females were also collected as virgins and kept in single-sex vials at a density of 8 females/vial. LH males were collected on the 12th day as adults from the culture vials using CO_2 anaesthesia.

Experimental design:

Experiment (1) Effect of different number of competitors on reproductive investment

Mating latency and Copulation duration assay – After virgin collection, males from each of the M and F populations were subjected randomly to one of three experimental treatments- a) 1 male/vial (b) 8 males/vial (c) 32 males/vial. For each of the treatments, 30 individuals were sampled. Males were held undisturbed in these treatments for 2 days. After two days, one conditioned male from every vial within each treatment was aspirated out randomly, 2 hours before the mating trials and held singly in individual vials. Individual virgin LHst females were presented to these males in the vials. Each pair was observed for mating latency and copulation duration. In all the experiments, flies that did not copulate within 2 hours were discarded.

Sperm defense ability assay (Proportion of progeny sired by the 1st male or P1**) -** After mating ended, males from each of the M and F selection regimes were removed using light CO_2 anaesthesia and females were held back. Following this, after about 1 hour, LHst females previously mated with M and F males were combined with control red-eyed LH

males in new vials for remating. These vials were kept undisturbed for the next two days. I did not observe the second mating of females with LH (red-eyed) males; therefore, my measure of P1 includes a composite measure of actual P1, i.e. direct sperm competition between the males in the FRT, as well as the first male's ability to prevent the female from re-mating. After two days, LH males were cleared from the vials and females were transferred individually to test tubes (12 mm × 75 mm) containing cornmeal-molasses-yeast food. Females were provided with a time period of 18 hours to oviposit in these test tubes, after which these females were discarded. After 12 days, when all the flies had eclosed in these test tubes, progenies within each test tube were scored for eye colour to determine paternity.

Experiment (2) Consequences of changing the identity of rival males

Virgin males from M and F regimes were combined randomly with the ancestral LH males according to the following three treatments

(a) 1 M/F male/vial

(b) 1 M/F male + 7 LH males = 8 males/vial

(c) 1 M/F male + 31 LH males = 32 males/vial

Males were retained in these treatments for two days. After two days, conditioned M/F males were pulled out from these treatments using light CO_2 anaesthesia before the mating assay. Then these conditioned M/F males were paired with common virgin LHst female for recording mating latency and copulation duration. Flies that did not mate within 2 hours of observation were disposed of. Sperm defense ability (P1) was quantified in the same way as described previously in experiment (1).

Experiment (3) Effect of increase in the time lag between removal of competitive cues and assessment of reproductive traits

To investigate this, I collected two different sets of males as virgins from each of the M and F

populations. After a conditioning period of 2 days in the three kinds of treatments (with rivals from their own respective regimes) described above, these two sets of males were assayed for reproductive investment at two different periods. After two days, males from both sets were pulled out from the treatments and were kept singly in individual vials.

Following this, these males from each of the M and F regimes were assayed at two different time periods:

(a) after 3 days of the removal of cues

(b) after 5 days of the removal of cues.

Reproductive assays were executed in the same way as described above (experiment 1) for assaying mating latency, copulation duration and sperm defense ability (P1).

Statistical analyses

All data analyses were executed in the R version 3.5.2, using the "lme4" (Bates et al. 2014) and "lmerTest" (Kuznetsova et al. 2017) packages. Data for each reproductive trait (mating latency, copulation duration and sperm defense ability) were analyzed using a mixed model analysis of variance (ANOVA). Treatment and selection were used as fixed factors, crossed with blocks as the random factor. We did not find any significant effect of block and its interactions with other fixed factors. The effect of block and its interactions with the selection and treatment has been summarized in Tables S1, S2, S3 and S4. Post-hoc Tukey's HSD tests were performed for multiple comparisons using the R package "emmeans" (Lenth et al. 2020). Linear mixed-effects models used for mating latency, copulation duration and sperm defense ability (P1) were the same for all the experiments and are summarized below-

1. Mating latency - ML ~ Selection + Treatment + (1 | Block) + (1 | Block:Selection) + (1|Block:Selection:Treatment) + (1 | Block:Treatment) + Selection:Treatment.

2. Copulation duration - CD ~ Selection + Treatment + (1 | Block) + (1 | Block:Selection) + (1|Block:Selection:Treatment) + (1 | Block:Treatment) + Selection:Treatment.
3. Sperm defense ability (P1) - P1 ~ Selection + Treatment + (1 | Block) + (1 | Block:Selection) + (1 | Block:Selection:Treatment) + (1 | Block:Treatment) + Selection:Treatment

Results

1) **Selection affected the reproductive investment pattern in response to different number of rivals**

No effect of selection or treatment on mating latency (ML) was observed (Figure.1a, Table. 1). However, a significant effect of selection, treatment and selection × treatment interaction for copulation duration (CD) (Figure.1b, Table.1) and sperm defense ability (P1) was observed (Figure.1c, Table.1). Overall, M males showed significantly higher CD and P1 than F males. However, M and F males showed different responses to the varying number of competitors in terms of their CD and P1. M males were found to initially show a significant increase in CD when the number of competitors changed from single male to eight males (Figure.1b, Table.1). However, in the case of the 32 males/vial treatment, M males showed a decline in their CD compared to the 8 males/vial treatment. For M males, the CD of the 32 males/vial treatment was not significantly different from that of the single male treatment. While the P1 of M males showed a trend similar to that of CD, we did not find significant differences between 1, 8 and 32 males/vial treatment (Figure.1c, Table.1). On the other hand, F males displayed a significant increase in CD from single male treatment to the 8 males/vial treatment and 8 male to 32 males/vial treatment. In the case of P1, a significant increase was found in the case of the 8 males/vial treatment to the 32 males/vial treatment for F males. In contrast, the single male/vial and the 8 males/vial treatments were not significantly different from each other.

2) **On changing the identity of rival males, the effect of selection gets diluted.**

In this experiment, neither treatment nor selection affected mating latency (Figure.2a, Table.2). For CD, we observed a significant treatment effect, but there was no effect of selection and no interaction between selection × treatment was found (Figure.2b, Table.2). Males from both of the M and F regimes showed a continuous increase in their CD with an increase in the number of competitors from 1 to 32. We observed a significant effect of selection and treatment for P1, but there was no interaction between selection × treatment (Figure.2c, Table.2). In M males, the P1 of single male/vial and 8 males/vial treatments were not significantly different from each other but the P1 of single male treatment was significantly lower than the 32 males/vial treatment. F males also followed a similar trend with no significant increase in P1 from single to 8 males/vial treatment. But, the P1 of the single male treatment was significantly lower than that of the 32 males/vial treatment.

3) Response to the number of rivals and the effect of selection changes with the time lag between the cue detection and resulting response

For this experiment, the ML, CD and P1 of males were assayed at different time points from the elimination of competitive cues, i.e., after 3 and 5 days of isolation. The summary of results is presented in Table 3 and Table 4. Mating latency (ML) was not observed to be significantly different across selection regime or treatment in any of the assay periods (i.e., 3 days or 5 days after treatment) {Figure.3 (T3), Table.3, Figure.4 (T5), and Table.4 respectively)}.

In the case of males assayed 3 days after the removal of competitive cues, for the CD we found a significant effect of treatment and a significant interaction between selection and treatment {Figure.4 (T3), Table.3}. In M males, we observed a significant increase in CD from single male/vial to 8 males/vial treatment and then a decrease afterwards in the 32 males/vial treatment. For P1, we found no effect of treatment, but a significant effect of

selection and a significant interaction between selection × treatment was observed {Figure.5 (T3), Table.3}. Altogether, M males showed significantly higher P1 compared to F males. We observed no significant difference for P1 between any of the treatments for M males. On the other side, the P1 of F males initially showed a significant increase from single male treatment to 8 males/vial treatment with no further increase in the case of 32 males/vial treatment.

In the case of males that were assayed 5 days after the removal of competitive cues, we found no effect of selection, treatment, selection × treatment interaction in ML or CD {Figure.3 (T5), Figure.4 (T5), and Table.4}. However, in the case of P1, we observed a significant effect of selection {Figure.5 (T5), Table.4}. Overall, P1 of M males was found to be significantly higher than F males. Within the M regime, we found no significant difference for P1 across treatments. Similarly, in the F regime also, P1 was not significantly different across different treatments.

Table.1 Main and interactive effects of selection and treatment on mating latency, copulation duration, and sperm defense ability (P1) immediately after removal of competitive cues in males of M and F regimes (experiment 1). Significant effects ($p < 0.05$) are marked with '*' and are shown in bold.

III Analysis of Variance Table with Satterthwaite's method							
Trait	**Effect**	**Sum Sq**	**Mean Sq**	**Num DF**	**Den DF**	**F value**	**Pr(>F)**
Mating latency (ML)	Selection	3.23	3.23	1	347.04	2.48	0.116
	Treatment	2.83	1.40	2	3.950	1.080	0.422
	Selection× Treatment	0.26	0.14	2	347.01	0.100	0.904
Copulation duration (CD)	Selection	14.50	14.50	1	2.00	2.974	0.226
	Treatment	292.3	146.2	2	7.95	29.96	**<0.0001*** **
	Selection× Treatment	89.98	44.94	2	7.95	9.21	**<0.0085** **
Sperm defense ability (P1)	Selection	1.098	1.098	1	351	22.3	**<0.0001*** **
	Treatment	0.215	0.107	2	351	2.11	0.114
	Selection× Treatment	0.411	0.207	2	353	4.98	**0.016***

Table.2 Main and interactive effects of selection and treatment on mating latency, copulation duration, and sperm defense ability (P1) of M and F regime males after changing identity of rival males (experiment 2). Significant effects ($p < 0.05$) are marked with '*' and are shown in bold.

Type III Analysis of Variance Table with Satterthwaite's method							
Trait	**Effect**	**Sum Sq**	**Mean Sq**	**Num DF**	**Den DF**	**F value**	**Pr(>F)**
Mating Latency (ML)	Selection	0.72	0.72	1	6.01	0.47	0.52
	Treatment	1.04	0.52	2	5.99	0.34	0.72
	Selection× Treatment	1.01	0.50	2	6.01	0.33	0.73
Copulation duration (CD)	Selection	57.85	57.85	1	1.99	8.61	0.99
	Treatment	795.1	397.5	2	3.99	59.20	**0.001****
	Selection× Treatment	12.04	6.02	2	3.99	0.89	0.47
Sperm defense ability (P1)	Selection	0.25	0.25	1	532.00	6.65	**0.010****
	Treatment	1.10	0.55	2	532.01	14.50	**<0.0001*****
	Selection× Treatment	0.004	0.002	2	532.01	0.064	0.94

Table.3 Main and interactive effects of selection and treatment on mating latency, copulation duration, and sperm defense ability (P1) of M and F regime males 3 days after the removal of competitive cues (experiment 3). Significant effects ($p < 0.05$) are marked with '*' and are shown in bold.

	Type III Analysis of Variance Table with Satterthwaite's method						
Trait	**Effect**	**Sum Sq**	**Mean Sq**	**Num DF**	**Den DF**	**F value**	**Pr(>F)**
Mating Latency (ML)	Selection	3.23	3.23	1	347.04	2.48	0.116
	Treatment	2.83	1.40	2	3.950	1.080	0.422
	Selection× Treatment	0.26	0.14	2	347.01	0.100	0.904
Copulation duration (CD)	Selection	14.50	14.50	1	2.00	2.974	0.226
	Treatment	292.3	146.2	2	7.95	29.96	**<0.0001*** **
	Selection× Treatment	89.98	44.94	2	7.95	9.21	**0.0085** **
Sperm defense ability (P1)	Selection	1.098	1.098	1	351	22.3	**<0.0001*** **
	Treatment	0.215	0.107	2	351	2.11	0.114
	Selection× Treatment	0.411	0.207	2	353	4.98	**0.016***

Table.4 Main and interactive effects of selection and treatment on mating latency, copulation duration, and sperm defense ability (P1) of males, 5 days after the removal of competitive cues (experiment 3). Significant effects ($p < 0.05$) are marked with '*' and are shown in bold.

	Type III Analysis of Variance Table with Satterthwaite's method						
Trait	**Effect**	**Sum Sq**	**Mean Sq**	**Num DF**	**Den DF**	**F value**	**Pr(>F)**
Mating Latency (ML)	Selection	0.233	0.233	1	2.00	0.154	0.732
	Treatment	0.302	0.151	2	4.01	0.099	0.907
	Selection× Treatment	2.278	1.139	2	345	0.750	0.473
Copulation duration (CD)	Selection	0.403	0.403	1	2.00	0.054	0.838
	Treatment	5.801	2.900	2	349	0.386	0.680
	Selection× Treatment	1.177	0.588	2	349	0.078	0.924
Sperm defense ability (P1**)**	Selection	1.214	1.214	1	3.99	26.13	**<0.007****
	Treatment	0.006	0.003	2	8.03	0.073	0.929
	Selection× Treatment	0.001	0.0009	2	8.03	0.021	0.979

Table.S1 Effect of random blocks and their interactions with the fixed factors on mating latency, copulation duration, and sperm defense ability (P1) after immediate removal of competitive cues in males of the M and F regimes (experiment 1).

Trait	Effect	Npar	logLik	AIC	LRT	DF	Pr(>Chisq)
Mating Latency (ML)	Block×Selection	10	-566.0	1152.1	0.77457	1	0.3788
	Block×Treatment	10	-565.6	1151.3	0.00000	1	1.0000
	Block×Selection×Treatment	10	-565.6	1151.3	0.00000	1	0.9996
	Block	11	-565.7	1151.4	0.05286	1	0.8182
Copulation duration (CD)	Block×Selection	10	-796.3	1612.8	0.0000	1	1.000
	Block×Treatment	10	-796.3	1612.8	0.0000	1	1.000
	Block×Selection×Treatment	10	-797.7	1615.6	2.7796	1	0.095
	Block	10	-796.8	1613.8	1.0006	1	0.317
Sperm defense ability (P1)	Block×Selection	10	44.29	-68.58	0.000	1	1.000
	Block×Treatment	10	44.03	-68.58	0.000	1	1.000
	Block×Selection×Treatment	10	44.29	-68.58	0.000	1	0.999
	Block	10	44.29	-68.06	0.514	1	0.473

Table.S2 Effect of random blocks and their interactions with the fixed factors on mating latency, copulation duration, and sperm defense ability (P1) after changing the identity of rival males on the M and F males (experiment 2).

Trait	Effect	Npar	LogLik	AIC	LRT	DF	Pr(>Chisq)
Mating Latency (ML)	Block×Selection	10	-892.2	1804.4	0.000	1	1.0000
	Block×Treatment	10	-892.8	1805.6	1.205	1	0.2723
	Block×Selection × Treatment	10	-892.2	1805.4	0.988	1	0.3200
	Block	11	-892.2	1804.4	0.000	1	1.0000
Copulation duration (CD)	Block×Selection	10	-1285.6	2591.2	0.014	1	0.9063
	Block×Treatment	10	-1285.7	2591.4	0.126	1	0.7221
	Block×Selection × Treatment	10	-1285.9	2591.8	0.529	1	0.4669
	Block	10	-1286.5	2592.9	1.701	1	0.1922
Sperm defense ability (P1)	Block×Selection	10	100.62	-181.2	0.000	1	1.0000
	Block×Treatment	10	100.62	-181.2	0.000	1	1.0000
	Block×Selection × Treatment	10	100.62	-181.2	0.000	1	1.0000
	Block	10	100.03	-180.0	0.000	1	0.2795

Table.S3 Effect of random blocks and their interactions with the fixed factors on mating latency, copulation duration, and sperm defense ability (P1) after 3 days of the removal of competitive cues (experiment 3).

Trait	Effect	Npar	logLik	AIC	LRT	DF	Pr(>Chisq)
Mating Latency (ML)	Block×Selection	10	-563.63	1147.3	0.000	1	0.999
	Block×Treatment	10	-563.74	1147.5	0.228	1	0.633
	Block×Selection×Treatment	10	-563.63	1147.3	0.000	1	0.999
	Block	11	-565.53	1151.0	3.797	1	0.051
Copulation Duration (CD)	Block×Selection	10	-803.37	1626.8	3.621	1	0.057
	Block×Treatment	10	-801.56	1623.1	0.000	1	0.999
	Block×Selection×Treatment	10	-802.29	1624.6	1.449	1	0.228
	Block	10	-802.17	1624.3	1.207	1	0.271
Sperm Defense Ability (P1)	Block×Selection	10	16.754	-13.508	0.000	1	1.000
	Block×Treatment	10	16.754	-13.508	0.000	1	1.000
	Block×Selection×Treatment	10	16.754	-13.508	0.000	1	1.000
	Block	10	16.051	-12.102	1.406	1	0.236

Table.S4 Effect of random blocks and their interactions with the fixed factors on mating latency, copulation duration, and sperm defense ability (P1) after 5 days of the removal of competitive cues (experiment 3).

Trait	Effect	Npar	logLik	AIC	LRT	DF	Pr(>Chisq)
Mating Latency (ML)	Block×Selection	10	-593.84	1207.7	2.102	1	0.147
	Block×Treatment	10	-593.32	1206.6	1.057	1	0.303
	Block×Selection×Treatment	10	-592.79	1205.6	0.000	1	1.000
	Block	11	-593.59	1207.2	1.613	1	0.204
Copulation Duration (CD)	Block×Selection	10	-871.69	1763.4	0.993	1	0.318
	Block×Treatment	10	-871.19	1762.4	0.000	1	1.000
	Block×Selection×Treatment	10	-871.19	1762.4	0.00	1	1.000
	Block	10	-871.23	1762.5	0.081	1	0.775
Sperm Defense Ability (P1)	Block×Selection	10	26.858	-33.71	0.002	1	0.959
	Block×Treatment	10	26.859	-33.71	0.000	1	0.998
	Block×Selection×Treatment	10	26.721	-33.44	0.275	1	0.599
	Block	10	26.859	-33.71	0.000	1	1.000

Figure.1 Effect of different densities of competitors experienced in early life on (a) mating latency, (b) copulation duration, and (c) sperm defense ability (P1) of M and F regime males. Dark grey boxes represent males from F populations, whereas light grey boxes indicate males from M populations (experiment 1). Boxplots show median and interquartile range. Whiskers indicate variability outside the upper and lower quartiles. Dots represent outliers.

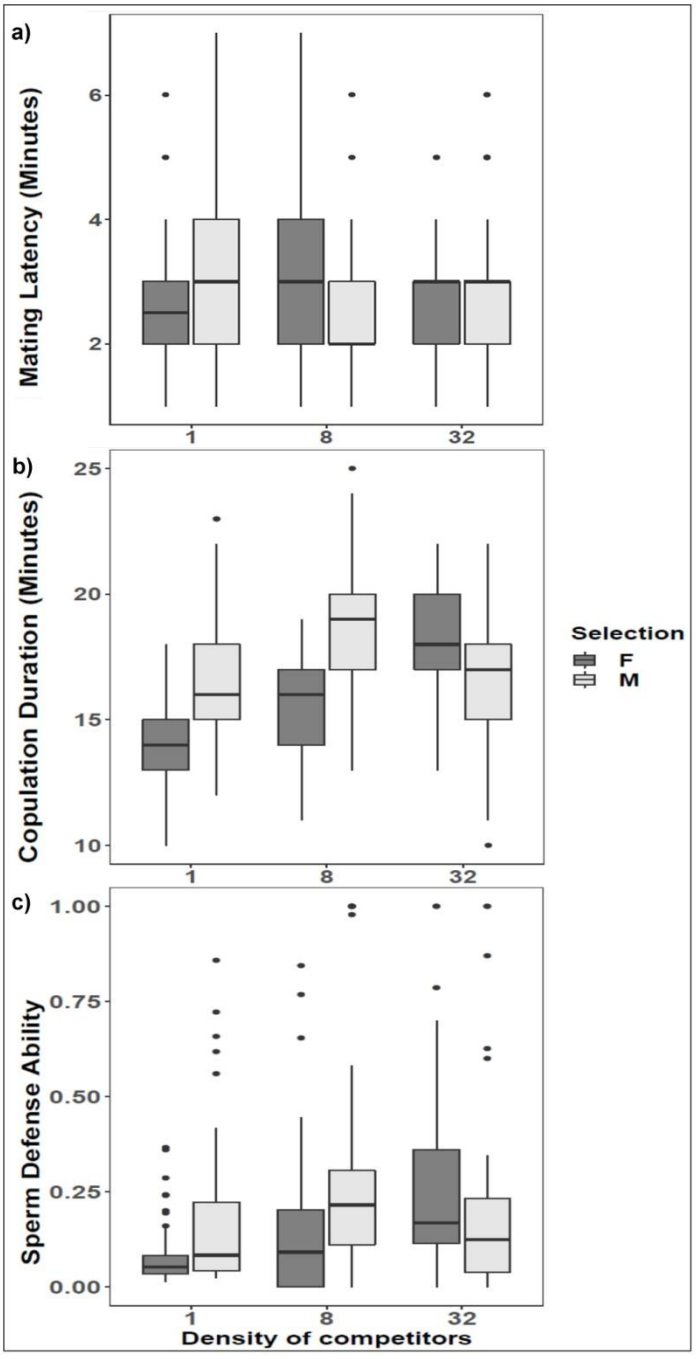


Figure.2 Effect of different densities of competitors from ancestral (LH) population experienced in early life on (a) mating latency (b) copulation duration (c) sperm defense ability (P1). Dark grey box indicates males from the F population, whereas the light grey box indicates males from the M population (experiment 2). Boxplots indicate median and interquartile range. Whiskers indicate variability outside the upper and lower quartiles. Dots represent outliers.

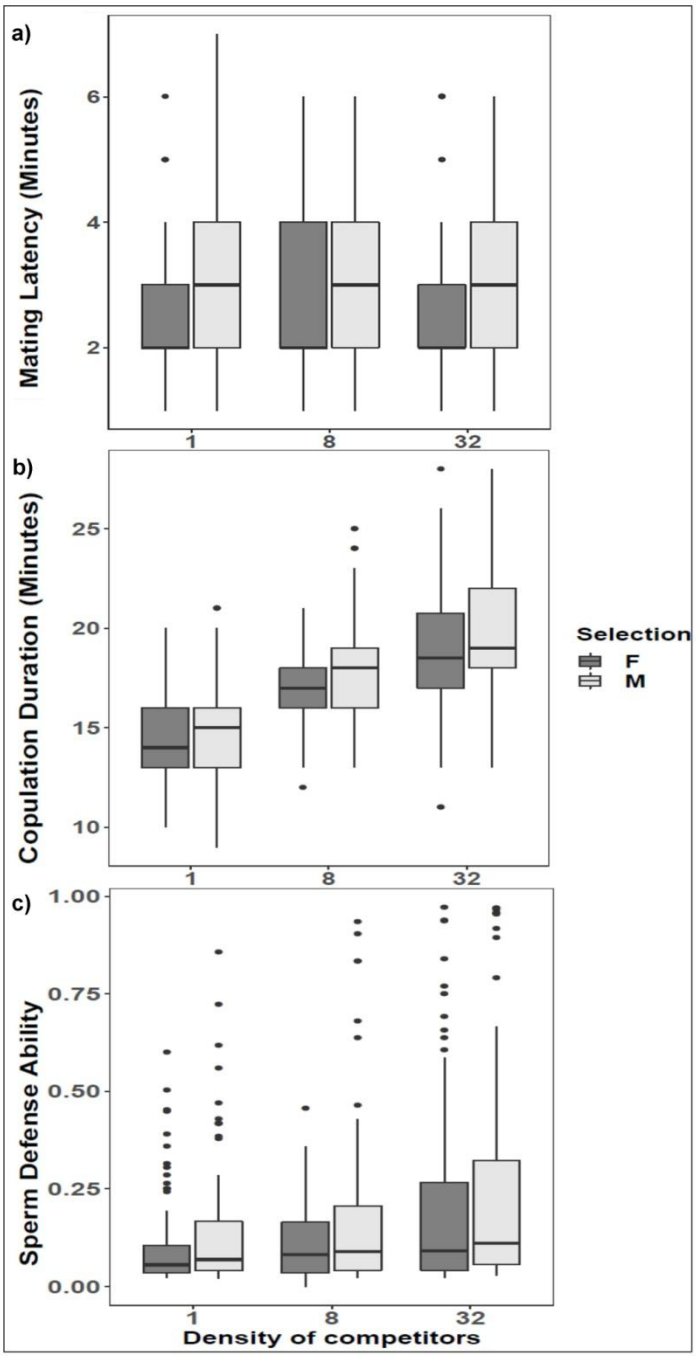


Figure.3 Mating latency of M (light grey boxes) and F (dark grey boxes) regime males in response to different numbers of rivals from their own regimes respectively (i.e., M males housed with M males and F males housed with F males) at different time periods from the exposure of competitive cues. Panel on the left represents mating latency after immediate exposure to rivals (at t=T0), the middle panel shows the mating latency after 3 days of the removal of competitive cues (at t= T3), and the panel on the right represents mating latency after 5 days (at t=T5) of removal of competitive cues.

