All's fair in Love and War.
Evolution of reproductive traits in populations of *Drosophila melanogaster* evolved under differential levels of sexual selection.

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A thesis submitted for the partial fulfillment of the degree of Doctor of Philosophy

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Dedicated to my parents.

Declaration

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

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

...……

(Tejinder Singh Chechi)

Date:

Place:

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

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Date:

Place:

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Even after leaving this place, I will always refer to EBL as MY LAB and IISER Mohali as MY INSTITUTE.

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Synopsis

Mating systems are expected to majorly affect the evolution of secondary sexual characters, especially in males. One of the most common ways to experimentally vary mating systems is to alter operational sex ratios or to enforce monogamy in an otherwise promiscuous system. Male biased populations (or promiscuity) are expected to impose strong intra- and inter-sexual selection on males leading to the rapid divergence of male fitness related traits at both the pre-and post-copulatory stages. A further result of promiscuity is inter-sexual conflict, defined as the conflict between the two sexes in the species because of the evolutionary difference in the optimal trait value for each sex. Such a conflict can result because the optimum outcome over direct male-female interactions can vary between sexes (for example, over mating rate, parental investment, etc.), or the expression of the same traits can lead to opposite fitness effects on the sexes. These are called inter-and intra-locus sexual conflicts, respectively. Altered levels of operational sex ratios can alter the degree of intersexual conflict and therefore affect the evolution of male fitness related traits.

In the present thesis, I used a set of Drosophila melanogaster populations maintained at two sex ratio regimes – in the male-biased (M) regime, the operational sex ratio (male: female) was 3:1, while in the female-biased (F), it was 1:3. There are three independent replicate populations in each of the two regimes. Previous doctoral works by Bodhisatta Nandy, Syed Zeeshan Ali, and Komal Maggu have demonstrated the higher intensity of sexual selection and conflict in the M regime compared to that in F, with M males having evolved higher courtship ability, locomotor activity, and both sperm defense and offense ability compared to F males (Nandy et al., 2013c, 2013a). Further, M females have become more resistant to male-induced harm than F females, which came at the cost of decreased base level fecundity and lifespan (Nandy et al., 2014).

Using these *Drosophila melanogaster* populations maintained at different operational sex ratios, I address a series of questions related to the evolution of mating success, mating rates, sperm competitive ability, and secondary sexual characters.

a) How do immediate and evolutionary differences in operational sex ratio affect mating rates of males and females?
b) Does evolution under altered operational sex ratio lead to differential mate choice?
c) Does male mating success evolve as a result of evolving under different operational sex ratios?
d) Does evolution under differential levels of sexual selection lead to divergence in secondary sexual traits of males?
e) Do male and female co-evolutionary history affect the outcome of sperm competitive ability?

In the first set of studies, I addressed the question of mating rates of males and females and the sexual conflict that arises over the mating rates. In promiscuous species like *Drosophila*, the optimal mating rates of males are expected to be much higher than that of females. While the males try to maximize their mating, females often resist rematings (Parker, 2006). This leads to a constant conflict between the two sexes over the control of the mating rates in the population. How the inter-sexual conflict over the mating rates plays out in a population would depend upon the male-female interaction and sexual selection present in the population. M and the F populations have evolved under differential levels of sexual selection via altered sex ratios. I measured the mating rates of the males and females in M and F populations when combined with ancestral females and males, respectively, at the three sex ratio treatments- male-biased, equal sex ratio, and female-biased- to examine a) how the mating rates have evolved as a result of evolutionary history and b) how the altered sex-ratio has an immediate effect

on mating rates. The mating rates of the M females were significantly lower than the mating rates of the F females. On the other hand, the mating rates of M males were significantly higher than the F males. While M females have evolved to resist remating, M males have evolved to maximize their mating potential. There was an overall significant effect of sex-ratio treatment, with male-biased sex ratio treatment having higher mating rates than equal sex ratio and female-biased sex ratios. I further compared the mating rate of each sex when combined with individuals of the opposite sex derived from the ancestral population or selected populations. For example, for the M population with a male-biased sex ratio, I compared the mating rates of M males combined with ancestral females, M males combined with M females, and M females combined with ancestral males. Similarly, in the female-biased sex ratio, mating rates were compared for F males combined with ancestral females, F males combined with F females, and F females combined with ancestral males. In the F population, we did not find any significant difference in the mating rate of males and females when combined with individuals from the F population or ancestral population. In the case of the M population, M males achieve significantly higher mating with ancestral females than with M females. The mating rates of the M females were similar to males from either the M population or ancestral population. This shows that while M males can achieve significantly higher mating rates, their mating rates are lowered when combined with M females. This suggests that M females have the upper hand in controlling the mating rates in the M population.

To a large extent, mating success can be argued to be the property of a mating pair. These include the resistance of the female to remate and the persistence of the males to obtain a mating. In addition, previous studies have shown that males can also mate

strategically, based on their own resource levels and female fitness (Edward and Chapman, 2011). In the second study, I investigated the male mating preference if given a choice between the M and the F females. For this experiment sperm depleted males from the M and the F populations were presented with a choice between the M and the F female. The sperm depleted males from both the M and the F populations mated with F females significantly more number of times. This could be the result of higher resistance to mating by the M females with resource-depleted males or due to the males choosing to mate with females from the F population that are less resistant to mating. For females, investing in resistance traits is expected to have a trade-off as resistance traits for females are expected to be energy expensive and, therefore, trade-off with other life-history traits. A previous study by Nandy et al., (2014) has shown that F females have a higher number of progeny after single mating with common males than M females. I examined the fecundity of M and the F females after single mating with common ancestral males. The fecundity results showed that the M females' fecundity is lower after single mating than the F females. The costly resistance trait in M females might have come at the expense of fecundity in the M females.

Overall reproductive success of males depend upon both mating success and fertilization success. Mating success is predicted to be a strong determinant of male reproductive success because mating success is the pre-requisite for fertilization success. The M population males are expected to be under strong selection for mating success as the male-male competition and female choice are intense in these populations. Therefore, the males in the M population are expected to evolve competitive mating strategies and traits that would ensure their mating success. I assayed the mating success of M and the F males under competitive conditions to test

whether M males had indeed evolved higher mating success. The assays were conducted with both the virgin females (common ancestral females, M females, and F females) and the mated females (M females and F females). I found that M males have higher mating success, irrespective of the evolutionary history of the females. Relative to F males, the mating success of M males was higher with previously mated females. Thus, having evolved under intense male-male competition, M males seem to have evolved to be better at remating previously mated females.

Knowing that M males have a higher mating success, in the next study, I examined the secondary sexual traits which might lead to this advantage in mating success. Male-male competition often leads to the evolution of secondary sexual traits in males. In *Drosophila* species, wings play an essential part in the courtship process (Tauber and Eberl, 2003). Males produce a courtship song through the wing vibrations to attract the females. Various studies have found that wing morphology (wing shape and size) influences males' mating success (Naseerulla and Hegde, 1992). So, I investigated if differential sexual selection in the M and the F population led to divergence in wing morphology of the M and the F males. I did not find any wing shape and size divergence across the selected populations. Further, the two selection regimes did not differ in their fluctuating asymmetry. Differential levels of sexual selection in M and F populations do not lead to divergence in wing morphometry and symmetry.

Along with wing morphology, I also studied a recently discovered phenomenon of wing interference pattern (WIP). Wing interference pattern (WIP) is a form of vivid coloration pattern on the otherwise transparent wings of the insects. WIPs are formed because of thin-film interference from the wings. Recent studies have identified that WIPs are a potential target for sexual selection as they might play a role in courtship

activity and might evolve under female choice (Hawkes et al., 2019; Katayama et al., 2014). Males with brighter and more colorful wind interference patterns have been found to have an advantage in female mate choice in *Drosophila melanogaster*. I examined the wing interference pattern for both the males and the females in the M and the F populations. I also measured the attractiveness index of males from the M and the F population in terms of mating latency. I found that the WIPs do evolve and diverge in the two populations, and there exists sexual dimorphism in WIPs. The males from the M populations had brighter and more colorful wings than the F males. However, I did not find any significant difference in the attractiveness index of the males from the M and the F populations, though there is a trend toward M males having a higher attractiveness score. While the WIPs are under selection in the M and the F population, it does not translate into an advantage for M males in terms of attractiveness.

In the final part of my thesis, I explored the male-female interaction in sperm competition. In promiscuous species like *Drosophila*, sperm competitive ability is a vital fitness trait influencing post-copulatory fertilization success. For quite a while, post-copulatory sexual selection studies looking at sperm competitive ability considered females as passive observers in the whole process. The female reproductive tract was considered as an inactive arena for the sperm competitive ability of males to play out. However, recent studies have shown that females play an active role in the outcome of the sperm competitive ability via cryptic female choice or by male-female interactions (Ala-Honkola and Manier, 2016; Eberhard, 1996; Lüpold et al., 2020). Previously, Nandy et al., (2013a) found that males from the M population have a fitness advantage in sperm competitive ability over the males from the F population. M and F populations provided a suitable system to test the hypothesis of male-female interaction

on sperm competitive ability. A full factorial sperm competitive assay was set up using the males and females from M, F, and the ancestral (LHst) populations for this set of studies. I measured sperm defense (P1) and sperm offense (P2) of males from M, F, and LHst populations (when competing with common male) when the female was from M, F, or LHst populations. M males, as expected, were better at sperm competitive ability with females from all the three populations. There was a significant effect of females on sperm competition. P2 proportions were significantly lower in assays with M females as compared to F and LHst females. The result also showed a significant male-female interaction in P2. M males have significantly higher P2 with M females than with other females while P2 proportion of F and LHst males is lower with M females as compared to F and LHst females. These results clearly show that co-evolutionary history of males and females has an important role to play in the outcome of sperm competition.

To sum up, my thesis addresses several essential questions regarding sexual selection and sexual conflict using the populations evolved under differential levels of sexual selection via altered sex-ratio. M males experience intense levels of male-male competition and female choice, which leads to conflict over mating rates in M populations, with M females controlling the mating rates, M males evolving to be better at competitive mating success with virgin and with mated females, F females getting preference over M females in male mate choice assay, M males having brighter and colorful wings without any difference in wing morphometry as compared to F males, and M male-M female co-evolutionary history playing a role in the outcome of sperm competition.

Introduction

Chapter 1

In his book 'On the origin of species' Darwin touched upon the topic of sexual selection; 'Sexual selection "..depends not on a struggle for existence, but on a struggle between males for possession of females; the result is not *death* to the unsuccessful competitor, but *few or no offspring*" (Darwin, 1859). Darwin went on to suggest that sexual selection is not as strong an evolutionary force as natural selection, and is limited mainly to one sex, generally males. This view of Darwin seems to have changed at a later stage when he himself emphasized the importance of sexual selection by devoting one entire book to discussing its role in organic evolution (Darwin, 1871). We now know that sexual selection can be much more rigorous than initially thought to be and affects both sexes, admittingly, to different degrees (Kirkpatrick, 1982; Svensson et al., 2006).

Darwin defined sexual selection as "the advantage which certain individuals have over others of the same sex and species solely in respect of reproduction" (Darwin, 1871). This particular type of "selection" answered, or at least sought to solve, a crucial question: how do features with little survival value, such as vivid coloration, expensive courting, horns, and antlers, evolve? In addition to describing sexual selection, Darwin emphasized that it can take two forms: (a) intra-sexual competition for mate access (intra-sexual selection) and (b) one sex imposing mate choice on the other sex. He went on to describe how males frequently compete and fight for access to mating partners while females are choosy.

The difference in two sexes of competition and choosiness can be explained in terms of the apparent reproductive investment gap between the sexes. This leads to intra- and intersexual selection. Intrasexual selection occurs when one sex, usually males, competes for access to the other sex and was once thought to be a precopulatory process.

Males establishing and defending territory to get access to females, male-male conflict for access to females, and scramble competition are all manifestations of precopulatory intrasexual selection (Andersson, 1994). Male lekking marine iguanas, *Amblyrhynchus cristatus,* for example, create and defend areas to obtain access to females (Partecke et al., 2002). Males create and defend territories well before the mating season begins to obtain the most excellent location for attracting and mating with females, and successful males have greater mating rates than failed males (Partecke et al., 2002). To get access to females, male kangaroos, *Macropus* genus, engage in combat with one another in order to form a hierarchy. When females are estrous and more receptive to mating, fighting rises (Warburton et al., 2013). The milkweed leaf beetle, *Labidomera clivicollis,* must compete with other males to discover and mate with females. Males spend the majority of their time looking for a mate and will battle other males to obtain access to females (Dickinson, 1992).

Intrasexual selection has recently been discovered to occur even after copulation, resulting in the evolution of features such as mate guarding, mating plugs, and bigger ejaculates when other males are present (Andersson, 1994; Gage, 1991). There are several examples of similar characteristics as well. For example, male whiptail lizards, *Aspidoscelis costata,* defend mated females to ensure they produce the most babies in the clutch. To increase their fertilization success, males would fiercely guard the female from other males and copulate with her many times (Ancona et al., 2010). Males of the scorpion *Vaejovis punctatus* use a mating plug to minimize sperm competition and female receptivity to remating (Contreras-Garduño et al., 2006). Females are unable to remove the plug and stay sterile until it dissolves, allowing the first mated male to father the majority of the progeny (Contreras-Garduño et al., 2006). Similarly, when

competitive males are present, male golden egg bugs, *Phyllomorpha laciniata*, copulate for longer periods in order to transmit more sperm to the female (García-González and Gomendio, 2004). When sperm competition rises, the mean mating duration increases by up to eight hours in order to optimize sperm transmission and boost the male's chances of fertilizing the egg (García-González and Gomendio, 2004). In *Drosophila* species, males transfer seminal fluids along with sperms in their ejaculates to manipulate females and increase their chances of siring a higher proportion of progeny (Ravi Ram and Wolfner, 2007; Sirot et al., 2011; Wolfner, 1997a).

While all these examples are the result of years of excellent work from researchers across the globe, the field of sexual selection was reignited into active research in the early twentieth century when basic theoretical models and unique experimental results forced the then great minds to dig deeper into the subject of sexual selection and its implications on the evolution as a whole.

Models of sexual selection

The first model for the genesis and development of female choice and male sexual features was offered by Ronald A. Fisher in 1930, which significantly advanced Darwin's notion of sexual selection. Fisher (1930) proposed a male-female co-evolutionary dynamics model in which female preference drove the development of male sexual traits, and at the same time, female preference evolved as male quality in the population evolved. Males are assumed to be selected for exhibiting features that females prefer through the mate-choice process, according to this concept. Assuming that there is a positive relationship between trait value and male mating success, such a condition can increase male trait value across generations. Females benefited from

selecting men with higher trait levels since their offspring are expected to inherit their father's 'attractive feature.' Notably, the offsprings are likely to acquire not only the male characteristics but also the preference trait of their mother. This link between the female-preference trait and the male sexual characteristic might theoretically lead to the male sexual trait being exaggerated. The opposing impacts of viability selection prevent this 'runaway' amplification of masculine features. This was the first of the now-famous "genetic-benefit" set of sexual selection ideas. Another set of hypotheses, known as "direct benefit," claimed that the "sexy son" advantage was insufficient to explain the evolution of female preferences and male characteristics (Kirkpatrick, 1985). According to this theory, female preference could only have evolved if there was an apparent fitness benefit to the females for exhibiting preference. The probable flaw in this theory was in the original inception and dissemination of the preference gene in females. Fisher (1930) proposed that the preference gene had an "initial advantage". Male-female coevolution can occur after the initial distribution of the preference and male trait. Preference may expand in a population if the favored male attribute is linked to male fitness, even if just tangentially, and can be passed down to the progeny. This indicates that the maternal preference gene, as well as the paternal 'sexy' characteristic and fitness relationship, will be passed down to the kids.

A non-co-evolutionary paradigm called "sensory exploitation" was presented as a substitute for these previously discussed co-evolutionary models (Basolo, 1995, 1990; Ryan, 1990). It has been suggested that female preference (or sensory bias for particular male traits) is a by-product of viability selection on the female sensory system. Sinervo, (1996) found that female preference originated before the preferred male trait in a swordtail fish species, *Xyphosura*. According to the sensory exploitation theory, males

are likely to be chosen for their ability to exploit females' pre-existing sensory bias in order to induce mating attraction.

Theory of Sexual conflict

A.J. Bateman in 1948 showed that in fruit flies, for mating frequency, males and females have distinct fitness optima (Bateman, 1948). Trivers (1972) expanded on this theory, indicating that there is a fundamental difference in fitness strategies between the two sexes. (Parker, 1979) established and re-expanded the idea of sexual selection, demonstrating how intra-sexual selection (competition among males) may have negative consequences for their mates, leading to intersexual conflict. Rice (1996, 1986), Arnqvist (1992a, 1992b, 1989) and Arnqvist and Rowe (1995) in a series of publications, developed a new paradigm in our understanding of male-female coevolution — sexually antagonistic coevolution (Rice and Chippindale, 2002).

Holland and Rice (1998) offered a thought-provoking concept of 'chase-away selection' to this new paradigm. The chase-away hypothesis proposes that females' prior sensory bias puts pressure on males to acquire a primary display characteristic that boosts their attraction to females, such as a modestly longer tail. These highly attractive males then get females to mate outside of their comfort zones. As a result, females face selective pressure to develop 'resistance' rather than 'preference' for the male display feature. Males are now under greater selective pressure to acquire a more intense display feature in order to overcome female resistance. It causes males and females to go through a cycle of adaptation and counter-adaptation, culminating in a sexually antagonistic coevolution process.

In contrast to 'preference,' females are chosen to evolve 'resistance' to male persistence in this process. Female resistance in model organisms, including bed bugs, water striders, and fruit flies, is now thoroughly studied (Arnqvist and Rowe, 1995; Crudgington and Siva-Jothy, 2000; Kuijper et al., 2006; Long et al., 2009; Nandy et al., 2014; Reinhardt et al., 2009). This concept appealed to researchers since it made no complicated assumptions and believed that any male trait that improves the frequency of mating in females is chosen for in males. It might be an essential kind of behavioral coercion or a cryptic form of modification (for example, sensory bias suggested by Basolo (1990)). Female fitness is projected to suffer as a result of the higher mating rate, causing females to evolve resistance to male stimulation and/or compulsion. This is thought to be the beginning of intersexual antagonistic coevolution (Rice, 2000). Because this process includes separate loci from males and females, which are typically sex-limited or biassed in their expression, it is also known as interlocus sexual conflict. A different type of sexual conflict occurs when the same allele is expressed in both sexes. Because each sex has a distinct ideal trait value, a genetic tug of war emerges between males and females in this instance. This is a type of intralocus sexual conflict (Prasad et al., 2007a; Rice and Chippindale, 2001a).

Taken together, sexual conflict can arise from either direct male-female conflicting interactions (Inter-locus conflict) or non-sex-limited expression of traits that have antagonistic fitness impacts in males and females (Intra-locus conflict). Sexual conflict may influence the fitness of both sexes by impacting life-history features and reproductive qualities. The evolutionary implications of the interlocus conflict, namely how it might influence the strategies associated with male and female reproductive behavior, is, however, debatable.

Sexual conflict and evolution of reproductive traits

Interlocus conflict is common and has been found in a variety of animals and taxa (Arnqvist and Rowe, 2013; Rice, 2000). Males' reproductive success in *D. melanogaster* is mainly determined by their capacity to mate with available females and their sperm competitive abilities. Males have evolved a range of important traits to improve their reproductive fitness (Clark and Civetta, 2000; Rice, 2000), which may result in a decline in the fitness of their mates. These traits can affect at precopulatory levels (i.e., behavioral), such as persistent courtship (Fowler and Partridge, 1989; Kuijper et al., 2006; Partridge and Fowler, 1990), or post-copulatory levles (i.e., physiological) effects, such as seminal fluid stimulation (Chapman et al., 1995; Wolfner, 1997b). Mate-harm is the collective term for all of these negative impacts of males on female fitness (Jiang et al., 2011). Natural selection is likely to work on females to acquire resistance to mate-harm, as explained above because mate-harm reduces female fitness. In *D. melanogaster*, developing resistance to mate harm entails a combination of mate rejection, genital extrusion, and unknown physiologic processes (Cook and Connolly, 1973; Rice et al., 2006; Wolfner, 2009). As a result, interlocus sexual conflict may be defined as the coevolution of mate-harm and mate-harm resistance. Using laboratory experimental evolution, researchers have attempted to explore the process of evolution under intralocus and interlocus sexual conflict.

Nandy et al., (2014, 2013) provided empirical evidence for the evolution of mate harm and mate harm resistance over 40-50 generations in *D. melanogaster* populations selected for different levels of interlocus sexual conflict (these are the same populations that I studied). Nandy changed the adult operational sex ratios in these populations to

modify the amount of interlocus sexual conflict. Changing the population's operational sex ratio affects the intensity of intermale competition as well as the male-female encounter rate. The degree of interlocus sexual conflict is likely to alter due to this skewed sex ratio. Male-biased sex ratio regimes are likely to have significant conflict, female-biased sex ratio regimes are expected to have low conflict, and an equal sex ratio is in the ancestral condition.

Similarly, for 33 generations, Wigby and Chapman, (2004) subjected duplicate populations of *D. melanogaster* to a skewed operational sex ratio. The only substantial response to selection was discovered in the female-biased regime, where females' resistance to mate-harm was dramatically reduced (Wigby and Chapman, 2004). Males from the female-biased regime were found to have acquired a slower ejaculate depletion pattern after 60-67 generations of selection (Linklater et al., 2007). In a similar study, researchers changed the operational sex ratio in flour beetles – *Tribolium castaneum* – for 20 generations and discovered that females in the female-biased regime were sensitive to multimale mating, resulting in a loss of fitness (mate-harm). In contrast, females in the male-biased regime showed no such effect (Michalczyk et al., 2011). This study found indications of divergence between populations with male-biased operational sex ratios and those with female-biased operational sex ratios.

Apart from changing the operational sex ratios, other methods can be used to control sexual selection or conflict levels. Rice (1996) employed a natural approach of 'male restricted evolution' in a population of *D. melanogaster*, where only males were permitted to evolve against a static female phenotype since females were not allowed to counter-adapt. As a result, higher mate-harming capacity in males evolved, coupled with improved male reproductive fitness and sperm competitive ability (Rice and

Holland, 1997). On the other hand, Jiang et al., (2011), using the same experimental technique, found no evidence of the development of mate-harm and sperm competitive ability in males, but did find an improvement in male fitness compared to controls (Prasad et al., 2007a).

Using laboratory experimental evolution technique, another way to investigate sexual conflict is to allow populations to evolve under experimentally imposed monogamous (relaxing sexual conflict) and polyandrous / polygynous / promiscuous (retaining sexual conflict) mating systems. Males with less toxic seminal fluid and females with enhanced vulnerability to mate-harm evolved in *D. melanogaster* populations with enforced monogamy (Holland and Rice, 1999a). Dung fly (*Sypsis cynapsea*) males forced to develop under monogamous conditions for 29 generations were found to be rather benign, but females from the same regime were shown to be more prone to mate-harm (Martin and Hosken, 2003a). Crudgington et al., (2009, 2005) used *D. pseudoobscura* to study the development of male and female-specific characteristics and found similar findings.

Thus, while laboratory experimental investigations are time-consuming, they have shown to be quite valuable in the field of sexual selection research. This field of research is highly complicated, and much more research needs to be done to fully comprehend the ramifications of this evolutionary process. I attempted to unearth certain facts concerning the function of sexual selection and conflict in the evolution of reproductive traits through my thesis, which was conducted using laboratory experimental evolution.

In the first series of experiments, I looked at male and female mating rates, as well as the sexual conflict that might occur as a result of these rates. Males are projected to have substantially greater optimum mating rates than females in promiscuous species like Drosophila. Females frequently oppose rematings, while males attempt to maximize their mating (Parker, 2006). As a result, there is a perpetual battle between the sexes for control of the population's mating rates. The male-female interaction and sexual selection existing in the population will determine how the inter-sexual struggle over mating rates plays out in the population. Through changed sex ratios, the Male biased (M) and Female biased (F) populations have developed under differing levels of sexual selection (Populations described in detail in chapter 2). I examined a) how mating rates have evolved as a result of evolutionary history and b) how the altered sex ratio has an immediate effect on mating rates in M and F populations when combined with ancestral females and males, respectively, at the three sex ratio treatments-male-biased, equal sex ratio, and female-biased.

Mating success may be argued to be a characteristic of a mating pair. These include the female's reluctance to remate and the males' perseverance to obtain a mate. Males can also mate strategically based on their own resource levels and female fitness, according to an earlier research (Edward and Chapman, 2011). For this study, I explored male mating preferences of sperm deficient males from the M and F groups when choosing between the M or the F female.

Male reproductive success is determined by both mating success and fertilization success. Because fertilization requires successful mating, mating success is expected to be a key driver of male reproductive success. Because male-male rivalry and female choice are fierce in these communities, males in the M population are likely to be under

significant selection for mating success. As a result, males in the M population should develop competitive mating techniques and features to assure their mating success. In this set of experiments, under competitive conditions, I compared the mating success of M and F males to see if M males had evolved better mating success. The assays were conducted on both virgin (common ancestral females, M females, and F females) and mated females (M females and F females).

I then looked into the secondary sexual traits that could contribute to the precopulatory advantage of males and might be under sexual selection. Males' secondary sexual traits are often evolved as a result of male-male competition. Wings are an important trait during the courting phase of Drosophila species (Tauber and Eberl, 2003). To attract females, males generate a courting song using wing vibrations. According to many studies, male wing morphology (wing form and size) impacts mating success (Naseerulla and Hegde, 1992). As a result, I looked at whether differing sexual selection in the M and F populations resulted in male wing morphological divergence.

I looked at a new phenomenon called wing interference pattern (WIP) along with wing morphology. Wing interference pattern (WIP) is a form of vibrant color pattern found on insect wings that are otherwise transparent. Thin-film interference from the wings allows WIPs to form. WIPs have been highlighted as a possible target for sexual selection in recent research because they may play a part in courting activities and may change under female choice(Hawkes et al., 2019; Katayama et al., 2014). In Drosophila melanogaster, males with brighter and more colorful wind interference patterns have an advantage in female mate choice. In both the M and F populations, I looked at the wing interference pattern in both males and females along with the male attractiveness, quantified as mating latency.

In the last section of my thesis, I looked at the male-female interaction in the outcome of sperm competition. Sperm competitive ability is a critical fitness trait impacting post-copulatory fertilization success in promiscuous species like Drosophila. Females were treated as passive observers in post-copulatory sexual selection research looking at sperm competitive ability for a long time. Males' sperm competing ability was thought to play out in an inert arena in the female reproductive system. Recent research has found that females influence the result of sperm competitive ability through cryptic female choice and male-female interactions (Ala-Honkola and Manier, 2016; Eberhard, 1996; Lüpold et al., 2020). Males from the M population have a fitness advantage in sperm competitive ability over males from the F population (Nandy et al., 2013a). The M and F populations were a good fit for testing the hypothesis of male-female interaction in the outcome of sperm competitive ability. For this series of experiments, a full factorial sperm competitive assay was set up utilizing males and females from the M, F, and ancestral (LHst) populations. With the female from M, F, or LHst populations, I examined sperm defense (P1) and sperm offense (P2) of males from M, F, and LHst populations (when competing with common males).

Experimental System

Chapter 2

There has been an ever-growing interest in understanding the evolutionary processes and their potential in shaping the species and biodiversity ever since Darwin's work on natural selection as an evolutionary force (Darwin's, 1859). Darwin suggested that evolution is a slow and gradual process, which is probably something he got wrong, as pointed out by (Garland and Rose, 2009). On the contrary, with sufficient genetic variation, one can find a rapid response to selection through experimental evolution.

In the form of artificial selection used by horse, dog, and pigeon breeders, experimental evolution has been around for a long time. Interestingly, Darwin himself got a lot of data supporting his theory of natural selection from artificial breeders. At the turn of the 20th century, researchers started gaining more and more interest in experimental evolution, and by the 21st century, it became the prominent force in evolutionary biology research to answer fundamental questions like the evolution of aging, immunity, sexual selection (Garland and Rose, 2009; Prasad and Joshi, 2003a).

Experimental evolution can be defined as "research in which populations are studied across multiple generations under defined and reproducible conditions (Garland and Rose, 2009). Laboratory selection is one of the crucial component of experimental evolution where a researcher sets up an isolated population, having sufficient additive genetic variations, in laboratory conditions, and monitors the evolution of such population under a well-defined selection force. Using the laboratory selection as a tool, particular conditions can be imposed on replicate populations which allow evolutionary modifications to occur in a confined and well-controlled environment that provides an advantage of repeatability, statistical power, and control over the selection environment. As a result, using laboratory selection, researchers have collected comprehensive data to study evolutionary responses in bacteria, yeast, Drosophila, and

mice, among other species (Bennett, 2003; Garland Jr et al., 2002; Hoffmann and Parsons, 1993; Kliman et al., 2003; Riehle et al., 2003; Travisano et al., 1995).

While there is no doubt that experimental evolution is a powerful tool to dissect the phenotypic and genetic correlation between different traits, it does come with limitations of its own, of which the researcher should be aware. As the field of experimental evolution developed, researchers have observed some drawbacks with selection studies (Gibbs, 1999; Harshman and Hoffmann, 2000). With the strict control over environmental conditions during selection, one would expect a canalized response, but that is not the case. Often, variability exists in response to selection where the researchers have enforced the same selection pressure and controlled for all other conditions. It can be argued that laboratory selection leads to complex and diverse responses, with chance and genetic diversity influencing the evolutionary process and mechanism (Garland Jr 2003; Folk and Bradley 2005). Along with this, other phenomena like the 'Cheshire Cat Syndrome' effects of inbreeding (Rose et al., 1996), and significant effects of gene X environment interaction can affect the experimental evolution and conceal the inferences to be drawn from the studies. Even with the limitations, experimental evolution is still a powerful tool in understanding the mechanisms and processes of evolution.

An ideal system for an experimental evolution study with laboratory selection would be a closed, isolated population of decent size with ample genetic variation and the ability to manipulate its organic and inorganic effectors. Laboratory adapted populations of *D. melanogaster* provide just this. In addition, the genetics and biochemistry of *D. melanogaster* are well defined and well-studied, making it an ideal model system to understand different evolutionary phenomena. In this chapter, I will

introduce the model system *Drosophila melanogaster,* discussing the general life-cycle of the flies, the laboratory population, and the selected populations used in this thesis.

Drosophila melanogaster

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

Drosophila melanogaster, commonly known as the fruit fly, is widely used as a model system in genetics, physiology, microbial pathogenesis, and life history evolution research. *D. melanogaster* is a holometabolous insect with four distinct life cycle phases: egg, larva, pupa, and adult. *Drosophila melanogaster* populations used for this study were maintained in the standard laboratory environment; 25^{o}C temperature and at 60-90% relative humidity. At these standard laboratory conditions, their life cycle follows the same route described below:

Eggs laid by the females hatch into the larva and go through three instars. After 4-5 days, upon reaching a "critical mass", the late third instar larva withdraws from feeding and moves out of the food. The third instar larva develops into a pupa by secreting a chitinous covering at an appropriate location (usually on the walls or cotton plugs of rearing vials). The adult fly appears from the pupal shell – a process commonly known as 'eclosion' after 4-5 days in the pupal stage. The adult males usually take ~8 hours to become reproductively mature and start the mating activity. During this window of 8 hours from eclosion, flies can be separated and held in single-sex groups as virgins. Once the adults become sexually mature, they can mate multiple times, and females can start laying eggs. Females can mate with multiple males and store sperms to fertilize the eggs at a later timepoint (Lefevre Jr and Jonsson, 1962; Manier et al., 2010a). Female fecundity primarily relies upon protein sources, e.g., yeast, the commonly used

protein source in laboratory cultures (Nandy et al., 2012b; Prasad and Joshi, 2003b; Stewart et al., 2005).

Laboratory baseline Populations: LH and derivatives

All the experiments in this thesis are carried out using the baseline population LH, LHst, and their derivatives. Lawrence Harshman established the LH population in 1991with 400 wild-caught *Drosophila melanogaster* females from central California, USA (Rice and Chippindale, 2001b). These populations are maintained on a 14-day discrete generation cycle on standard cornmeal-yeast-molasses fly food (Table) and at standard laboratory conditions; 25^0C, 60 -80% relative humidity, and 12/12 light/day cycle. These populations are maintained in glass vials with the dimensions of 25mm diameter × 90mm height. In 8-10ml of standard cornmeal-yeast-molasses fly food, larvae are cultured at the density of ~150 eggs in these vials every generation. Adult flies are cultured on the 12th day post egg collection into 16 mating pairs per vial under light CO_2 anaesthesia in fresh vials containing food supplemented with a fixed amount of live yeast. These vials are left undisturbed for two days, during which the males and females interact and mate, and females compete for access to the limited amount of live yeast. After two days, the flies in these vials are transferred to fresh 'oviposition vials' containing 8-10ml of fresh food, and the females are allowed to oviposit for 18 hours. The adults are discarded, and the egg density is controlled at a density of ~150 eggs per vial by scooping the extra eggs. These vials now become the rearing vials for the next generation. 60 such vials are maintained for the LH population. Every generation, all the vials are reshuffled and mixed before the culture on the 12^{th} day post egg collection. Another baseline population, called LHst, was derived from the LH population by

inserting the autosomal-recessive trait scarlet-eye colored marker ('st') by repeated back cross (Prasad et al., 2007b). LHst population is maintained in a similar protocol as that of LH. 30 vials of LHst population are maintained. To maintain the genetic uniformity with the LH population, LHst is regularly backcrossed with the LH population. The LH and LHst populations have been maintained in constant laboratory conditions for more than 600 generations. Therefore they are assumed to have adapted to the laboratory regime by now. The 14 day discrete generation cycle and 18-hour oviposition window allow for defining important time windows that determine the fitness of the individuals in these populations.

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

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, 2006; Pischedda and Chippindale, 2006), intralocus sexual conflict (Pischedda and Chippindale, 2006), and, most recently sexually antagonistic coevolution through alteration of sex ratio- the selection lines used in all the experiments in my thesis. 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., 2013d). Below, I will provide a brief introduction and maintenance protocol for these populations.

Maintenance

The population consists of nine subpopulations – three sex ratio regimes (Male-biased, equal sex ratio, and female-biased), each with three replicates– male-biased (M1-3), equal sex ratio (C1-3), and female-biased (F1-3) regime. Populations sharing the same numerical subscript are more closely related through a common ancestral population than populations with different subscripts (Figure 2.1). For example, M1 is more closely related to C1 and F1 than to M2 or M3. M1, C1, and F1 subpopulations constitute the 'Block-1', M2, F2 and C2 make 'Block-2', similarly, M3, F3, and C3 constitute 'Block-3'. Selection regimes from the same block are handled together during stock maintenance and experimentation. Except for the adult operational sex ratio, all aspects of the maintenance regime were kept the same across the regimes. Like the ancestral LHst population, these populations are maintained in 2-week discrete generation cycles and standard laboratory conditions; 25^{o}C temperature, 60-80% relative humidity, and 12-hours light / 12-hours dark. Eggs are cultured in standard cornmeal-yeast-molasses fly food at a density of 140-160 eggs / 8-10ml of food in 8-dram vials. On the 9th -10th-day post egg collection, adult flies start eclosing. In the period of 6 hours post eclosion, virgin flies are collected and kept in single-sex vials at a density of 8 flies per vial. On the 12th day post egg collection, the virgin flies are combined in sex ratio according to the selection regime – male-biased (24 males: 8 females) for M-populations, equal sex ratio (16 males: 16 females) for C-populations, and female-biased (8 males: 24 females) for F-populations. The combined flies are provided with food vials containing a fixed amount (0.467mg/female) of live yeast smeared on the food. The rest of the

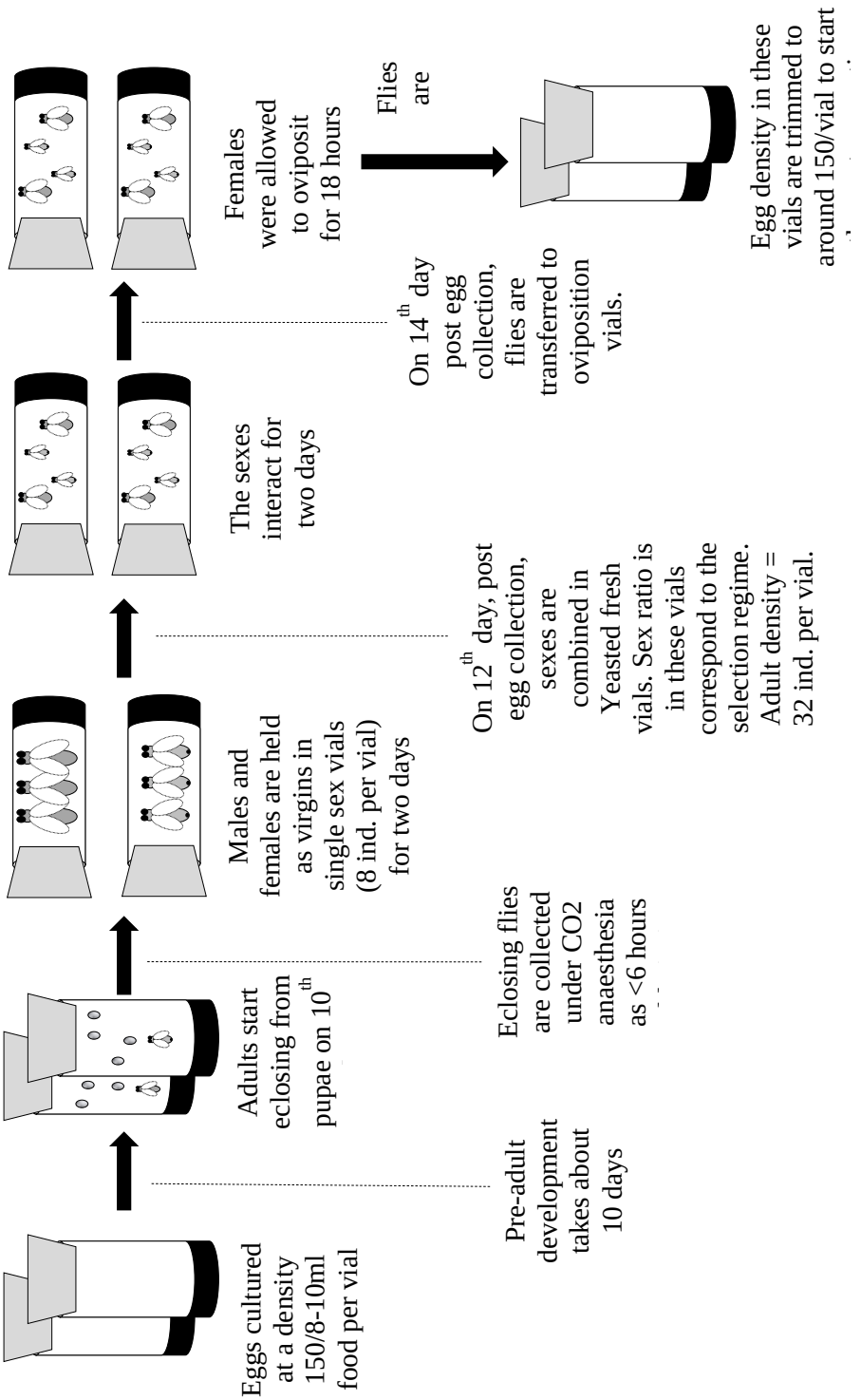

Figure 2.1: General maintenance protocol for the selected populations (M_{1-3}, C_{1-3}, and F_{1-3})
Figure Credits: (Nandy et al., 2013a)

maintenance protocol is the same as for the LH population. For each population, the effective population size was maintained at around 450. 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).

Sl. No.	Ingredient	Amount (per litre of food)
1.	Water	1100 ml
2.	Agar powder	14.8 gm
3.	Molasses	100 ml
4.	Corn meal	100 gm
5.	Baker's Yeast	41.2 gm
6.	Propionic acid	8 ml
7.	p-Hydroxymethyl benzoate	2.25 gm
8.	Ethanol	22.5 ml

Table 2.1: Composition of Cornmeal-molasses-yeast food.

Standardization: To Exclude parental effects

It is necessary to differentiate genetic changes due to selection from non-genetic parental effects while conducting the experiment using selection regimes. This was achieved by imposing all the populations to pass through one generation of maintenance under standard conditions, a process known as standardization (Michael R Rose, 1984), to neutralize the parental effects across different regimes. During standardization, egg density and the developmental phase remain the same, but the adult flies were allowed

to develop until the 12th day post egg collection instead of a virgin collection. On the 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 standard food and a paste of live yeast smeared on it. For a window of 6 hours, a fresh food plate is provided for oviposition, from which eggs are collected at the exact density of 150 eggs per vial to generate experimental flies.

What is known from these populations so far?

As mentioned before, these populations were established in 2009 by Bodhisatta Nandy. Over the last 12 years, numerous experimental studies on these populations have contributed considerably to answering important questions regarding sexual selection and conflict. I have summarised some of the important results from these populations below-

- Males from the M regime have evolved significantly higher sperm competitive ability (sperm defense - P1, offense - P2), with M regime males having increased P1 relative to that of males of the F regime. An increase in P1 was correlated with increased copulation duration, possibly suggesting more significant ejaculate investment by these males (Nandy et al., 2013b). However, this was not reflected in terms of evolutionary changes in either testis and accessory gland size or their depletion patterns (Chechi et al., 2017b).
- M regime males have evolved higher locomotor activity and courtship frequency which came at the expense of increased rates of aging and a decrease in mean lifespan (Nandy et al., 2013d).