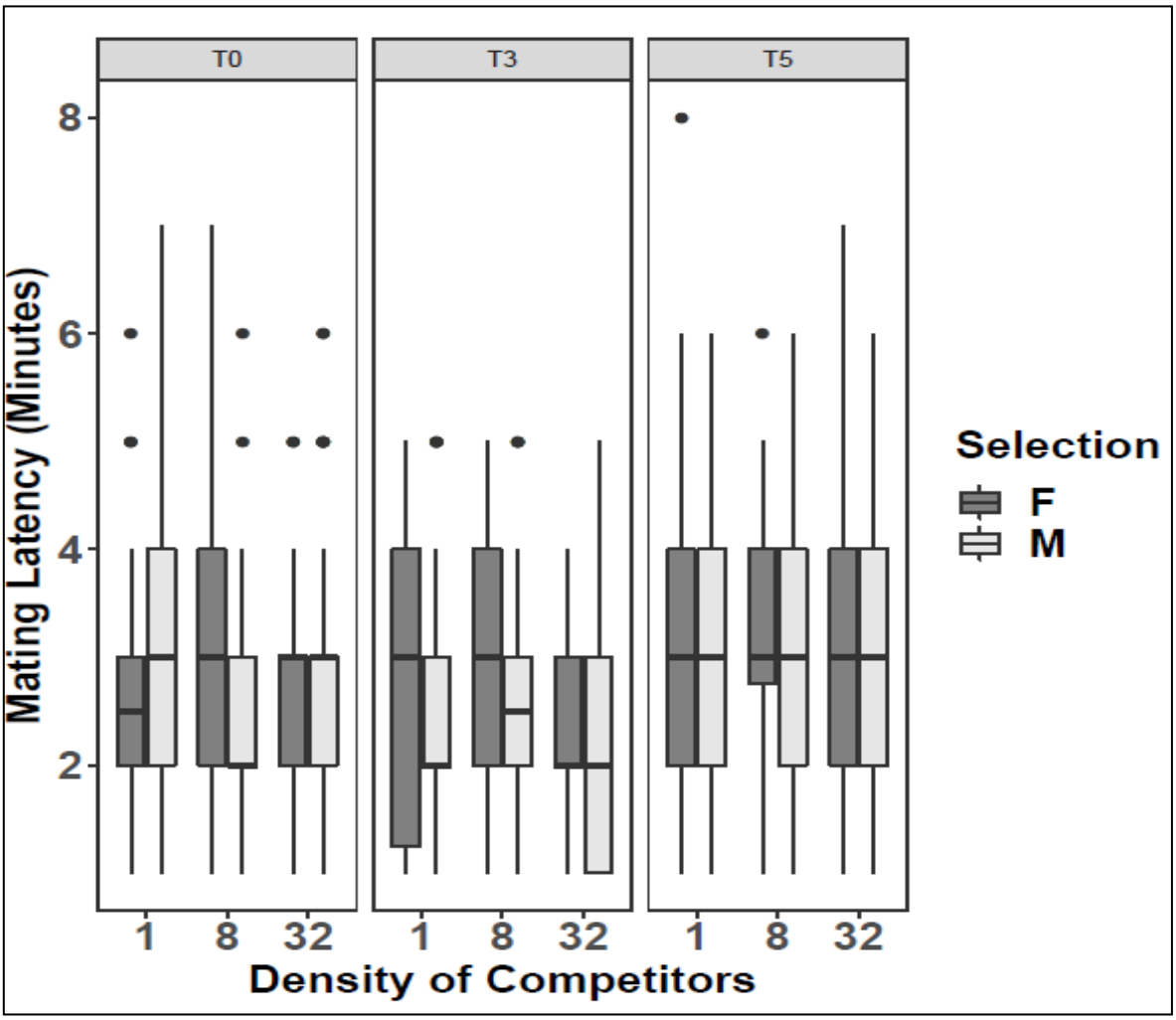


Figure.4 Copulation duration of M (light grey boxes) and F (dark grey boxes) regime males in response to different numbers of rivals from their own regimes respectively (i.e., M males housed with M males and F males housed with F males) at different time periods from the exposure of competitive cues. Panel on the left represents copulation duration after immediate exposure to rivals (at t=T0), the middle panel shows the copulation duration after 3 days of the removal of competitive cues (at t= T3), and the panel on the right represents copulation duration after 5 days (at t=T5) of removal of competitive cues.

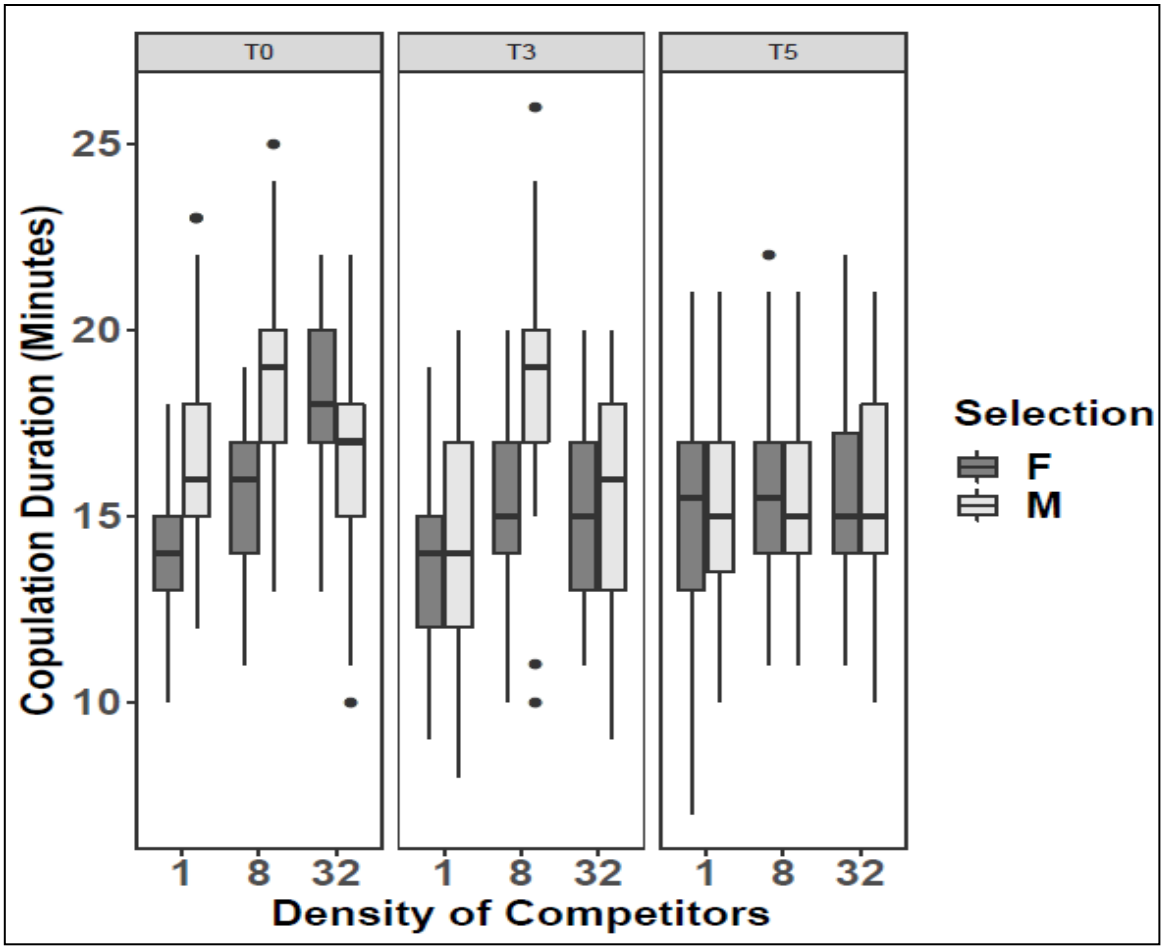
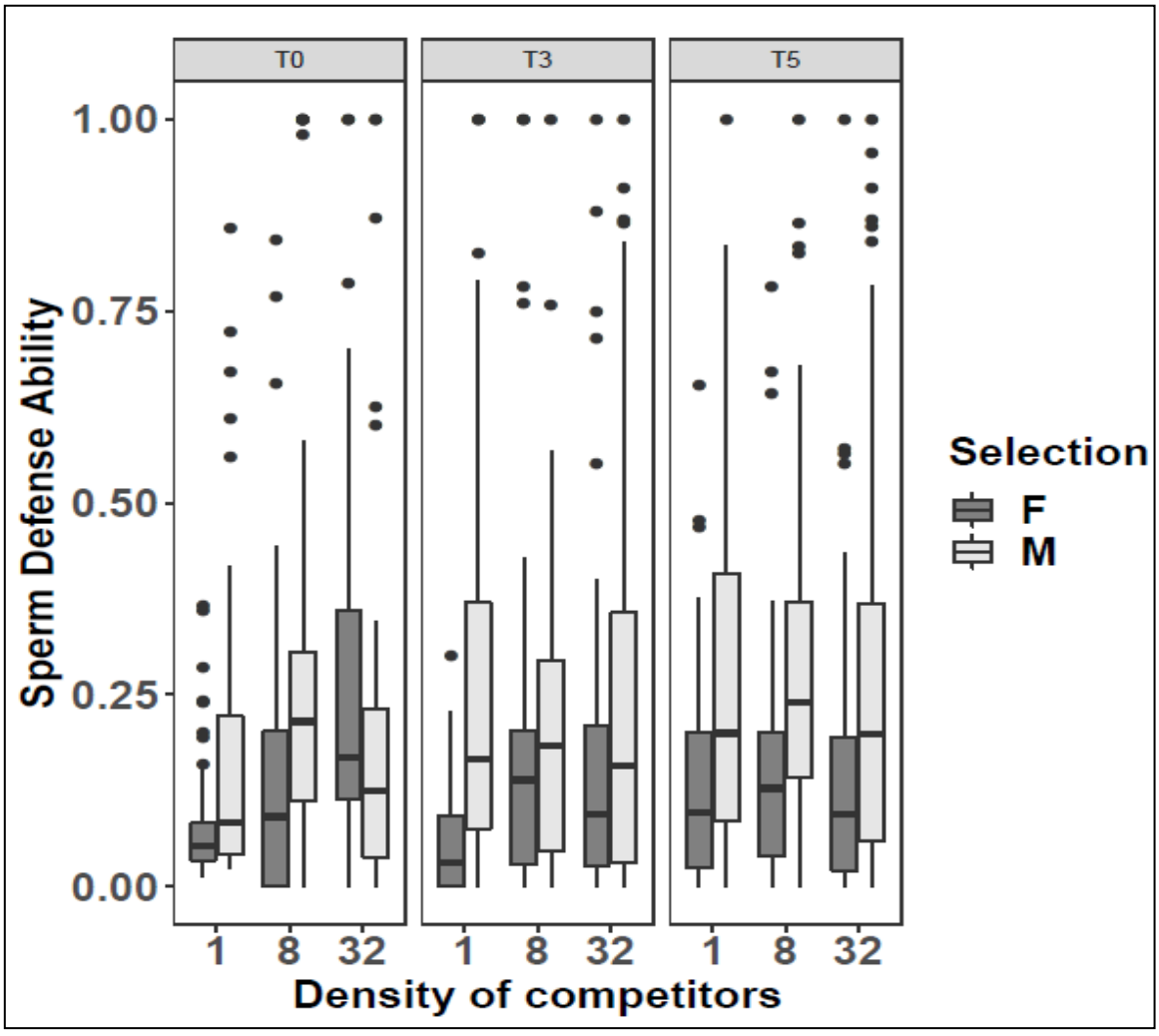


Figure.5 Sperm defense ability (P1) of M (light grey boxes) and F (dark grey boxes) regime males in response to different numbers of rivals from their own regimes respectively (i.e., M males housed with M males and F males housed with F males) at different time periods from the exposure of competitive cues. Panel on the left represents sperm defense ability after immediate exposure to rivals (at t=T0), the middle panel shows the sperm defense ability after 3 days of the removal of competitive cues (at t= T3), and the panel on the right represents sperm defense ability after 5 days (at t=T5) of removal of competitive cues.



Discussion

In this study, I investigated whether the evolutionary history of the populations (i.e. evolution under male-biased and female-biased sex ratios) and manipulation in the immediate socio-sexual environment significantly affected the reproductive investment patterns of the males. Males under differential levels of sexual selection showed different kinds of reproductive investment pattern in response to varying numbers of early-life competitors. Additionally, the pattern of reproductive investment was also observed to be sensitive to the identity (evolutionary history) of the competitor males. Another interesting inference from this study was that sexual selection intensity also affected the memory retention of the early-life cues (exposure to competitors). The memory formed in response to exposure to different numbers of competitors in early life subsided at different rates between M and F regimes.

In various insect species, males have been shown to increase their reproductive investment when exposed to rivals prior to or during mating (Gage and Baker 1991; Parker et al. 1996; Wedell and Cook 1999; Martin and Hosken 2002; Neff et al. 2003; Siva-Jothy and Stutt 2003; Pound and Gage 2004; Reichard et al. 2004; Friberg 2006; Carazo et al. 2007; Bretman et al. 2009). However, in most of these studies, only the presence of rivals instead of their numbers has been reported to affect the reproductive plastic strategies in males. For example, Bretman et al. (2009) tested the ejaculate investment of males in response to different numbers of rivals (i.e. 0, 1 and 4). They showed that ejaculate investment of males increased in response to the presence of a rival, but they observed no significant effect of increasing the number of rivals beyond 1. Conversely, in LH (the ancestral population of M and F regimes), an increase in copulation duration and sperm defense ability (P1) was observed in males when the number of competitors increased from 1 to 16 and then a decrease when the numbers increased further to 32 (Nandy and Prasad 2011). In my study, M males displayed a

pattern similar to the ancestral (LH) population as M males showed an increase in reproductive investment when the numbers of competitors increased from 1 to 8, and afterwards, a decline was observed in the case of the 32 male treatment. On the other hand, for F males, we observed a continuous increase in reproductive investment with the increasing number of competitors. A handful of studies have tested the evolution of plastic patterns in reproductive behavior in response to sexual selection (Edward et al. 2010; Dore et al. 2020). My results from experiment 1 show that males from populations evolving under different levels of sexual selection displayed different density specific responses to rivals from their own population (in terms of CD and P1). With an increase in the number of rivals, M males (evolving under higher sexual selection) showed a "tent-like" pattern of reproductive investment while F males (evolving under lower sexual selection) showed an increasing pattern of reproductive investment.

A plausible explanation for these differences in the reproductive investment patterns could be that sensitivity to the number of competitors has itself evolved in M and F regime males. According to maintenance regime conditions, M males are maintained under male-biased sex- ratios, i.e. 24 males: 8 females and F males are maintained under female-biased sex ratios, i.e. 8 males: 24 females, respectively. However, males are subjected to these sex-ratio treatments only on the 12^{th}-day after egg collection. By that time, males from both M and F regimes are 2 to 3 day old as adults. Now to note here, M and F males both are collected as virgins at a density of 8 males/vial in their early life. Accordingly, for both the M and F males, the early life density of competitors is the same in the course of their evolutionary history. But, in later life (when males are combined with the females), male density for M males becomes three times the early life density (i.e., 24 males), while for F males, density remains constant in early and later life (i.e. 8 males). Thus, it is possible that M males might have evolved to become more sensitive towards the changes in the number of rivals

compared to F regime males. Furthermore, it is quite likely that there may be a certain threshold beyond which increasing the ejaculate investment does not result in an increase in fitness returns. This threshold for reproductive investment can be expected to be different for M and F males as they have evolved in different levels of intrasexual selection and intersexual conflict for more than 150 generations. Also, since F males are exposed to a constant male density (i.e., 8 males/vial) throughout their life, it is possible that the ability to sense the threshold capacity of resource investment has declined in F males, which led to the differences in responses to the number of competitors by M and F males.

A study on *Drosophila melanogaster* populations showed that males evolving under a male-biased sex ratio had lower mating latencies when exposed to rivals in comparison to the males evolving under a female-biased sex ratio (Edward et al. 2010). In another study, *Drosophila melanogaster* populations were evolved under three sex ratios (male-biased, female-biased and equal) and two dietary regimes. This study showed that males from the male-biased populations displayed a novel plastic behavioral pattern that involved reduced courtship and increased mating latency upon exposure to a rival male (Dore et al. 2020). On the contrary, ancestral males and males from the other sex-ratio regime did not show such a pattern. Thus, my results are, majorly, in agreement with those of Edward et al. (2010) and Dore et al. (2020), suggesting that in general, patterns of male reproductive investment in response to the presence of rivals can diverge between populations evolving under male-biased or female-biased sex-ratios.

One of the exciting results of this study was that the identity of early-life competitors influenced the pattern of reproductive investment in M and F males in different ways. When M and F males were held with 0, 7 or 31 early-life competitors from the ancestral LH populations (experiment 2), their reproductive investment showed a similar pattern and increased with increasing numbers of competitors. This pattern exhibited by M males is

different from the one when M males were housed with rival males of their own population (experiment 1). On the other hand, the reproductive investment pattern for the F males was similar in the two conditions (continuous increase in reproductive investment with an increase in the number of competitors). These results suggest that the pattern of reproductive investment of M males was sensitive to the change in the identity of the competitors.

Theory predicts that the intensity of intrasexual social interactions can affect reproductive traits (Lizé et al., 2014). Therefore, the difference in the pattern of reproductive investment shown by M males when housed with rivals of their own kind (M males) versus ancestral LH rivals could possibly be because of different intensities of intrasexual interactions in these two conditions (i.e. M - M versus M - LH). One important point to note here is that F males exhibited almost similar patterns of reproductive investment in both the experiments (experiment 1, experiment 2), irrespective of the identity of rival males. Therefore, it can be concluded that M and F populations have evolved divergent responses to different kinds of rival males. These results can be explained on the basis of two alternative explanations.

First, it is quite likely that M males have evolved a pattern of reproductive investment that is different from the pattern exhibited by F males and is highly specific to and therefore only invoked by exposure to males from their own population. M males have been evolving under intense intersexual conflict and sexual selection for more than 150 generations. During this period, their reproductive traits have gone through rapid evolution (Nandy et al. 2013a,b). Consequently, it has resulted in the evolution of post-mating pre-zygotic reproductive barriers between the three replicates of the M regime, whereas no such isolation has been observed between the replicates of the F regime (Syed et al., 2017). Therefore, it is reasonable to expect that M males have evolved responses that are fine-tuned to the particular environment of their respective populations. Hence, they exhibited a “tent-like” pattern of reproductive

investment in response to different numbers of early-life competitors only when those competitors were from their own population.

Alternatively, it is plausible that both M and F males can show both kinds of reproductive investment patterns observed in my experiments and the pattern they actually display depends on the perceived intensity of competition. In experiment 1, M males competed with other M males, and therefore were exposed to stronger competition than F males who competed with other F males. According to this explanation, the patterns of reproductive investment have not evolved between M and F populations. But it is the increased intensity of male-male competition that has evolved to be stronger in the M population, prompting M males to show a pattern of reproductive investment that is distinct from the one shown by F males. Unfortunately, results from these experiments are not sufficient to distinguish between these two possibilities. However, my results strongly suggest the role of the intensity of sexual selection in generating these different kinds of reproductive investment patterns in M and F males.

I also assayed reproductive investment of males in response to different numbers of competitors (from their own population) at different time points from the time of exposure to rival males. Patterns of reproductive investment in terms of copulation duration and sperm defense ability shown by M and F regime males immediately after removing the competitive cues were different from those after 3 days and after 5 days of removal of competitive cues. Therefore, the response of M and F males depended on the time lag between cue detection and time of assessment of reproductive traits. A crucial observation from my copulation duration results is that M males retained the impact of early-life cues for longer time than F males. After 3 days of the removal of competitive cues, the response (in terms of reproductive investment) displayed by M males to different numbers of competitors was almost similar to the response observed in the assay performed immediately after the removal

of cues. On the other hand, F males did not exhibit any trace of early-life competitive cues even three days after the removal of cues. One of the possible explanations for the observed responses in M and F regime males could be the force of sexual selection acting on M males. It has been shown that the intensity of sexual selection can influence memory retention in males (Hollis and Kawecki 2014). *D. melanogaster* males under polygamous conditions (i.e., under higher level of sexual selection) evolved to become better at cognition tasks (courtship learning and olfactory learning ability) than the monogamous males (i.e., males under a lower level of sexual selection (Hollis and Kawecki 2014). Therefore, it is possible that M males have evolved to efficiently assess and then process the experienced cues in order to increase their reproductive success. Contrary to this, for the F regime males with relaxed male-male competition, it is possible that the adaptive value of cognitive performance has declined, resulting in reduced memory retention of experienced cues in these males. In conclusion, through this study, I demonstrated that the responses generated in M and F males following a change in socio-sexual environment depended on density as well as the evolutionary history/identity of rival males. Further, I also showed that evolution at different operational sex ratios could affect the memory retention of the competitive cues experienced in early life. Results from this study support the idea that intense sexual selection can possibly lead to the evolution of adaptive plastic reproductive responses under varying socio-sexual conditions.

Chapter -4

Sexual selection and the evolution of courtship learning ability in males

Introduction

Courtship is an important aspect of male reproductive behaviour that can influence the reproductive success of males. Though reproductive behaviour is genetically programmed in an individual, but it can be manipulated by factors such as environment unpredictability, social interactions, and sexual experiences (Dukas 2005a; Villella and Hall 2008; Bretman et al. 2009b). As an instance, *Drosophila* males possess an innate behaviour of courting potential mates in their surroundings. But social encounters with the conspecific or heterospecific individuals can alter their reproductive strategies in the context of courtship (Dukas 2005b). Studies also suggest that males can learn from their past experience to refrain from courtship towards unreceptive individuals (Siegel and Hall 1979; Gailey et al. 1982, 1985). After an unsuccessful courtship of non-receptive/unreceptive mated females, males withdraw their courtship efforts towards unreceptive females for a short duration, a phenomenon known as 'Courtship conditioning' (Siegel and Hall 1979).

Courtship conditioning assay is well characterized as an associative learning paradigm and is extensively used for testing learning and memory in some of the *Drosophila* species (Siegel and Hall 1979; Kamyshev et al. 1999; McBride et al. 1999; Keleman et al. 2007). Males depend on a complex network of sensory systems for evaluation of the suitability of the females. This network assesses the visual, gustatory, tactile, olfactory, and auditory information before choosing a mate (Bretman et al., 2011b). Studies also suggest that courtship experience with previously mated/unreceptive females enabled males to filter their courtship behaviour. Following courtship conditioning, conditioned males suppressed their courtship effort towards the mated/unreceptive females for a longer time compared to the virgin receptive females (Dukas, 2005a). Another study in *D. melanogaster* males documented that previous experience at courting unreceptive, heterospecific *Drosophila simulans* females resulted in suppressed courtship towards other *D. simulans* females but not

towards receptive *D. melanogaster* females (Dukas 2004). Above mentioned examples indicate that courtship conditioning can play a significant role in increasing the mating success of males.

Since courtship learning/conditioning may have an adaptive value as it enables males to assess the receptivity of females, it can be presumed that courtship learning can itself evolve with respect to certain ecological/social pressures. Also, the evolutionary force of sexual selection can lead to improved cognition abilities if organisms with better cognitive ability relish the highest mating success (Miller and Cohen 2001; Boogert et al. 2011; Hollis and Kawecki 2014). Very few studies have investigated this idea using experimental evolutionary approaches in few systems. As an example, Hollis and Kawecki (2014) tested for cognitive performance of *Drosophila melanogaster* populations maintained under monogamous and polygamous conditions in two complex learning tasks– (a) ability to avoid an odour previously paired with aversive shock (b) and the ability to distinguish between a receptive and unreceptive female in a complex environment with several unreceptive females and a single receptive female. Polygamous males outperformed monogamous males in both cognitive tasks (Hollis and Kawecki 2014). In seed beetles, *Callosobruchus maculates,* cognitive abilities were tested in males and females from polygamous and monogamous lines using different spatial learning tasks within an arena: males were tested for discriminating between female conspecifics and male conspecifics. Females were scored for locating the seeds that were preferable for egg-laying among non-preferable seeds (Baur et al. 2019). Overall, monogamous males were less successful than polygamous males, whereas in the case of females, there was no difference between polygamous and monogamous females.

Thus, there is little evidence about the role of sexual selection in the evolution of cognitive abilities, and current evidence is not enough to speculate a broad conclusion about the impact of sexual selection intensity on cognitive abilities, specifically on courtship learning ability in

males. Therefore, in the present study, I mainly analysed the influence of sexual selection on courtship learning ability using *Drosophila melanogaster* populations evolving under contrasting levels of sexual selection for more than 150 generations.