- M regime females have evolved increased mate harm resistance quantified in terms of both longevity and fitness, which again traded off with an increased rate of aging. 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).
- Evidence for sexual selection could serve as a mediator in the process of speciation. Increased levels of sexually antagonistic coevolution resulted in the evolution of early stages of reproductive isolation at (a) premating and (b) postmating prezygotic stages. 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. (Syed et al., 2017).
- Evolution of senescence in components of competitive fitness, secondary sexual traits, and correlated mate harming ability in male *D. melanogaster* (Ali, 2018).
- No evidence of a trade-off between reproduction and immune components was observed between the males evolving under differential levels of sexual selection (Syed et al., 2020).
- Evolution of female influence on male competitive fertilization in response to the sexual conflict (Ali, 2018).
- Evolution of plastic response in their reproductive investment to varying density of rival males and identity of the competitors. M males initially increased their reproductive investment as the number of competitors increased from 1 to 7, but

after exposure to a higher number of competitors (31), these males decreased their reproductive investment. On the other hand, the F regime males continually increased their reproductive investment with an increasing number of competitors. In the case of the competitors being LH ancestral males, M males' reproductive investment pattern changed compared to when they were housed with males of their own kind (Maggu et al., 2021).

- No difference in the courtship learning ability of males from the two populations. However, males from M populations are better at recognizing and courting receptive females, even when they were not previously exposed to unreceptive females (Maggu et al., 2022).
- Evidence shows the evolution of transgenerational maternal effects in response to sexual selection and conflict. Daughters sired by multiply mated F regime mothers suffered a decline in their fecundity compared to daughters sired by singly mated F mothers. 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 (Maggu and Prasad, 2021).

Mating rates

Chapter 3

Introduction

In nature, we find a whole range of distribution of mating systems, from strictly monogamous to highly promiscuous (Andersson, 1994). In most species, anisogamy gives rise to a difference in the mating capacities of males and females, leading to promiscuity (Bulmer and Parker, 2002). Variation in the number of mates and matings in promiscuous populations is often defined by various evolutionary and ecological forces (Taylor et al., 2014), like sexual conflict, natural selection, and social organization of individuals within the population. Mating rates of the individuals affect the evolution of various life-history and reproduction traits within the population (Gavrilets and Hayashi, 2005; Parker, 2006; Simmons, 2019; Wedell et al., 2006).

The mating strategies of males and females rarely align and leads to the evolutionary conflict between the two. Mating rates in a population are dependent upon the interaction between the two sexes. In general, the mating rate of the population is defined as the number of matings secured by individuals in the population (but also see (Höglund et al., 1995; Kokko et al., 2014). The distribution of matings in a population depends upon the strength of selection on individuals to mate more (or less) often (Kokko et al., 2014). Males in the population continue to gain fitness advantage with every sequential mating (Bateman, 1948). On the other hand, after a minimal number of matings, females do not gain any advantage unless there is shortage of sperms from the previous matings (Parker, 2006). Also, frequent remating leave females vulnerable to mate harm, increased predation, and disease risks (Parker, 1970a; Rowe, 1994). Thus, both sexes are in conflict with the optimal mating rates wherein males want to acquire as many mates as possible, and females try and resist remating (Kokko et al.,

2014; Parker, 1979). This conflict over mating can lead to exaggerated sexual traits and behavior in males and more discriminating females (Holland and Rice, 1998a). Males evolve to be more persistent in acquiring more mating through increased aggression, rigorous courtship, elevated harm during mating, or exaggerated secondary sexual traits to attract females. This puts a further cost on females remating, and females evolve resistance traits to resist remating and reduce the harm inflicted through mating.

Males have to be persistent enough to overcome the female threshold resistance to mating to secure a mating. In a population with an equal sex ratio, for mating to occur, an individual male needs to have minimal threshold persistence to overcome female resistance to mating (Gavrilets et al., 2001). However, the scenario becomes much more complicated in the case of manipulated operational sex ratio. The spatial and temporal distribution of mates and the encounter rate affect the population's mating rates and sexual selection (Emlen and Oring, 1977; Kokko and Mappes, 2013; Trivers, 1996). In a male-biased sex ratio, the females encounter males at a higher rate. Therefore per female mating frequency is expected to be higher in male-biased populations. On the other hand, females encounter males at a much lower rate in the female-biased sex ratio. Various theoretical models have predicted that the operational sex ratio would affect the mating system of the population and thus the mating rates (Clutton-Brock and Parker, 1992; Kokko and Monaghan, 2001; Parker and Simmons, 1996). The above argument is also supported by (Wigby and Chapman, 2004) study, which found that per female mating rates were higher in females evolved in males biased population as compared to the female-biased population. At the same time, the females from the male-biased population had higher mate harm resistance ability without any difference in

male mate harm ability across the two populations, suggesting that the female's response to resist male harm is in response to the higher mating frequency.

Resolving conflict over mating rates is not straightforward, as the optimal trait values are dependent on the interaction between the two sexes. This can result in one sex reaching close to its fitness optima at the cost of the other sex, or both the sexes reaching a compromise to an intermediate trait value (Parker, 2006). Various theoretical models have found that the optimal mating rates for females are an intermediate value which is much lower than the optimal mating rate for the males (Arnqvist and Nilsson, 2000; Gavrilets et al., 2001; Holland and Rice, 1998a; Parker, 1974; Rice, 1996a). In nature, how this conflict plays out depends on species, evolutionary history, and various other ecological and environmental factors like density and operational sex ratio.

Using the experimental evolution approach, studies have found that mating rates can drive the evolution of various phenotypic traits like mating behavior (Crudgington et al., 2009; Klemme and Firman, 2013; Martin and Hosken, 2003b; S Pitnick et al., 2001), sperm competition (Hosken et al., 2001; Klemme and Firman, 2013), gonad size (Hosken and Ward, 2001; House et al., 2013; S. Pitnick et al., 2001), mate harm and mate harm resistance (Gay et al., 2011; Martin and Hosken, 2003c; Wigby and Chapman, 2004), and cognitive function (Hollis and Kawecki, 2014). Thus, mating rates become an essential trait to investigate in order to understand the evolutionary dynamics in the population and persistence and resistance traits in males and females, respectively.

In the current study, I measured the mating rates of the M and F populations. With more males than females in the M population, the levels of male-male competition and female

choice are higher, thus increasing the levels of sexual conflict in the population. On the other hand, in the female-biased (F) population, adult interaction leads to lower levels of sexual conflict. Males and females from M populations are expected to be in conflict over mating rates. Given the higher intensity of sexual selection in the M population, I hypothesized that the M population would have higher mating rates than the F population. To test this hypothesis, I measured the mating rates of these populations in their standard conditions. Next, males from M and F populations were combined with common ancestral females at three different sex ratios, and their mating rates were measured. Similarly, females from M and F populations were combined with common ancestral males at three different sex ratios their mating rates were measured. Sex ratio treatments a) male-biased, b) equal sex ratio c) female-biased were included to capture the effect of sex ratios and evolutionary selection history on the mating rates.

Materials and Methods

Standardizing and generating experimental flies

The flies from the selection population were standardized for experiments by maintaining them without sex-ratio manipulation for one generation. This standardization process is done to control for any non-genetic parental effects that might affect the results.

Eggs are collected from these standardized flies of M, F, and LH_{st} populations, at a density of 150±2 eggs per vial. On day 9^{th} -10^{th} from the egg collection, the virgin flies are collected in a single sex vial at 8 flies per vial density. The adult flies are combined on the 12th day from egg collection for the mating rate assay.

Population Mating rates assay of M and F populations in their respective sex-ratios

Mating rates of M and F populations were measured in their standard maintenance conditions of altered sex ratios. Five vials were set up with males and females from the M population in a male-biased ratio (3:1:: male: female). Similarly, 5 vials were set up with males and females from the F regime in a female-biased ratio. Physical observation ensures the first mating of all the females in a vial in M population and all males in a vial in the F population. Every hour, the vials were scanned for two days, and the number of mating pairs in each vial was recorded. 5 vials were set up for each M and F population for each replicate. In total, for three replicated of two selected populations, data from 30 vials was used for further analysis.

M and F male mating rates assay with common females

Virgin flies from M, F, and LH_{st} populations were collected in single-sex vials at the density of 8 flies per vial. For measuring the mating rates of males, on the day of the experiment, virgin males from M and F populations are combined with LH_{st} females in three different sex-ratio treatments: male-biased (3:1:: male: female), equal sex ratio (1:1:: male: female) and female-biased (1:3:: male: female). Each vial contains in total of 32 flies, with male: female ratio depending on the sex-ratio treatment. Once the vials were set up, the first mating of all individuals of limiting sex in each vial was observed. For male-biased treatment, females per vial are less in number than males, making it the limiting sex, so the first mating of all the females (8 females) was observed. The males are the limiting sex for female-biased treatment, so first mating was observed for all the males in the vial. For equal sex ratio treatment, the first mating of all the flies are ensured. For the next two days, every hour the vials were scanned, the number of

mating pairs in each vial was recorded. Assays for selected populations from the same replicate line were performed on the same day. 5 vials were set up per sex-ratio treatment for each of the M and F selection populations. This gives 45 vials for M males over three treatments and three replicate blocks and 45 vials for F males over three treatments and three replicate blocks. In total, for three replicate blocks and two selected populations, data from 90 vials was used for further analysis.

M and F female mating rates assay with common males

A similar design as above was followed to measure females' mating rates. On the day of the experiment, the virgin females from M and F populations were combined with LH_{st} males in three different sex-ratio treatments: male-biased (3:1:: male: female), equal sex ratio (1:1:: male: female), and female-biased (1:3:: male: female). First mating of all the individuals of limiting sex was observed and ensured. Every hour, the vials were scanned for two days, and the number of mating pairs in each vial was recorded. 45 vials with M females and LH_{st} males were set up over three sex-ratio treatments and three replicate blocks. In total, for three replicate blocks and two selected populations, data from 90 vials was used for further analysis.

Statistical analysis

Since females are usually the limiting factor in mating, for analysis of mating rates, per female mating (PFM) data were extracted from the mating data by dividing the total number of matings by the number of females in the vial. PFM is a better variable for comparison across the sex-ratio treatments where the number of males and females is different, but the number of individuals is the same. Both the total number of matings(TM) and per female mating (PMF) were used for the analysis.

$$\text{Per female mating (PFM)} = \frac{\text{Total number of mating per vial}}{\text{Total number of females per vial}}$$

All statistical tests were performed in the R statistical environment (v4.0.2, R Core Team 2020), using "lmer4" package and the "CAR" package.

For population-level mating rates, males and females from the same population are left to interact in the sex ratio they have evolved in. Total matings (TM) and per female mating (PFM) were analyzed using analysis of variance (ANOVA) with the population as fixed factors crossed with block as a random factor.

For analysis of mating rates of males and females from the M and F populations, across the three sex-ratio treatments, the total number of matings (TM) and per female mating (PFM) were analyzed using analysis of variance (ANOVA) with the male selection regime (or female selection regime), and the sex-ratio treatment as fixed factors crossed with block as a random factor.

Results

Population mating rates

Mating rates of the M and F populations were measured in their respective sex ratio conditions. The total number of mating over two days was significantly higher in the F population as compared to the M population (Table 3.1, Figure 3.1). These mating rates were measured in different sex ratios. Therefore I used the per female mating (PFM) rate for better comparison. PFM was significantly higher in the M population as compared to the F population (Table 3.2, Figure 3.2).

M and F male mating rates

Mating rates of males from the M and F populations with common females were measured at three different sex ratio treatments. There was a significant effect of treatment on total matings (Table 3.3, Figure 3.3) without any significant effect of the selection regime of males or the interaction of treatment or selection. The total number of matings was highest in equal sex ratio and lowest in male-biased sex ratio treatment. In per female mating (PFM), there was a significant effect of the treatment and selection regime of the males (Table 3.4, Figure 3.4). Across the sex-ratio treatments, PFM was highest in male-biased sex ratio treatment and lowest in females-biased sex ratio treatment.

M and F female mating rates

M and F female's mating rates were measured with common males at three different sex ratios. There was a significant effect of treatment and selection regime of females and their interaction on both total matings (Table 3.5, Figure 3.5) and per female mating (PFM) (Table 3.6, Figure 3.6). Total matings were higher for F females as compared to M females. Total matings were highest in equal sex ratio and lowest in male-biased sex ratio. Per female mating (PFM) was also higher for F females than M females across the sex-ratio treatments.

Comparing the mating rates at a male-biased sex ratio between M males (with ancestral females), M females (with ancestral males), and M populations (M males with M females) shows that per female mating was significantly higher for M males with common females than with M females (Table 3.8, Figure 3.8). On the other hand, M females with common males, and M males have similar mating rates (Figure 3.8). On

comparing mating rates at a female-biased sex ratio between F males, F females, and F population, total matings and matings per female were not significantly different (Table 3.8, Figure 3.9).

	Sum Sq	Mean Sq	Num DF	Den DF	F value	Pr(>F)
Population	26.133	26.133	1	26	10.54	0.00321**

Table 1: Results of ANOVA for total number of matings in M and F populations. Population here describes the M and F populations.

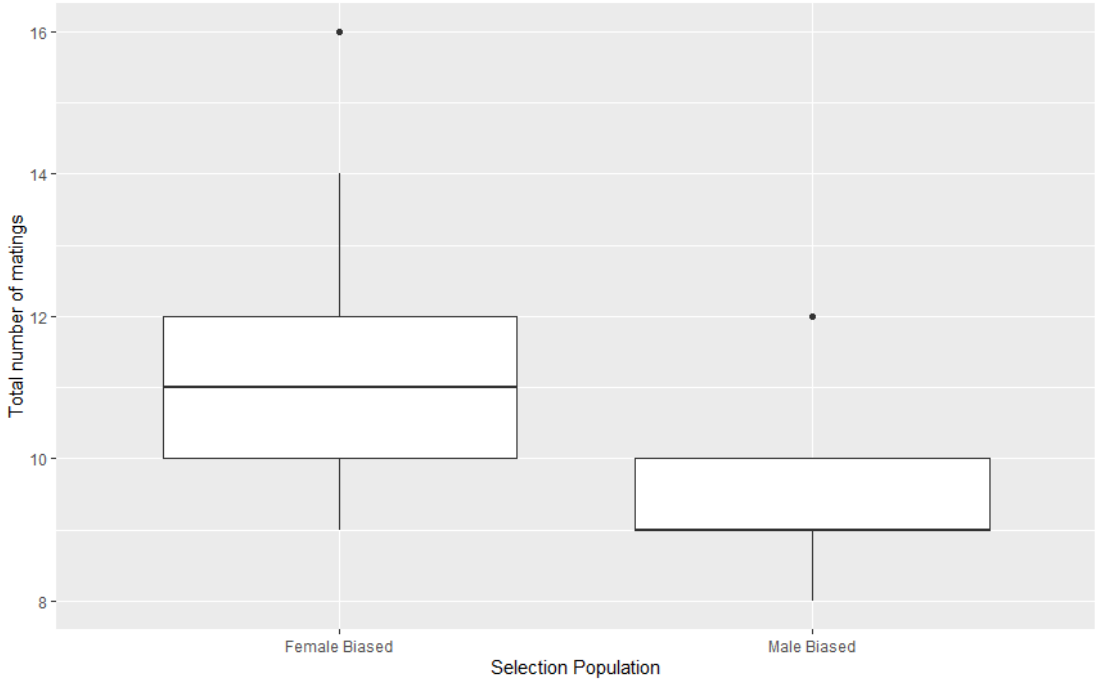

Figure 3.1: Representing the comparison of total number of mating (y- axis) across the two population (X-axis). Boxplots indicate median and interquartile range. Whiskers indicate variability outside the upper and lower quartiles. Dots represent outliers.

	Sum Sq	Mean Sq	NumDF	DenDF	F value	Pr(>F)
Population	3.7336	3.7336	1	28	247.46	$1.98e^{-15}$***

Table 3.2: Results of ANOVA for per female mating in the M and the F populations. Population here describes the M and F populations.

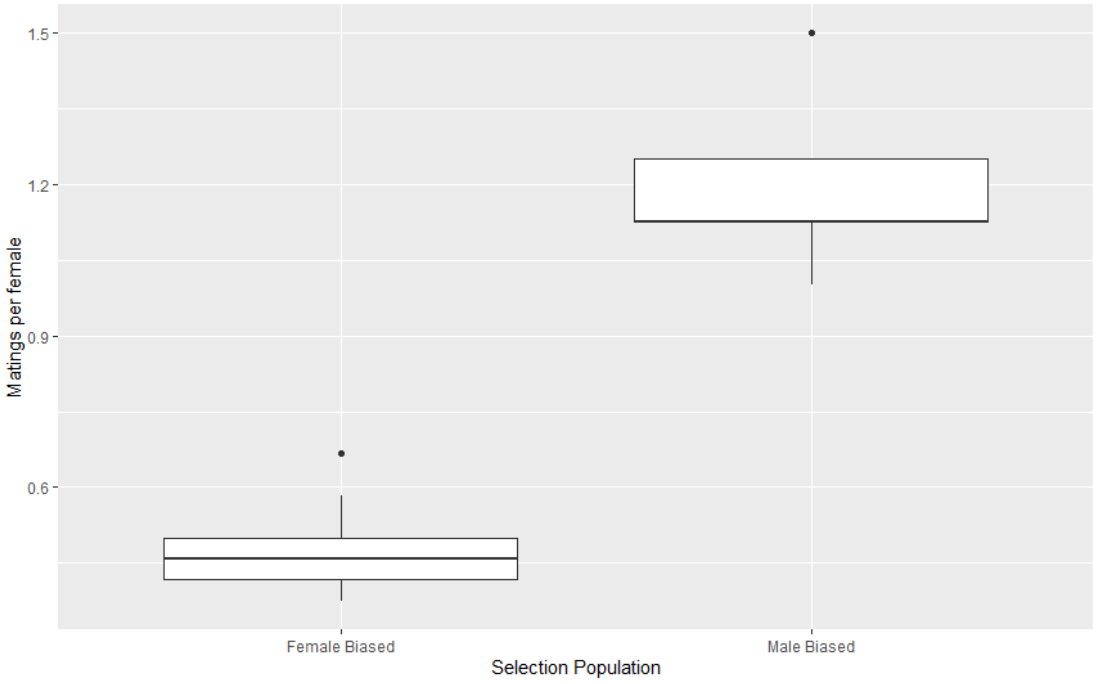

Figure 3.2: Representing the comparison of per female mating (y- axis) across the two population (x-axis). Boxplots indicate median and interquartile range. Whiskers indicate variability outside the upper and lower quartiles. Dots represent outliers.

	Sum Sq	Mean Sq	Num DF	Den DF	F value	Pr(>F)
Treatment	710.13	355.06	2	4	91.5566	0.0004569***
Selection	47.71	47.71	1	2	12.3021	0.0725518
Treatment:Selection	0.87	0.43	2	76	0.1117	0.8944239

Table 3.3: Results of ANOVA for total number of mating of males from M and F population. Here treatment describes the male-biased, female biased and equal sex ratio treatments and selection describes the two selected populations –M and F from which males were selected.

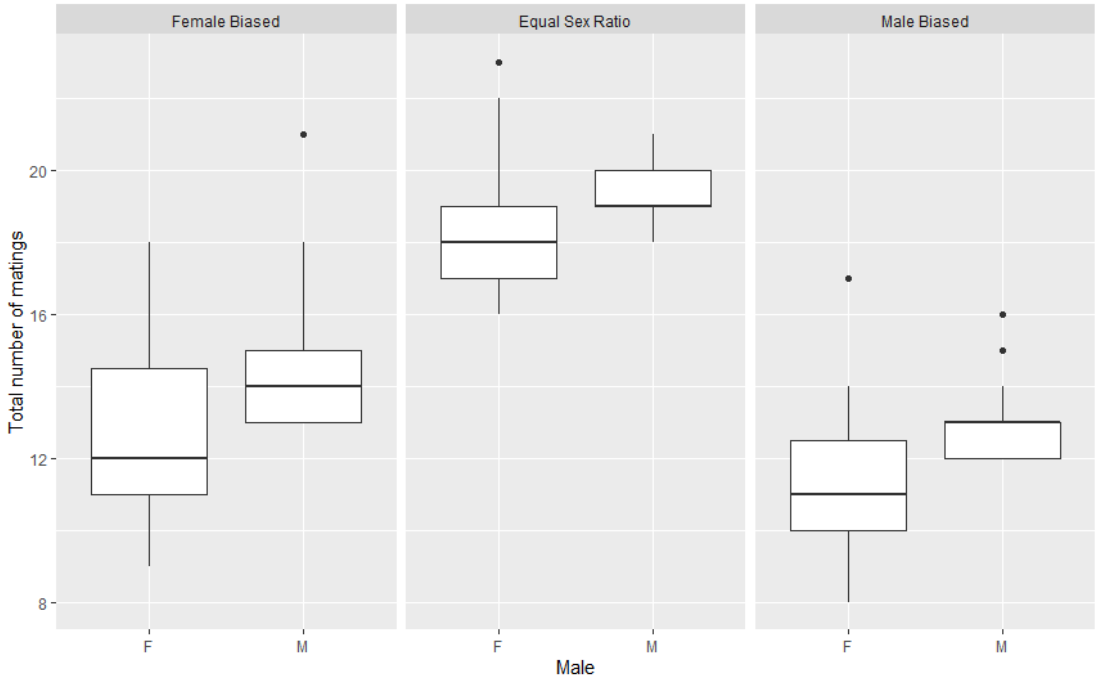

Figure 3.3: Representing the comparison of total number of mating (y- axis) of the males from M and F populations (X-axis), with common females, at three different sex ratios (grids). Boxplots indicate median and interquartile range. Whiskers indicate variability outside the upper and lower quartiles. Dots represent outliers.