M regime males have been shown to evolve greater courtship frequency than the F regime males (Nandy et al., 2013c). Further, a recent study (results from the previous chapter) showed that M regime males are more efficient in processing the information received in the form of complex cues from variable early life socio-sexual environment. This study also reported that M males retained the memory of early life experience for a more extended period than F regime males (Maggu et al. 2021). Therefore, following this, I hypothesised that as M regime males, in general, have a higher courtship activity which allow an opportunity to gather information about the mating target, M males might have evolved to retain and use this information to enhance their reproductive success. To investigate this, I assigned both M and F regime males to the learning phase by housing them with unreceptive females. Following the conditioning phase, we then tested the males from both the regimes for their courtship learning abilities, when paired with one receptive and 4 unreceptive females i.e. if they learned to suppress their courtship towards unreceptive females on again encountering them and to direct their courtship towards the receptive female. I also noted courtship latency to check if the amount of courtship, if suppressed, differed between M and F regime males. Mating latency was also noted to examine which type of males were faster to start the mating. Lower mating latencies suggest better learning as the males who would find the receptive female earlier would also initiate the mating earlier.

Particularly, I asked the following questions-

1) If courtship conditioning can affect the ability of males under divergent levels of sexual selection to distinguish between receptive and unreceptive females.

2) If courtship latency was affected by courtship conditioning treatment and sexual selection.
3) If courtship experience enabled conditioned M and F males to start mating with the receptive female earlier than the unconditioned males.

Materials and methods

Before experimental egg collection, all the populations were subjected to one generation of standardisation to eliminate the possibility for any non-genetic parental effects on my experimental observations. In the standardisation process, populations were sustained under the cultural conditions of the ancestral population, LHst. These populations are referred to as standardised populations. For the experiments, I collected eggs from these standardised populations at a density of 150 eggs per vial and incubated them under standard laboratory conditions. After this, on 9-10th day post egg collection, I collected males and females from these populations (M, F and LHst) as virgins. I kept these males and females in single-sex vials at a density of 8 flies per vial. After two days, i.e. on the 12th-day post egg collection, when flies were 2-3 days old as adults, I performed experimental reproductive assays on these flies.

Generating unreceptive LHst females

Studies have reported that if, after first mating, females are not allowed to mate for a few hours, they might display a strong physiological refractory period between matings that can continue up to 24 hours (Manning 1962; Manning 1967; Brown et al. 2004). For my experiment, I used this principle to generate unreceptive LHst females. To achieve this, I combined eight virgin LHst females with 10 LHst males in a vial and allowed them to mate for one hour. During that period, I ensured that all the females in a vial mated only once by continuously observing those vials. After that, males were removed from the vials using light CO_2 anaesthesia and females were held back and were not allowed for any kind of sexual

exposure for the next 6 hours. After this period, these females were used for the experiments. My observations showed that these females resisted mating and remained receptive.

Experimental assay

On the 12th-day post egg collection, M and F males were randomly assigned to one of the following two treatments- (a) Conditioned treatment - Males were exposed to unreceptive females for a period of two hours, and (b) Unconditioned treatment- Males were not exposed to unreceptive and any kind of females. In the conditioning phase, a single, virgin M or F male was exposed to five unreceptive LHst females per vial with ad libitum food. I maintained sixty such vials for each of the population and within each treatment. Males were subjected to the conditioning phase for 2 hours. During these 2 hours, males continuously courted the females while females consistently rejected the mating attempts of the males. Vials were observed manually, and if in any vial, mating happened, those vials were discarded (mating happened only in approximately 5% of total vials). After 2 hours, I aspirated out the conditioned males and transferred them to fresh food vials. These males were then allowed to rest for 30 minutes before going forward to the next stage of the experiment. During those 2 hours, when conditioned males were with the unreceptive females, the males from the unconditioned treatment were kept as single flies in individual vials without exposure to any females.

After 2 hours, individual M and F males from both conditioned and unconditioned treatments were paired with four unreceptive and a single receptive LHst female per vial for a short duration of 15 minutes. The unreceptive and receptive females were marked with fluorescent dust of different colours (reciprocal markings were also done to control for the effect of the colour). The unreceptive females used in this test phase of the experiment were different from those used in the experiment's conditioning phase. I set up sixty such vials for each of the population. Vials were observed manually, and readings were taken every 30 seconds. During

this period, numbers of courtship bouts to the unreceptive and receptive females were noted. I calculated the courtship index (CI) by taking a fraction of the total number of courtship bouts towards the receptive female to the total number of courtship bouts attempted by the male.

$$Courtship\ Index = \frac{Number\ of\ courtship\ bouts\ directed\ by\ the\ male\ towards\ the\ receptive\ female}{Total\ number\ of\ courtship\ bouts\ attempted\ by\ the\ male}$$

Where,

a) Courtship bout refers to any courtship related activity such as mounting, orientation, wing vibration, following etc. If a male displayed any of these behaviours or a series of such activities towards a female, it was counted as one bout. For example, if a male oriented towards a female and then broke off the interaction, it constituted one bout. If a male oriented, vibrated the wings, attempted to mount and then broke off the interaction, it was also counted as one bout.

b) The total number of courtship bouts is the sum of the numbers of courtship bouts directed to the receptive and unreceptive females by a male and referred to as courtship intensity.

Courtship latency was calculated as the time taken by a male to start courtship from the time it was presented with a female into the vial. I calculated mating latency as the time spent by a male to initiate mating from the time it was introduced into the vial with the female.

Statistical Analysis

For statistical investigation of courtship learning, I used the package "glmmTMB" (Brooks et al. 2017) to set up a generalised linear mixed-effects model with a beta-binomial error structure. Courtship index was used as the dependent variable, with selection regime (M vs F) and treatment (conditioned or unconditioned) and their interaction as fixed factors. Block and its interactions with other fixed predictors were used as random effects and are specified

below in the model formulae. I did not observe any significant effect of block and its interactions with other fixed factors. I performed an analysis of variance on the model to test the fixed effects using a Wald Chi-square test from the package "car"(Fox and Weisberg 2018).

For calculating courtship latency, mating latency and courtship intensity, I set up linear mixed-effects models using the package "lme4" (Bates et al. 2014) with courtship latency/mating latency/courtship intensity as the dependent variable. Selection regime, treatment, and their interaction were taken as fixed effects. The random effects are specified below in the model formulae. Models used for different traits are summarized below-

1. Courtship index - CI ~ Selection + Treatment + (1 | Block) + (1 | Block:Selection) + (1|Block:Selection:Treatment) + (1 | Block:Treatment) + Selection:Treatment.
2. Courtship latency- CL ~ Selection + Treatment + (1 | Block) + (1 | Block:Selection) + (1|Block:Selection:Treatment) + (1 | Block:Treatment) + Selection:Treatment.
3. Mating latency – ML ~ Selection + Treatment + (1 | Block) + (1 | Block:Selection) + (1 | Block:Selection:Treatment) + (1 | Block:Treatment) + Selection:Treatment
4. Courtship intensity – CI ~ Selection + Treatment + (1 | Block) + (1 | Block:Selection) + (1 | Block:Selection:Treatment) + (1 | Block:Treatment) + Selection:Treatment

Results

Selection led to an increase in the courtship towards the receptive female

I compared the courtship index between the conditioned and unconditioned treatments of M regime and F regime males. Here, I found a significant effect of selection and treatment (Figure.1, Table.1). Conditioned males performed better in assessing female receptivity and showed higher courtship towards the virgin/receptive females in both regimes. Overall, M regime males showed significantly higher numbers of courtship bouts towards the receptive

female irrespective of the treatment than F regime males. However, no significant effect of selection× treatment interaction was observed, indicating no significant difference in the courtship learning ability between the M and F regime males.

Treatment affected courtship latency but no effect of selection

I also tested for any differences in the time taken to start courting the females and to see if courtship suppressed to different extents in the two regimes. I observed no significant effect of selection and selection × treatment interaction (Figure.2, Table.2). Both kinds of males (M and F males) took almost similar time to initiate courting the females. However, I found a significant effect of treatment here, and males from conditioned treatment took longer to initiate the courting relative to the males from unconditioned treatment.

No effect of selection on mating latency

In the experimental design, four unreceptive and one receptive female were presented to each kind of male in the test phase. Out of 60 vials for each treatment, males in approximately 52-57 vials started mating from both the M and F regime by the end of observation time (15 min). Therefore, I noted the mating latency to observe that between the M and F regime males, which type of males, were faster in recognising the receptive female and to start mating (Figure.3, Table.3). Here also, similar to courtship latency I didn't find any significant effect of selection and selection× treatment interaction. I found that both types of males were equally faster in initiating the mating. However, I found a significant effect of treatment for mating latency. Conditioned males from both M and F males took less time than the unconditioned males to start mating with the receptive female.

Courtship conditioning resulted in suppression of courtship intensity

Total courtship bouts attempted by the males were significantly lower in the conditioned treatment as compared to unconditioned treatment. However, I did not observe any

significant effect of selection and selection × treatment interaction on total courtship intensity (Figure.4, Table.4).

Table.1 Summary of results from a generalized linear mixed model with beta-binomial error structure showing the main and interactive effects of selection, treatment and block on courtship index. Significant values ($p<0.05$) are marked with '*' and are shown in bold.

	Chi sq	Df	Pr(>Chisq)
(Intercept)	2.862	1	0.09065
Selection	31.62	1	**<0.0001*** **
Treatment	69.04	1	**< 0.0001*** **
Selection×Treatment	2.502	1	0.11370
Groups	Name	Variance	Std.Dev.
Treatment×Selection×Block	(Intercept)	0.00978	0.0985
Selection×Block	(Intercept)	7.32×10^{-9}	8.55×10^{-5}
Block	(Intercept)	3×10^{-13}	5.48×10^{-7}
Block×Treatment	(Intercept)	3.155×10^{-12}	1.77×10^{-6}

Table.2 Main and interactive effects of selection, treatment and block on courtship latency in males of M and F regimes. Significant values ($p<0.05$) are marked with '*' and are shown in bold.

Type III Analysis of Variance Table with Satterthwaite's method						
	Sum Sq	**Mean sq**	**Num DF**	**Den df**	**F value**	**Pr(>F)**
Selection	4.12	4.12	1	2.85	3.81	0.1506
Treatment	156.6	156.6	1	3.04	144.7	**<0.001*** **
Selection × Treatment	1.688	1.688	1	664.0	1.559	0.212
	N par	**Loglik**	**AIC**	**LRT**	**Df**	**Pr(>F)**
Null model	9	-989.5	1997.2			
(1 | Block)	8	-989.5	1995.2	0.000	1	1.000
(1 | Block×Selection)	8	-989.7	1995.5	0.368	1	0.543
(1 | Block×Selection ×Treatment)	8	-989.5	1995.2	0.000	1	0.999
(1 | Block×Treatment)	8	-989.8	1995.7	0.492	1	0.482

Table.3 Main and interactive effects of selection, treatment and block on mating latency in males of M and F regimes. Significant values ($p<0.05$) are marked with '*' and are shown in bold.

Type III Analysis of Variance Table with Satterthwaite's method						
	Sum Sq	**Mean sq**	**Num Df**	**Den Df**	**F value**	**Pr(>F)**
Selection	1.40	1.40	1	4.00	0.313	0.605
Treatment	1428.6	1428.6	1	666.1	319.6	**<0.001*** **
Selection ×Treatment	0.01	0.01	1	666.1	0.001	0.992
	N par	**Loglik**	**AIC**	**LRT**	**Df**	**Pr(>F)**
<none>	9	-1465.3	2948.5			
(1 | Block)	8	-1465.3	2946.5	0.000	1	1.000
(1 | Block× Selection)	8	-1467.2	2950.3	3.808	1	0.051
(1 | Block× Selection ×Treatment)	8	-1465.3	2946.5	0.000	1	1.000
(1 | Block×treatment)	8	-1466.8	2946.5	0.000	1	1.000

Table.4 Main and interactive effects of selection, treatment and block on courtship intensity or the total number of courtship bouts in males of M and F regimes. Significant values ($p<0.05$) are marked with '*' and are shown in bold.

Type III Analysis of Variance Table with Satterthwaite's method						
	Sum Sq	**Mean sq**	**Num Df**	**Den df**	**F value**	**Pr(>F)**
Selection	0.148	0.148	1	2.41	0.011	0.921
Treatment	207.6	207.6	1	2.69	16.75	**0.032***
Selection × Treatment	26.59	2.59	1	664.3	2.14	0.143
	N par	**Loglik**	**AIC**	**LRT**	**Df**	**Pr(>F)**
Null model	9	-1807.6	3633.2			
(1 | Block)	8	-1807.6	3631.2	0.000	1	1.000
(1 | Block×Selection)	8	-1808.0	3631.9	0.753	1	0.385
(1 | Block× Selection× Treatment)	8	-1807.6	3631.2	0.000	1	1.000
(1 | Block×Treatment)	8	-1808.1	3632.2	1.015	1	0.313

Figure.1 Effect of courtship conditioning on courtship index (mean ± 95% CI) for M and F regime males. Circles represent males from the F regime, and triangles represent males from the M regime. Courtship index (CI) refers to the fraction of courtship bouts to the receptive female out of the total courtship bouts attempted by the male.

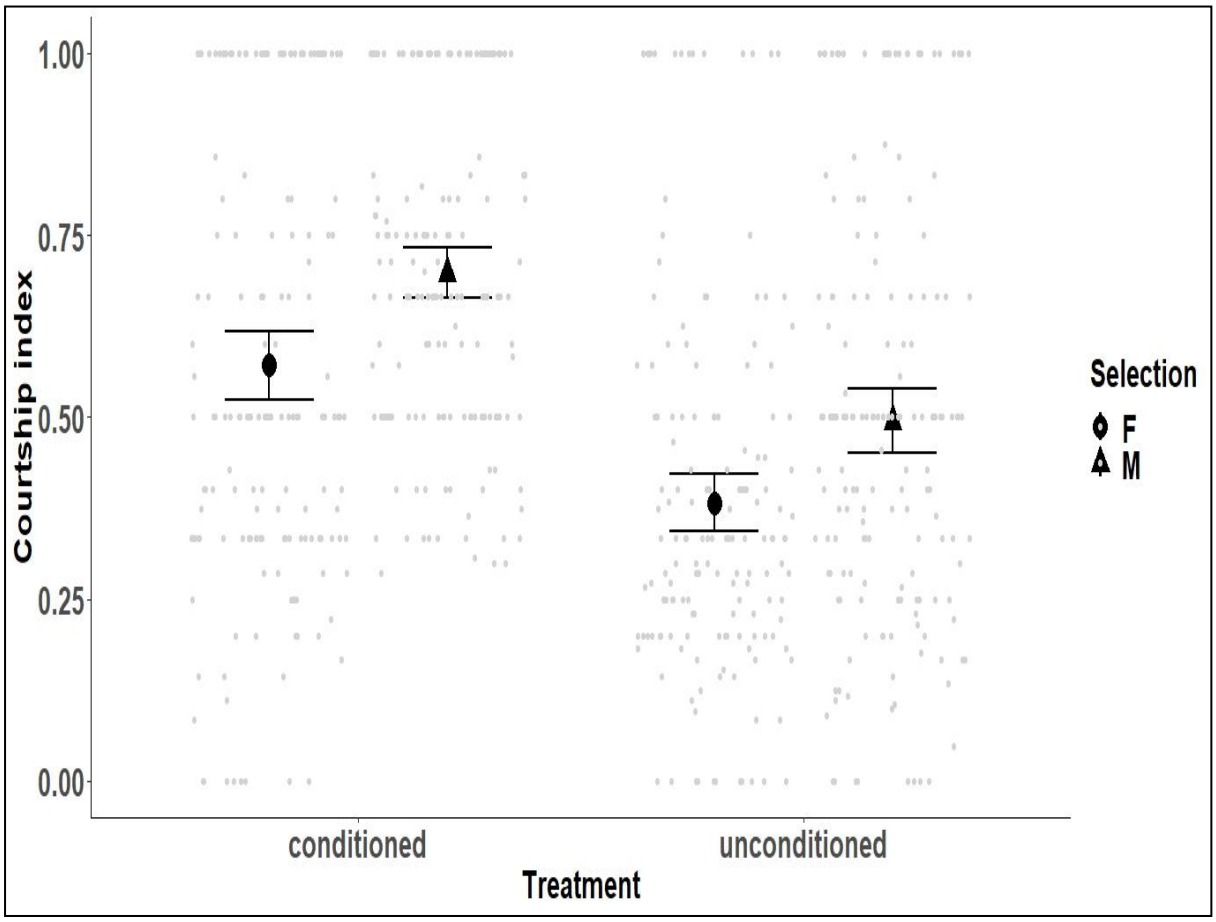


Figure.2 Courtship latency (minutes) (mean ± 95% CI) for M and F regime males compared between conditioned and unconditioned (control) treatments. Circles represent males from the F regime, and triangles represent males from the M regime.

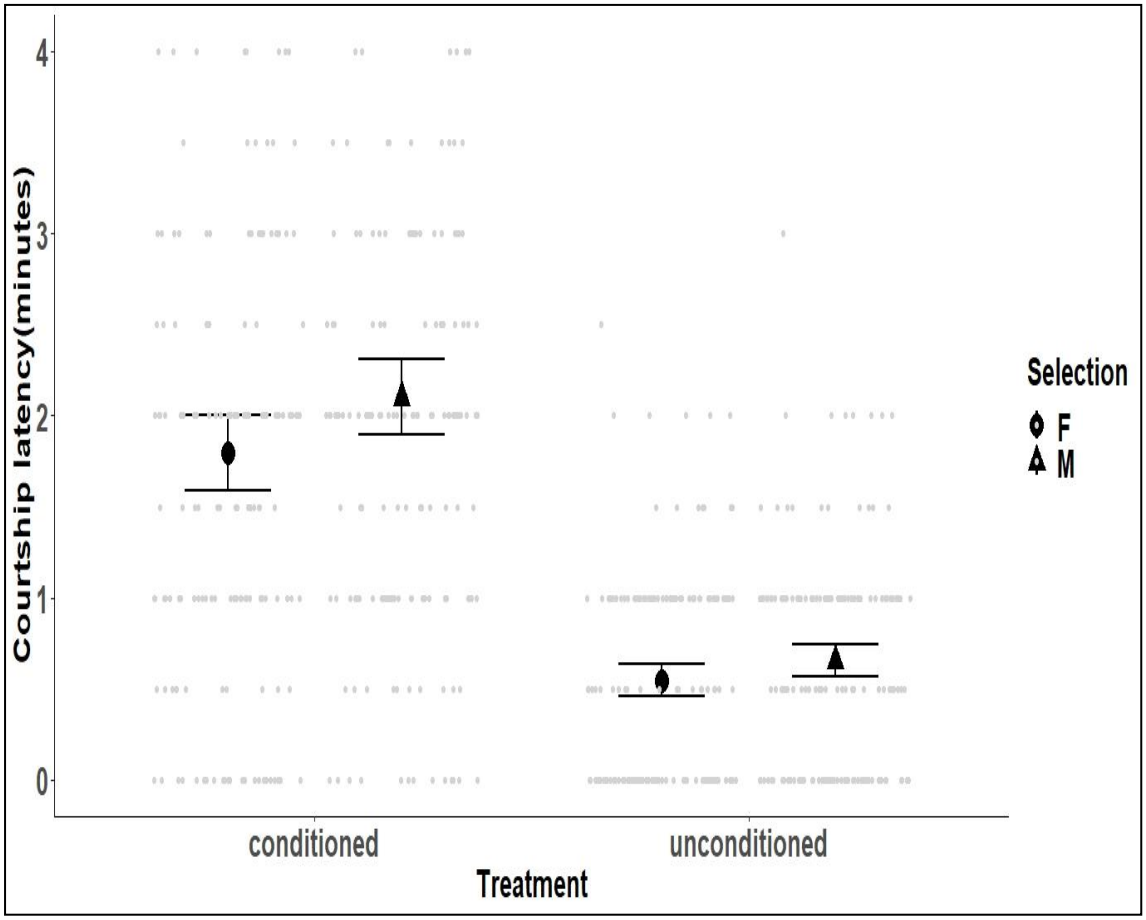


Figure.3 Mating latency (mean ± 95% CI) for M and F regime males compared between conditioned and unconditioned (control) treatments. Circles represent males from the F regime, and triangles represent males from the M regime.

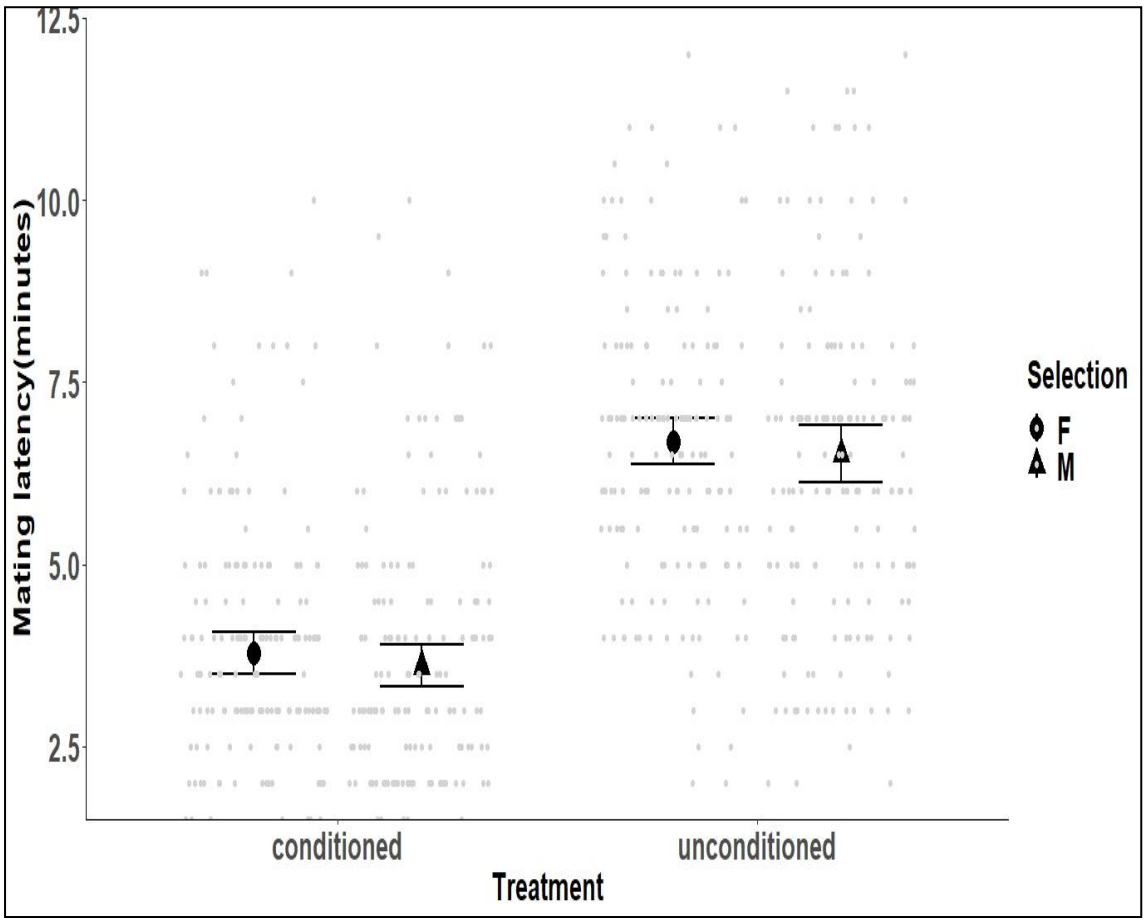
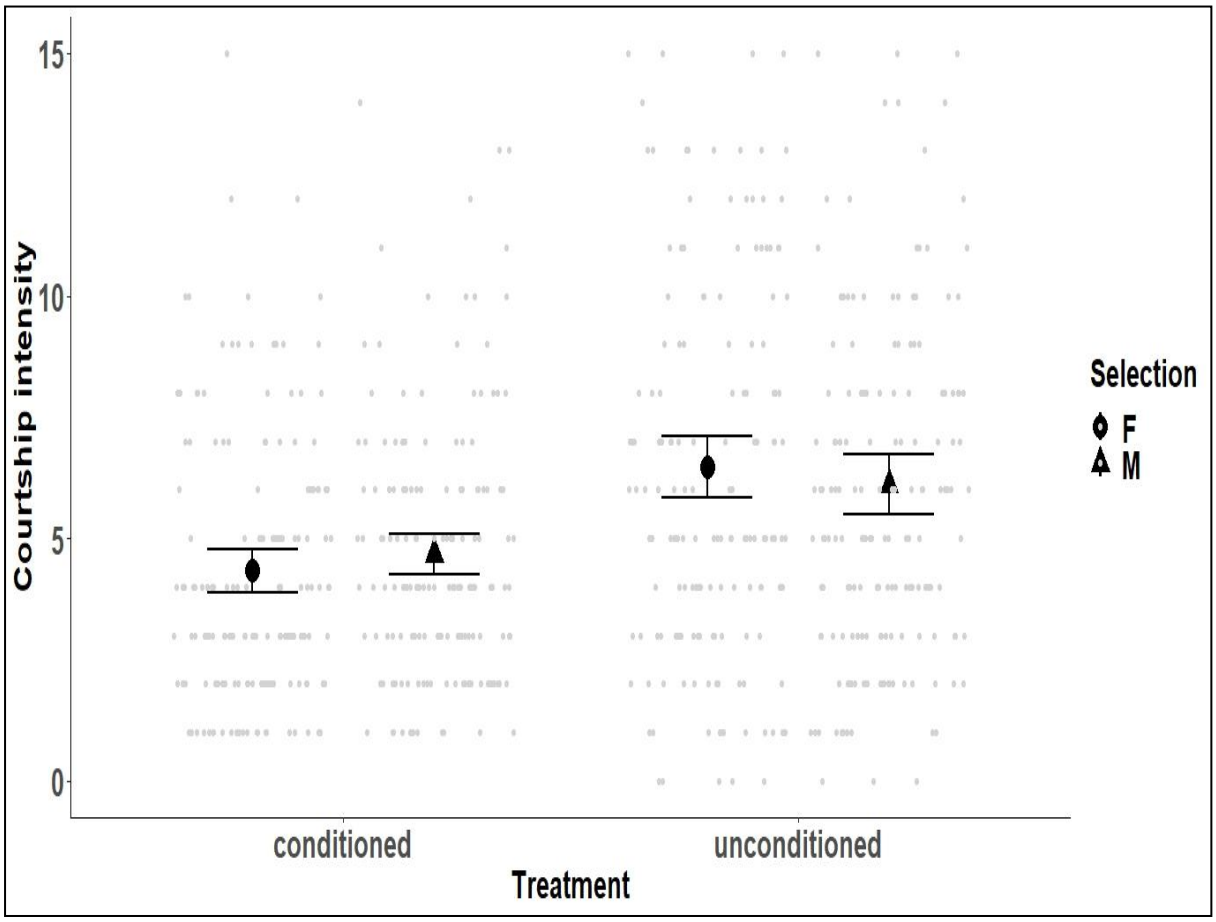


Figure.4 Effect of courtship conditioning on courtship intensity (mean ± 95 % CI) or total courtship bouts attempted by the males for M and F regime males compared between conditioned and unconditioned (control) treatments. Circles represent males from the F regime, and triangles represent males from the M regime.



Discussion

Through this study, using *Drosophila melanogaster* populations evolving under variable levels of sexual selection, I showed that males under higher level of sexual selection (M males) evolved to become inherently better at determining the receptivity of females. Prior experience with the unreceptive females resulted in the refinement of courtship behaviour of males from both populations. Males from conditioned treatment from each of the M and F selection regimes were more superior at recognising the receptive female relative to unconditioned males from the respective selection regimes. Overall, males from the M selection regime directed their courtship towards the receptive female more frequently than the F selection regime irrespective of the treatment. However, the courtship learning ability was not significantly different between M and F selection regime males. Additionally, the total number of courtship bouts, courtship latency and mating latency was significantly affected by the treatment rather than the selection history of males. Conditioned males showed decreased courtship intensity, took longer to initiate courting but took less time to begin mating with the receptive females than unconditioned males.

Although courtship behaviour is genetically programmed in males, this courtship behaviour can be modified by social encounters with same-sex or opposite-sex individuals (Siegel and Hall 1979; Gailey et al. 1982, 1985; Dukas 2010). One of the classic examples of such studies includes 'courtship conditioning' whereby courtship of males is suppressed for a short duration by training with the unreceptive females (Siegel and Hall 1979). Results from my study suggest that experience gained through courtship conditioning enabled both M and F regime males to learn and analyse the receptivity of females in a complex mating environment. As an effect of conditioning, males from both the M and F regime showed decreased courtship effort (indicated by courtship learning index) towards the unreceptive females when they were presented with both the receptive and unreceptive females. This fact

clearly suggests that both M and F regime males learnt from their prior social experience with the unreceptive females. Further, mating latency and courtship latency data show that conditioned males from both M and F regimes took more time to start the courting but were very quick to initiate the mating with the receptive female in comparison to unconditioned males. This again indicates the effect of experience based learning, as conditioned males had interacted with the mated / unreceptive females earlier in the experience phase.

One of the interesting inferences of this study was that I observed no difference in the rate of learning between the males of M and F regimes. Conditioned males from both the treatments showed learning at almost similar rates as I did not observe any interaction selection regime and treatment. It has been reported that sexual selection can affect the evolution of cognitive abilities. As a piece of evidence, Hollis and Kawecki (2014) reported that sexual selection resulted in increased performance in terms of cognition in *Drosophila melanogaster* as males from the polygamous line were more successful when focussing on the receptive female in comparison to males from monogamous lines. Another study reported similar results in seed beetles maintained under monogamous and polygamous conditions, with polygamous males showing overall better performance than monogamous males in complex spatial learning tasks (Baur et al. 2019). My findings from this study suggest that males from the regime under the higher force of sexual selection had increased ability to discriminate between receptive versus unreceptive females even when they were not subjected to conditioning. Conditioning led to an improvement in the courtship learning of both types of males (as indicated by a significant effect of treatment on courtship index). But, I did not find any clear evidence of differences in learning abilities of the M and F regime males (as indicated by a lack of significant selection by treatment interaction on courtship index). Although courtship latency, mating latency and the total number of courtship bouts (courtship intensity), did not differ between the M and F males, the prior experience influenced these traits in both types of

males (M and F) in an identical fashion. It again suggests that both kinds of males were equally good at learning from previous experience. One of the possibilities for M males showing overall higher courtship towards receptive females than the F males could be because M males have evolved to have higher courtship ability and locomotor activity than the F males (Nandy et al. 2013c). Consequently, it is possible that M males, in general, attempted a higher number of courtship bouts towards the females than the F males, eventually leading to the higher number of courtship bouts towards the receptive female. But this possibility can be ruled out as I observed no significant difference in the total number of courtship bouts or courtship intensity between the M and F males.

M males were better than the F males in recognising the receptive female even in the unconditioned treatment. This observation points out the possibility of an inherent ability in M males to determine the receptivity of females. One of the potential explanations for this could be the difference in the maintenance regime of the M and F regime males. Every generation for two days, during which males and females are allowed to interact in the selection regimes, M regime males are maintained at 24 males and 8 females (sex ratio of 3male:1female; a total of 32 flies per vial) per vial and F males are maintained at 24 females and 8 males (sex ratio of 1 male: 3 female; a total of 32 flies per vial). Considering these sex ratios, it can be expected that there will be more variability in the mating status of the females across the F regime than the M regime. However, as there is intense male-male competition in the M regime, the benefits of locating a female earlier than the other males would be higher in the M regime relative to the F regime. Any M male that has the ability to recognise and court a receptive female and rapidly mate with her will have a selective advantage over other males. Hence, it is possible that M males have evolved to recognise receptive females more quickly as compared to the F males.

Additionally, given that M males are expected to have a greater exposure with the unreceptive females in the course of their evolutionary history, and it has been shown that cuticular hydrocarbon profiles play a significant role in recognition between receptive and unreceptive females (Siwicki et al. 2005), one of the possibilities for the improved ability of M males to discriminate between receptive and unreceptive females could be M males being more familiar with the CHCs of the unreceptive females. It is quite possible that M males might have evolved a greater aversion to chemosensory cues from CHCs of unreceptive females as a consequence of being exposed to them every generation.

Another potential explanation of this observation could be the evolution of a more sensitive sensory system in M males. Increased cognitive performance depends on accurate perception of complex cues to gather information about the surroundings. It has been suggested that in vertebrates the social complexity of the environment can lead to the evolution of brain size and cognition abilities (Dunbar 1998). M males have been evolving in a complex mating environment in terms of increased male-male competition than relaxed intra male competition in F males. Thus, it is also possible that M males have evolved to become more sensitive towards such complex cues and hence showed an increased ability to discriminate between receptive and unreceptive females in conditioned and unconditioned treatments.

In conclusion, in this study, I show that in a complex mating environment, while prior experience can influence courtship learning ability in males, no clear evidence for the effect of sexual selection intensity on the same was observed. Also, other important components of reproductive behaviour, such as courtship latency, courtship intensity and mating latency required for increased reproductive fitness, were not affected by sexual selection in this study, whereas the immediate experience of males was observed to affect these traits. However, through this study, I found a novel consequence of sexual selection to drive the evolution of inherent ability in males to determine the mating status of the females. Accurate

determination of female mating status can significantly influence the reproductive success of males.

Chapter-5

Effect of sexual selection and conflict on the relationship between reproductive activity and immune function

Introduction

Empirical evidence from many taxa indicate that phenotypic tradeoffs exist between reproductive effort and immune response in both males and females (Sheldon and Verhulst 1996; Zuk 1996; Moret and Schmid-Hempel 2000; Ahmed et al. 2002; Lazzaro et al. 2008; McKean et al. 2008; Bashir-Tanoli and Tinsley 2014; Howick and Lazzaro 2014). For example, wolf spider males increased their drumming rates at the expense of lytic activity (estimates the concentration of antimicrobial peptides formed in response to an infection in the heamolymph) when presented with females (Ahtiainen et al. 2005). In bush-crickets, encapsulation rate and both call syllable number and spermatophore size were found to be negatively correlated (Barbosa et al. 2016). Activation of the immune system through injections of lipopolysaccharides resulted in a decreased rate of daily mating calls in decorated crickets (Jacot et al. 2005). Furthermore, in *Drosophila melanogaster,* females, bacterial or fungal infections resulted in reduced fecundity (Zerofsky et al. 2005; McKean et al. 2008; Bashir-Tanoli and Tinsley 2014; Howick and Lazzaro 2014). Identical effects have been noticed in Orthoptera, where fecundity is drastically decreased after mounting an immune response against heat-killed bacteria or bacterial cell wall components in house crickets (Bascuñán-García et al. 2010), Wellington tree weta (*Hemideina crassidens*) (Kelly 2011), and the Texas field cricket (*Gryllus texensis*) (Stahlschmidt et al. 2013). Thus, there exists enough evidence in favour of a correlation between immune activation and reproductive performance.

Theory suggests that sexual selection can influence the relationship between reproductive traits and immune traits (Gustafsson et al. 1994; Norris and Lampe 1994; Richner et al. 1995). Accordingly, a handful of studies have tried to test this idea using an experimental-evolutionary framework by subjecting different intensities of sexual selection to replicate populations. In one of such studies on red flour beetles, *Tribolium castaneum* evolving under

different operational sex ratios, no evidence of genetic trade-offs between reproduction and immunity traits was found (Hangartner et al., 2013). Males and females from male-biased and female-biased regimes were assessed for both immune measurement (phenoloxidase activity) and host resistance in terms of survival against microsporidian *Nosema whitei*. Both phenoloxidase activity and host resistance were not different between the male-biased and female-biased lines (Hangartner et al., 2013). In a more direct approach, in *Drosophila melanogaster* effect of bacterial infection was measured on the reproductive output of males and females evolving under monogamous and promiscuous regimes. Males and females from each of the monogamous and promiscuous regimes were challenged with heat-killed bacterial mixture of a gram-negative bacterium *Escherichia coli*, and a gram-positive bacterium, *Micrococcus luteus*. This study reported that males evolving under the promiscuous regime, whether bacterially challenged or not, were reproductively more successful in terms of progeny production than males from the monogamous regime (Nystrand et al. 2018). Also, no effect of selection was observed on the reproductive success of females between monogamous and promiscuous regimes.

While there is growing evidence for phenotypic trade-offs between immunity and post-copulatory reproductive traits, evolutionary consequences of immune system activation in response to sexual selection are less well documented. Therefore, for this study, I used laboratory populations of *Drosophila melanogaster* populations evolving under differential levels of sexual selection. One of the previous studies on the same populations has shown that there is no significant effect of selection on survivorship between males under higher sexual selection regime, i.e. male-biased regime (M), and males under lower sexual selection regime, i.e. female-biased regime (F) after being immune challenged by live *Pseudomonas entomophila* (Syed et al. 2020). Additionally, it has been shown that M males have evolved to invest more in sexually selected traits (copulation duration, sperm defense ability and

courtship frequency) than the F males (Nandy et al. 2013a). Since sexual selection promotes any trait that increases the reproductive fitness of males even at a cost to survival, I predicted that M males would not invest much into immune traits and might continue their investment towards reproductive functions even though being challenged with heat-killed bacteria. On the other hand, the predictions about the response of females to the immune response activation are not straightforward. If immune traits in males and females are positively correlated, I would expect the M females to invest less in immune traits and therefore maintain their reproductive output. However, it has been reported that M females invest more in mate harm resistance mechanisms (Nandy et al. 2014). But, it is not yet reported if there are any costs of maintaining these mate harm resistance mechanisms and whether these mechanisms as a by-product, can also protect against pathogen infections. Therefore, the effect of immune activity in females can also be influenced by the balance of these costs and benefits.

Furthermore, in several promiscuous species, males and females are expected to allocate resources in different ways, as the fitness of males is usually limited by the number of matings, whereas female fitness is predicted by the number of offspring produced during a lifetime (Bateman 1948; Trivers 1972; Clutton-Brock et al. 1988; Rolff and Siva-Jothy 2002). Consistent with this, various studies have documented the scope for sex differences to appear in immune responses in vertebrates (Nunn et al., 2009; Klein and Flanagan, 2016) as well as invertebrates (Nunn et al. 2009; Kelly et al. 2018). Nevertheless, it is still not well understood which sex should be more susceptible to immune challenge. Henceforth, to test this idea, we used virgin males and females from male-biased (M) and female-biased (F) regimes of *Drosophila melanogaster* populations and challenged them with the heat-killed *Pseudomonas entomophila* and assayed their reproductive investment. Using this experimental framework, we particularly asked whether -

1. There is any trade-off between reproductive investment and immune response in males and females of male-biased and female-biased regimes?
2. There are any sex-specific differences in reproductive investment pattern in response to infection with heat-killed bacteria in these two regimes?

To answer these questions, we set up an experimental design where both male and female flies from two regimes with contrasting levels of sexual selection were first challenged with a heat-killed gram-negative bacterium *Pseudomonas entomophila* and then tested for their reproductive performance in comparison to control flies which were not subjected to any kind of pathogen exposure.

Materials and methods

Bacterial culture

In this study, we used a gram-negative bacterium *Pseudomonas entomophila* L48 (Vodovar et al. 2005) for infecting the adult flies. This bacterium was isolated from wild-caught *Drosophila*. To prepare the bacterial suspension for infections, we grew an overnight primary bacterial culture at $27^{\circ C}$ and 147rpm (Pe) till OD = 1.0 ± 0.1 from a glycerol stock maintained at -80^{oC}. Next morning, we used primary culture to set up a fresh secondary culture that was allowed to grow for 3-4 hours (to ensure that the bacterial cells are in the actively growing phase). After this, bacterial culture was suspended into the centrifuge, and bacterial cells were pelleted down and homogenised in an equal volume of 10 mM MgSO4 to prepare final OD = 1.0 ± 0.1 before infection. For infections, the bacterium was heat-killed by putting microcentrifuge tubes containing bacterial slurry in a water bath for 30 minutes at 72^{0C}. Heat-induced inactivation of bacteria was assured by plating bacterial solutions on LB agar followed by overnight incubation at 27^{0C}. On the next morning, LB agar plates were scanned

for any bacterial colonies. Absence of bacterial colonies on plates confirmed the heat inactivation of bacteria.

Experimental protocol Experiment was conducted in the following sequential manner

1) Egg collection - Before egg collection, M and F populations were passed through one generation of standardisation. For standardisation, these flies were maintained in ancestral conditions for one generation to rule out the possibility of any non-genetic parental effects affecting the results. For the generation of experimental flies, eggs were collected from each of the M and F populations at densities of 150 eggs per vial containing 8-10 ml cornmeal-molasses yeast food. We also collected eggs from the LHst population at densities of 150 eggs per vial to generate males and females to be used in the mating trials.

2) Virgin collection – On 9-10th day post egg-collection, virgin males and females were collected from M, F and LHst population using light CO_2 anaesthesia and were placed in single sex vials containing standard cornmeal- molasses food at a density of 8 flies /vial.

3) Infecting the flies –On the 12th day, male and female flies from M and F regimes were anaesthetized using light CO_2 gas and randomly assigned to one of the three treatments with a sample size of 35 vials per treatment. Males and females from LHst population were only anaesthetised and separated and kept as individually into new food vials.

Three kinds of experimental treatment were

(a) Infected (I),where M and F regime flies were infected in the thorax (Gupta et al. 2013) by pricking with a needle (Minutein pin 0.1 mm, Fine Science Tools, CA) dipped in bacterial (non-infectious/heat-killed) suspension (bacteria suspended in 10 mM MgSO4).

(b) Sham(S) infected in which flies were pricked with a needle dipped in sterile 10mM MgSO4 solution, and these flies served as injury controls.

(c) Unhandled (U) or control where flies were not exposed to pricking and only subjected to anaesthesia.

Reproductive assays for males – Mating latency, copulation duration and competitive fertilization assay

After infection, flies were allowed to rest for a refractory period of 6 hours. After 6 hours, males from each selection regime and within each treatment were combined with a single LHst female and observed for mating latency and copulation duration.

For determining the competitive fertilization success of males, four males from each treatment of the M and F regime were placed with eight LH males (red-eye colour marker) and eight LHst females per vial. These vials were kept undisturbed for two days, and after two days all the males were discarded, and single females were transferred to test tubes (12 mm × 75 mm) to lay eggs for a window of 18 hours. After 18 hours, females were discarded, and after 12 days, progeny which came out of these eggs, were scored for eye colour to determine the competitive fertilization success of males.

Reproductive assay for females – Fecundity assay

After 6 hours of infection with heat-killed bacteria, females from each selection regime and each treatment at a density of 8 females /vial were combined with LHst males at a density of 10 males /vial. Five such vials were set up for each of the six treatments (making a total of 8×5=40 data points for each treatment). Mating of all the eight females in the vials was assured by observing them, and as soon as mating ended, males were removed, and females were transferred individually into fresh food vials and were provided with a window of 18 hrs for egg-laying. After 18 hrs, females were disposed of and eggs in each of 6 kinds of treatments were counted under a microscope.

Statistical Analysis

All analyses were completed in the R version 3.5.2, using the “lme4” (Bates et al. 2014) and “lmerTest” (Kuznetsova et al. 2017) packages. The dependent variable for the male model was the mating latency (ML), copulation duration (CD) and competitive fertilization success (CFS). For females, the dependent variable was fecundity, and for both the male and female models, the explanatory variables were the “selection treatment” (low vs high sexual selection), “bacterial treatment”, and all possible interactions between these effects. Block was used as the random factor, while selection and treatment were fixed factors. Data for each of the traits (mating latency, copulation duration, competitive fertilization and fecundity) were analyzed using a mixed model analysis of variance (ANOVA) using Satterthwaite’s method. Post-hoc Tukey's HSD tests were performed using the R package "emmeans"(Lenth et al. 2020). The results for each sex were analyzed separately because the reproductive traits measured in males and females were not directly comparable and were obtained from different assays.

Results

Selection affected the correlation between immune function and reproductive investment in males.

For males, three reproductive traits were assayed – Mating latency, copulation duration and competitive fertilization success. In the case of mating latency results, we found a significant effect of treatment (Figure.1, Table.1) as males from I treatment from both M and F regimes took longer to mate with the females as compared to the other two treatments, whereas no effect of selection was found and both kinds of males spent an almost similar amount of time to gain access to matings. Also, no interaction was found between selection and bacterial treatment.

For copulation duration, a significant effect of selection and selection × treatment interaction was observed (Figure.2, Table.2). However, no significant effect of treatment was observed. We reported that males of infected (I) treatment from the M regime were not different from sham and unhandled treatments. On the other hand, F regime males from infected (I) treatment mated for the lowest duration and differed significantly from sham and unhandled treatments of F regime males. Further, there was no difference between sham and unhandled treatment males for M and F regime males.

In the case of competitive fertilization success, we found a significant effect of selection and selection× treatment interaction (Figure.3, Table.3), but the effect of treatment was not significant. There was no significant difference among the three treatments within the M regime. On the other hand, F regime males showed a similar pattern of investment as of copulation duration as in F regime males from I treatment showed the lowest competitive fertilization success. Also, no significant difference in competitive fertilization was observed between sham and unhandled treatment in the case of males from each of the regimes.

Females showed a similar pattern of reproductive investment after infection with the heat-killed bacteria irrespective of selection regimes.

In females, a significant effect of selection and treatment was observed for fecundity (Figure.4, Table.4). In both M and F regime females, the number of eggs laid decreased because of heat-killed bacterial challenge and fecundity observed was higher in the case of F regime females in all the treatments in comparison with M regime females. There was no difference between sham and unhandled treatment for F as well as M regime females. No significant effect of selection × treatment interaction was found.

Table.1 Summary of results from the ANOVA analysis on mating latency of males using selection and bacterial treatment as the fixed factors crossed with each other and with block as the random factor. Statically significant values ($p<0.05$) are marked with '*' and shown in bold.

Type III Analysis of Variance Table with Satterthwaite's method						
	Sum Sq	Mean Sq	NumDF	DenDF	F value	Pr(>F)
Selection	6.768	6.768	1	1.9964	0.8337	0.4577
Treatment	197.711	98.855	2	8.0974	12.177	**0.0036 ****
Selection×Treatment	3.999	2.000	2	8.0974	0.2463	0.7873
	Npar	logLik	AIC	LRT	Df	Pr(>Chisq)
<none>	11	-1391	2805.4			
(1 |Block)	10	-1391	2803.4	0.0243	1	0.8760
(1 |Block×Selection)	10	-1392	2804.2	0.7920	1	0.3735
(1|Block×Selection×Treatment)	10	-1393	2806.5	3.1377	1	0.0765
(1 |Block×Treatment)	10	-1391	2803.4	0.0000	1	0.9996

Table.2 Summary of results from the ANOVA analysis on copulation duration of males using selection and bacterial treatment as the fixed factors crossed with each other and with block as the random factor. Statically significant values ($p<0.05$) are marked with '*' and shown in bold.

Type III Analysis of Variance Table with Satterthwaite's method						
	Sum Sq	Mean Sq	NumDF	DenDF	F value	Pr(>F)
Selection	169.58	169.58	1	10.104	28.245	**0.0003*** **
Treatment	42.61	21.30	2	10.106	3.5487	0.0680
Selection×Treatment	147.93	73.96	2	10.104	12.320	**0.0019** **
	Npar	logLik	AIC	LRT	Df	Pr(>Chisq)
<none>	11	-1304	2631.7			
(1 |Block)	10	-1305	2631.3	1.5702	1	0.2102
(1 |Block×Selection)	10	-1304	2629.7	0.0000	1	1.0000
(1|Block×Selection×Treatment)	10	-1305	2630.8	1.0828	1	0.2981
(1 |Block×Treatment)	10	-1304	2629.7	0.0000	1	1.0000

Table.3 Summary of results from the ANOVA analysis on the competitive fertilisation success of males using selection and bacterial treatment as the fixed factors crossed with each other and block as the random factor. Statistically significant values ($p<0.05$) are marked with '*' and shown in bold.

Type III Analysis of Variance Table with Satterthwaite's method						
	Sum Sq	Mean Sq	NumDF	DenDF	F value	Pr(>F)
Selection	0.5975	0.5975	1	3.87	13.0763	**0.02372 ***
Treatment	0.4468	0.2234	2	4.44	4.8890	0.07553
Selection×Treatment	0.3525	0.1762	2	525.74	3.8579	**0.02171 ***
	Npar	logLik	AIC	LRT	Df	Pr(>Chisq)
<none>	11	48.777	-75.553			
(1|Block)	10	48.777	-77.553	0.0000	1	1.0000
(1 |Block×Selection)	10	46.955	-73.910	3.6430	1	0.0563
(1|Block×Selection×Treatment)	10	48.777	-77.550	0.0000	1	1.0000
(1 |Block×Treatment)	10	48.630	-77.261	0.2926	1	0.5886

Table.4 Summary of results from the ANOVA analysis on the fecundity of females using selection and bacterial treatment as the fixed factors crossed with each other and with block as the random factor. Statically significant values ($p<0.05$) are marked with '*' and shown in bold.