	Sum Sq	Mean Sq	Num DF	Den DF	F value	Pr(>F)
Treatment	14.0233	7.0116	2	82	294.7563	<2.2e-16***
Selection	0.2918	0.2918	1	82	12.2684	0.0007487***
Treatment:Selection	0.0545	0.0273	2	82	1.1458	0.3229967

Table 3.4: Results of ANOVA for per female mating of males from M and F populations. Here treatment describes the male-biased, female biased and equal sex ratio treatments and selection describes the two selected populations –M and F from which males were selected.

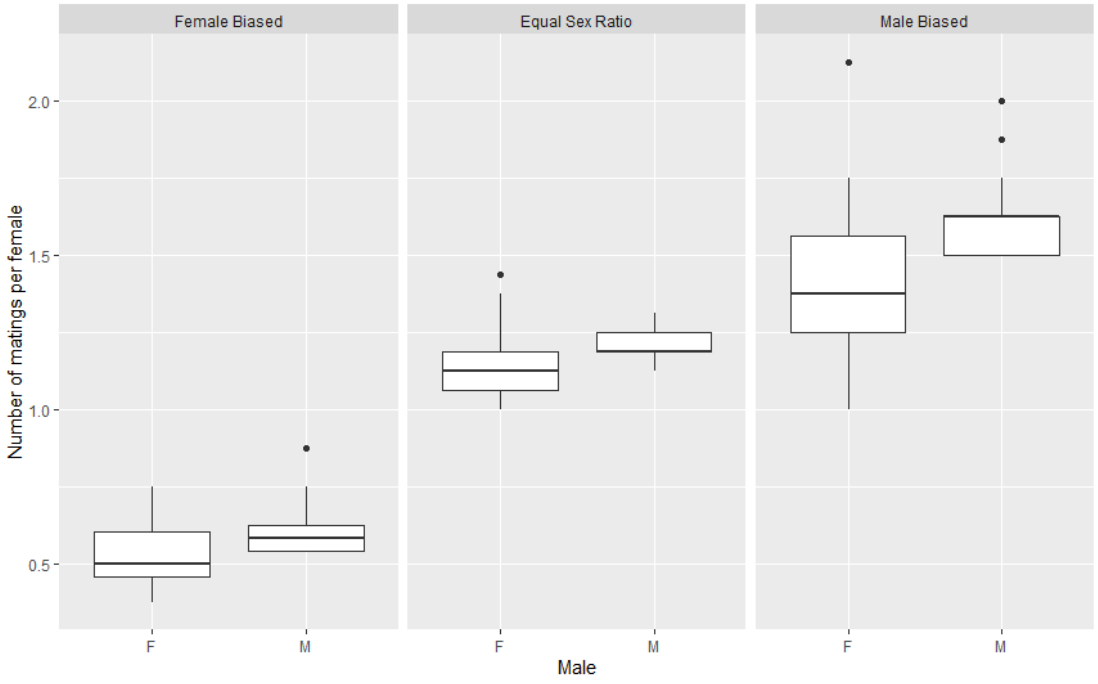

Figure 3.4: Representing the comparison of per female mating (y- axis) of the males from M and F populations (X-axis) with common females, at three different sex ratios (grids). Boxplots indicate median and interquartile range. Whiskers indicate variability outside the upper and lower quartiles. Dots represent outliers.

	Sum Sq	Mean Sq	Num DF	Den DF	F value	Pr(>F)
Treatment	232.539	116.270	2	4	73.9932	0.0001292 ***
Selection	63.662	63.662	1	2	40.5140	0.0092291**
Treatment:Selection	28.956	14.478	2	76	9.2136	0.0002620***

Table 3.5: Results of ANOVA for total number of mating for females from M and F populations. Here treatment describes the male-biased, female biased and equal sex ratio treatments and selection describes the two selected populations –M and F from which females were selected.

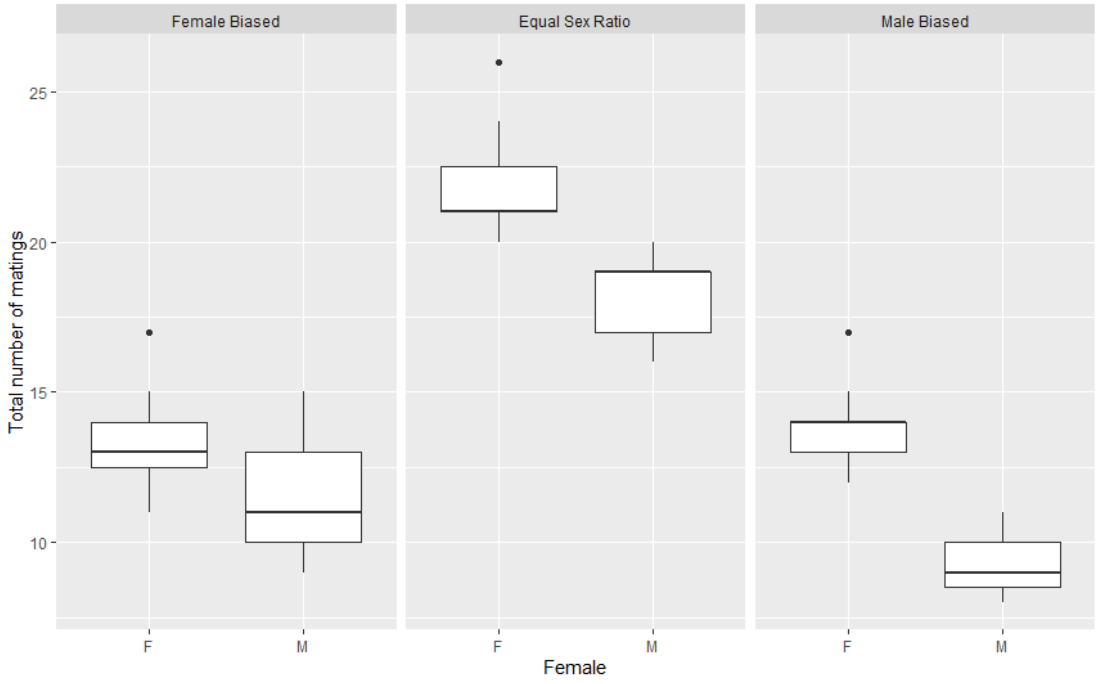

Figure 3.5: Representing the comparison of total number of mating (y- axis) of the female from M and F populations (X-axis) with common males, at three different sex ratios (grids). Boxplots indicate median and interquartile range. Whiskers indicate variability outside the upper and lower quartiles. Dots represent outliers.

	Sum Sq	Mean Sq	Num DF	Den DF	F value	Pr(>F)
Sex ratio Treatment	14.1635	7.0818	2	82	606.888	$<2.2e^{-16}$***
Selection	1.7477	1.7477	1	82	149.773	$<2.2e^{-16}$***
Treatment:Selection	0.9.93	0.4546	2	82	38.962	$1.276e^{-12}$***

Table 3.5: Results of ANOVA for per female mating for females from M and F populations. Here treatment describes the male-biased, female biased and equal sex ratio treatments and selection describes the two selected populations –M and F from which females were selected.

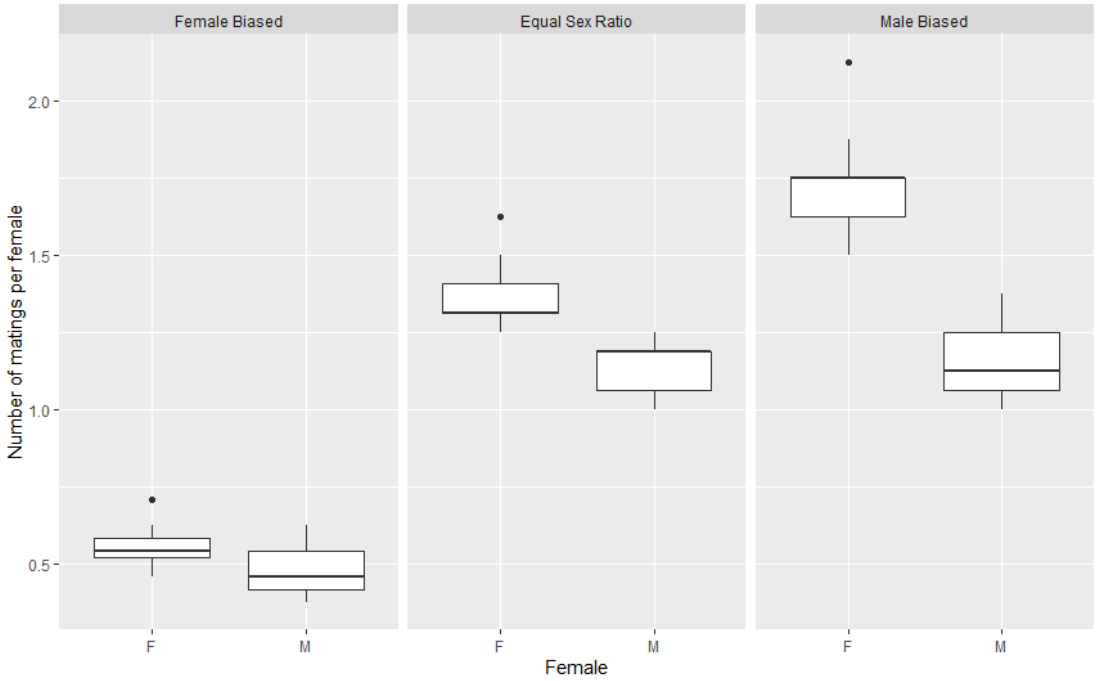

Figure 3.6: Representing the comparison of per female mating (y- axis) of the females from M and F populations (X-axis) with common males, at three different sex ratios (grids). Boxplots indicate median and interquartile range. Whiskers indicate variability outside the upper and lower quartiles. Dots represent outliers.

	Sum Sq	Mean Sq	NumDF	DenDF	F value	Pr(>F)
M population	1.9882	0.9941	2	4	44.635	4.04^{e-11}***
F population	0.030911	0.015455	2	4	2.4206	0.2047

Table 3.6: Results of ANOVA for mating rate comparison between males and females of M population and of F population. Here M population describes the comparison of mating rates of M males with common females, M females with common males and M males with M females, in male biased sex-ratio. Similarly, F population describes the comparison of mating rates of F males with common females, F females with common males and Fmales with F females, in female biased sex-ratio.

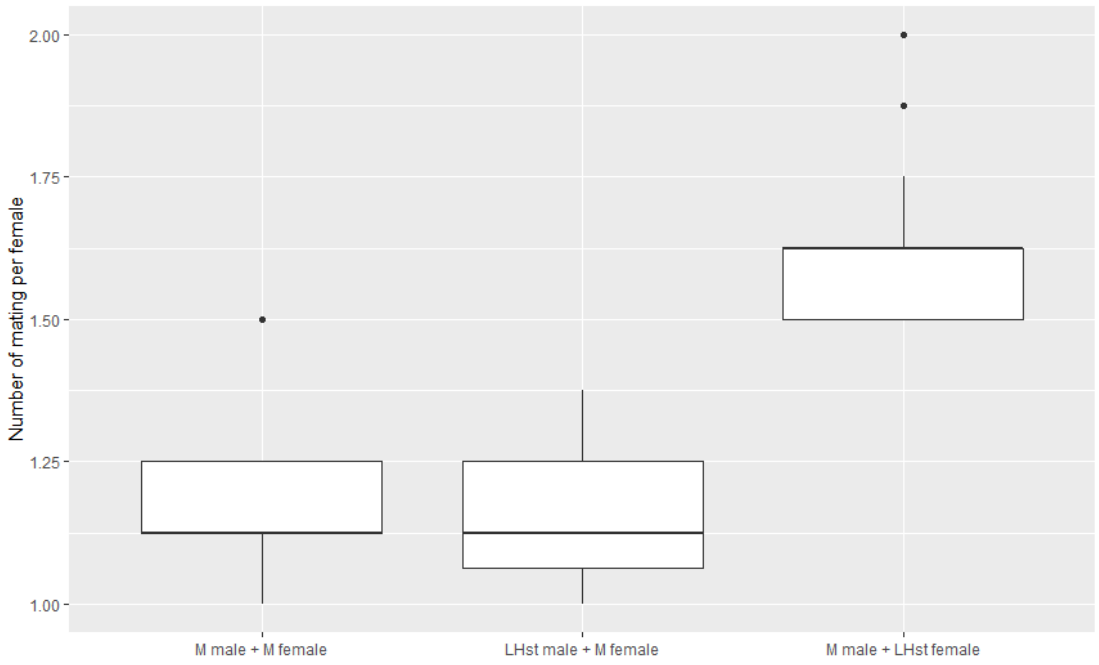

Figure 3.7: Representing the comparison of total number of matings (y-axis), in male biased sex ratio, of three different mating pairs consisting of either, both males and females from M population, or one sex of M population and a common male/female. Boxplots indicate median and interquartile range. Whiskers indicate variability outside the upper and lower quartiles. Dots represent outliers.

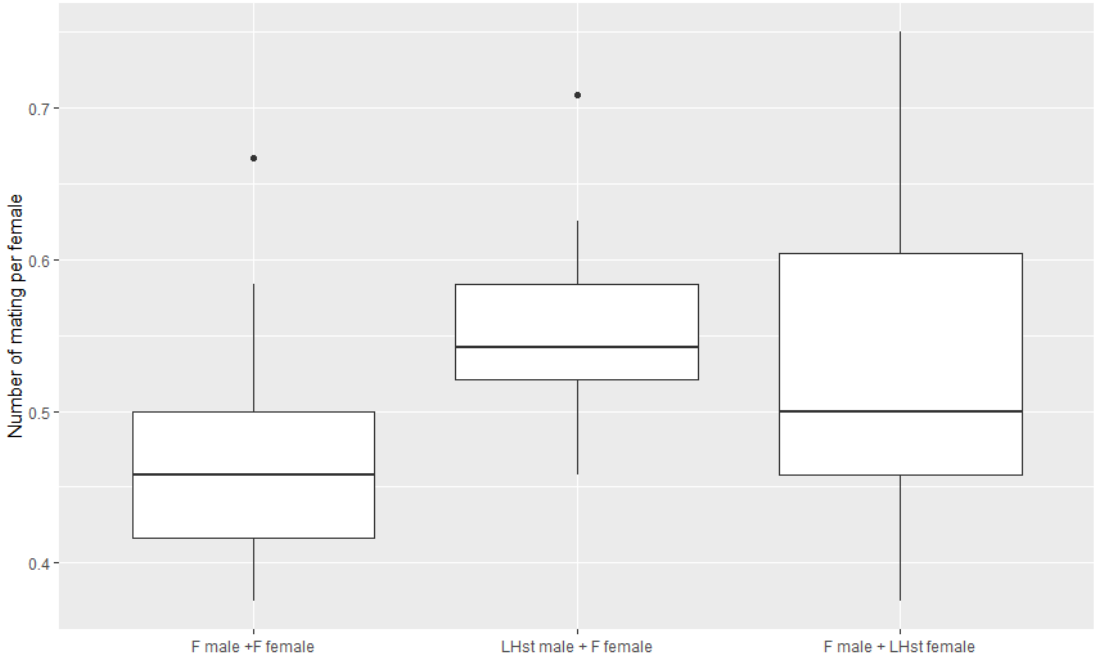

Figure 3.8: Representing the comparison of total number of matings (y-axis), in female biased sex ratio, of three different mating pairs consisting of either, both males and females from F population or one sex of F population and a common male/female. Boxplots indicate median and interquartile range. Whiskers indicate variability outside the upper and lower quartiles. Dots represent outliers.

Discussion

In this study, I measured the mating rates of the populations that evolved under altered sex ratios in their standard conditions. Mating rates of the M population are measured with males and females from the M population combined in a male-biased sex ratio. Similarly, mating rates of the F population are measured with males and females from the F population combined in a female-biased sex ratio. We also measured male mating rate and female mating rate separately, in M and F populations. Males from the male-biased (M) and female-biased (F) populations were combined with common ancestral females in three different sex ratios of 3:1 male-biased, 1:1 equal sex ratio, and 1:3 female-biased. Similarly, females from the M and F population were combined with common ancestral males in the three different sex-ratio treatments mentioned above. The total number of matings was measured over a 2 day interaction period, and per female mating (PFM) was calculated.

The results show that at the population level, the female-biased (F) population has more total number of matings than the male-biased (M) population. Interestingly, the M population has significantly higher per female mating (PFM). It is important to note here that the mating rates of the M and F populations are measured at their respective sex ratios under which they have evolved. Considering that females are the limiting factor in mating, the total number of matings is expected to be higher in the F population, given that there are more females per vial. This result is in accordance with the result from (Wigby and Chapman, 2004) study, which also found that the male-biased population has higher mating rates per female. Wigby and Chapman (2004) found that females from the male-biased population evolved greater mate harm

resistance in their population. This increased mate harm resistance was in response to higher mating rates, as males from the male-biased and the female-biased populations did not differ in their mate harming ability. Unlike their population, in M and F populations males have been found to differ in their mate harm ability (Nandy et al., 2014). Therefore, increased mate harm resistance in females from the M population could be a response to increased mate harm and not increased mating rates. More males than females in a male-biased sex ratio leads to higher male-male competition and female choice. This would result in higher persistence of males to acquire mating adding to the mate-harm abilities of the males. More males acquiring mating in a male-biased sex ratio would lead to higher per female mating, increasing mate-harm and mate harm resistance in males and females, respectively.

When comparing the males' mating rates with common ancestral females, we found a significant difference in per female mating rates of males from the M and F populations across the three sex-ratio treatments. This result suggests that males from M and F populations, having evolved under differential levels of sexual conflict, have diverged in their mating capacities. According to the Bateman principle, males try to maximize their mating capacity to increase their reproductive fitness (Bateman, 1948). While males from both M and F populations try to maximize their mating capacity, M males obtain more mating per female than F males. M males are expected to have evolved higher persistence in courting the females as in male-biased sex ratio; males have to be ready to court and try to mate with the fewer females available. On the other hand, in the F population, males do not need to be as persistent as M males, as the number of females available is more and the male-male competition is also less.

There was a significant effect of the sex-ratio treatment in the male mating rate assay. PFM across the sex-ratio treatment was highest in male-biased treatment, followed by equal sex-ratio and female-biased sex ratio. The significant effect of sex-ratio treatments shows that the availability of females limits the mating rates, and sex-ratio manipulation affects the mating rates (Kokko and Mappes, 2013).

When comparing the females' mating rates from M and F populations with common ancestral males, the total number of matings and PFM were significantly lower in females from the M population compared to the F population females. These results show that the females from the M population are more resistant to mating than the F population. M females have evolved in the male-biased sex ratio and in a higher male-male competition scenario. In order to restrict the number of matings, and the mate harm, they have to become more resistant to mating. The results show that females evolved in the male-biased sex ratio, under higher levels of sexual conflict, evolved to be more resistant to mating.

The mating rates for females across the sex-ratio treatment were also significantly different. Total mating over two days in equal sex ratio treatment was significantly higher than male-biased and female-biased treatments. Similarly, PFM in female-biased sex ratio treatment was significantly lower than in equal sex ratio and male-biased sex ratio treatments. The significant difference in the treatments in female mating rates, like in males, further provides evidence that encounter rates and sex ratio treatments affect the mating rates of the population, along with the evolutionary history.

When analyzed separately, I found that while M males have increased mating rate, M females have decreased mating rates. However, in the M population, M males and

females interact and define the mating rate of the population. This points towards sexual conflict over mating rates in the M population. Therefore, I further compared the population mating rates with the mating rates of males and females from the respective population in their respective sex ratios. M females have similar mating rates when put together with the ancestral males or M males, in a male-biased sex ratio. On the other hand, M males' mating rate is significantly higher when mating with ancestral females as compared to when mating with M females in a male biased-sex ratio. This result shows that conflict over mating rates exists in the M population. While M males can maximize their mating to a higher value, they are limited by the M females to sub-optimal mating rates. This, together with the fact that M female mating rates with ancestral males are similar to population mating rates, suggests that M females dictate the mating rates in the population. On the other hand, in the F population, we did not find any significant difference in the population mating rates with the mating rates of F females with ancestral males or F males with ancestral females. Sexual conflict in the F population over mating rates seems to be absent or low enough not to be observed in our assays.