Type III Analysis of Variance Table with Satterthwaite's method						
	Sum Sq	Mean Sq	NumDF	DenDF	F value	Pr(>F)
Selection	5929.6	5929.6	1	2.0173	337.7	**0.0028****
Treatment	4489.2	2244.6	2	3.9767	127.8	**<0.001*****
Selection×Treatment	107.6	53.8	2	3.9609	3.063	0.1570
	Npar	logLik	AIC	LRT	Df	Pr(>Chisq)
<none>	11	-1621	3265.2			
(1|Block)	10	-1621	3263.3	0.0640	1	0.8003
(1 |Block×Selection)	10	-1621	3263.4	0.1290	1	0.7195
(1|Block×Selection×Treatment)	10	-1621	3263.2	0.0021	1	0.9633
(1 |Block×Treatment)	10	-1621	3263.4	0.1568	1	0.6920

Figure.1 Effect of heat-killed bacterial treatment on mating latency of M and F regime males. Dark grey boxes represent males from F populations, whereas light grey boxes indicate males from M populations (experiment 1). Boxplots indicate median and interquartile range. Whiskers indicate variability outside the upper and lower quartiles. Dots represent outliers.

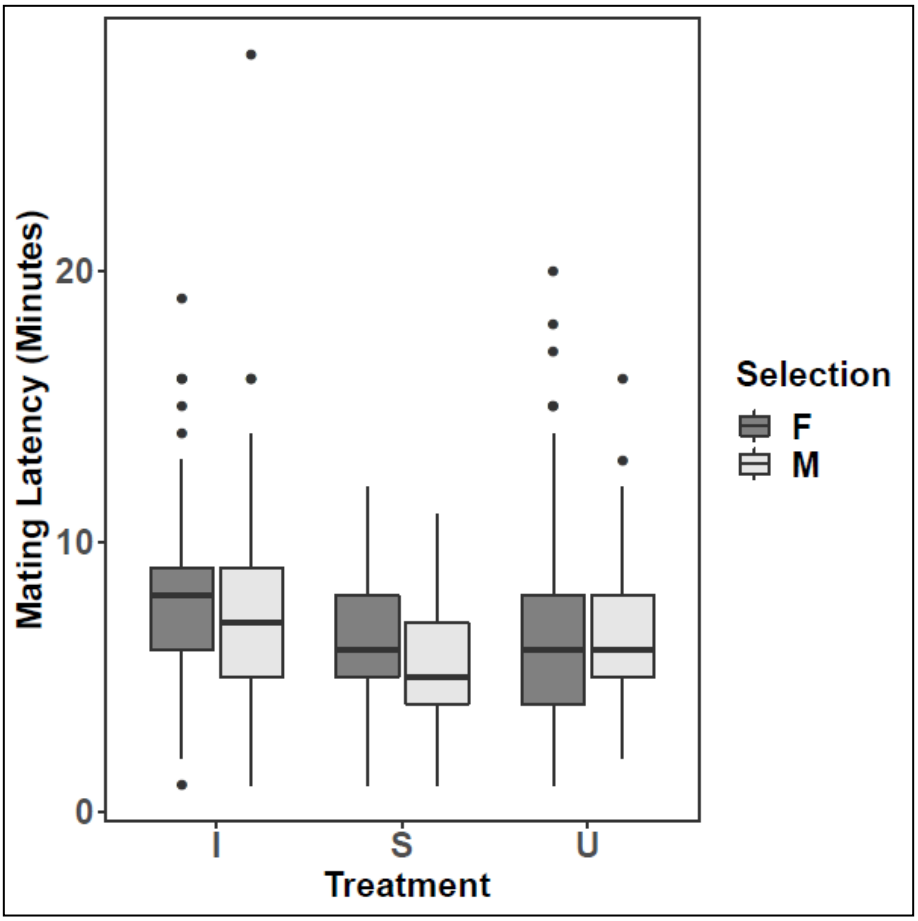


Figure.2 Effect of heat-killed bacterial treatment on copulation duration of M and F regime males. Dark grey boxes represent males from F populations, whereas light grey boxes indicate males from M populations. Boxplots indicate median and interquartile range. Whiskers indicate variability outside the upper and lower quartiles. Dots represent outliers.

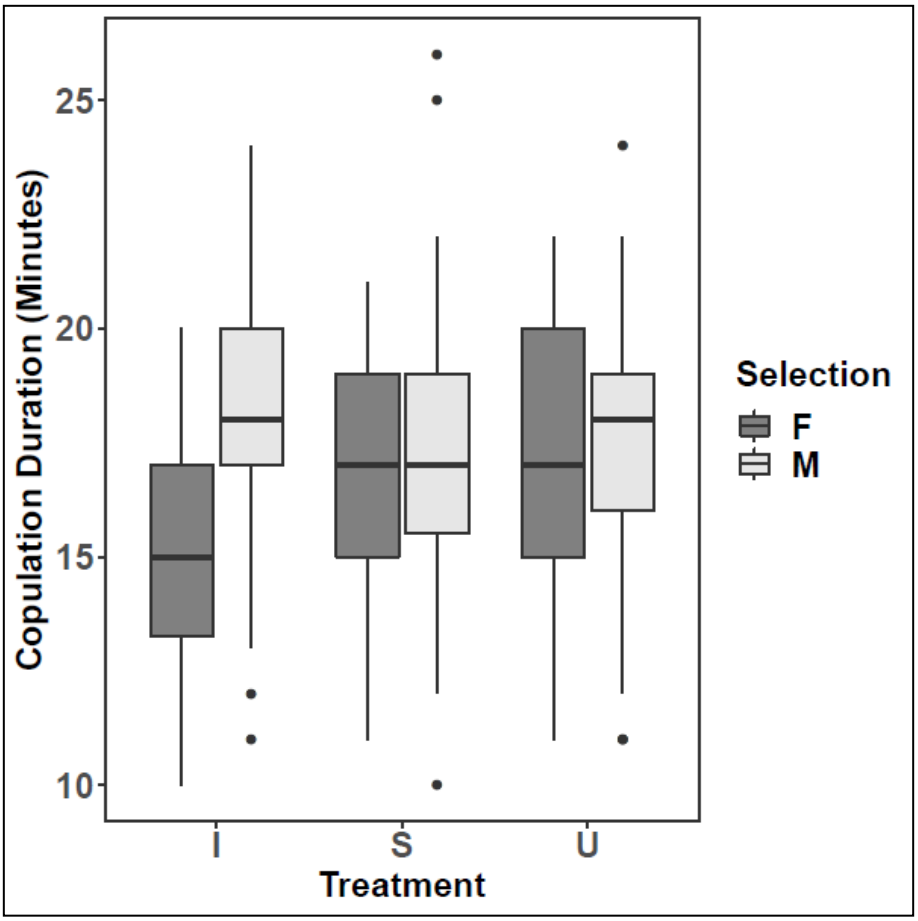


Figure.3 Effect of heat-killed bacterial treatment on competitive fertilization success of M and F regime males. Dark grey boxes represent males from F populations, whereas light grey boxes indicate males from M populations. Boxplots indicate median and interquartile range. Whiskers indicate variability outside the upper and lower quartiles. Dots represent outliers.

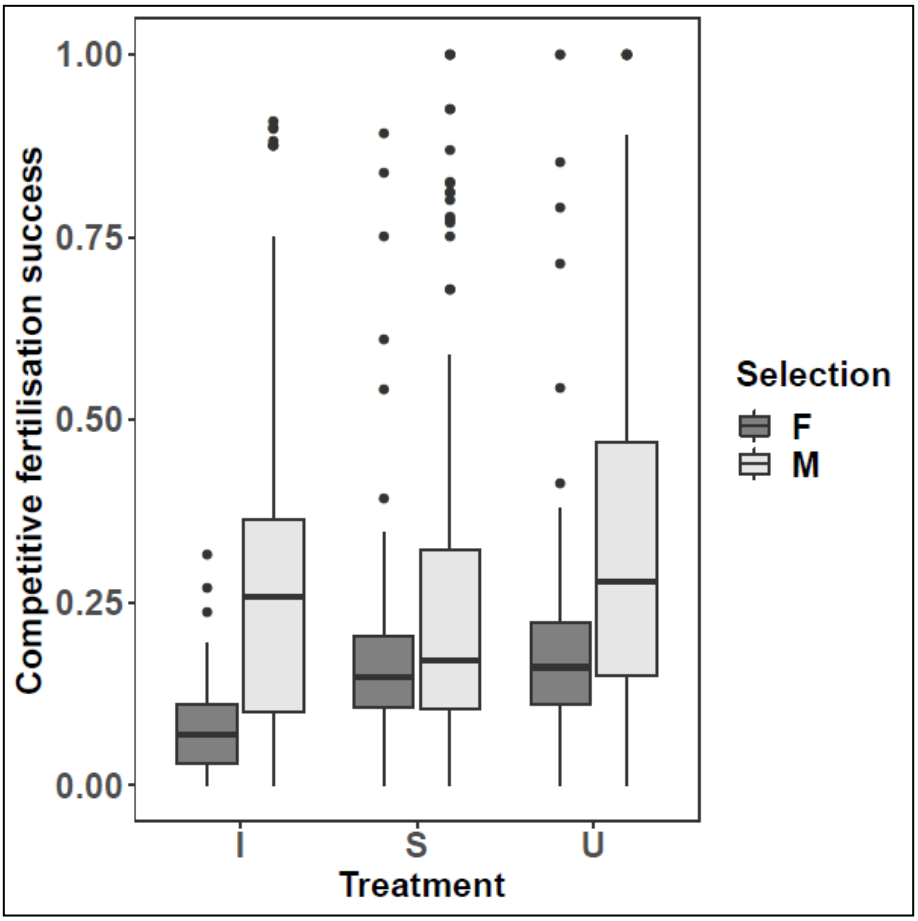
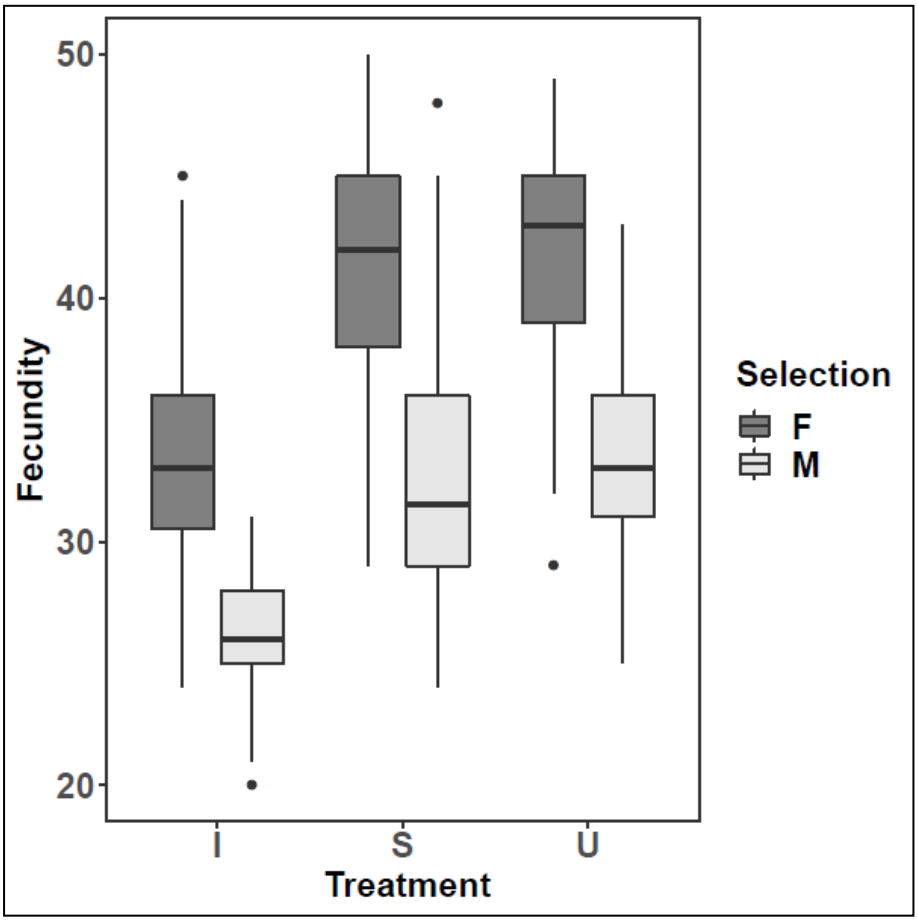


Figure.4 Effect of heat-killed bacterial treatment on the fecundity of M and F regime females. Dark grey boxes represent males from F populations, whereas light grey boxes indicate males from M populations. Boxplots indicate median and interquartile range. Whiskers indicate variability outside the upper and lower quartiles. Dots represent outliers.



Discussion

In the present study, I tried to explore the relationship between immunological function and sexually selected traits by examining the effect of heat-killed infection with *Pseudomonas entomophila* on reproductive performance of *Drosophila melanogaster* males and females evolving under contrasting levels of sexual selection. Males under higher sexual selection (M population males) maintained their reproductive performance following exposure to heat-killed bacteria. Males from the lower sexual selection regime (F population males) showed a decrease in reproductive performance when exposed to heat-killed bacteria. In the case of females, both M and F regime females suffered a decline in their fecundity after infection with heat-killed bacteria. Therefore, through this study I report that the association between reproductive investment and investment in immune traits is significantly influenced by the evolutionary history of the organisms.

Sexually selected traits and immune traits are both costly to produce and are expected to trade-off with each other (Folstad and Karter 1992; Zuk and Stoehr 2002; McKean et al. 2008; Dowling and Simmons 2012). All else being equal, males from both M and F populations can be expected to decrease their reproductive performance post-exposure to heat-killed bacteria. We found that F regime males infected with heat-killed bacteria showed a decline in their reproductive performance compared to F regime males subjected to sham and uninfected treatments. But, interestingly, males from M populations did not show a decline in their reproductive performance after exposure to heat-killed bacteria as compared to M males subjected to sham and uninfected treatments. Many previous studies have reported adverse effects of pathogen exposure on individual traits related to male reproductive performance. For example, in decorated crickets, *Gryllodes sigillatus*, immune challenged males produced significantly smaller sized spermatophores; in field crickets, *Telegryllus oceanicus* (Simmons 2012) and *D. melanogaster* (Radhakrishnan and Fedorka

2012), a negative correlation was observed in sperm viability and immune system activation. In our study, we found that the males from M populations did not suffer a decrease in either progeny production or the component traits (copulation duration) post-infection with heat-killed bacteria. However, the F males did suffer a decline in progeny production, and component traits (mating latency and copulation duration) post-infection with heat-killed bacteria.

The difference in the response of M and F males to heat-killed bacterial challenge is probably due to the difference in sexual selection experienced by these two kinds of males. In the M regime, there are three males to every female, and hence these males experience intense intrasexual selection (compared to F males), leading to the evolution of males with increased investment in reproductive performance. Indeed, previous studies on these populations show that M males have evolved to have increased sperm defense ability (P1) and increased copulation duration than the F regime males (Nandy et al. 2013a,b). Thus, M regime males have evolved to invest more in reproduction. Therefore, it is possible that M regime males, in general, channel their resources into reproduction even under conditions of immune challenge. Our results for M regime males are in agreement with the results from Nystrand et al.(2018) where heat-killed infection did not result in a decrease in reproductive output of males that were evolving under higher sexual selection (Nystrand et al. 2018) and Barthel et al. (2015) where in moths, *Heliothis virescens*, the mating success of males was not affected when immune challenged with entomopathogenic bacterium *Serratia entomophila.* (Barthel et al. 2015)

Unlike males, no different patterns of reproductive effort were observed between females of M and F regimes. Females from both the M and F regimes showed a decline in their fecundity on exposure to heat-killed bacteria. Fecundity of females from infected treatments from each of the M and F regimes was depressed when compared to females from sham and

uninfected treatment within each of the respective regimes. However, the overall fecundity of F regime females was higher than M regime females in case of all the treatments. Previous studies have shown that F females have higher fecundity compared to M females post single mating (Nandy et al. 2014), and our results are in agreement with that of Nandy et al. (2014). The decline in fecundity post-infection with heat-killed bacteria indicates that both kinds of females suffered a cost of immune activation. These results are consistent with the previous studies that show a decrease in fecundity of females in response to immune activation in *D. melanogaster* (McKean et al. 2008; Bashir-Tanoli and Tinsley 2014; Nystrand et al. 2018). In this study, the absence of selection × bacterial treatment interaction for the fecundity of M and F females indicates that the association between reproduction and immunity is not dependent upon the evolutionary history of females.

Noticeably, we observed that within the M regime, males and females responded differently in terms of reproductive investment to heat-killed infection. In response to immune system activation, M males maintained their reproductive investment, whereas M females showed a reduction in their fecundity. From an evolutionary point of view, since males and females have divergent life histories, strategies for resource investment for achieving an optimal immune response following infection can be expected to be sex-specific (Folstad and Karter 1992; Rolff 2002; Zuk and Stoehr 2002). Though males and females were assayed for different reproductive traits that are not comparable but from the results, we can conclude that sexual selection shaped a different kind of correlation between reproductive investment and immune function among males and females of the M regime.

In conclusion, in this study, we report here that there is an association between immune response and reproductive efficiency and in the case of males, this relation appears to be dependent upon evolutionary history. We report evidence of a trade-off between immunity and reproduction in the case of males as well as females under the lower load of sexual

selection and females under higher sexual selection pressure. Although there are various evidence of phenotypic trade-offs between investments in immunity and reproduction, but this study demonstrates the evolution of sex-specific responses to immune activation in the regime under a higher level of sexual selection. Under higher sexual selection, the reproductive performance of males was not affected, whereas females suffered a decline in reproductive performance after infection with heat-killed bacteria. However, we propose that the kind of responses observed in response to immune activation may depend on the type of immune challenge used, the kind of traits addressed, and the conditions under which the traits are quantified.

Chapter-6

Sexual conflict and the evolution of maternal effects

Introduction

Typically, in many promiscuous species, females are forced to mate beyond their optimum by the males. The increased mating rate has been shown to have adverse effects on females at physiological as well as the morphological level (Arnqvist and Nilsson 2000; Crudgington and Siva-Jothy 2000; Gavrilets et al. 2001; Blanckenhorn et al. 2002). However, multiple mating is universal in most animal species in nature. To elucidate the existence of polyandry despite its substantial costs, various adaptive explanations have been suggested. These may be divided into those in which costs of polyandry in females are balanced through direct benefits (parental care, nuptial gifts or access to territories) and others in which the female is benefited indirectly through the elevated fitness of her offspring principally through good genes processes (Jennions and Petrie 2000; Birkhead and Pizzari 2002). In a wide range of taxa, it has been shown that indirect benefits could result in elevated offspring viability and attractiveness, genetic heterogeneity and phenotypic diversity (Tregenza and Wedell 2002; Foerster et al. 2003; Head et al. 2005; Rundle et al. 2007; Barbosa et al. 2010; Garcia-Gonzalez and Simmons 2010; Gowaty et al. 2010). On the contrary, some studies suggest that the probability of indirect genetic benefits to outweigh the direct costs suffered by females is very low (Cameron et al. 2003) and this has been established by a range of empirical studies (Arnqvist and Nilsson 2000), such as in the common lizard *Lacerta vivipara* (Marquis et al. 2008) and in fruit fly *D. melanogaster* (Brommer et al.2012; Orteiza et al. 2005; Stewart et al. 2008).

Recent studies suggest that the effects of multiple mating by the females can spill over to the subsequent generations through non-genetic maternal or paternal or paternally induced maternal effects (García-González and Simmons 2007; Priest et al. 2008; Brommer et al. 2012; Gasparini et al. 2012; Dowling et al. 2014; Zajitschek et al. 2018). Priest et al. (2008) showed paternally induced benefits of multiple mating for the females in terms of increase in

daughter fitness. They showed that daughters sired by the mothers who were exposed to multiple mating with the males who lacked production of main cell Acps (accessory gland proteins) as well as those sired by mothers who were exposed to multiple mating with sterile males (unable to produce Acps) had higher fecundity than the daughters sired by singly mated mothers. Conversely, some of the studies demonstrate that direct costs of multiple mating to females can be aggravated by transgenerational costs and harm from multiple matings can span the future generations also (Gasparini et al. 2012; Dowling et al. 2014), or may give rise to antagonistic effects across different generations (Brommer et al. 2012). More interestingly, such transgenerational effects might be mediated through the non-sire mates of the female also (Garcia-Gonzalez and Dowling 2015). Taken together, these results clearly suggest the occurrence of transgenerational effects of maternal sexual history, even though the evidence for the role of such effects in maintaining polyandry is equivocal.

Given that non-genetic effects related to maternal sexual history have important consequences for progeny fitness, it is not unreasonable to expect such effects to evolve, given suitable variation and selection. A previous study shows the occurrence of such non-genetic, transgenerational effects in a baseline population (LHm bw) that is closely related to our baseline population (LH st) (Garcia-Gonzalez and Dowling 2015). Thus, it is possible that such effects are present in our baseline populations also as our selection M and F regimes are derived from the LHst baseline population. In our M and F populations, the females experience very different sexual environments every generation. The M females are expected to be multiply mated and are expected to experience greater male harassment (relative to F females). If the female mating rate (or the total number of mating per female) has consequences for progeny fitness, given that mating rates are very different in the M and F females, we would expect such effects to evolve differently in the M and F populations. For example, if multiple mating by females decreases offspring fitness, all else being equal, we

would expect selection to act against such effects through the evolution of mechanisms to ameliorate such effects. Alternatively, if such maternal effects enhance the fitness of the offspring, we would expect selection to favour such effects.

To test this, I subjected mothers from both male-biased and female-biased regimes to variable sexual environments to see if maternal selection history and maternal mating history influences offspring fitness. Also, since some studies have reported that maternal effects can be sex-specific (Walzer and Schausberger 2015; Nystrand et al. 2016), I also looked for any sex-specific maternal effects between male and female offspring.

Particularly I asked the following questions-

1. Do maternal effects due to differential maternal sexual history affect offspring fitness in our populations?
2. Does the evolutionary history of females (evolving under high versus low mating rate environments) affect such maternal effects?
3. Are there any sex-specific effects of maternal treatment on offspring fitness?

Materials and methods

Generating experimental flies

Before generating experimental flies, one generation of standardization of populations was followed to eliminate the possibility of any unwanted non-genetic potential parental effects to influence the results. During standardization, populations were maintained in ancestral conditions excluding virgin collection and the sex ratio was not manipulated. Egg collection was done on the same day at a density of 150 eggs/vial for each of the M regime, F regime and LHst population. After egg collection, eggs from all the populations were incubated under standard laboratory conditions.

Maternal treatments- On the 9th-10th day post egg collection, virgin females were collected from the M and F regimes at a density of 8 females/vial and virgin males were collected from the LHst regime at a density of 10 males/vial. After that, on the 12th day post egg collection, virgin females from M and F regimes were combined with virgin LHst males. This was done by combining one vial of females from M/F regimes with one vial of LHst males into fresh food vials. For each of the M and F females, 20 such vials were set up, out of which ten vials were assigned to single mating (SM) treatment, and the other ten vials were subjected to multiple mating (MM) treatment. Taken together, we had four types of parental crosses -

a) M♀_SM × LHst♂
b) M♀_MM × LHst♂
c) F♀_SM × LHst♂
d) F♀_MM × LHst♂

After combination, males and females were allowed to mate only once for single mating treatment. Single mating was assured by continuously observing the vials. In each of the ten vials set up for single mating treatment, all the females mated only once successfully. After the mating ended, females were retained while males were discarded using light CO_2 anaesthesia. After this, females from single mating treatment were held in single-sex vials and transferred to fresh food vials every alternate day until the 4th day after the treatment.

For multiple mating treatment, females and males were kept together in the vials for four days. These flies were transferred to fresh food vials every alternate day till the 4th day from the start of the treatment. On the 4th day, females from both the single mating and multiple mating treatments were transferred to oviposition vials and allowed to oviposit for a window of 18 hours. After 18 hours, females were discarded, and numbers of eggs were trimmed down to a density of 150 eggs/vial in the same way as the stock populations. On the same day, egg collection was done at a density of 150 eggs/vial from the LHst population to

generate males and females for mating trials with the female and male offspring and from the LH population to use the males for sperm defense ability assay in the contest with the male offspring. These eggs were then incubated under standard laboratory conditions. The progeny eclosing from these eggs were collected as virgins on the 9-10th day and were assayed on the 12th day for reproductive traits.

Reproductive traits assay

Male offspring were assayed for mating latency, copulation duration and sperm competitive ability. On the 12th day, post egg collection, virgin males generated from all the four types of crosses were combined with single virgin LHst females in individual vials and observed manually for mating latency (ML) and copulation duration (CD). For sperm defense ability (P1) assay females were first allowed to mate with the focal males. After the end of mating, these males were discarded, and females were then combined with LH males (red-eye colour) and kept undisturbed with them for two days. After two days, LH males were disposed, and single females were transferred to test tubes (12 mm × 75 mm) for oviposition. After 12 days, when all the progenies had eclosed, these were scored for eye colour to check for sperm defense ability.

Female offspring from all four parental crosses were assayed for fecundity. On the 9th-10th day post egg collection, female offspring were collected as virgins. On the 12th day post egg-collection, virgin female offspring were allowed to interact with LHst males in food vials with 8 females and 10 males per vial for 1 hour. Five such vials were set up for the female offspring from each of the four types of parental crosses. In about a period of one hour, all the female offspring from different kinds of maternal treatments mated successfully with the LHst males. Single mating was assured by continuous observation of the vials. On the same day, after mating, males were discarded, and female offspring were transferred to fresh food vials and allowed to oviposit for a window of 18 hours. After 18 hours, i.e. on the 13th day

post egg -collection, female offspring were discarded, and the vials with the eggs were frozen at -80^{0}C and counted later under a microscope to determine the early life fecundity of female offspring.

Statistical analysis

All analyses were performed in the R version 3.5.2. Reproductive traits of males offspring (ML, CD and P1) and fecundity of females offspring were analysed using the “lme4” (Bates et al. 2014) and “lmerTest” (Kuznetsova et al. 2017) packages. Data for each of the traits (mating latency, copulation duration, sperm defense ability and fecundity) were analyzed using a mixed model analysis of variance (ANOVA) with treatment and selection as fixed factors crossed with blocks as a random factor. Post-hoc Tukey's HSD tests were performed using the R package "emmeans" (Lenth et al. 2018)

Results

Reproductive fitness of male offspring was not influenced by maternal selection history and maternal treatment

No effect of selection, treatment or interaction between selection and treatment was observed for any of the three reproductive traits quantified- mating latency (ML) (Figure.1, Table.1), copulation duration (CD) (Figure.2, Table.2) and sperm defense ability (P1) (Figure.3, Table.3). Also, no interaction with random factors was observed.

Selection and maternal treatment affected reproductive fitness of female offspring

For fecundity, selection and selection × treatment interaction significantly affected the fecundity of female offspring (Figure.4, Table.4) but no significant effect of treatment was observed. Overall, female offspring from F mothers had greater fecundity than M regime mothers. Female offspring sired by F regime mothers from single mating treatment had the highest fecundity; it was significantly greater than fecundity of female offspring sired by F regime as well as M regime mothers from multiple mating treatment and singly mated M

regime mothers. Fecundity of female offspring sired by M and F mothers from single mating treatment and M mothers from multiple mating treatment were not significantly different from each other.

Table.1 Summary of results of mixed-model ANOVA on mating latency of sons generated from the crosses- (a) M♀_SM × LHst♂ (b) M♀_MM × LHst♂ (c) F♀_SM × LHst♂(d) F♀_MM × LHst♂ and mated with baseline LHst females, treating selection regime and treatment as the fixed factor crossed with block as a random factor. Significant values ($p<0.05$) are shown in bold.

Type III Analysis of Variance Table with Satterthwaite's method						
	Sum Sq	Mean Sq	NumDF	DenDF	F value	Pr(>F)
Selection	18.150	18.150	1	2.00	3.3225	0.2099
Treatment	18.095	18.095	1	352.09	3.3126	0.0696
Selection×Treatment	2.8807	2.8807	1	352.06	0.5273	0.4682
	Npar	logLik	AIC	LRT	Df	Pr(>Chisq)
<none>	9	-817.3	1652.7			
(1|Block)	8	-817.5	1651.1	0.3993	1	0.5274
(1 |Block×Selection)	8	-817.3	1650.7	0.0006	1	0.9812
(1|Block×Selection×Treatment)	8	-817.3	1650.7	0.0000	1	1.0000
(1 |Block×Treatment)	8	-817.3	1650.7	0.0000	1	1.0000

Table.2 Summary of results of mixed-model ANOVA on copulation duration of sons generated from the crosses- (a) M♀_SM × LHst♂ (b) M♀_MM × LHst♂ (c) F♀_SM × LHst♂(d) F♀_MM× LHst♂ and mated with baseline LHst females, treating selection regime and treatment as the fixed factor crossed with block as the random factor. Significant values ($p<0.05$) are shown in bold.

Type III Analysis of Variance Table with Satterthwaite's method

	Sum Sq	Mean Sq	NumDF	DenDF	F value	Pr(>F)
Selection	6.3417	6.3417	1	1.9999	1.7863	0.3132
Treatment	4.7996	4.7996	1	3.9897	1.3519	0.3097
Selection×Treatment	0.3094	0.3094	1	3.9897	0.0872	0.7825

	Npar	logLik	AIC	LRT	Df	Pr(>Chisq)
<none>	9	-745.7	1509.4			
(1|Block)	8	-746.1	1508.3	0.8831	1	0.3473
(1 |Block×Selection)	8	-745.8	1507.6	0.1869	1	0.6655
(1|Block×Selection×Treatment)	8	-746.7	1509.5	2.1068	1	0.1466
(1 |Block×Treatment)	8	-745.7	1507.4	0.0000	1	1.0000

Table.3 Summary of results of mixed-model ANOVA on sperm defense ability(P1) of sons generated from the crosses- (a) M♀_SM × LHst♂ (b) M♀_MM × LHst♂ (c) F♀_SM × LHst♂(d) F♀_MM × LHst♂ and mated with baseline LHst females, treating selection regime and treatment as the fixed factor crossed with random blocks. Significant values ($p<0.05$) are shown in bold.

Type III Analysis of Variance Table with Satterthwaite's method						
	Sum Sq	Mean Sq	NumDF	DenDF	F value	Pr(>F)
Selection	0.0045	0.0045	1	2	0.0729	0.8125
Treatment	0.1376	0.1376	1	2	2.1967	0.2765
Selection×Treatment	0.0058	0.0058	1	350	0.0935	0.7599
	Npar	logLik	AIC	LRT	Df	Pr(>Chisq)
<none>	9	-24.43	66.865			
(1|Block)	8	-25.08	66.175	1.30912	1	0.2526
(1 |Block×Selection)	8	-24.49	64.989	0.12318	1	0.7256
(1|Block×Selection×Treatment)	8	-24.43	64.865	0.00000	1	1.0000
(1 |Block×Treatment)	8	-24.67	65.339	0.47401	1	0.4912

Table.4 Summary of results of mixed-model ANOVA on the fecundity of daughters generated from the crosses- (a) M♀_SM × LHst♂ (b) M♀_MM × LHst♂ (c) F♀_SM × LHst♂(d) F♀_MM × LHst♂ and mated with baseline LHst males, treating selection regime and treatment as the fixed factor crossed with random blocks. Significant values ($p<0.05$) are shown in bold.

Type III Analysis of Variance Table with Satterthwaite's method						
	Sum Sq	Mean Sq	NumDF	DenDF	F value	Pr(>F)
Selection	409.74	409.74	1	4.017	16.990	**0.0144***
Treatment	162.09	162.09	1	4.014	6.7214	0.0603
Selection×Treatment	444.60	444.60	1	4.017	18.435	**0.0125***
	Npar	logLik	AIC	LRT	Df	Pr(>Chisq)
<none>	9	-1453	2925.8			
(1|Block)	8	-1453	2923.8	0.00049	1	0.9823
(1 |Block×Selection)	8	-1453	2923.8	0.00000	1	1.0000
(1|Block×Selection×Treatment)	8	-1454	2925.1	1.30849	1	0.2527
(1 |Block×Treatment)	8	-1453	2923.8	0.00049	1	0.9823

Figure.1 Effect of maternal treatment on the mating latency of sons produced from different crosses-(a) M♀_SM × LHst♂ (b) M♀_MM × LHst♂ (c) F♀_SM × LHst♂ (d) F♀_MM × LHst♂ and mated with baseline LHst females. Dark grey boxes indicate sons from the F regime, and light grey boxes indicate sons from the M regime. Boxplots indicate median and interquartile range. Whiskers indicate variability outside the upper and lower quartiles. Dots represent outliers.

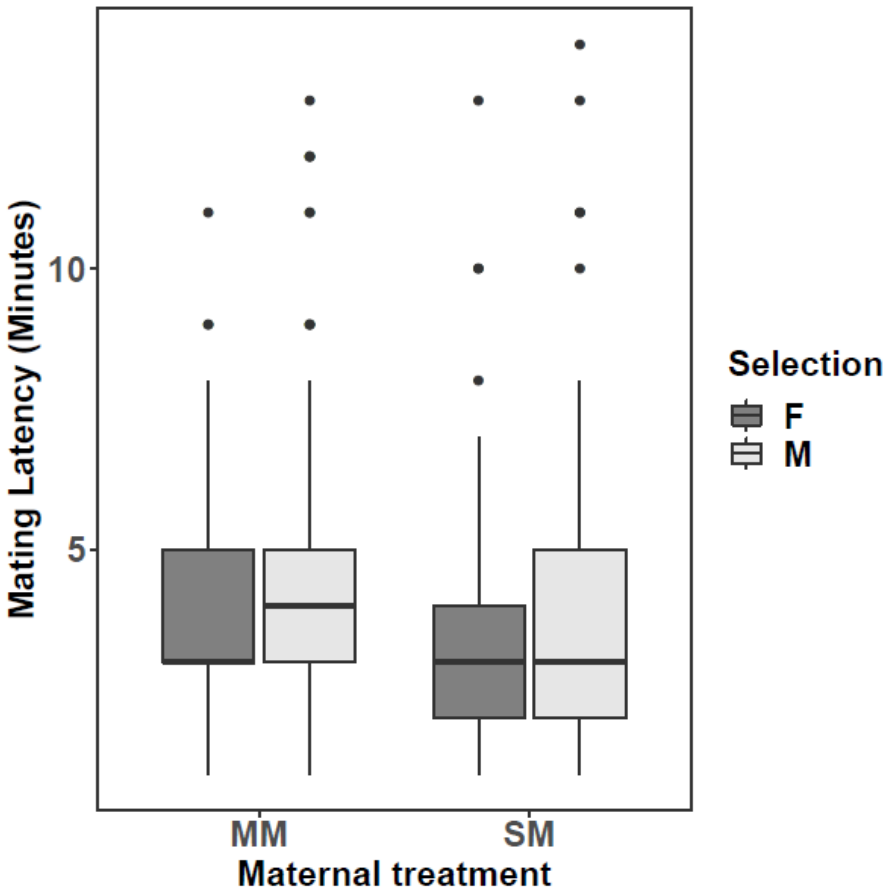


Figure.2 Effect of maternal treatment on the copulation duration of sons produced from different crosses-(a) M♀_SM × LHst♂ (b) M♀_MM × LHst♂ (c) F♀_SM × LHst♂(d) F♀_MM × LHst♂ and mated with baseline LHst females. Dark grey boxes indicate sons from the F regime, and light grey boxes indicate sons from the M regime. Boxplots indicate median and interquartile range. Whiskers indicate variability outside the upper and lower quartiles. Dots represent outliers.

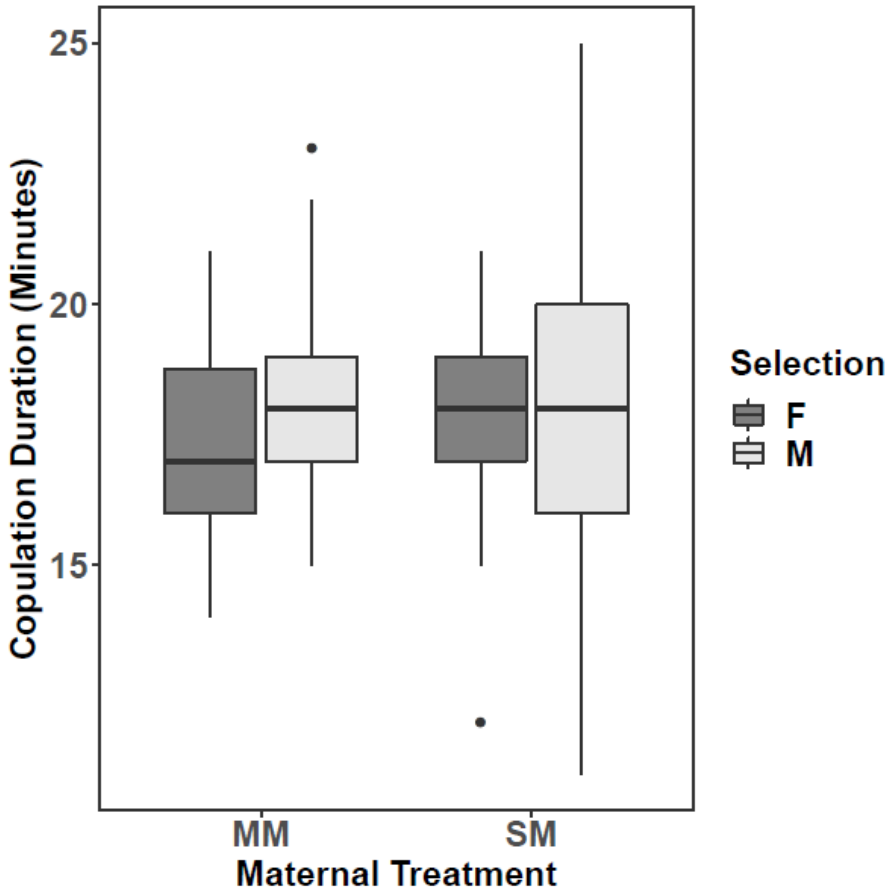


Figure.3 Effect of maternal treatment on the sperm defense ability of sons produced from different crosses-(a) M♀_SM × LHst♂ (b) M♀_MM × LHst♂ (c) F♀_SM × LHst♂(d) F♀_MM × LHst♂ and mated with baseline LHst females. Dark grey boxes indicate sons from the F regime, and light grey boxes indicate sons from the M regime. Boxplots indicate median and interquartile range. Whiskers indicate variability outside the upper and lower quartiles. Dots represent outliers.

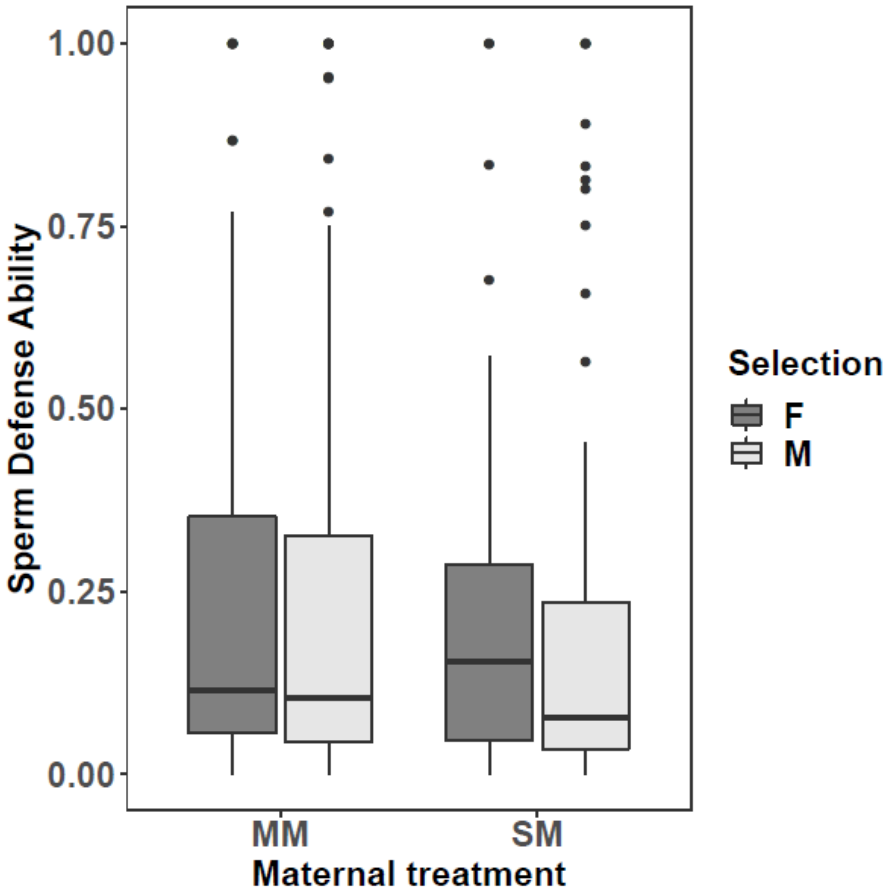
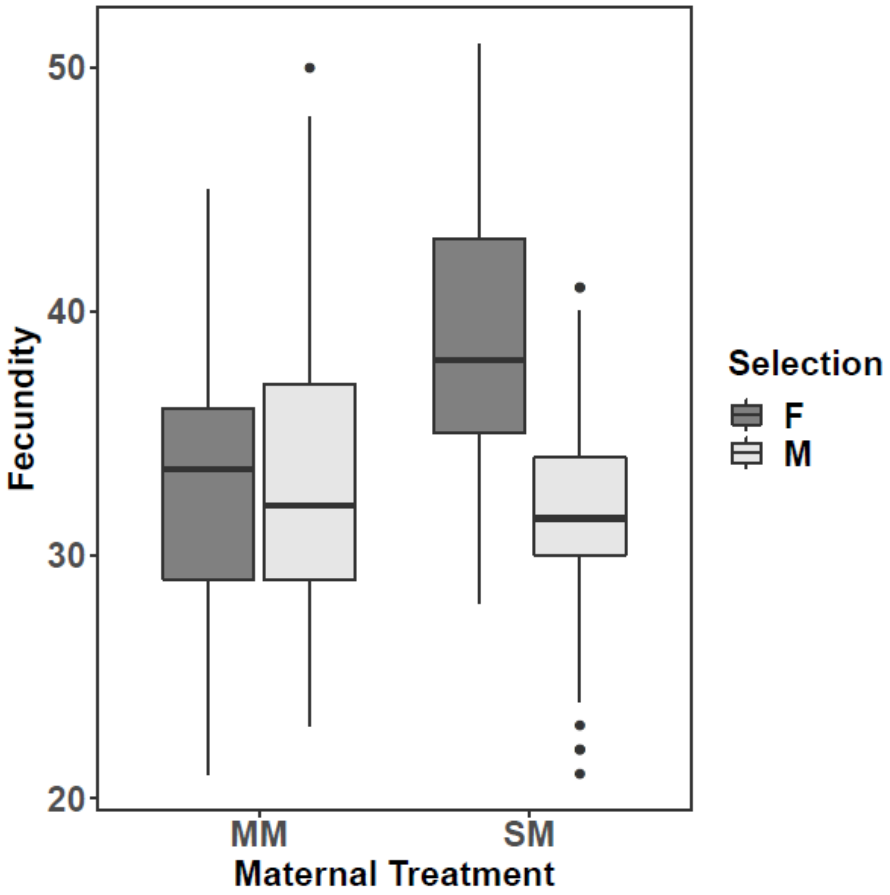


Figure.4 Effect of maternal treatment on the fecundity of daughters produced from different crosses-(a) M♀_SM × LHst♂ (b) M♀_MM × LHst♂ (c) F♀_SM × LHst♂(d) F♀_MM × LHst♂ and mated with baseline LHst males. Dark grey boxes indicate daughters from the F regime, and light grey boxes indicate daughters from the M regime. Boxplots indicate median and interquartile range. Whiskers indicate variability outside the upper and lower quartiles. Dots represent outliers.



Discussion

Multiple mating has been documented to be harmful to females in a diverse variety of taxa (McKinney et al. 1983; Arnqvist 1989; Fowler and Partridge 1989; Burpee and Sakaluk 1993; Crudgington and Siva-Jothy 2000; Moore et al. 2001). Recently, it has been suggested that multiple mating can be adaptive for females in terms of indirect benefits to future generations. While testing this idea, some studies have reported positive transgenerational effects on offspring fitness components in response to increased maternal, sexual interactions (Konior et al. 2001; Head et al. 2005; Fisher et al. 2006; Rundle et al. 2007; Priest et al. 2008; Taylor et al. 2008; Garcia-Gonzalez and Simmons 2010; Firman and Simmons 2012), whereas a few studies have also reported negative effects for offspring fitness as a result of multiple mating by females (Brommer et al. 2012; Gasparini et al. 2012; Dowling et al. 2014). In the present study, I attempted to test this idea using *D. melanogaster* females evolving under male-biased and female-biased regimes to see the effect of maternal mating history on offspring fitness. I showed that the fitness of the daughters in terms of fecundity, sired by females from populations under higher sexual selection, was not reduced when their mothers were exposed to multiple mating treatment compared to daughters sired by singly mated females. In contrast, daughters of females from populations under lower sexual selection suffered the costs of multiple mating by the mothers and showed a decline in fecundity. Additionally, for sons, we did not find any effect of multiple mating and selection history of mothers on their reproductive fitness. Thus, my results find no evidence in support of the hypothesis that multiple mating can be adaptive in terms of indirect fitness benefits to the females (in terms of increase in offspring fitness) as multiple mating did not result in an increase in fitness of daughters or sons from any type of females (M or F). On the contrary, daughters sired by F regime females from multiple mating treatment suffered a decline in fecundity. Also, we found a significant interaction between maternal treatment and maternal

selection history for female offspring fecundity. As per my knowledge, this is the first experimental evidence to show the evolution of maternal effects themselves in response to sexual selection.

In the case of female offspring, different responses (in terms of fecundity) to maternal sexual history were observed between the daughters sired by M and F regime mothers. There can be two different possibilities for the observed results. One possibility could be that our results were driven by the differences in the evolutionary history of M and F regime females and maternal effects have themselves evolved in the respective regimes per se. According to maintenance protocol, males and females of M and F regimes are combined on the 12^{th} day post egg collection according to their respective sex ratio regimes and are provided a time of 2 days for sexual interactions. Sex-ratio for the F regime is 1 male: 3 female with 24 females and 8 males in a vial. Thus, during a period of two days, most of the F females probably get a chance to mate only once. So, F females have little or no exposure to multiple mating throughout their evolutionary history. On the contrary, the sex-ratio for the M regime is 3 male: 1 female, i.e. there are 8 females and 24 males in a vial for two days. Therefore, almost every M female is more likely to get more than one mating in that period of two days. Hence, M females are exposed to multiple mating every generation. Since M and F mothers experience different levels of sexual environment every generation, it is possible that these experience-based socio-sexual cues are passed on to the offspring by M and F mothers leading to the evolution of maternal effects. Accordingly, we observed no additional costs of multiple mating for female offspring sired by M regime mothers subjected to multiple mating treatment, whereas a decline in the fecundity of female offspring sired by F regime mothers exposed to multiple mating.

Alternatively, these results could also be the outcome of the evolution of differential abilities to sustain the mate harm between the M and F regime females. As explained above, M

females are exposed to more number of males every generation than F females. Also, M males have evolved to be more mate harming than the LHst males and F regime males. Hence, M females are subjected to mate harm every generation. Therefore, it is clear that M females are exposed to a harsher sexual environment than F females every generation. As a consequence of this, M females have been shown to evolve mate harm resistance (Nandy et al. 2014). In the experimental setup, since LHst males were used for mating trials with M and F regime females, it is possible that LHst males would have appeared benign than M males to the M females. Moreover, in the maintenance regime, 8 M females are housed with 24 M males in a single vial, whereas in the experimental treatment, 10 LHst males and 8 M females were housed per vial. Thus, it is possible that experimental multiple mating treatment was not that much harsher for M females and therefore, the daughter fitness was not affected when these females were subjected to multiple mating treatment. On the contrary, F females are not exposed to mate harm throughout their evolutionary history. Additionally, the ability to resist mate harm has degenerated in F regime females (Nandy et al. 2014). Therefore, multiple mating treatment would have been intensely harsher for F regime females resulting in a decrease in offspring fitness. Unfortunately, our results cannot distinguish between these two possibilities. But, it is clear that maternal effects are influenced by the evolutionary history of mothers. Further, as it has been suggested that there is an optimal mating rate for female insects (Arnqvist and Nilsson 2000), it is possible that this optimal mating rate is different for M and F regime females. Multiple mating by F regime females might have crossed the optimum level of mating to the extent that negative effects of multiple mating spanned the F1 generation also.

Maternal sexual history has been shown to affect the reproductive fitness of sons. Multiple mating by the mothers resulted in fitness benefits to the sons in the house cricket *Acheta domesticus* (Head et al. 2005) and *D. melanogaster* (Rundle et al. 2007). However, in a study

in seed beetles, instead of sons from multiple mating treatment, sons of monandrous females showed higher reproductive fitness in terms of increased sperm competitive ability (Hook 2018). In the present study, we did not find any effect of maternal treatment and selection history of mothers on reproductive fitness of sons. There was no difference in mating latency, copulation duration and sperm defense ability between the sons sired by M and F regime females from single mating or multiple mating treatment. Given that along with non genetic effects, parents can also have genetic influence on offspring phenotype, one of the possibilities for the observed affect on sons could be the genetic contribution from the maternal and paternal genome. On population level, it has been shown that M males have evolved to have increase reproductive investment (in terms of increased CD and P1) than the F regime males. In this study, we did not observe any difference in reproductive investment by males offspring sired by M and F regime females when mated with LHst males. This suggests the role of paternal genome on the reproductive success of sons. It is possible that beneficial alleles responsible for male mating success are recessive and sons sired by M♀/LHst♂, and F♀/LHst♂ are heterozygous for the same allele. This could be the reason that we did not find any difference in the reproductive success of sons. Also, the pattern of reproductive success in the case of female offspring sired by M and F regime females suggest that alleles responsible for female fitness are dominant and are therefore expressed in daughters sired by M and F regime females.