More often than not, the two sexes' mating strategies and reproductive investments mismatch in a population (Andersson, 1994; Kokko et al., 2014; Parker, 2006). Sexual conflict over mating rates between males and females is expected in a population. While optimal mating rates for males are as high a number as they can achieve, the optimal number of matings is much lower for females. As a result of the conflict over mating rates, males might get their way by persistent courting and cohering the females to remating. However, the number of matings for males is often limited by the search for a mate and female reluctance to frequent mating (Kokko et al., 2014). While the males

try to increase their mating frequency, females are more reluctant to remate. This brings down the overall mating rates of the population. While in the F population evolved under lower levels of sexual conflict, I did not see any sexual conflict over the mating rates; in the M population, the conflict is very clear. From the results, it is clear that at this point of time in the evolution of the M population, females have the upper hand in the conflict over mating rates in the M population. While M males have the potential to achieve higher matings, it is brought down to the average M population mating rates by the M females. An explanation for this result could be found in (Gavrilets et al., 2001) study, where they incorporate the cost of sexual conflict to both the sexes in their model and predicts that the system evolves towards stable equilibrium with a stable limit cycle. In some cases, based on initial conditions and natural selection acting in the population, the exaggerated male traits do not cause females to deviate from optimal mating rates.

Another explanation for the results we found could be that we are looking at a one-time point in the evolutionary process. Sexual conflict over mating rates and various other traits is a continuous process where males and females continuously co-evolve to adapt to and outcompete the other sex (Arnqvist and Rowe, 2002; Chapman and Partridge, 1996; Gavrilets et al., 2001; Partridge and Hurst, 1998; Rice, 1996a). In such a case, these results are a snapshot of the process at a given time point. The M and F population have evolved under differential sexual selection for over 200 generations. Previously, at some point in the selection process, it might have happened that the M males had the upper hand in conflict over the mating rates in the population. However, at this point in the process, the M females control the mating rates of the population and have the upper hand over males in the conflict over mating rates.

In conclusion, my study shows that in populations with high levels of sexual selection, the conflict over mating rates appears as a result of the difference in mating rates of the individual sexes. This conflict over the mating rates can be resolved if one sex dominates the mating rates of the population, which my result shows could be females, restricting the population levels in the population to sub-optimal levels to that the males can achieve.

Mate Preference

Chapter 4

Introduction

In many species, females are typically the choosier sex because of their higher investment per offspring and lower variance in realized fitness than males. In species with sex-role reversal, male mate choice has been observed. Even in populations where females are typically the choosier sex, males can exercise choice if the situation so arises. If there is a substantial cost to mating, males do exercise choice. Pre-mating rituals and ejaculate production are much more energy expensive than previously thought to be (Dewsbury, 1982). Ejaculate exhaustion in males puts a constraint on the male mating rate (Linklater et al., 2007; Wedell et al., 2002). In such a case, males benefit by exercising choice over random mating (Byrne and Rice, 2006).

When the males are forced to exercise choice, the choice will depend upon the difference between the fitness of the females to choose from and the male's ability to sense that fitness differences. Fecundity difference in females is often seen as the trait upon which such a choice relies (Edward and Chapman, 2011; LeBas et al., 2003). A male can also sense female fitness based on the honest signals that reflect overall female fitness like body size, CHC profile, and female ornamentation (Clutton-Brock, 2009, 2007; Kraaijeveld et al., 2007); those traits can act as the medium for choice. Similarly, males can benefit from exercising mate choice when the investment in courtship and mating is high, as in the case of nuptial gifts or energetically demanding courtship rituals, which limits the number of matings for males (Baruffaldi and Andrade, 2015; Judge and Brooks, 2001; Morse, 2010; Simmons et al., 1993; Wedell et al., 2002).

While certain traits can help the male choose the female, some traits can help the male choose to steer clear of certain females. For example, for a male to mate with a female

with higher threshold resistance to mating, he will have to invest more energy and resources in obtaining a successful mating. For a resource-depleted male, investing in courtship and pre-mating rituals towards mated unreceptive females or females with higher resistance to mating would be a bad investment in terms of energy and resources. Thus, males' preference for mating depends upon the fitness and behavioral traits of the females available to mate.

D. melanogaster is the classic example of species where males maximize their fitness by increasing their mating frequency (Bateman, 1948). However, even in D. melanogaster, male mate choice has been observed (Arbuthnott et al., 2017; Byrne and Rice, 2006; Edward and Chapman, 2012; Khan and Prasad, 2013; Long et al., 2009; Nandy et al., 2012a) as is in other drosophila species like *D. pseudoobscura* (Gowaty et al., 2003, 2002). Male mate choice is favored in these species potentially because ejaculate exhaustion constraints the male mating rates (Linklater et al., 2007; Wedell et al., 2002).

In the population, females usually evolve resistance traits to resist the mating attempts from the males. While higher mating rates are beneficial for male fitness, it decreases females' reproductive fitness. Such sexually antagonistic co-evolution between the sexes leads to a conflict between the sexes. The conflict has been proposed to affect a wide range of female traits, including female behavior and life history (Rice 2000, Promislow 2003, Wedell et al. 2006, Bonduraiansky et al. 2008). In populations with higher levels of conflict, male adaptations (such as persistent courtship, toxic ejaculate, spiky genitalia, or other forms of traumatic inseminations that harm females) are predicted to increase extrinsic mortality rates of females. This can lead to the evolution of faster intrinsic rates of aging (Promoslow 2003, Maklakov et al. 2005, 2006, 2007,

Bonduriansky et al. 2008) through 'mutation accumulation (Medawar 1952) or 'antagonistic pleiotropy' (Williams 1957). In addition, females are selected for resistance to counter the mate harm later (Bonduriansky et al. 2008). Such resistance traits have been documented in many species and often take the form of changes in behavior, morphology, or physiology (Birkhead et al. 1988, Rowe et al. 1994, Arnqvist and Rowe 1995, Bonduriansky 2003, Andersson et al. 2004, Snook and Hosken 2004). The resistance traits are expected to be costly to the females. For example, females in water striders have been shown to bear the ecological cost of resistance, in the form of increased risk of predation (Rowe 1994) and physiological cost of resistance, in the form of increased energy expenditure (Watson et al. 1998). In fruit flies (*Drosophila melanogaster*), specifically in the laboratory populations, female resistance is expressed in the form of an intense pre-mating struggle between the sexes (Rice et al. 2006), including such female behavior as kicking, flicking, and extrusion of genitalia (Connolly and Cook 1973). Given that organisms are limited by the availability of resources, the evolution of resistance to mate harm is predicted to come at the cost of other life-history traits, such as aging and life span, fecundity, etc. (Promislow 2003). While a number of empirical studies have addressed the evolution of female resistance to mate harm (Holland and Rice 1999, Martin and Hosken 2003, Wigby and Chapman 2004, Crudington et al. 2005, Michalczyk et al. 2010), few have tried to test the predictions on the life-history consequences of such female adaptations (Maklakov et al. 2007).

In the present study, I used laboratory-adapted populations of *Drosophila melanogaster,* evolved under differential levels of sexual selection. With the difference in female and male fitness across the M and F populations, I hypothesize that sperm-

depleted males would exercise choice towards F females, which have a lower resistance threshold to mating than the M females. To test the hypothesis, I conducted two-way choice experiments with sperm depleted males from the selected population, given a choice between M and F females. The evolution of resistance traits in M females is expected to trade off with other traits. To test for this trade-off, I quantified the fecundity of females from M and F populations after single mating.

Materials and Methods

Standardizing and generating experimental flies

Experimental flies are subjected to a standardization process to avoid non-genetic parental effects on the experiments' outcome (Michael R. Rose, 1984). The flies are maintained for one generation without selection, i.e., not subjected to the virgin collection and biased adult sex ratio. For the experiments in this study, eggs were collected from these standardized flies at the exact density of 150 ±2 per vial in cornmeal molasses food. On days 9-10, post egg collection, virgin flies were collected in a single-sex vial at 8 flies per vial density. For all the experimental assays, 12-13 days (post egg collection) old flies from $M_{1\text{-}3}$, $F_{1\text{-}3}$, LH, and LH_{st} populations were used.

Two-way mate preference assay

For the mate preference assay, 13 days (post egg collection) old adult flies were used in a two-way choice setup. A day before the mate-choice assay, the males from the M and F population were combined with females from the LH population. In each vial, 8 males were combined with 20 females to ensure that males had more females than their

mating capacity. On the day of the mate choice assay, the resource-depleted males were separated from LH females under light CO_2 anesthesia.

For the assay, one M_i female and F_i (where i represent the replicate population) female were combined with one resource-depleted male from either M or F population in a single vial. Note that flies from populations with identical subscripts were combined; i.e., M_1 males and F_1 were given a choice between M_1 females and F_1 females, and so on. Females were colored with either pink or green fluorescent dust (Day-Glo Corp.) before the assay to allow observers to distinguish between them visually. To account for a possible color bias, reciprocal coloration was performed. Vials were observed to record the female that the male chooses to mate with first, along with the latency to mate and duration of copulation. Vials in which no successful mating occurred for 60 minutes were discarded. Eighty such vials were set up for each M and F replicate population. A similar design was followed for all the replicate populations (M_1-F_1, M_2-F_2, and M_3-F_3). In total, 480 such vials were set up, and data from 415 vials in which successful mating was observed within 30 minutes was used for further analysis.

Fecundity assay for M and F females

To quantify female fecundity from M and F populations, 8 females per vial from the $M_{1\text{-}3}$ or $F_{1\text{-}3}$ population were combined in a vial with 10 males from the LHst population. Ten such vials were set up for each M and F population. These vials were observed to ensure all females were singly mated. As soon as the matings ended for all the females in a vial, males and females were separated under light CO2 anesthesia, and females were transferred to fresh food vials for oviposition. Females were discarded from the oviposition vials after 18 hours, and the vials were frozen to count the number of eggs

laid. The same setup was repeated for all the three replicate populations of M_{1-3} and F_{1-3}.

Statistical analysis

Female fecundity was analyzed using a linear mixed-effects model using the "lme4" package (Bates et al., 2018), in R (ref) with the number of eggs as the response variable, selection as the fixed predictor, and replicate block for each population as the random effect.

To analyze mate preference in M and F regime males, I set up a Bayesian generalized linear mixed-effects model with binomial errors using the package "blme" (Chung et al., 2015). A binomial test revealed that there was a color bias in the mate choice assay of M and F males (more green-colored females were chosen than expected by random chance, pbinom < 0.001, Table 4.1). Therefore, I included female coloring in the model to test for mate choice of M and F males. I set up a Bayesian generalized linear mixed-effects model with binomial errors. A new random variable was created, where a value of 1 was assigned to the female, which successfully mated, and 0 for failures. This random variable was used as the response variable, with female type, female color, and their interaction as fixed predictors. Vial identity was taken as a random effect to account for the non-independence of two females in the same vial, nested within the replicate block of the population of origin (M_{1-3} or F_{1-3}). A likelihood ratio test revealed that dropping the male type from the model did not make a difference and resulted in a model with lower AIC, showing that the selected population of origin of males does not affect the outcome of the male choice assay.

To analyze mating latency, I set up a generalized linear mixed-effects model with Poisson errors using the package "lme4" (Bates et al., 2018), taking mating latency as the response variable and female type as the fixed predictor. Replicate block for populations was used as a random factor. The fixed predictors were male type, female type, and their interaction.

In order to analyze copulation duration, I set up a linear mixed-effects model using the package "lme4" (Bates et al., 2018), taking copulation duration as the response variable and female type as the fixed predictor. Block was used as a random factor, and the fixed predictors were male type, female type, and their interaction.

All analysis was conducted in R v4.0.2 (Team, 2020).

Results

Mate preference assay

There was no effect of the male type on the outcome of mating preference assay . There was a significant effect of the female type, with F females getting more matings than M females (Table 4.2, Figure 4.2). There was an effect of female coloring where a male choice was exerted only on green-colored females (Table 4.3). I used a reverse coloration design for this specific scenario to mitigate any effects of color bias, where an equal number of M and F females were colored in pink and green.. F females got 54% of total matings with M and F males.

There was an effect of male type on mating latency (Table 4.4, Figure 4.3), with M males having lower mating latency than F males (Figure 4.4). There was no effect of female type or male x female interaction on mating latency.

There was no male or female effect on copulation duration (Table 4.5, Figure 4.5), but the male X female interaction was significant (Table 4.5). M males had a higher copulation duration with M females, and F males had a higher copulation duration with F females (Figure 4.5).

Fecundity assay for M and F females

There was a significant effect of selection on fecundity (Table 4.7, Figure 4.6) measured after single mating with common ancestral males from the LH_{st} population. Females from the F regime were found to have higher fecundity than M regime females after a single mating with common ancestral males (Figure 4.6).

	Female ID	**Frequency**
1	F female	222
2	M female	193
	Female color	**Frequency**
1	Green	247
2	Pink	168
Pbinom < .001		

Table 4.1: Frequency of preferred female, based on selection history and color used in the assay. Pbinomial test shows that there is a color bias in the mating preference.

	Estimate	Std. Error	value	Pr(>|z|)
(Intercept)	4.17E-06	1.005E-01	0.000	0.99997
Male	1E-01	7.128E-02	1.8095	0.7707
Female	1E-01	7.128E-02	1.403	0.16066
ColorFem	-1.754E-06	7.177E-02	0.000	0.99998
Female x ColorFem	2.196E-01	7.129E-02	3.081	0.00206***

Table 4.2: Results of the analysis of deviance tests on the Bayesian generalised mixed effects model testing for mate choice of M or F males between M or F females. This model includes female color. Here, Male describes the selection lines of males used for the assay, M and F males. Female describes the M and F females, ColorFem describes the two color treatments (green and pink) used to distinguish the females, and Female X ColorFem interaction, showing the effect of color in the outcome of mate preference of M and F males.

contrast	estimate	SE	z.ratio	p.value
Green F female - Pink F female	0.439	0.202	2.171	0.1313
Green F female - Green M female	0.639	0.183	3.501	0.0026***
Green F female - Pink M female	0.200	0.202	0.989	0.7560
Pink F female - Green M female	0.200	0.202	0.989	0.7559
Pink F female - Pink M female	-0.239	0.219	-1.092	0.6942
Green M female - Pink M female	-0.439	0.202	-2.171	0.1313

Table 4.3: Pairwise contrasts for female and color effect in the male mate choice model of M or F males.

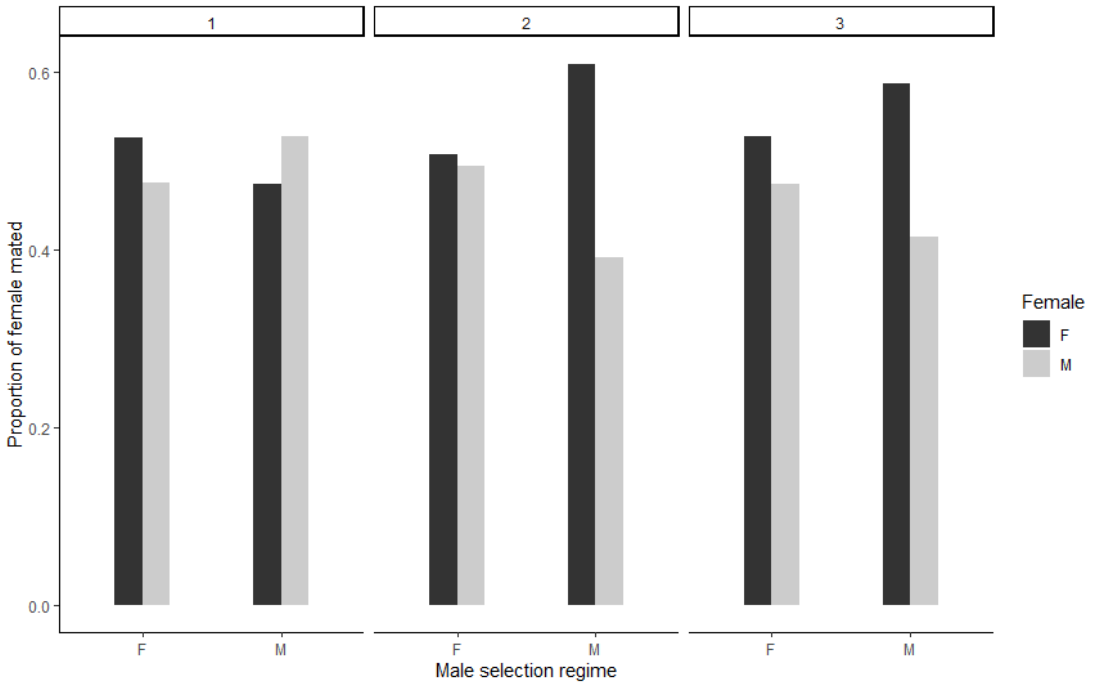

Figure 4.1: Representing the proportion of total matings (y-axis) obtained by F females (Black bars) and M females (grey bars), with males from the M and F populations (x-axis), across the three blocks (grids).

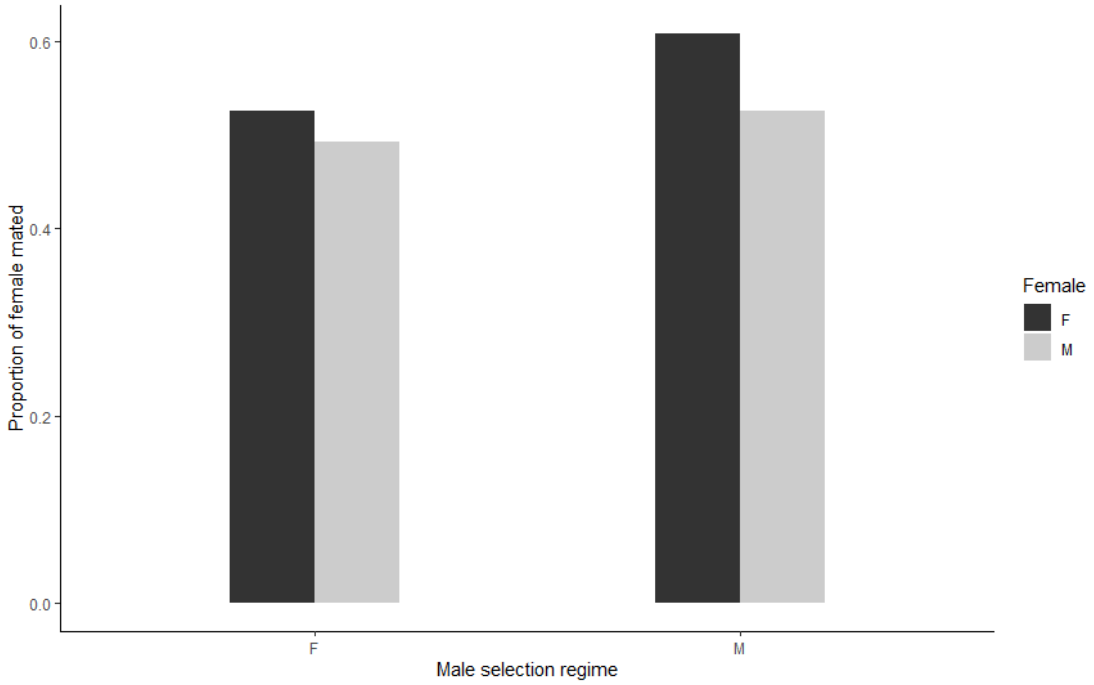

Figure 4.2: Representing the proportion of total matings (y-axis) obtained by F females (Black bars) and M females (grey bars), with males from the M and F populations (x-axis), combined over three blocks.

	Sum Sq	Mean Sq	NumDF	DenDF	F value	Pr(>F)
Male	127.803	127.803	1	409.14	5.8634	0.01589*
Female	2.474	2.474	1	409.39	0.1135	0.73639
Male:Female	0.853	0.853	1	409.54	0.0391	0.84327

Table 4.4: Results of ANOVA for mating latency for mating preference assay. Here, Male describes the M and F males used in the assay, Female describes the M and F females in the assay.

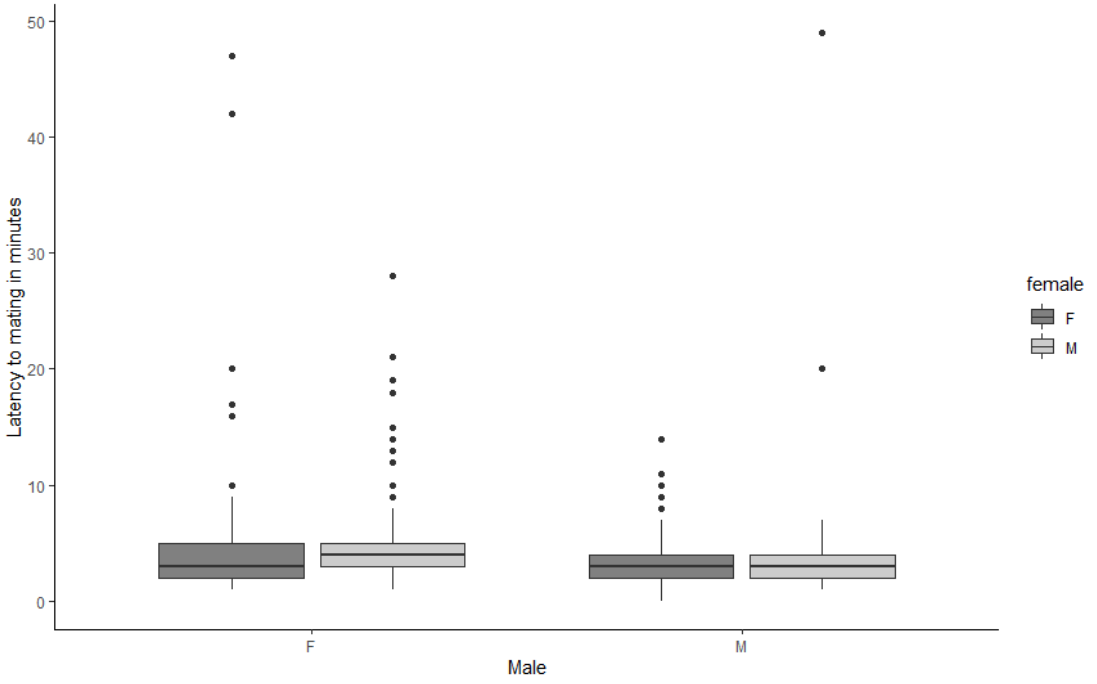

Figure 4.3: Representing the mating latency (y-axis) of F females (black boxes) and M females (grey boxes) with males from the M and F populations (x-axis). Boxplots indicate median and interquartile range. Whiskers indicate variability outside the upper and lower quartiles. Dots represent outliers.

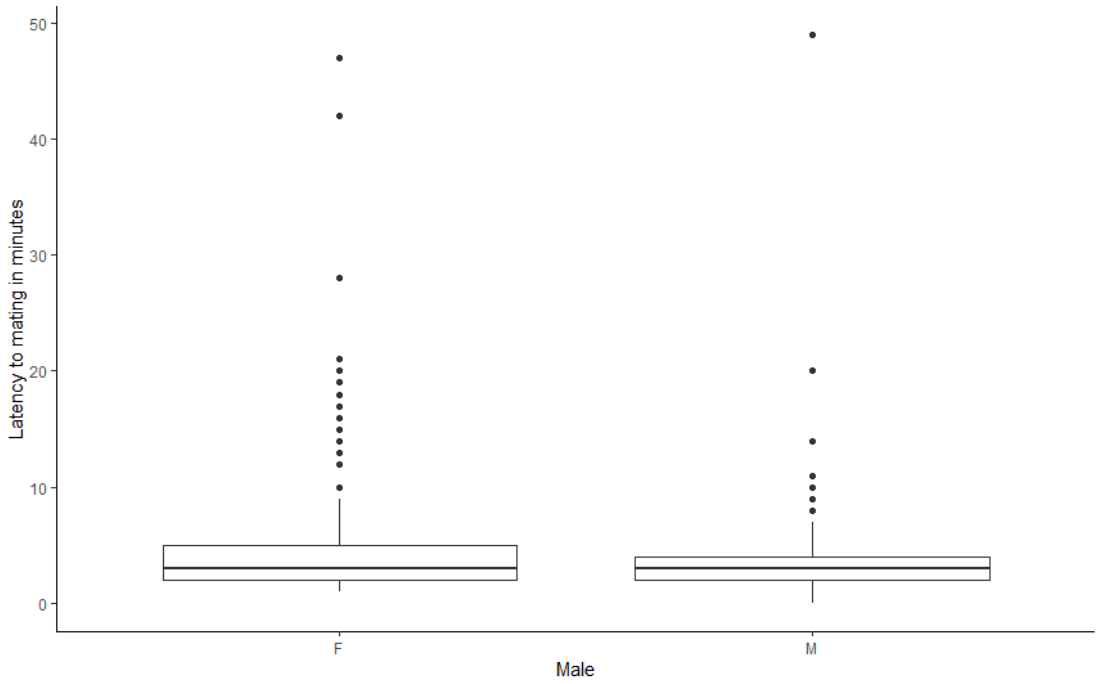

Figure 4.4: Representing the mating latency (y-axis) of males from M and F populations (x-axis). Boxplots indicate median and interquartile range. Whiskers indicate variability outside the upper and lower quartiles. Dots represent outliers.

	Sum Sq	Mean Sq	NumDF	DenDF	F value	Pr(>F)
Male	0.516	0.516	1	1.85	0.0206	0.90004
Female	0.129	0.129	1	409	0.0051	0.94292
Male:Female	98.114	98.114	1	408.98	3.9108	0.04865*

Table 4.5: Results of ANOVA for copulation duration for the mate preference assay. Here, Male describes the M and F males used in the assay, Female describes the M and F females in the assay.

contrast	estimate	SE	df	t.ratio	p.value
F F - M F	1.0929	0.923	3.5	1.185	0.6695
F F - F M	0.9427	0.689	407.11	1.369	0.5196
F F - M M	0.0793	0.954	3.95	0.083	0.9998
M F - F M	-0.1502	0.933	3.65	-0.161	0.9982
M F - M M	-1.0136	0.714	410.32	-1.419	0.4884
F M - M M	-0.8634	0.964	4.11	-0.896	0.8087

Table 4.6: Results of pairwise comparison (Male-Female) of copulation duration. For pairwise comparison in copulation duration, the two letters used, describe the populations of male and female pair (first letter for male and second letter for female). For example FF-MF describes the pairwise comparison between F male-F female and M male and F female.

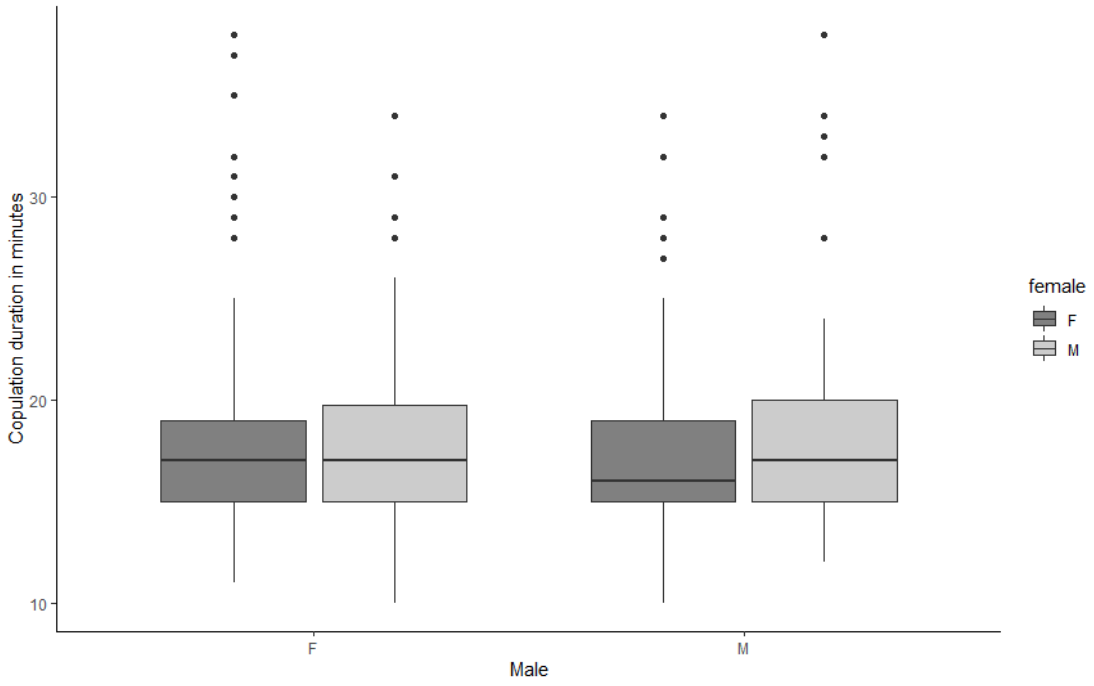

Figure 4.5: Representing the copulation duration (y-axis) of F female (black boxes) and M female (Grey boxes) with males from M and F population (x-axis). Boxplots indicate median and interquartile range. Whiskers indicate variability outside the upper and lower quartiles. Dots represent outliers.

	Sum Sq	Mean Sq	NumDF	DenDF	F value	Pr(>F)
Female	15715	15715	1	84.007	23.289	6.16E-06***

Table 4.7: Results of ANOVA for fecundity of M and F females after single mating with common males. Here, Female describes the females from M and F population.

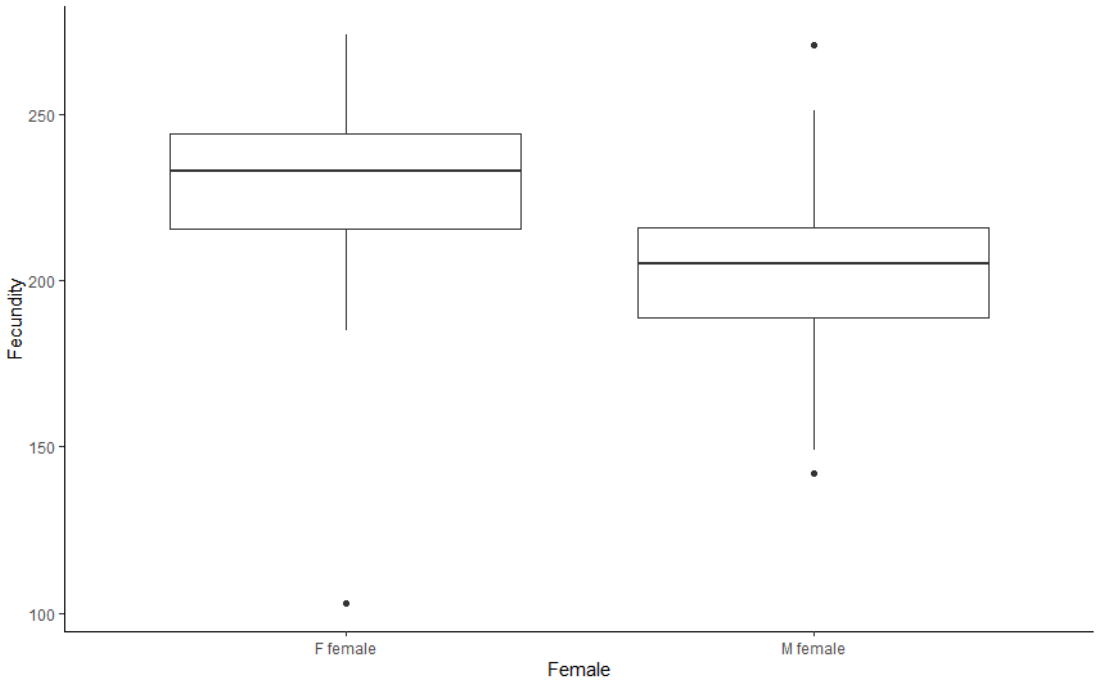

Figure 4.6: Representing the fecundity (y-axis) of females from M and F populations (x-axis) after single mating with common males. Boxplots indicate median and interquartile range. Whiskers indicate variability outside the upper and lower quartiles. Dots represent outliers.

Discussion

In this study, I investigated the mating preference of males between M and F population females in a two-way male mate choice setup. Sperm-depleted males from M and F populations were used in the assay as they are more likely to exhibit choice when the mating opportunity arises because, in a resource-limited state, the mating cost would be high (Byrne and Rice, 2006). Each sperm-depleted male from the M and F population was given a choice between females from the M and F population. I also measured female fecundity after single mating in these populations as a variable in female fitness and potential trait for a trade-off with resistance trait in the M females. The results show that males from the selected populations, both M and F populations, prefer females from the F regime (Figure 4.2). Females from the F population also have higher fecundity than M females after a single mating (Figure 4.6).

Female body size affects male mate choice and female fecundity across many species (Andersson, 1994; Clutton-Brock, 1988; Long et al., 2009). (Nandy et al., 2014) found body size difference in the females when freshly eclosed, which disappeared after 3-4 days (upon sexual maturity). The fecundity assay and mate choice assays used 3 days old post eclosion females from M and F populations. Recent unpublished data also show that M and F population females do not differ in their body size. Therefore, female body size does not play a role in either fecundity or mate choice assays in this study.

I found clear evidence of mating bias towards females from the F population from the male mate choice assay (Figure 4.2). Sperm-depleted males from both the selection lines chose to mate significantly more times with F regime females when presented a choice between M and F regime females. Interestingly, there was no male effect in the

outcome of the mate choice assay, which essentially means that both M and F males prefer the F population females. Male preference towards F population females could result from the higher threshold resistance to mating in M population females. In the previous chapter, I showed that M females' mating rates were significantly lower than the mating rates of F females. If M females have higher threshold resistance to mating, the resource-depleted males would have to invest more energy and resources in courting. Thus, it could be the M female's higher threshold resistance to mating, resulting in male preference against it.

Another possible explanation for the male preference results could be the fecundity difference between M and F population females. The fecundity assay for M and F females after single mating to common ancestral males shows that F females have higher fecundity than M females after single mating. Previous results by (Nandy et al., 2014) also found that the progeny production of F females was higher than M females after a single mating. These two results together show that females from the F regime have higher fitness than M regime females. In previous studies of male mate choice in *D. melanogaster,* males have been shown to prefer females with higher fecundity (Byrne and Rice, 2006; Edward and Chapman, 2012; Long et al., 2009; Nandy et al., 2012a) but also see (Candolin and Salesto, 2009; Edward and Chapman, 2013). The difference in female fecundity across the selection regimes results from the difference in male-mate harm that the females experience in the two selection regimes. Male mate harm negatively affects fecundity in *Drosophila* females (Holland and Rice, 1999a; Partridge et al., 1986; Ravi Ram and Wolfner, 2007). Females from the M regime face higher mate harm and have evolved higher mate harm resistance (Nandy et al., 2014, 2013c). There might be a trade-off between mate harm resistance and fecundity in M

females. On the other hand, F females do not face similar mate-harm and thus invest in their fecundity. The difference in female fecundity across the selection regime suggests that female fecundity variability does exist in these populations and could be under indirect selection from males.

Although the males from both M and F populations prefer females from F populations, my experimental design could not pinpoint the exact reason for this choice. The higher threshold to mating resistance in M females or the higher fecundity after single mating in F females could be the contributing factors. However, these two factors are not independent of each other. The evolution of a higher resistance threshold to mating and mate harm would have led to a trade-off with the fecundity of M males.

Similarly, one of the drawback of two-way choice assays is that it is almost impossible to dissect out the contribution of each sex in the outcome of the assay. In the present design, it is difficult to conclude whether it is the males making a biased mating preference towards F females or that the M females are the one resisting to mate with the sperm depleted males. Previous studies have shown than females can detect the mating status of males from different cues and use the information in future matings to avoid already mated males (Harris and Moore, 2005; Muller et al., 2016; Scarponi et al., 2015). (Loyau et al., 2012) showed that *Drosophila melanogaster* females avoid mating with semen depleted males. Therefore, in the current assay, it might be the choice of the certain females, most likely M females to avoid mating with sperm depleted males, rather than F females being preferred by the males. Further experiments with a better experimental design might shed more light on the decision making process involved in the assay.

For males to show mate preference, they need to be able to assess fitness differences in the females available. Male can choose to target pre-mating rituals towards one female more than the other (Edward and Chapman, 2011; Hoefler et al., 2008; South et al., 2012). These results suggest that males from both M and F populations can access female fitness differences between M and F regime females. In the male-biased (M) regime, each mating effort is costly because of intense male-male competition and female choice. As receptive females are far fewer than males in the M regime, a male who is quick to correctly access female fitness and mate with a high fitness female will have an advantage over other males and reward for the mating efforts. In the F regime, males have ample mating opportunities. Therefore males can choose to discriminate against and mate with high fitness females first. In both cases, the males benefit from choosing to mate with higher fitness females. This argument is supported by the mating latency data from the mate choice assay. M males have lower mating latency than F males in matings with females from both the regimes (Figure 4.3). M males might be faster than F males at assessing the fitness of the females and making a choice to mate. In normal maintenance regime conditions, M males face higher male-male competition than F males; therefore, it pays to be quick to correctly assess the female and decide to mate or not before more males enter the competition.

In copulation duration, I found male X female interaction to be significant. There is a trend of higher copulation duration towards the co-evolved female (Figure 4.5). M males have a higher copulation duration with M females, and F males have a higher copulation duration with F females. However, pairwise comparisons did not reveal any significant difference in copulation durations (Table 4.6). In the presence of rival males, longer copulation duration is the plastic response by males, leading to higher investment

in mating (Bretman et al., 2011, 2010). However, (Dore et al., 2020) found no reproductive fitness benefit from prolonged copulation duration in their latest finding.

In conclusion, results from this study show that resource-depleted males exercise choices, given there are fitness differences in females' available choices. While the selection does not affect the choice of the males, the females with lower resistance to mating and higher fecundity are preferred by the males.

Competitive Mating Success

Chapter 5

Introduction

The overall reproductive success of males is governed by both mating success (pre-copulatory) and fertilization success (post-copulatory). In males, reproductive success generally increases with the number of successful matings (Bateman, 1948; Janicke et al., 2016; Trivers, 1972). Higher variance in mating success, in part, drives pre-copulatory sexual selection in males leading to the evolution of behavioral and morphological traits related to male mating success (Andersson, 1994). Thus, pre-copulatory sexual selection is predicted to be a strong determinant of male reproductive success, as mating success is the prerequisite for fertilization success. However, in polyandrous species, multiple matings set the arena for post-copulatory sexual selection to act (Hosken and Ward, 2001; Parker and Birkhead, 2013). In promiscuous species, post-copulatory sexual selection as a result of polyandry can reduce paternity even after mating. Females can store ejaculate from different males they mate with (Lefevre and Jonsson, 1962), leading to sperm competition, where sperms from different males compete inside the female reproductive tract to fertilize the eggs (Parker, 1970a; Wedell et al., 2002). Therefore, traits that increase the post-copulatory success of males are also likely to be under selection.

In many species of birds and invertebrates (including *D. melanogaster*), post-copulatory competition is further complicated by the last male sperm precedence, where the last male to mate sires a majority of the progeny (Boorman and Parker, 1976; Harshman and Clark, 1998; Schnakenberg et al., 2012). However, mated females are often resistant to re-mating and are choosier (Kohlmeier et al., 2021; Wigby et al., 2020). Therefore, in order to take advantage of the last male sperm precedence, males

are expected to invest in courting and coercing females to remate. Pischedda and Rice, (2012) found that after controlling for the mating order, fertilization success could explain only 2% of overall male reproductive success in *Drosophila melanogaster*, indicating that mating order is an important determinant of male reproductive success. Thus, male mating success is likely to be under strong selection in many species (like *Drosophila*), where the post-copulatory success of males is also affected by the order of mating.

From a female point of view, mating status-dependent choosiness would be a beneficial trait because it allows the females to avoid the risk of dying a virgin without compromising the offspring's quality (Kokko and Mappes, 2005). For a virgin female, mating can be a random event based on encounter rate as the virgin female runs a risk of not getting a mate by being choosy. On the other hand, mated females can afford to be choosy to mate with a male of high quality or attractiveness. Elevated postmating choosiness is particularly beneficial in species with last male precedence (Kokko and Mappes, 2005). Kohlmeier et al., (2021) found that *Drosophila melanogaster* choosiness is higher in mated than in virgin females. A trait like mating status-dependent choosiness in females can be under selection in certain cases, where the females have enough choice of mates. For males competing for the mating with already mated females, the attractiveness and fitness difference between the two males would be more of a deciding factor than with virgin females.

Very few studies have investigated the evolution of male mating success either directly or via a female preference. In guppies, *Poecilia reticulata,* Hall et al., (2004) did not see any response in male mating success under direct selection on male attractiveness

based on female preference. Similarly, McGuigan et al., (2008) did not find any response in male mating success in *Drosophila bunnanda* populations selected for male attractiveness based on female choice. These studies argue that the persistent directional selection on male traits by female choice can erode the standing genetic variation, leaving little room for evolution to act. In contrast, selecting for male mating success using female preference in *D. melanogaster*, Dugand et al., (2018) found that competitive male mating success can evolve. Thus, the evidence for the evolution of male mating success is mixed.

Other than direct selection for male mating success, evolution under increased male-male competition via altered mating systems (monogamy vs. polygamy) or altered operational sex ratios can potentially lead to the evolution of male mating success. Male-male competition has been shown to be an evolutionary force capable of driving divergence in reproductive traits and, ultimately speciation (Tinghitella et al., 2018). Similarly, interlocus sexual conflict, because of sexually antagonistic coevolution, can result in divergence in populations and allopatric speciation (Gavrilets, 2014; Parker, 2006; Qvarnström et al., 2012; Rönn et al., 2007). Thus, male mating success in populations evolving under differential levels of male-male competition can provide insights into the evolutionary process, which on a bigger evolutionary timescale can potentially cause divergence in the populations and lead to speciation.

Under enforced monogamy or female-biased sex-ratios, males face relaxed male-male competition and female choice. On the other hand, males under polygamy or male-biased sex-ratios face intense male-male competition to get access to females and, at the same time, face a stronger female choice. Under this increased selection pressure,

males are likely to evolve traits and strategies to improve mating success and fertilization success. In *D. melanogaster* populations evolved under enforced monogamy and polygamy, Wensing et al., (2017) found that polygamous males get a higher proportion of matings than monogamous males in direct competition over mating success. In the flour beetle *Tribolium castaneum*, Michalczyk et al., (2011) found that males that evolved in a male-biased regime have higher mating success over males that evolved in the female-biased regime. In *D. melanogaster*, males from polygamous populations evolved higher courtship frequency (Holland and Rice, 1999a). In *D. pseudoobscura*, the polygamous treatment males evolved courtship strategies (Snook et al., 2005) along with mating capacity, without any change in sperm investment (Bacigalupe et al., 2008; Crudgington et al., 2009).