Further, theory suggests that maternal effects can be sex-specific, and mothers can invest differently in sons and daughters. For instance, in mites, *Phytoseiulus persimilis* and *Neoseiulus californicus,* females are of larger body size than males. When mothers from *Phytoseiulus persimilis* and *Neoseiulus californicus* were exposed to food stress, it resulted in variation in offspring sex ratios and mothers were found to invest more in producing sons than the daughters and the body size of the daughters was also smaller (Walzer and

Schausberger 2015). In *D. melanogaster*, when mothers were immune challenged with a mixture of non-infectious pathogens –*Escherichia coli.* and *Micrococcus luteus*, they produced daughters who showed decreased offspring viability compared to daughters sired by control females, whereas no effect of maternal treatment was observed on the reproductive success of sons in terms of offspring viability (Nystrand et al. 2016). In our study, daughters and sons produced by M regime females subjected to single mating treatment and multiple mating treatment did not show any difference in the pattern of their reproductive success. However, daughters and sons produced by the F regime females under multiple mating treatment showed sex-specific responses in their reproductive investment. Daughters sired by harassed mothers from the F regime showed decreased fecundity, whereas sperm defense ability and copulation duration of males was not affected due to maternal treatment. As of now, I cannot speculate any exact mechanism that can explain the evolution of sex-specific maternal effects in sons and daughters of F regime females, but it is clear that maternal effects can shape the fitness of the offspring.

In conclusion, this study highlights the importance of selection history along with the mating history of mothers in shaping the offspring phenotype. I document here that sexual selection imposes a baseline cost on the females but no additional costs of maternal sexual harassment for the offspring. Through this study, I show that difference in maternal evolutionary trajectories induced by the variable intensity of sexual selection and conflict can lead to the evolution of maternal effects.

Chapter-7

Conclusion

Asymmetry in the evolutionary interests of males and females sets the stage for the evolution of sexual conflict. These conflicts can occur over relative parental effort, mating frequency, female remating behaviour, fertilization, female reproductive rate, clutch size, etc. The model of intersexual co-evolution through sexual conflict chase-away selection has gained a lot of attention because of its contribution in commencing and propelling the open-ended arms race between the sexes. Both sexes are predicted to evolve a range of sexually antagonistic adaptations that favours the outcome towards their own interests (Holland and Rice 1998). There is substantial evidence for the role of sexual conflict in the evolution of a suite of life history and reproductive traits in both sexes. Despite our astounding success so far, there remains much to be accomplished in the sexual selection and conflict research area, and a lot of questions still needs to be addressed.

In this thesis, I predicted that in addition to the direct implications of sexual selection and conflict theories (which have been tested by many studies), there could be some indirect implications of sexual selection and conflict on evolutionary dynamics of reproductive traits. To address these indirect consequences, I used the approach of laboratory experimental evolution. The operational sex ratio of the populations was varied to accomplish differential levels of sexual selection, thereby changing the level of male-male competition. Male- biased (M) regime is under higher level of sexual selection, and female-biased (F) regime is under lower level of sexual selection. Here, I present some distinct and novel outcomes of the effect of sexual selection and conflict on reproductive traits of males (such as mating latency (ML), copulation duration (CD), and (P1) sperm defense ability) and females which have not been explored yet. In this chapter, I will summarize this thesis's unique findings and discuss how sexual selection is involved in making room for their origination.

Major findings of the thesis are summarised as follows:

Not only the presence but the numbers and identity of competitors also matters

Sexual conflict theories suggest that intersexual conflict along with sexually antagonistic selection imposes intense intrasexual selection on males at both pre and post-copulatory level (Bateman 1948) Parker 1979; Simmons 2001; Crudgington et al. 2009). My results show that the males evolving under differential levels of sexual selection and hence different intensities of sperm competition evolved different kinds of reproductive investment pattern (in terms of ML, CD and P1) in response to variable socio-sexual environment experienced early in life. My results are, majorly, in agreement with those of Edward et al. (2010) and Dore et al. (2021), indicating that in general, male reproductive investment patterns in response to rivals' presence can diverge between populations evolving under male-biased or female-biased sex-ratios. However, my study showed that along with presence, the density and identity of rivals are also important factors that can signal the varying levels of sperm competition and hence can shape the reproductive investment patterns in males. The most plausible explanation for the difference in the responses between M and F regime males could be that sensitivity to the number and identity of competitors has itself evolved in these regimes. M males were better at sensing the changes in the density as well as the identity of competitors to fine-tune their reproductive investment with the changing environment.

Sexual selection and conflict affected the memory retention of early life competitive cues

One of the novel findings of my thesis is that males under different levels of sexual conflict retained the cues experienced from early life competitive cues for different times. Males under higher sexual conflict (M males) retained the same reproductive investment pattern (in terms of CD) in response to early life competitive cues for a longer time (3 days after the removal of competitive cues) than their lower selection counterparts (F males). This is the 1st

comprehensive evidence of the effect of sexual selection on the memory retention of competitive cues by males.

M males evolved to become inherently better at assessing the mating status of females

Identifying appropriate mating partners is a critical factor that can affect a male's reproductive success (Ejima et al. 2005). Courtship conditioning is a phenomenon that enables males to analyse the mating status of females and modify their courtship behaviour accordingly (Siegel and Hall 1979). My results suggest that males from both the M and F regimes were equally good at learning from their prior experience with unreceptive females. Both the M and F regime males from conditioned treatment directed more of their courtship towards the receptive females when they were presented with both receptive and unreceptive females. Also, conditioned males took longer to initiate courtship, but were faster in starting the mating, again indicating the effect of courtship learning/conditioning. However, M regime males were better at discriminating between receptive and unreceptive females and directing courtship towards the receptive female than the F regime males in both conditioned and unconditioned treatments. It indicates that M males, in general are better at recognising receptive females.

One of the potential explanations for the observed results could be the difference in complexity of the sexual environment experienced by M and F males in terms of exposure to females of different receptivities driven by different levels of intersexual conflict. M males are exposed to a greater number of unreceptive females in their maintenance regime conditions; hence it is possible that M males, in order to increase their reproductive success, have evolved to discriminate between receptive and unreceptive females. Therefore, this study shows that along with courtship displays intersexual conflict can drive the evolution of innate abilities in males to successfully recognise the appropriate mating partners, which is an important phenomenon which can contribute to increase the reproductive fitness of males.

Under higher sexual selection and conflict, M males evolved to invest more in reproduction even under the conditions of immune system activation

In this study, males under the higher level of sexual conflict did not show a decline in their reproductive performance following exposure with heat-killed bacteria (*Pseudomonas entomophila*). However, males under lower levels of sexual conflict showed decreased reproductive performance upon infection with the same heat-killed bacteria. On the other hand, both kinds of females, i.e. female from regime under higher levels of intersexual conflict and lower levels of sexual conflict, showed a drop in their fecundity following infection with heat-killed bacteria. My results showed that in the males, the relationship between the reproductive performance and immune response was shaped by sexual conflict. In response to the higher level of intersexual conflict, males have been shown to evolve increased investment in reproduction. Thus, it is possible that males that have evolved to invest more in reproductive activities continue to invest in the same even in the face of challenges such immune system activation.

Sex-specific resource investment strategies evolved in the males and females under higher intersexual conflict

It has been suggested that sexual conflict can lead to the evolution of sexual dimorphism (Zuk 1990; Zuk and McKean 1996; Rolff 2002). In the present study, I show sex-specific responses in terms of different patterns of reproductive performance following exposure to heat-killed bacteria between males and females evolving under male-biased regime. Theories suggests that since males and females have divergent evolutionary interests, resource investment strategies for achieving an optimal immune response following infection can be expected to be sex-specific (Folstad and Karter 1992; Rolff 2002; Zuk and Stoehr 2002). Through this study, I present a piece of empirical evidence that sexual conflict can model a

different kind of correlation between reproductive investment and immune function among males and females.

Evolving under increased intersexual conflict diluted the costs of multiple mating by mothers on the offspring fitness

This study highlights the contribution of the evolutionary history of mothers in the evolution of maternal effects and their influence on offspring phenotype. To my knowledge, this is the 1st empirical evidence to show the evolution of maternal effects in response to the increased levels of intersexual conflict. Sexual harassment through multiple mating did not result in a decrease in the fitness of sons and daughters sired by mothers under male-biased regime. Contrarily, the fitness of daughters sired by mothers subjected to multiple mating treatment from female-biased regime was decreased, whereas fitness of the sons was not affected sired by the same mothers. Females from male-biased regimes are subjected to multiple mating and mate harm every generation, whereas, females from female-biased regimes are not much exposed to multiple mating or mate harm in the course of their evolutionary history. Therefore, it is possible that cues for the sexual harsher environment from M mothers are passed on to their offspring. Consequently, offspring of M mothers possibly are primed for the sexual harsher environment that resulted in no adverse effect of multiple mating by M mothers on offspring fitness in this study. Whereas in daughters sired by F, females who did not experience such cues in their selection history suffered a cost of multiple mating by their mothers. I also observed sex-specific effect of maternal sexual treatment only on daughters and sons of females evolving under female-biased regime. Currently, we cannot explain any exact reason for these sex-specific responses; however, it is clear that maternal effects are the primary cause for the observed responses. Therefore, through this study, I highlight the importance of sexual conflict in driving the evolution of such transgenerational maternal effects.

In conclusion, through this thesis, I attempted to uncover some novel and extended implications of sexual selection and conflict on the reproductive traits of males and females which have not been previously addressed. I used experimental evolution to answer the questions that have not been explored yet (such as the evolution of plastic responses in male reproductive behaviour in response to sexual conflict, the evolution of maternal effects) and investigated ideas that are disputed with unclear results (like the role of sexual conflict in shaping the relationship between reproductive traits and immune traits and evolution of courtship learning in males under different levels of sexual selection). In the end, I sincerely hope my thesis will contribute a fresh insight towards sexual selection and conflict research area and will be useful to understand the complexity of this interesting subject.

Literature cited

Ahmed, A. M., S. L. Baggott, R. Maingon, and H. Hurd. 2002. The costs of mounting an immune response are reflected in the reproductive fitness of the mosquito *Anopheles gambiae*. Oikos 97:371–377.

Ahtiainen, J. J., R. V. Alatalo, R. Kortet, and M. J. Rantala. 2005. A trade-off between sexual signalling and immune function in a natural population of the drumming wolf spider *Hygrolycosa rubrofasciata*. Journal of Evolutionary Biology 18:985-991.

Andersson, M. 1994. Sexual Selection. Princeton University Press, Princeton, New Jersey.

Andersson, M., and Y. Iwasa. 1996. Sexual selection. Trends in ecology & evolution 11:53–58.

Arnqvist, G. 1989. Multiple mating in a water strider: mutual benefits or intersexual conflict? Animal Behaviour 38:749–756.

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

Arnqvist, G., and L. Rowe. 2002. Antagonistic coevolution between the sexes in a group of insects. Nature Publishing Group. Nature 415:787–789.

Arnqvist, G., and L. Rowe. 2005. Sexual conflict. Princeton University Press.

Arnqvist, G., and L. Rowe. 1995. Sexual conflict and arms races between the sexes: a morphological adaptation for control of mating in a female insect. Proceedings of the Royal Society of London. Series B: Biological Sciences 261:123–127.

Barbosa, F., D. Rebar, and M. D. Greenfield. 2016. Reproduction and immunity trade-offs constrain mating signals and nuptial gift size in a bushcricket. Behavioral Ecology 27:109–117.

Barbosa, M., M. Dornelas, and A. E. Magurran. 2010. Effects of polyandry on male phenotypic diversity. Journal of Evolutionary Biology 23:2442–2452.

Barthel, A., H. Staudacher, A. Schmaltz, D. G. Heckel, and A. T. Groot. 2015. Sex-specific consequences of an induced immune response on reproduction in a moth. BMC Evolutionary Biology 15:282.

Bascuñán-García, A. P., C. Lara, and A. Córdoba-Aguilar. 2010. Immune investment impairs growth, female reproduction and survival in the house cricket, *Acheta domesticus*. Journal of Insect Physiology 56:204–211.

Bashir-Tanoli, S., and M. C. Tinsley. 2014. Immune response costs are associated with changes in resource acquisition and not resource reallocation. Functional Ecology 28:1011–1019.

Basolo, A. L. 1990. Female preference predates the evolution of the sword in swordtail fish. Science 250:808–810.

Basolo, A. L., and J. A. Endler. 1995. Sensory biases and the evolution of sensory systems. Trends in Ecology & Evolution 10:489.

Bateman, A. J. 1948. Intra-sexual selection in *Drosophila.* Heredity 2:349–368.

Bates, D., M. Mächler, B. Bolker, and S. Walker. 2014. Package Lme4: Linear Mixed-Effects Models Using Eigen and S4.

Baur, J., J. d'Amour Nsanzimana, and D. Berger. 2019. Sexual selection and the evolution of male and female cognition: A test using experimental evolution in seed beetles*. Evolution 73:2390–2400.

Bennett, A. F. 2003. Experimental evolution and the Krogh principle: generating biological novelty for functional and genetic analyses. Physiological and Biochemical Zoology 76:1–11.

Birkhead, T. R., and T. Pizzari. 2002. Postcopulatory sexual selection. Nature Reviews Genetics 3:262–273.

Bjork, A., W. T. Starmer, D. M. Higginson, C. J. Rhodes, and S. Pitnick. 2007. Complex interactions with females and rival males limit the evolution of sperm offence and defesne. Proceedings of the Royal Society B: Biological Sciences 274:1779–1788.

Blanckenhorn, W. U., D. J. Hosken, O. Y. Martin, C. Reim, Y. Teuschl, and P. I. Ward. 2002. The costs of copulating in the dung fly *Sepsis cynipsea*. Behavioral Ecology 13:353–358.

Boogert, N. J., R. C. Anderson, S. Peters, W. A. Searcy, and S. Nowicki. 2011. Song repertoire size in male song sparrows correlates with detour reaching, but not with other cognitive measures. Animal Behaviour 81:1209–1216.

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

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

Bretman, A., J. D. Westmancoat, M. J. G. Gage, and T. Chapman. 2011b. Males use multiple, redundant cues to detect mating rivals. Current Biology 21:617–622.

Brommer, J. E., C. Fricke, D. A. Edward, and T. Chapman. 2012. Interactions between genotype and sexual conflict environment influence transgenerational fitness in *Drosophila melanogaster.* Evolution 66:517–531.

Brown, W. D., A. Bjork, K. Schneider, and S. Pitnick. 2004. No evidence that polyandry benefits females in *Drosophila melanogaster*. Evolution; international journal of organic evolution 58:1242–1250.

Burpee, D. M., and S. K. Sakaluk. 1993. Repeated matings offset costs of reproduction in female crickets. Evolutionary Ecology 7:240–250.

Cameron, E., T. Day, and L. Rowe. 2003. Sexual conflict and indirect benefits. Journal of Evolutionary Biology 16:1055–1060.

Candolin, U., and J. D. Reynolds. 2002. Why do males tolerate sneakers? Tests with the European bitterling, *Rhodeus sericeus*. Behavioral Ecology and Sociobiology 51:146–152.

Carazo, P., E. Font, and E. Desfilis. 2007. Chemosensory assessment of rival competitive ability and scent-mark function in a lizard, *Podarcis hispanica.* Animal Behaviour 74:895–902.

Chapman, T., L. F. Liddle, J. M. Kalb, M. F. Wolfner, and L. Partridge. 1995. Cost of mating in *Drosophila melanogaster* females is mediated by male accessory gland products. Nature Publishing Group. Nature 373:241–244.

Chechi, T. S., Z. A. Syed, and N. G. Prasad. 2017. Virility does not imply immensity: Testis size, accessory gland size and ejaculate depletion pattern do not evolve in response to experimental manipulation of sex ratio in *Drosophila melanogaster.* Journal of insect physiology 98:67–73.

Chippindale, A. K., J. R. Gibson, and W. R. Rice. 2001. Negative genetic correlation for adult fitness between sexes reveals ontogenetic conflict in *Drosophila.* Proceedings of the National Academy of Sciences 98:1671–1675.

Civetta, A., and A. G. Clark. 2000. Correlated effects of sperm competition and postmating female mortality. Proceedings of the National Academy of Sciences 97:13162–13165.

Clutton-Brock, T. H., D. Green, M. Hiraiwa-Hasegawa, and S. D. Albon. 1988. Passing the buck: resource defesne, lek breeding and mate choice in fallow deer. Behavioral Ecology and Sociobiology 23:281–296.

Cook, R., and K. Connolly. 1973. Rejection responses by female *Drosophila melanogaster:* their ontogeny, causality and effects upon the behaviour of the courting male. Behaviour 44:142–165.

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

Crudgington, H. S., S. Fellows, N. S. Badcock, and R. R. Snook. 2009. Experimental manipulation of sexual selection promotes greater male mating capacity but does not alter sperm investment. Evolution 63:926–938.

Crudgington, H. S., and M. T. Siva-Jothy. 2000. Genital damage, kicking and early death. Nature 407:855–856.

Darwin, C. 1871. The Descent of Man and Selection in Relation to Sex. John Murray, London.

Darwin's, C. 1859. On the origin of species. published on 24.

Dawkins, R. 1986. The Blind Watchmaker, WW. W. Norton& Co., New York.

Dore, A. A., W. G. Rostant, A. Bretman, and T. Chapman. 2021. Plastic male mating behavior evolves in response to the competitive environment. Evolution 75:101–115.

Dowling, D. K., and L. W. Simmons. 2012. Ejaculate economics: Testing the effects of male sexual history on the trade-off between sperm and immune function in Australian crickets. Plos one 7:e30172.

Dowling, D. K., B. R. Williams, and F. Garcia-Gonzalez. 2014. Maternal sexual interactions affect offspring survival and ageing. Journal of Evolutionary Biology 27:88–97.

Dukas, R. 2010. Causes and consequences of male-male courtship in fruit flies. Animal Behaviour 80:913–919.

Dukas, R. 2004. Evolutionary biology of animal cognition. Annual review of ecology, evolution, and systematics 35:347–374.

Dukas, R. 2005a. Experience improves courtship in male fruit flies. Animal Behaviour - 69:, 1203-1209.

Dukas, R. 2005b. Learning affects mate choice in female fruit flies. Behavioral Ecology 16:800–804.

Dunbar, R. I. M. 1998. The social brain hypothesis. Evolutionary Anthropology: Issues, News, and Reviews 6:178–190.

Eberhard, W. G. 1996. Female Control: Sexual Selection by Cryptic Female Choice. Princeton University Press, Princeton.

Edward, D. A., C. Fricke, and T. Chapman. 2010. Adaptations to sexual selection and sexual conflict: insights from experimental evolution and artificial selection. Philosophical Transactions of the Royal Society B: Biological Sciences 365:2541–2548.

Ejima, A., B. P. C. Smith, C. Lucas, J. D. Levine, and L. C. Griffith. 2005. Sequential learning of pheromonal cues modulates memory consolidation in trainer-specific associative courtship conditioning. Current Biology 15:194–206.

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

Firman, R. C., and L. W. Simmons. 2012. Male house mice evolving with post-copulatory sexual selection sire embryos with increased viability. Ecology Letters 15:42–46.

Fisher, D. O., M. C. Double, S. P. Blomberg, M. D. Jennions, and A. Cockburn. 2006. Post-mating sexual selection increases lifetime fitness of polyandrous females in the wild. Nature Publishing Group. Nature 444:89–92.

Foerster, K., K. Delhey, A. Johnsen, J. T. Lifjeld, and B. Kempenaers. 2003. Females increase offspring heterozygosity and fitness through extra-pair matings. Nature 425:714–717.

Folk, D. G., and T. J. Bradley. 2004. The evolution of recovery from desiccation stress in laboratory-selected populations of *Drosophila melanogaster*. Journal of Experimental Biology 207:2671–2678.

Folstad, I., and A. J. Karter. 1992. Parasites, bright males, and the immunocompetence handicap. The American Naturalist 139:603–622.

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

Friberg, U. 2006. Male perception of female mating status: its effect on copulation duration, sperm defesne and female fitness. Animal Behaviour 72:1259–1268.

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

Gage, M. J. 1991. Risk of sperm competition directly affects ejaculate size in the Mediterranean fruit fly. Animal Behaviour 42:1036–1037.

Gage, M. J.., and R. R. Baker. 1991. Ejaculate size varies with socio-sexual situation in an insect. Ecological Entomology 16:331–337.

Gailey, D. A., J. C. Hall, and R. W. Siegel. 1985. Reduced reproductive success for a conditioning mutant in experimental populations of *Drosophila melanogaster*. Genetics 111:795–804.

Gailey, D. A., F. R. Jackson, and R. W. Siegel. 1982. Male courtship in *Drosophila*: the conditioned response to immature males and its genetic control. Genetics 102:771–782.

Gallone, B., J. Steensels, T. Prahl, L. Soriaga, V. Saels, B. Herrera-Malaver, A. Merlevede, M. Roncoroni, K. Voordeckers, and L. Miraglia. 2016. Domestication and divergence of *Saccharomyces cerevisiae* beer yeasts. Cell 166:1397-1410.

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

Garcia-Gonzalez, F., and D. K. Dowling. 2015. Transgenerational effects of sexual interactions and sexual conflict: non-sires boost the fecundity of females in the following generation. Biology Letters 11:20150067.

Garcia-Gonzalez, F., and L. W. Simmons. 2010. Male-induced costs of mating for females compensated by offspring viability benefits in an insect. Journal of Evolutionary Biology 23:2066–2075.

García-González, F., and L. W. Simmons. 2007. Paternal indirect genetic effects on offspring viability and the benefits of polyandry. Current Biology 17:32–36.

Garland Jr, T. 2003. Selection experiments: an under-utilized tool in biomechanics and organismal biology. Vertebrate biomechanics and evolution 23–56.

Garland Jr, T., M. T. Morgan, J. G. Swallow, J. S. Rhodes, I. Girard, J. G. Belter, and P. A. Carter. 2002. Evolution of a small-muscle polymorphism in lines of house mice selected for high activity levels. Evolution 56:1267–1275.

Garland, T., and M. R. Rose. 2009. Experimental evolution: concepts, methods, and applications of selection experiments. Berkeley, CA: University of California Press.

Gasparini, C., A. Devigili, and A. Pilastro. 2012. Cross-generational effects of sexual harassment on female fitness in the guppy. Evolution 66:532–543.

Gavrilets, S., G. Arnqvist, and U. Friberg. 2001. The evolution of female mate choice by sexual conflict. Proceedings of the Royal Society of London. Series B: Biological Sciences 268:531–539.

Gibbs, A. G. 1999. Laboratory selection for the comparative physiologist. Journal of Experimental Biology 202:2709–2718.

Gomulkiewicz, R., and M. Kirkpatrick. 1992. Quantitative genetics and the evolution of reaction norms. Evolution 46:390–411.

Gowaty, P. A., Y.-K. Kim, J. Rawlings, and W. W. Anderson. 2010. Polyandry increases offspring viability and mother productivity but does not decrease mother survival in *Drosophila pseudoobscura.* Proceedings of the National Academy of Sciences, 107: 13771-13776.

Gubry-Rangin, C., C. Kratsch, T. A. Williams, A. C. McHardy, T. M. Embley, J. I. Prosser, and D. J. Macqueen. 2015. Coupling of diversification and pH adaptation during the evolution of terrestrial *Thaumarchaeota*. Proceedings of the National Academy of Sciences 112:9370–9375.

Gupta, V., Z. S. Ali, and N. G. Prasad. 2013. Sexual activity increases resistance against *Pseudomonas entomophila* in male *Drosophila melanogaster*. BMC Evolutionary Biology 13:185.

Gustafsson, L., D. Nordling, M. S. Andersson, B. C. Sheldon, A. Qvarnström, W. D. Hamilton, and J. C. Howard. 1994. Infectious diseases, reproductive effort and the cost of reproduction in birds. Philosophical Transactions of the Royal Society of London. Series B: Biological Sciences 346:323–331.

Hangartner, S., S. H. Sbilordo, Ł. Michalczyk, M. J. G. Gage, and O. Y. Martin. 2013. Are there genetic trade-offs between immune and reproductive investments in *Tribolium castaneum?* Infection, Genetics and Evolution 19:45–50.

Harshman, L. G., and A. A. Hoffmann. 2000. Laboratory selection experiments using *Drosophila:* what do they really tell us? Trends in Ecology & Evolution 15:32–36.

Head, M. L., J. Hunt, M. D. Jennions, and R. Brooks. 2005. The indirect benefits of mating with attractive males outweigh the direct costs. Plos Biology 3:e33.

Hoffmann, A. A., and P. A. Parsons. 1993. Direct and correlated responses to selection for desiccation resistance: a comparison of *Drosophila melanogaster* and *D. simulans*. Journal of Evolutionary Biology 6:643–657.

Holland, B., and W. R. Rice. 1999. Experimental removal of sexual selection reverses intersexual antagonistic coevolution and removes a reproductive load. Proceedings of the National Academy of Sciences 96:5083–5088.

Holland, B., and W. R. Rice. 1998. Perspective: chase-away sexual selection: antagonistic seduction versus resistance. Evolution 52:1–7.

Hollis, B., and T. J. Kawecki. 2014. Male cognitive performance declines in the absence of sexual selection. Proceedings of the Royal Society B: Biological Sciences 281:20132873.

Hook, K. A. 2018. No support for the sexy-sperm hypothesis in the seed beetle: Sons of monandrous females fare better in post-copulatory competition. Ecology and evolution 8:11742–11753.

Hosken, D. J., and P. I. Ward. 2001. Experimental evidence for testis size evolution via sperm competition. Ecology Letters 4:10–13.

Houston, A. I., and J. M. McNamara. 1992. Phenotypic plasticity as a state-dependent life-history decision. Evolutionary Ecology 6:243–253.

Howick, V. M., and B. P. Lazzaro. 2014. Genotype and diet shape resistance and tolerance across distinct phases of bacterial infection. BMC Evolutionary Biology 14:1-13.

Hug, L. A., B. J. Baker, K. Anantharaman, C. T. Brown, A. J. Probst, C. J. Castelle, C. N. Butterfield, A. W. Hernsdorf, Y. Amano, and K. Ise. 2016. A new view of the tree of life. Nature microbiology 1:1–6.

Jacot, A., H. Scheuber, J. Kurtz, and M. W. G. Brinkhof. 2005. Juvenile immune status affects the expression of a sexually selected trait in field crickets. J Evolutionary Biology18:1060–1068.

Jennions, M. D., and M. Petrie. 2000. Why do females mate multiply? A review of the genetic benefits. Biological Reviews 75:21–64.

Jiang, P.-P., S. Bedhomme, N. G. Prasad, and A. Chippindale. 2011. Sperm competition and mate harm unresponsive to male-limited selection in *Drosophila*: an evolving genetic architecture under domestication. Evolution: International Journal of Organic Evolution 65:2448–2460.

Kamyshev, N. G., K. G. Iliadi, and J. V. Bragina. 1999. *Drosophila* conditioned courtship: two ways of testing memory. Learning and Memory 6:1–20.

Kawecki, T. J., R. E. Lenski, D. Ebert, B. Hollis, I. Olivieri, and M. C. Whitlock. 2012. Experimental evolution. Trends in ecology & evolution 27:547–560.

Kawecki, T. J., and S. C. Stearns. 1993. The evolution of life histories in spatially heterogeneous environments: Optimal reaction norms revisited. Evolutionary Ecology 7:155–174.

Keleman, K., S. Krüttner, M. Alenius, and B. J. Dickson 2007. Function of the *Drosophila* CPEB protein Orb2 in long-term courtship memory. Nat ure Neuroscience 10:1587–1593.

Kelly, C. D. 2011. Reproductive and physiological costs of repeated immune challenges in female *Wellington tree weta* (Orthoptera: Anostostomatidae). Biological Journal of the Linnean Society 104:38–46.

Kelly, C. D., A. M. Stoehr, C. Nunn, K. N. Smyth, and Z. M. Prokop. 2018. Sexual dimorphism in immunity across animals: a meta-analysis. Ecology Letters 21:1885–1894.

Kirkpatrick, M. 1985. Evolution of female choice and male parental investment in polygynous species: The Demise of the" Sexy Son". The American Naturalist 125:788–810. .

Kirkpatrick, M. 1982. Sexual selection and the evolution of female choice. Evolution 1–12.

Klein, S. L., and K. L. Flanagan. 2016. Sex differences in immune responses. Nature Rev iewa Immunology 16:626–638.

Kliman, R. M., N. Irving, and M. Santiago. 2003. Selection conflicts, gene expression, and codon usage trends in yeast. Journal of molecular evolution 57:98–109.

Konior, M., J. Radwan, and M. Kolodziejczyk. 2001. Polyandry increases offspring fecundity in the bulb mite. Evolution 55:1893–1896.

Kuijper, B., A. D. Stewart, and W. R. Rice. 2006. The cost of mating rises nonlinearly with copulation frequency in a laboratory population of *Drosophila melanogaster*. Journal of Evolutionary Biology 19:1795–1802.

Kuznetsova, A., P. B. Brockhoff, and R. H. B. Christensen. 2017. lmerTest Package: Tests in Linear Mixed Effects Models. Journal of Statistical Software 82:1-26.

Lazzaro, B. P., H. A. Flores, J. G. Lorigan, and C. P. Yourth. 2008. Genotype-by-environment interactions and adaptation to local temperature affect immunity and fecundity in *Drosophila melanogaster*. PLoS Pathog 4:e1000025.

Lefevre Jr, G., and U. B. Jonsson. 1962. Sperm transfer, storage, displacement, and utilization in *Drosophila melanogaster*. Genetics 47:1719.

Lenth, R., H. Singmann, J. Love, P. Buerkner, and M. Herve. 2020. Package ‘emmeans’. R package version 1.4.6. 34:216–221.

Lizé, A., T. A. R. Price, C. Heys, Z. Lewis, and G. D. D. Hurst. 2014. Extreme cost of rivalry in a monandrous species: male–male interactions result in failure to acquire

mates and reduced longevity. Proceedings of the Royal Society B: Biological Sciences 281:20140631.

Long, A., G. Liti, A. Luptak, and O. Tenaillon. 2015. Elucidating the molecular architecture of adaptation via evolve and resequence experiments. Nature Reviews Genetics 16:567–582.

Maggu, K., N. Ahlawat, M. Geeta Arun, A. Meena, and N. G. Prasad. 2021. Divergence of responses to variable socio-sexual environments in laboratory populations of *Drosophila melanogaster* evolving under altered operational sex ratios. Evolution 75:414–426.

Manier, M. K., J. M. Belote, K. S. Berben, D. Novikov, W. T. Stuart, and S. Pitnick. 2010. Resolving mechanisms of competitive fertilization success in *Drosophila melanogaster*. science 328:354–357.

Manning, A. 1962. A Sperm Factor Affecting the Receptivity of *Drosophila Melanogaster* Females. Nature 194:252–253.

Manning, A. 1967. The control of sexual receptivity in female *Drosophila*. Animal Behaviour 15:239–250.

Marquis, O., M. Massot, and J. F. Le Galliard. 2008. Intergenerational effects of climate generate cohort variation in lizard reproductive performance. Ecology 89:2575–2583.

Martin, O., and D. J. Hosken. 2002. Martin OY, Hosken DJ. Strategic ejaculation in the common dung fly *Sepsis cynipsea*. Animal Behaviour 63:541–546.

Martin, O. Y., and D. J. Hosken. 2004. Copulation reduces male but not female longevity in *Saltella sphondylli (*Diptera: Sepsidae). Journal of Evolutionary Biology 17:357–362.

Martin, O. Y., and D. J. Hosken. 2003. Costs and benefits of evolving under experimentally enforced polyandry or monogamy. Evolution 57:2765–2772.

McBride, S. M., G. Giuliani, C. Choi, P. Krause, D. Correale, K. Watson, G. Baker, and K. K. Siwicki. 1999. Mushroom body ablation impairs short-term memory and long-term memory of courtship conditioning in *Drosophila melanogaster.* Neuron 24:967–977.

McKean, K. A., C. P. Yourth, B. P. Lazzaro, and A. G. Clark. 2008. The evolutionary costs of immunological maintenance and deployment. BMC Evolutionary Biology 8:1-19

McKinney, F., S. R. Derrickson, and P. Mineau. 1983. Forced copulation in waterfowl. Behaviour 86:250–294.

Michalczyk, Ł., A. L. Millard, O. Y. Martin, A. J. Lumley, B. C. Emerson, and M. J. G. Gage. 2011. Experimental evolution exposes female and male responses to sexual selection and conflict in *Tribolium castaneum*. Evolution 65:713–724.

Miller, E. K., and J. D. Cohen. 2001. An Integrative Theory of Prefrontal Cortex Function. Annual Review of Neuroscience 24:167–202.

Moatt, J. P., C. Dytham, and M. D. F. Thom. 2014. Sperm production responds to perceived sperm competition risk in male *Drosophila melanogaster*. Physiology & Behavior 131:111–114.

Mohorianu, I., A. Bretman, D. T. Smith, E. K. Fowler, T. Dalmay, and T. Chapman. 2017. Genomic responses to the socio-sexual environment in male *Drosophila melanogaster* exposed to conspecific rivals. RNA 23:1048–1059.

Møller, A. P. 1998. 2 - Sperm Competition and Sexual Selection. Pp. 55–90 *in* T. R. Birkhead and A. P. Møller, eds. Sperm Competition and Sexual Selection. Academic Press, San Diego.

Moore, A. J., P. A. Gowaty, W. G. Wallin, and P. J. Moore. 2001. Sexual conflict and the evolution of female mate choice and male social dominance. Proceedings of the Royal Society of London. Series B: Biological Sciences 268:517–523.

Moret, Y., and P. Schmid-Hempel. 2000. Survival for immunity: The price of immune system activation for bumblebee workers. Science 290:1166-1168.

Nandy, B., P. Chakraborty, V. Gupta, S. Z. Ali, and N. G. Prasad. 2013a. Sperm competitive ability evolves in response to experimental alteration of operational sex ratio. Evolution 67:2133–2141.

Nandy, B., V. Gupta, S. Sen, N. Udaykumar, M. A. Samant, S. Z. Ali, and N. G. Prasad. 2013b. Evolution of mate-harm, longevity and behaviour in male fruit flies subjected to different levels of interlocus conflict. BMC Evolutionary Biology 13:1-16.

Nandy, B., V. Gupta, N. Udaykumar, M. A. Samant, S. Sen, and N. G. Prasad. 2014. Experimental evolution of female traits under different levels of intersexual conflict in *Drosophila melanogaster.* Evolution 68:412–425.

Nandy, B., A. Joshi, Z. Syed, S. Sen, and N. Prasad. 2012. Degree of adaptive male mate choice is positively correlated with female quality variance. Scientific reports 2:1-8.

Nandy, B., and N. G. Prasad. 2011. Reproductive Behavior and Fitness Components in Male *Drosophila melaogaster* are Non-Linearly Affected by the Number of Male Co-Inhabitants Early in Adult Life. Journal of Insect Science 11:1–9.

Neff, B. D., P. Fu, and M. R. Gross. 2003. Sperm investment and alternative mating tactics in bluegill sunfish (*Lepomis macrochirus*). Behavioral Ecology 14:634–641.

Norris, T., and D. Lampe. 1994. Healthy communities, healthy people: A challenge of coordination and compassion. National Civic Review 83:280–289.

Nunn, C. L., P. Lindenfors, E. R. Pursall, and J. Rolff. 2009. On sexual dimorphism in immune function. Philosophical Transactions of the Royal Society B: Biological Sciences 364:61–69.

Nystrand, M., E. Cassidy, and D. Dowling. 2016. Transgenerational plasticity following a dual pathogen and stress challenge in fruit flies. BMC Evolutionary Biology 16:1-11.

Nystrand, M., E. J. Cassidy, and D. K. Dowling. 2018. The effects of a bacterial challenge on reproductive success of fruit flies evolved under low or high sexual selection. Ecology and Evolution 8:9341–9352.

Parker, G. A. 1998. 1 - Sperm Competition and the Evolution of Ejaculates: Towards a Theory Base. Pp. 3–54 *in* T. R. Birkhead and A. P. Møller, eds. Sperm Competition and Sexual Selection. Academic Press, San Diego.

Parker, G. A. 1979. Sexual selection and sexual conflict. Sexual selection and reproductive competition in insects 123:166.

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

Parker, G. A. 1990a. Sperm competition games: raffles and roles. Proceedings of the Royal Society of London. Series B: Biological Sciences 242:120–126.

Parker, G. A. 1990b. Sperm competition games: sneaks and extra-pair copulations. Proceedings of the Royal Society of London. Series B: Biological Sciences 242:127–133.

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

Parker, G. A. 1982. Why are there so many tiny sperm? Sperm competition and the maintenance of two sexes. Journal of Theoretical Biology 96:281–294.

Parker, G. A., M. A. Ball, P. Stockley, and M. J. G. Gage. 1997. Sperm competition games: a prospective analysis of risk assessment. Proceedings of the Royal Society of London. Series B: Biological Sciences 264:1793–1802.

Parker, G. A., M. A. Ball, P. Stockley, and M. J. G. Gage. 1996. Sperm competition games: individual assessment of sperm competition intensity by group spawners. Proceedings of the Royal Society of London. Series B: Biological Sciences 263:1291–1297.

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

Perez-Staples, D., and M. Aluja. 2006. Sperm allocation and cost of mating in a tropical tephritid fruit fly. Journal of Insect Physiology 52:839–845.

Pilastro, A., M. Scaggiante, and M. B. Rasotto. 2002. Individual adjustment of sperm expenditure accords with sperm competition theory. Proceedings of the National Academy of Sciences of the United States of America 99:9913–9915.

Pischedda, A., and A. K. Chippindale. 2006. Intralocus Sexual Conflict Diminishes the Benefits of Sexual Selection. Plos Biology 4:e356.

Pitnick, S., G. T. Miller, J. Reagan, and B. Holland. 2001. Males' evolutionary responses to experimental removal of sexual selection. Proceedings of the Royal Society of London. Series B: Biological Sciences 268:1071–1080.

Pizzari, T., C. K. Cornwallis, H. Løvlie, S. Jakobsson, and T. R. Birkhead. 2003. Sophisticated sperm allocation in male fowl. Nature 426:70–74.

Pound, N., and M. J. G. Gage. 2004. Prudent sperm allocation in Norway rats, *Rattus norvegicus*: a mammalian model of adaptive ejaculate adjustment. Animal Behaviour 68:819–823.

Prasad, N. G., S. Bedhomme, T. Day, and A. K. Chippindale. 2007. An evolutionary cost of separate genders revealed by male-limited evolution. The American Naturalist 169:29–37.

Prasad, N. G., and A. Joshi. 2003. What have two decades of laboratory life-history evolution studies on *Drosophila melanogaster* taught us? Journal of genetics 82:45–76.

Priest, N. K., L. F. Galloway, and D. A. Roach. 2008. Mating Frequency and Inclusive Fitness in *Drosophila melanogaster*. The American Naturalist 171:10–21.

Radhakrishnan, P., and K. M. Fedorka. 2012. Immune activation decreases sperm viability in both sexes and influences female sperm storage. Proceedings of the Royal Society B: Biological Sciences, 279:3577–3583.

Reichard, M., C. Smith, and W. C. Jordan. 2004. Genetic evidence reveals density-dependent mediated success of alternative mating behaviours in the European bitterling (*Rhodeus sericeus*). Molecular Ecology 13:1569–1578.

Reinhardt, K., E. Harney, R. Naylor, S. Gorb, and M. T. Siva-Jothy. 2007. Female-limited polymorphism in the copulatory organ of a traumatically inseminating insect. The American Naturalist 170:931–935.

Rice, W. R. 2000. Dangerous liaisons. Proceedings of the National Academy of Sciences 97:12953–12955.

Rice, W. R. 1996. Sexually antagonistic male adaptation triggered by experimental arrest of female evolution. Nature 381:232–234.

Rice, W. R., J. E. Linder, U. Friberg, T. A. Lew, E. H. Morrow, and A. D. Stewart. 2005. Inter-locus antagonistic coevolution as an engine of speciation: assessment with hemiclonal analysis. Proceedings of the National Academy of Sciences 102:6527–6534.

Rice, W. R., A. D. Stewart, E. H. Morrow, J. E. Linder, N. Orteiza, and P. G. Byrne. 2006. Assessing sexual conflict in the *Drosophila melanogaste*r laboratory model system. Philosophical Transactions of the Royal Society B: Biological Sciences 361:287–299.

Richner, H., P. Christe, and A. Oppliger. 1995. Paternal investment affects prevalence of malaria. Proceedings of the National Academy of Sciences 92:1192-1194.

Riehle, M. M., A. F. Bennett, R. E. Lenski, and A. D. Long. 2003. Evolutionary changes in heat-inducible gene expression in lines of *Escherichia coli* adapted to high temperature. Physiological genomics 14:47–58.

Rolff, J. 2002. Bateman's principle and immunity. Proceedings of the Royal Society of London. Series B: Biological Sciences 269:867–872.

Rolff, J., and M. T. Siva-Jothy. 2002. Copulation corrupts immunity: A mechanism for a cost of mating in insects. Proceedings of the National Academy of Sciences 99:9916–9918.

Rose, M. R. 1984. Laboratory evolution of postponed senescence in *Drosophila melanogaster.* Evolution 38:1004–1010.

Rouse, J., and A. Bretman. 2016. Exposure time to rivals and sensory cues affect how quickly males respond to changes in sperm competition threat. Animal Behaviour 122:1-8.

Rouse, J., K. Watkinson, and A. Bretman. 2018. Flexible memory controls sperm competition responses in male *Drosophila melanogaster*. Proceedings. Biological sciences 285: 20180619.

Rundle, H. D., A. Odeen, and A. Ø. Mooers. 2007. An experimental test for indirect benefits in *Drosophila melanogaster*. BMC Evolutionary Biology 7:36–36.

Ryan, M. J. 1990. Sexual selection, sensory systems and sensory exploitation. Oxford surveys in evolutionary biology 7:157–195.

Sakaluk, S. K., and J. K. Müller. 2008. Risk of sperm competition mediates copulation duration, but not paternity, of male burying beetles. Journal of Insect Behavior 21:153–163.

Sasaki, A., and G. de Jong. 1999. Density dependence and unpredictable selection in a heterogeneous environment: compromise and polymorphism in the ess reaction norm. Evolution 53:1329–1342.

Schaus, J., and S. Sakaluk. 2001. Ejaculate expenditures of male crickets in response to varying risk and intensity of sperm competition: Not all species play games. Behavioral Ecology 12:740–745.

Sheldon, B. C., and S. Verhulst. 1996. Ecological immunology: costly parasite defesnes and trade-offs in evolutionary ecology. Trends in Ecology and Evolution 11:317–321.

Siegel, R. W., and J. C. Hall. 1979. Conditioned responses in courtship behavior of normal and mutant *Drosophila*. Proceedings of the National Academy of Sciences 76:3430–3434.

Simmons, L.W. 2001. Sperm Competition And Its Evolutionary Consequences In The Insects.

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

Simmons, L. W., and F. García-González. 2008. Evolutionary reduction in testes size and competitive fertilization success in response to the experimental removal of sexual selection in dung beetles. Evolution: International Journal of Organic Evolution 62:2580–2591.

Simmons, L. W., and C. Kvarnemo. 1997. Ejaculate expenditure by male bushcrickets decreases with sperm competition intensity. Proceedings of the Royal Society B: Biological Sciences 264:1203–1208.

Sinervo, B., and A. L. Basolo. 1996. Testing adaptation using phenotypic manipulations. Adaptation 149:185.

Siva-Jothy, M. T., and A. D. Stutt. 2003. A matter of taste: direct detection of female mating status in the bedbug. Proceedings of the Royal Society of London. Series B: Biological Sciences 270:649–652.

Siwicki, K. K., P. Riccio, L. Ladewski, F. Marcillac, L. Dartevelle, S. A. Cross, and J.-F. Ferveur. 2005. The role of cuticular pheromones in courtship conditioning of *Drosophila males.* Learning & memory12:636–645.

Smith, C., M. Reichard, and P. Jurajda. 2003. Assessment of sperm competition by European bitterling, *Rhodeus sericeus*. Behavioral Ecology and Sociobiology 53:206–213.

Stahlschmidt, Z. R., N. Rollinson, M. Acker, and S. A. Adamo. 2013. Are all eggs created equal? Food availability and the fitness trade-off between reproduction and immunity. Functional Ecology 27:800–806.

Stearns, S. C., and J. C. Koella. 1986. The evolution of phenotypic plasticity in life-history traits: predictions of reaction norms for age and size at maturity. Evolution 40:893–913.

Stewart, A. D., E. H. Morrow, and W. R. Rice. 2005. Assessing putative interlocus sexual conflict in *Drosophila melanogaster* using experimental evolution. Proceedings of the Royal Society B: Biological Sciences 272:2029–2035.

Svensson, E. I., F. Eroukhmanoff, and M. Friberg. 2006. Effects of natural and sexual selection on adaptive population divergence and premating isolation in a damselfly. Evolution 60:1242–1253.

Syed, Z. A., M. Chatterjee, M. A. Samant, and N. G. Prasad. 2017. Reproductive isolation through experimental manipulation of sexually antagonistic coevolution in *Drosophila melanogaster*. Scientific Reports 7:1-8.

Syed, Z. A., V. Gupta, M. G. Arun, A. Dhiman, B. Nandy, and N. G. Prasad. 2020. Absence of reproduction-immunity trade-off in male *Drosophila melanogaster* evolving under differential sexual selection. BMC Evolutionary Biology 20:1-10.

Taylor, M. L., C. Wigmore, D. J. Hodgson, N. Wedell, and D. J. Hosken. 2008. Multiple mating increases female fitness in *Drosophila simulans.* Animal Behaviour 76:963–970.

Thornhill, R. 1983. Cryptic female choice and its implications in the scorpionfly *Harpobittacus nigriceps.* American Naturalist 122:765–788.

Travisano, M., F. Vasi, and R. E. Lenski. 1995. Long-term experimental evolution in *Escherichia coli. III.* Variation among replicate populations in correlated responses to novel environments. Evolution 49:189–200.

Tregenza, T., and N. Wedell. 2002. Polyandrous females avoid costs of inbreeding. Nature 415:71–73.

Trivers, R. 1972. Parental Investment and Sexual Selection. P. 378 *in* Sexual Selection and the Descent of Man.

Van Tienderen, P. H. 1991. Evolution of generalists and specialists in spatially heterogeneous environments. Evolution 45:1317–1331.

Via, S., and R. Lande. 1985. Genotype-environment interaction and the evolution of phenotypic plasticity. Evolution 39:505–522.

Villella, A., and J. C. Hall. 2008. Neurogenetics of courtship and mating in *Drosophila.* Advances in genetics 62:67–184.

Vodovar, N., M. Vinals, P. Liehl, A. Basset, J. Degrouard, P. Spellman, F. Boccard, and B. Lemaitre. 2005. *Drosophila* host defense after oral infection by an entomopathogenic *Pseudomonas* species. Proceedings of the National Academy of Sciences 102:11414-11419.

Walzer, A., and P. Schausberger. 2015. Food stress causes sex-specific maternal effects in mites. Journal of Experimental Biology 218:2603–2609.

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

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

Wigby, S., and T. Chapman. 2004. Female resistance to male harm evolves in response to manipulation of sexual conflict. Evolution 58:1028–1037.

Wigby, S., L. K. Sirot, J. R. Linklater, N. Buehner, F. C. F. Calboli, A. Bretman, M. F. Wolfner, and T. Chapman. 2009. Seminal fluid protein allocation and male reproductive success. Current Biology 19:751–757.

Wolfner, M. F. 2009. Battle and ballet: molecular interactions between the sexes in *Drosophila*. Journal of Heredity 100:399–410.

Wolfner, M. F. 1997. Tokens of love: functions and regulation of *Drosophila* male accessory gland products. Insect biochemistry and molecular biology 27:179–192.

Zajitschek, S. R. K., D. K. Dowling, M. L. Head, E. Rodriguez-Exposito, and F. Garcia-Gonzalez. 2018. Transgenerational effects of maternal sexual interactions in seed beetles. Heredity 121:282–291.

Zerofsky, M., E. Harel, N. Silverman, and M. Tatar. 2005. Aging of the innate immune response in *Drosophila melanogaster.* Aging Cell 4:103–108.

Zuk, M. 1996. Disease, Endocrine-Immune Interactions, and Sexual Selection. Ecology 77:1037–1042.

Zuk, M. 1990. Reproductive strategies and disease susceptibility: an evolutionary viewpoint. Parasitology Today 6:231–233.

Zuk, M., and K. A. McKean. 1996. Sex differences in parasite infections: Patterns and processes. International Journal for Parasitology 26:1009–1024.

Zuk, M., and A. M. Stoehr. 2002. Immune Defense and Host Life History. The American Naturalist 160:S9–S22.