However, to test for the male traits in the selected populations, most of the studies mentioned above use females from stock populations that have not been subjected to any selection, unlike the focal males in the studies. Various studies have shown that male and female phenotype and genotype interaction can affect the outcome of males' pre-copulatory (Pischedda et al., 2012; Reinhart et al., 2015; Turiegano et al., 2013) as well as post-copulatory fitness (Chow et al., 2010; Clark et al., 1999; Lüpold et al., 2020). Under experimental evolution (like monogamy vs. polygamy or altered sex ratio), males and females co-evolve over generations. Both male and female traits co-evolve as a result of selection. Therefore, it is quite possible that the fitness of one sex is affected by the other sex that it has co-evolved with. The reproductive success of males might be different with the common stock population females compared to the females they have co-evolved with and adapted to over generations.

Courtship is one of the pre-copulatory traits through which males can improve their competitiveness (Andersson, 1994). Investing in courtship activity gives a male advantage over other males in terms of showcasing quality to females and obtaining a mate. While competition between the males over courtship towards a female is an important determinant for mating success (Partridge et al., 1987a), the relationship between courtship and mating success is not a straightforward one (Bedhomme et al., 2008; S Pitnick et al., 2001).

In the current study, I use M and F populations of *D. melanogaster* populations to investigate how male mating success evolves in response to differential levels of male-male competition. Males from both the populations were allowed to directly compete to obtain a successful mating with a) virgin females from ancestral LH_{st}, M, and F populations and b) singly mated females from M and F populations. I also measured the courtship frequency of males towards singly mated M and F females to establish a correlation between courtship frequency and mating success.

Methods

Standardizing and generating experimental flies

To ensure that non-genetic parental effects have little to no role, all populations were subjected to a generation of standardization in which they were kept as their ancestral LH_{st} population - equal sex ratios and no virgin collection (Michael R. Rose, 1984). Flies maintained in this manner for one generation are called standardized flies. To generate experimental flies, eggs were collected from these standardized flies in standard rearing vials containing 6 mL of cornmeal-yeast-molasses food at an exact

density of 150 ± 2 per vial. On the 9-10th day post egg collection, males and females were collected as virgins and held separately, at a density of 8-10 individuals per vial containing 2 mL of food. All experimental assays were performed with old flies for 12 days (post egg collection).

Male mating success when competing for virgin ancestral females

To assay male mating success with virgin ancestral females in a competitive scenario, 12 day old (post egg collection) flies were used. One virgin male from M, one virgin male from F, and one virgin female from LH_{st} were transferred to a fresh food vial. Males were colored with either pink or green fluorescent dust (Day-Glo Corp.) prior to the assay to allow observers to visually distinguish between them. To account for a possible color bias, reciprocal coloration was performed. Vials were observed, and the male which mated successfully was recorded, along with the mating latency and copulation duration. Vials in which no successful mating occurred after an hour were discarded. 80 vials were set up for each replicate line of the M and F population. In total, 240 such vials were set up, and the data from 227 vials (in which successful matings were observed) was used for further analysis.

Male mating success when competing for virgin females from M and F populations

To assay male mating success with virgin females from M and F populations in a competitive scenario, a similar design as the previous assay was used. I combined one M_1 male and F_1 male with one M_1 or F_1 female in a single vial. A similar design was followed for all the replicate populations (M_{1-3} and F_{1-3}). All flies were 12 day old (post

egg collection). 80 vials per replicate line were set up for F female and 80 vials per replicate line for M female. In total, 480 vials (160 for each replicate of M_{1-3} and F_{1-3} population) were set up, and the data from 456 vials (in which successful matings were observed) was used for further analysis.

Male mating success when competing for singly mated females from M and F populations

To assay male mating success with singly mated females from M and F populations under direct competition, M and F females were first mated to common ancestral LH_{st} males. 8 M or F females were combined with 10 LH_{st} males into fresh food vials containing 2 mL of food. Vials were observed until all mating pairs were formed, and males were separated under light CO_2 anesthesia after all matings ended. Females were held in fresh vials for 1-2 hours. Singly mated females from M or F populations were then combined into fresh food vials with one virgin M male and one virgin F male. Males were colored with either pink or green fluorescent dust, with reciprocal coloration for each treatment combination. Unlike virgin females, singly mated females are less receptive, and the latency to mate with them is much higher. All mating trial vials were observed for 8-9 hours in the day-light period spanning from 3.5 hours to 11.5 hours from the time of lights on. 80 vials per replicate line were set up for F female, and likewise 80 vials per replicate line for M female. In total, 480 such vials (160 vials for each replicate of M_{1-3} and F_{1-3} population) were set up, and the data from 290 vials (in which successful matings were observed) was used for further analysis. Males and females from the same replicate line were used in the assay. Since the replicate lines

are independent, mating assays were distributed across three days, with each replicate line handled on a different day.

M and F male courtship frequency towards singly mated females

The courtship data was recorded from the same vials used in the previous experimental set up used to measure mating success competing for singly mated females from selection populations. At three different time points during mating observations, 5 hours from light on, 7.5 hours from light on, and 9 hours from lights on, vials were scanned every minute for an hour, to record courtship activity. Each vial was scanned for the presence or absence of courtship activity every minute. If any of the males in a vial displayed courtship behavior (orientation, chasing, wing flapping, licking, and attempted copulation), it was recorded as one courtship bout, entered with the color identity of the male or else zero activity for the vial was recorded. The proportion of courtship bouts performed by each male in a vial was calculated from this data of total courtship bouts in each vial. The vials in which mating occurred before the time of courtship observations were not included in the data.

Statistical analysis

All statistical tests were performed in the R statistical environment (v4.0.2, R Core Team 2020).

A Bayesian generalized linear mixed-effects model with binomial errors and default priors was constructed to analyze male mating success using the package "blme" (Chung et al., 2015). Mating success (0 = failure, 1 = success) was used as a response variable, with the male type (M or F), female type (M or F) wherever applicable, and

their interaction as fixed predictors. Vial identity was taken as a random effect to account for the non-independence of two males in the same vial, nested within the replicate population of origin (M_{1-3} or F_{1-3}). The same model was used for analyzing the mating success data from the three experiment assays separately.

Mating latency and copulation duration were analyzed using linear mixed-effects models using the "lme4" package (Bates et al. 2014). A generalized linear mixed model was constructed for the virgin female assay with poisson errors (log link) to analyze mating latency and a linear mixed-effects model with gaussian errors (identity link) to analyze copulation duration. In both these models, male and female types and their interactions were taken as fixed predictors and replicated population of origin as a random effect. Similar models were set up to analyze mating latency for virgin ancestral females, except there was only one fixed predictor, i.e., male type. For the singly mated female assay, linear mixed-effects models were constructed to analyze mating latency and copulation duration, assuming gaussian errors (identity link), taking male and female types and their interaction as fixed predictors and replicate population of origin as a random effect. ANOVA tests were performed on all models using the package "car" (Fox and Weisberg 2018).

To analyze courtship activity, the proportion of courtship bouts by M males was computed. First, we performed a mixed-model ANOVA with "female type" as the main effect and replicate population as a random effect (using the "ANOVA" function in R) to test whether the selection history of females affected the proportion of courtship bouts. A bootstrapped one-sample hypothesis test was performed using the package "wBoot" (Weiss 2016). The null expectation was set to 0.5, i.e., M males and F males

court equally. 100,000 bootstrap replicates were run to estimate bootstrapped 95% CIs and p-values.

To test whether courtship activity predicts mating success, I set up a model to estimate linear selection differentials of courtship activity on mating success. I set up models separately for M and F males and a generalized mixed-effects model with a binomial error structure. Mating success (1 = success, 0 = failure) was the response variable, and the proportion of courtship bouts by M (or F) males was the fixed predictor, with the replicate population of origin as a random effect. The predictor variable (i.e., the proportion of courtship bouts) was standardized (i.e., normalized) separately in M males and F males so that the models yield standardized selection differentials.

Results

Male mating success when competing for virgin ancestral females

When M and F males compete for a successful mating with the virgin ancestral female, the male type (Table 5.1, Figure 5.1) had a significant effect. There was no effect of male coloring on the outcome. Out of the three replicate lines, in replicate lines 1 and 2, M males outcompete the F males in successfully mating with the LH_{st} female, while in the replicate line 3, the F males obtained more matings than M males (Figure 5.2). Overall, M males were more successful across the three replicate lines than F males in obtaining a mating with the ancestral (LH_{st}) female (Table 1). M males got 56% of the total matings with virgin ancestral LH_{st} females.

There was no effect of male type on mating latency (Table 5.4, Figure 5.6) or copulation duration (Table 5.5, Figure 5.7).

Male mating success when competing for virgin females from M and F populations

In the experimental assay where M and F males compete for a successful mating with a virgin female from M and F populations, there was a significant effect of male type, with M males getting more matings than F males (Table 5.2, Figure 5.1). There was no effect of female type or male X female interaction on the outcome of male mating success. M males are equally likely to have higher mating success with females from the M population and females from the F population. Out of the three replicate lines, in replicate lines 1 and 2, M males recorded higher mating success than F males with both M and F females. In replicate lines 3, with both M and F females, F males obtained a higher number of matings than M males (Figure 5.3). Overall, M males got 54% of total matings across three replicate lines with virgin M and F females. Male coloring did not affect the outcome.

There was no effect of the female type, male type, or interaction on mating latency (Table 5.6, Figure 5.8) or copulation duration (Table 5.7, Figure 5.9).

Male mating success when competing for singly mated females from M and F populations

In the assay where M and F males competed for successful mating with a singly mated M and F female, there was a significant effect of male type, with M males being more successful at mating than F males (Table 5.3, Figure 5.1). There was no effect of female type or male X female interaction on the outcome of mating success. M males had a higher mating success than F males, with both mated M and F females, in all three

replicate lines (Figure 5.4). M males were successful over F males in obtaining mating with singly mated females in 64% of the mating trials. There was no effect of male coloring on the outcome.

There was no effect of the female type, male type, or interaction on mating latency (Table 5.8, Figure 5.10) or copulation duration (Table 5.9, Figure 5.11).

A likelihood ratio test was performed to test whether the slopes of the two models set up for mating success with virgin M or F females or singly mated M or F females were different. The least complex models (i.e., without female type as a fixed predictor) set up for the virgin and singly mated female assays were compared, and the effect of male type was stronger in the singly mated female assay (Table 5.13, Figure 5.5). These results, taken together, indicate that in comparison to F males, M males have higher mating success with both virgin and mated females, and the effect is stronger in the case of singly mated females.

M and F male courtship frequency towards singly mated females

A one-way ANOVA revealed no effect of female type on courtship proportion (Table 5.11). The proportion of courtship bouts by M males and F males was computed, and a bootstrapped one-sample hypothesis test revealed that M males significantly courted more times than F males (Table 5.10, Figure 5.12). To test whether there was a correlation between higher courtship of M males and their higher mating success, standardized selection differentials were computed for M males and F males which were not significant (Table 5.12).

	Estimate	Std. Error	Z value	Pr(>|z|)
(Intercept)	$3.289e^{-07}$	$1.345e^{-01}$	0.000	1.00000
Male type	$-2.586e^{-01}$	$9.496e^{-02}$	-2.723	0.00646**

Table 5.1: Results of the full Bayesian generalized mixed effects model for mating success of M and F males with ancestral (LH_{st}) virgin females. Here, Male type describes the males from M and F population used in the competitive mating success assay.

	Estimate	Std. Error	Z value	Pr(>|z|)
(Intercept)	$2.738e^{-07}$	$9.426e^{-02}$	0.000	1.0000
Male type	$-1.507e^{-01}$	$6.656e^{-02}$	-2.265	0.0235**
Female type	$3.882e^{-06}$	$6.668e^{-02}$	0.000	1.0000
Male:Female	$5.511e^{-02}$	$6.656e^{-02}$	0.828	0.4077

Table 5.2: Results of the full Bayesian generalized mixed effects model for mating success of M and F males with virgin females from selected populations (M or F). Here, Male type describes the M and F population males used in the assay and Female type describes the M and F population females.

	Estimate	Std. Error	Z value	Pr(>|z|)
(Intercept)	$-7.956e^{-08}$	$1.243e^{-01}$	0.000	1.000
Male type	$-5.873e^{-01}$	$8.702e^{-02}$	-6.749	$1.48e^{-11}$***
Female type	$3.173e^{-07}$	$8.725e^{-02}$	0.000	1.000
Male:Female	$9.908e^{-02}$	$8.698e^{-02}$	1.139	0.255

Table 5.3: Results of the full Bayesian generalized mixed effects model for mating success of M and F males with singly mated females from selection population (M or F) females. Here, Male type describes the M and F population males used in the assay and Female type describes the M and F population females.

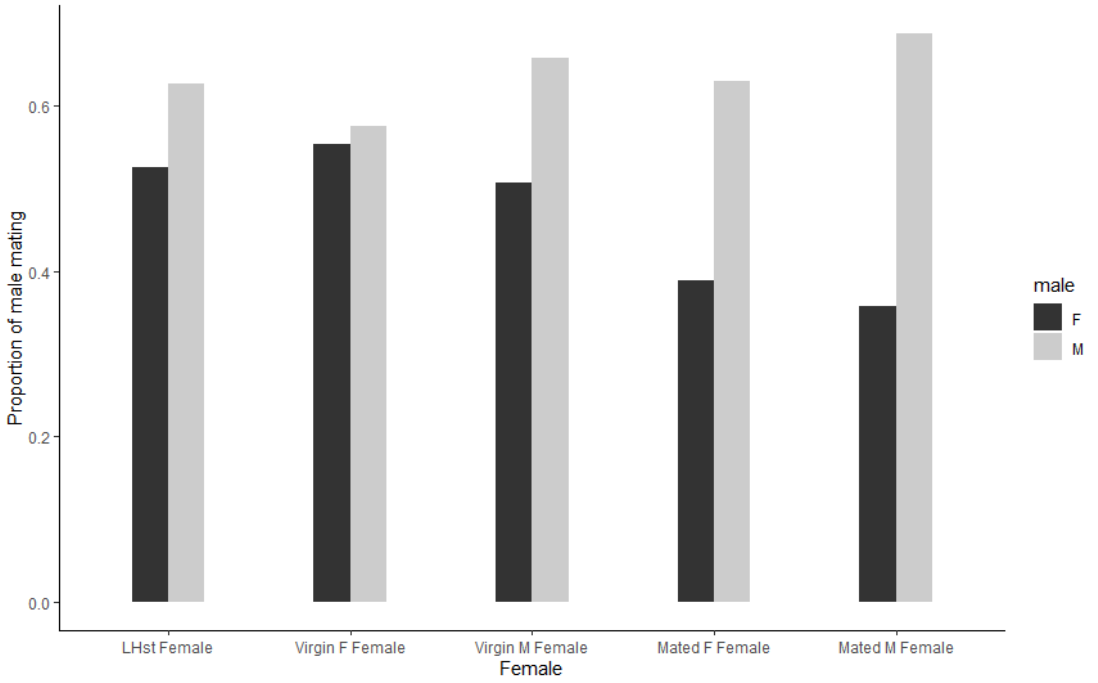

Figure 5.1: Representing the proportion of mating success (y-axis) of F males (Black bars) and M males (Grey bars), when in direct competition over females (x-axis) from different populations and mating status.

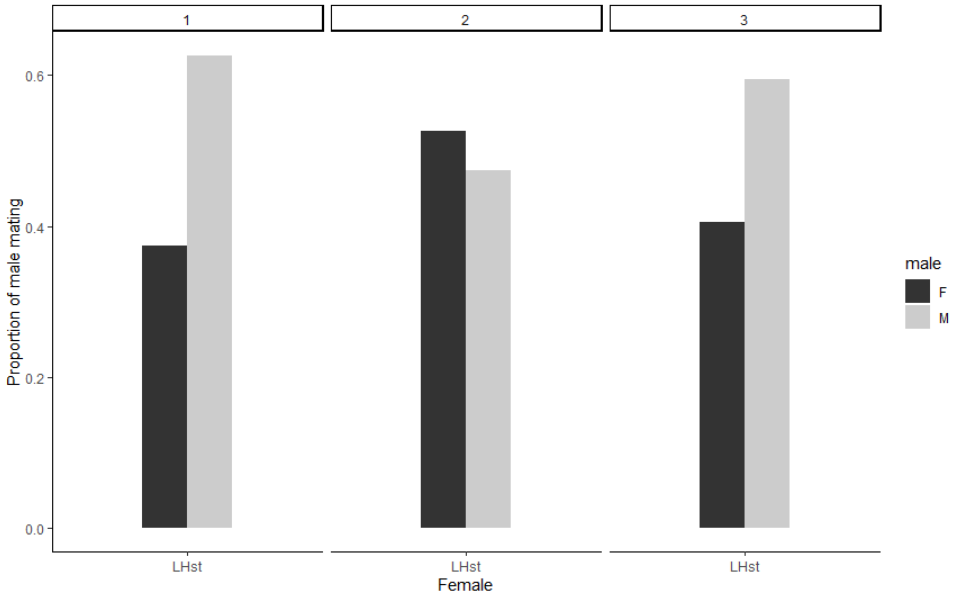

Figure 5.2: Representing the proportion of mating success (y-axis) of F males (Black bars) and M males (Grey bars), when in direct competition over virgin LH_{st} females (x-axis) across the three independent blocks (grids).

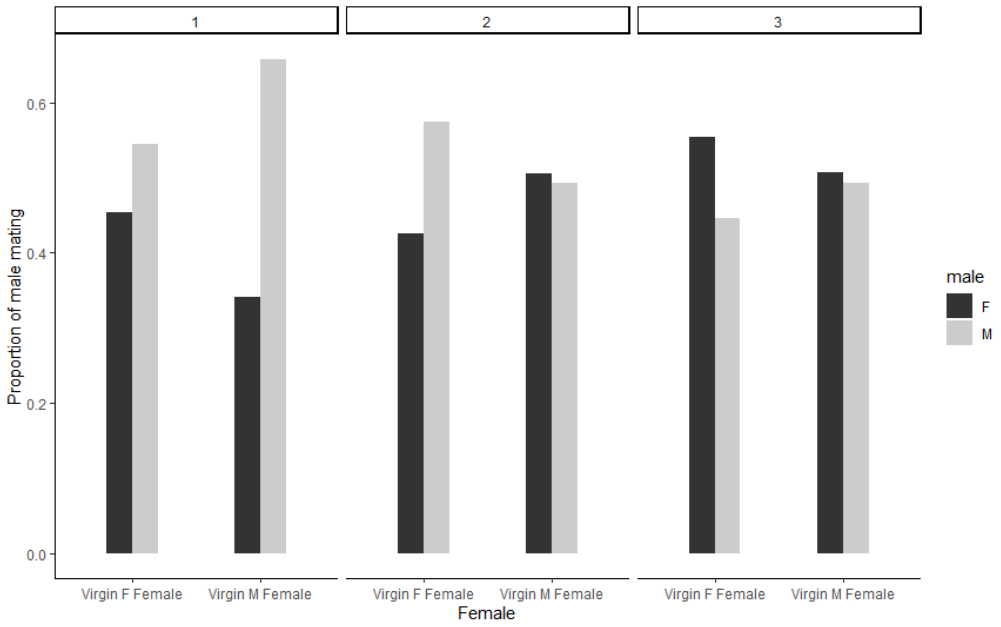

Figure 5.3: Representing the proportion of mating success (y-axis) of F males (Black bars) and M males (Grey bars), when in direct competition over virgin M and F population females (x-axis) across the three independent blocks (grids).

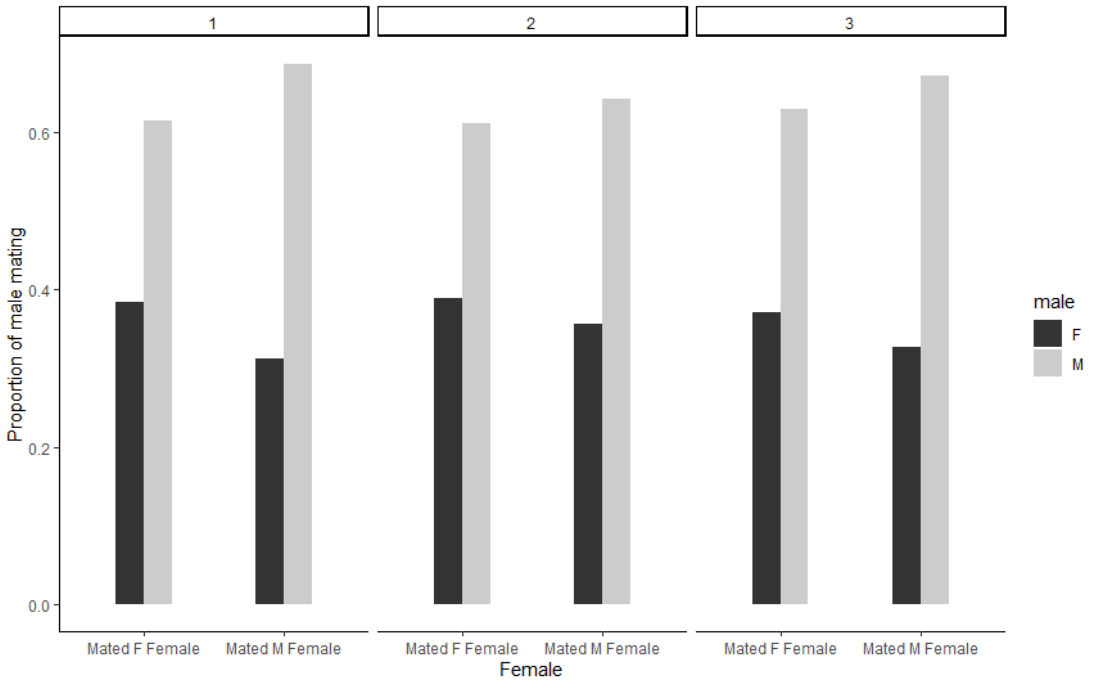

Figure 5.4: Representing the proportion of mating success (y-axis) of F males (Black bars) and M males (Grey bars), when in direct competition over singly mated females from M and F populations (x-axis) across the three independent blocks (grids).

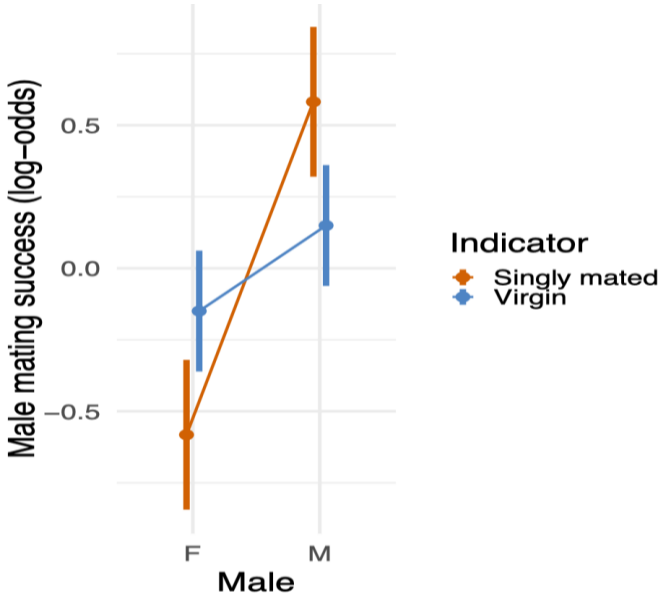

Figure 5.5: Male mating success in the assays with M or F virgin or singly mated females. The comparison reveals an increase in difference between competitive mating success of M and F males when the females are mated. The y-axis plots log-odds of male mating success with 95% confidence limits.

	Chisq	Df	Pr(>Chisq)
Intercept	410.7410	1	<2e-16 ***
Male type	0.0013	1	0.9713

Table 5.4: Analysis of deviance table for male mating latency with ancestral (LHst) virgin females. Here male type describes the M and F males.

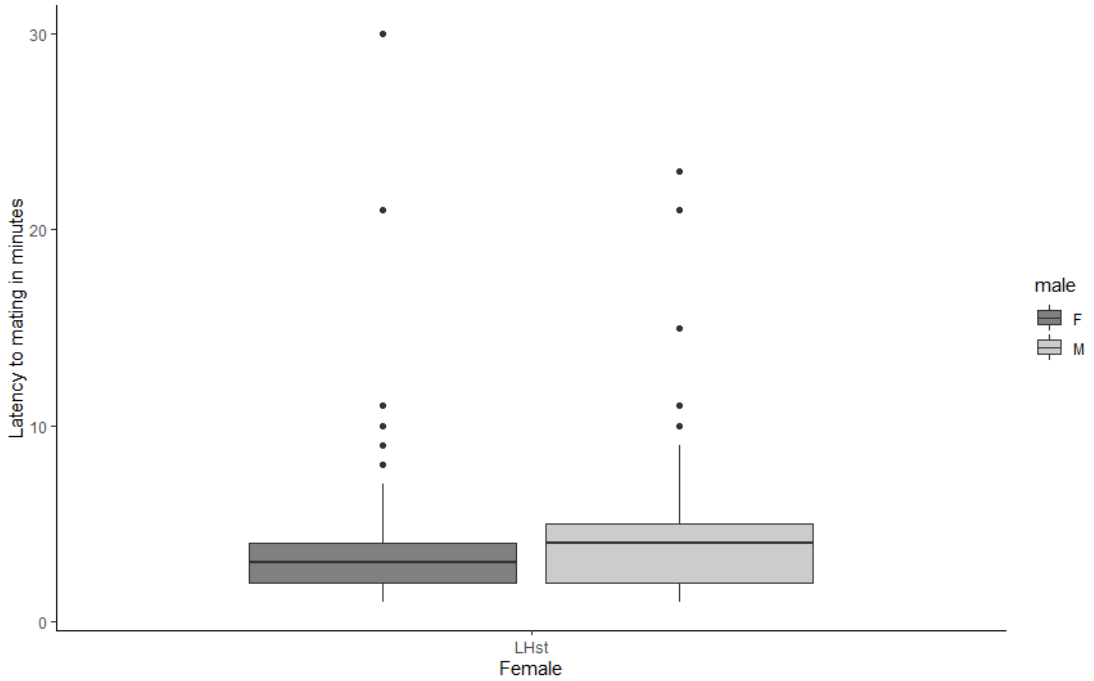

Figure 5.6: Representing the mating latency (y-axis) of F males (black boxes) and M males (grey boxes) with virgin LHst females (x-axis). Boxplots indicate median and interquartile range. Whiskers indicate variability outside the upper and lower quartiles. Dots represent outliers.

	Chisq	Df	Pr(>Chisq)
Intercept	193.7919	1	< 2e-16 ***
Male type	3.4714	1	0.06244

Table 5.5: Analysis of deviance table for copulation with ancestral (LHst) virgin females. Here male type describes the M and F males.

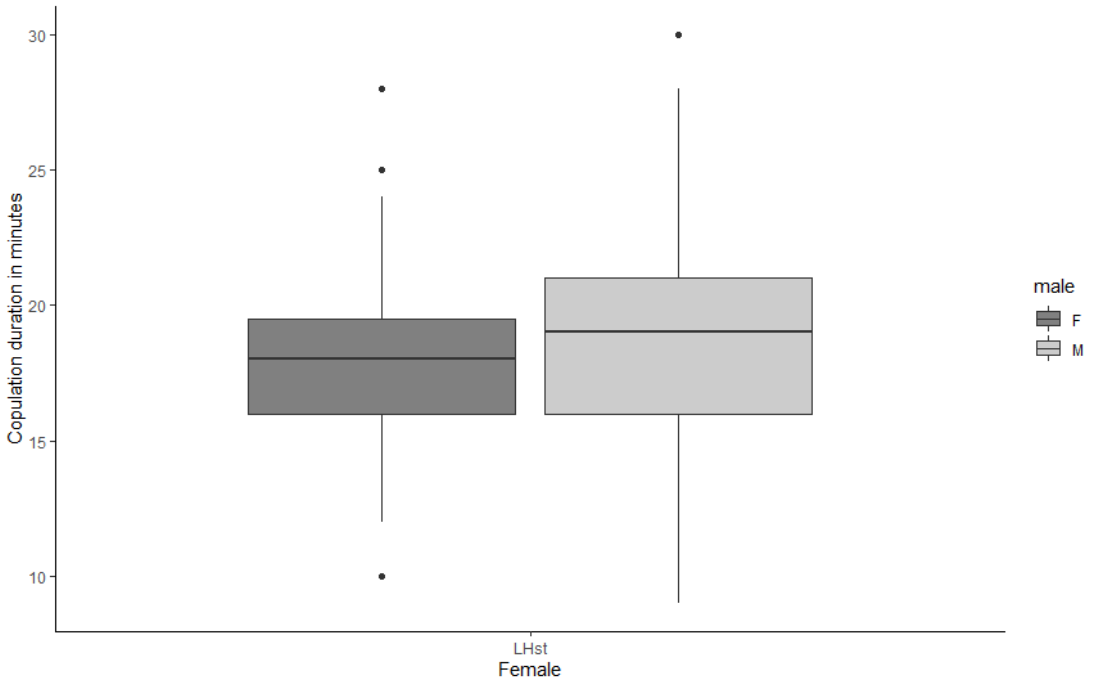

Figure 5.7: Representing the copulation duration (y-axis) of F males (black boxes) and M males (grey boxes) with virgin LHst females (x-axis). Boxplots indicate median and interquartile range. Whiskers indicate variability outside the upper and lower quartiles. Dots represent outliers.

	Chisq	Df	Pr(>Chisq)
(Intercept)	168.837	1	<2e-16***
Female Type	0.3342	1	0.5632
Male Type	0.7660	1	0.3815
Female: male	0.4776	1	0.4895

Table 5.6: Analysis of deviance table for male mating latency with virgin (M or F) females. Here female type describes the females from M and F populations and male type describes the males from M and F populations.

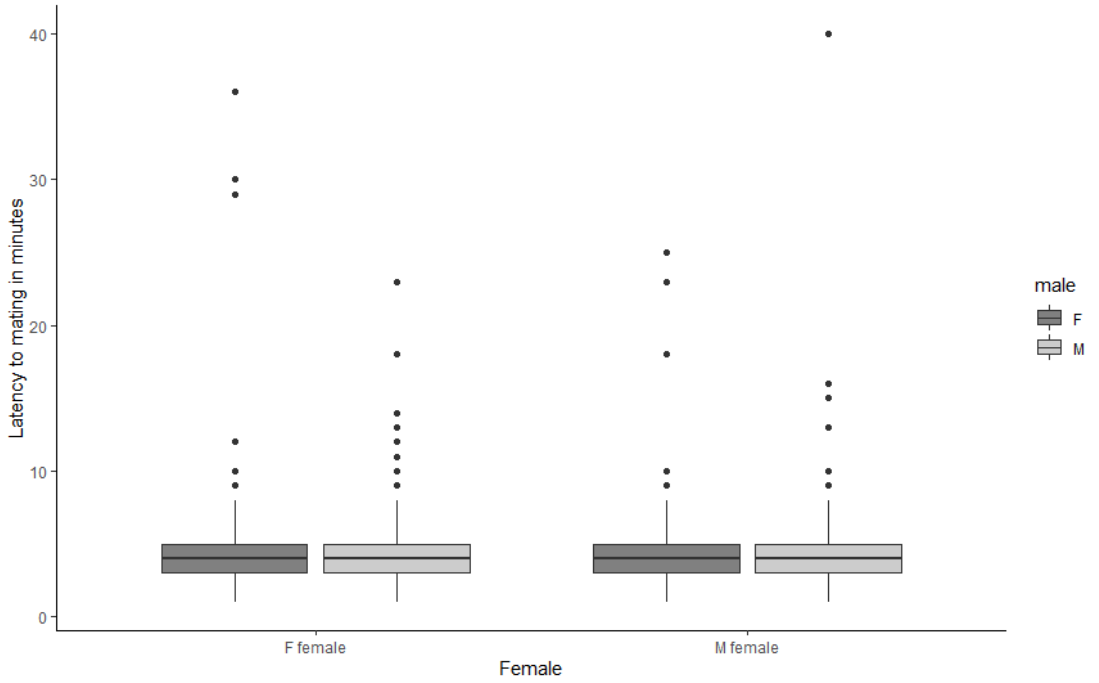

Figure 5.8: Representing the mating latency (y-axis) of F males (black boxes) and M males (grey boxes) with virgin females from M and F populations (x-axis). Boxplots indicate median and interquartile range. Whiskers indicate variability outside the upper and lower quartiles. Dots represent outliers.

	Chisq	Df	Pr(>Chisq)
(Intercept)	304.534	1	<2e-16***
Male Type	0.5112	1	0.4746
Female Type	2.3571	1	0.1247
Male:Female	0.0003	1	0.9857

Table 5.7: Analysis of deviance table for male copulation duration with virgin (M or F) females. Here female type describes the females from M and F populations and male type describes the males from M and F populations.

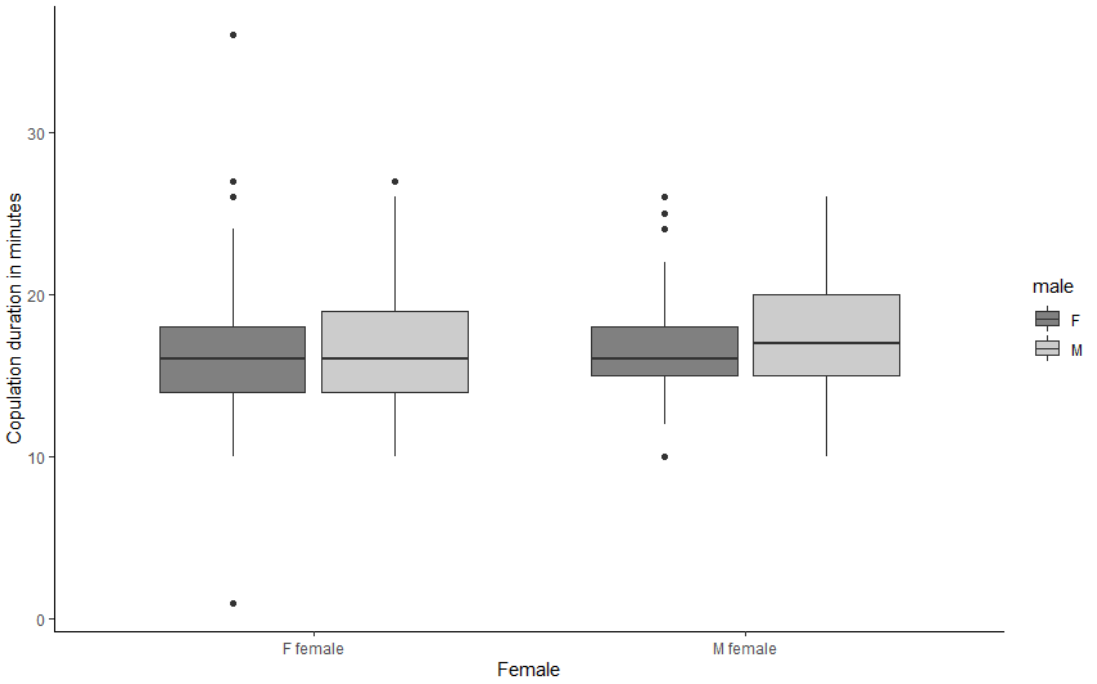

Figure 5.9: Representing the copulation duration (y-axis) of F males (black boxes) and M males (grey boxes) with virgin M and F females (x-axis). Boxplots indicate median and interquartile range. Whiskers indicate variability outside the upper and lower quartiles. Dots represent outliers.

	Chisq	Df	Pr(>Chisq)
(Intercept)	1021.1026	1	<2e-16***
Male Type	0.1866	1	0.6658
Female Type	0.1931	1	0.6604
Male:Female	0.8188	1	0.3655

Table 5.8: Analysis of deviance table for male mating latency with singly mated (M or F) females. Here female type describes the females from M and F populations and male type describes the males from M and F populations.

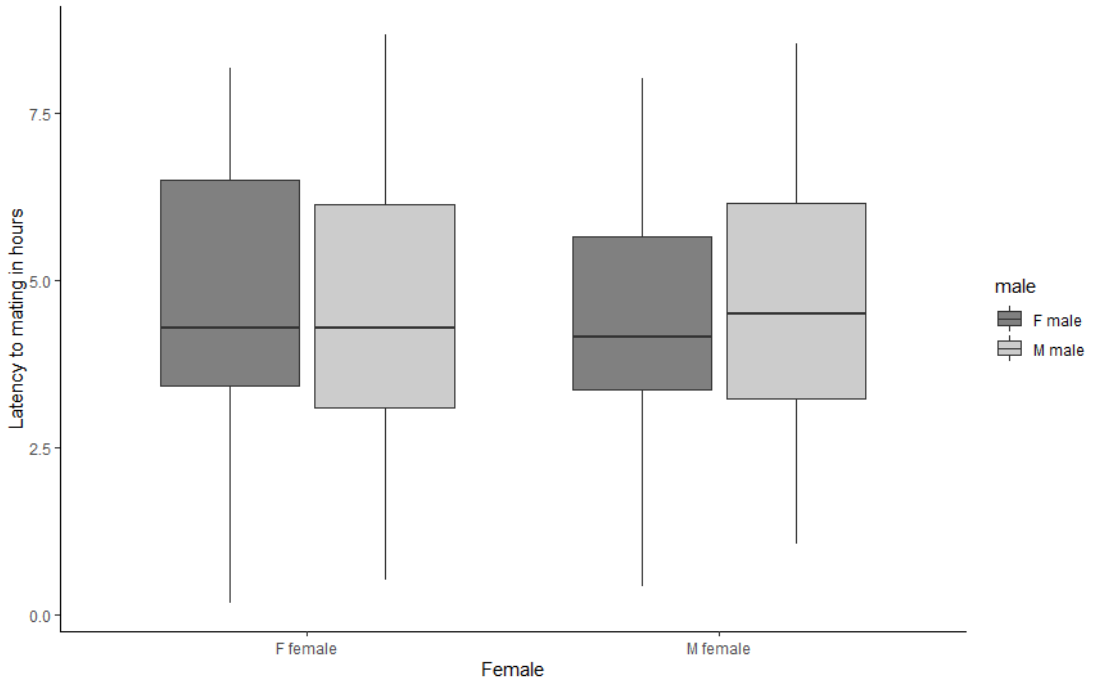

Figure 5.10: Representing the mating latency (y-axis) of F males (black boxes) and M males (grey boxes) with singly mated females from M and F populations (x-axis). Boxplots indicate median and interquartile range. Whiskers indicate variability outside the upper and lower quartiles. Dots represent outliers.

	Chisq	Df	Pr(>Chisq)
(Intercept)	229.7595	1	<2e-16
Male Type	1.1830	1	0.2767
Female Type	0.0064	1	0.9361
Male:Female	0.4641	1	0.4957

Table 5.9: Analysis of deviance table for male copulation duration with singly mated (M or F) females. Here female type describes the females from M and F populations and male type describes the males from M and F populations.

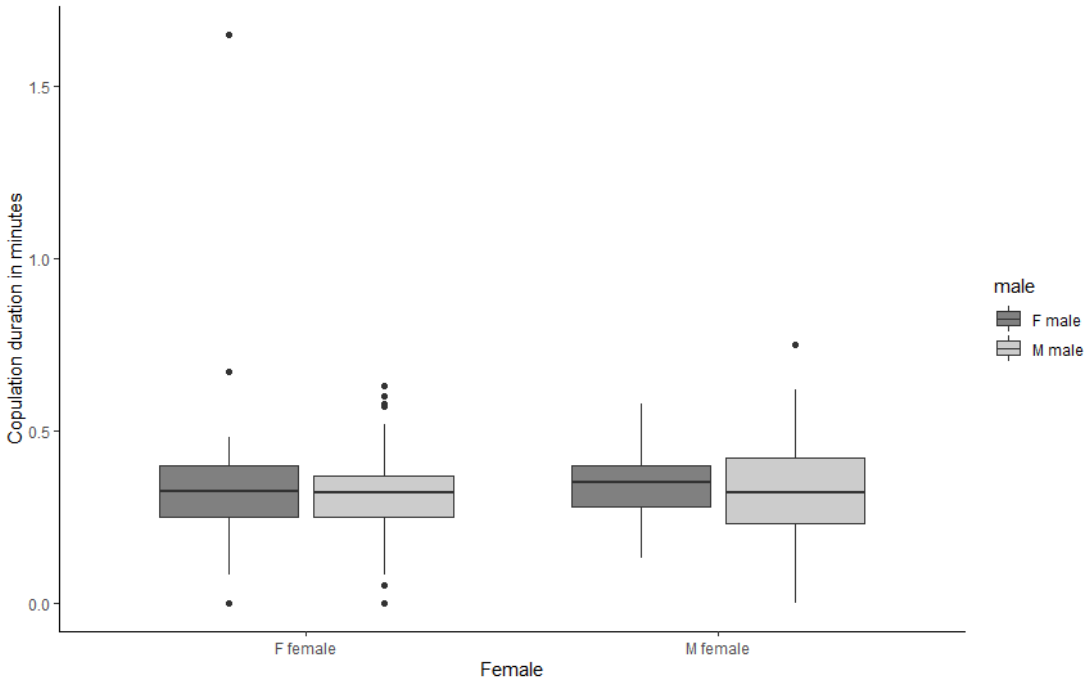

Figure 5.11: Representing the copulation duration (y-axis) of F males (black boxes) and M males (grey boxes) with singly mated M and F females (x-axis). Boxplots indicate median and interquartile range. Whiskers indicate variability outside the upper and lower quartiles. Dots represent outliers.

Courtship: Bootstrap t-test results:	
Null hypothesis: Prop M courtship = 0.5	
95% CI (0.5146, 0.5604)	**P = 0.0017**

Table 5.10: Results of Bootstrap t-test on courtship frequency data.

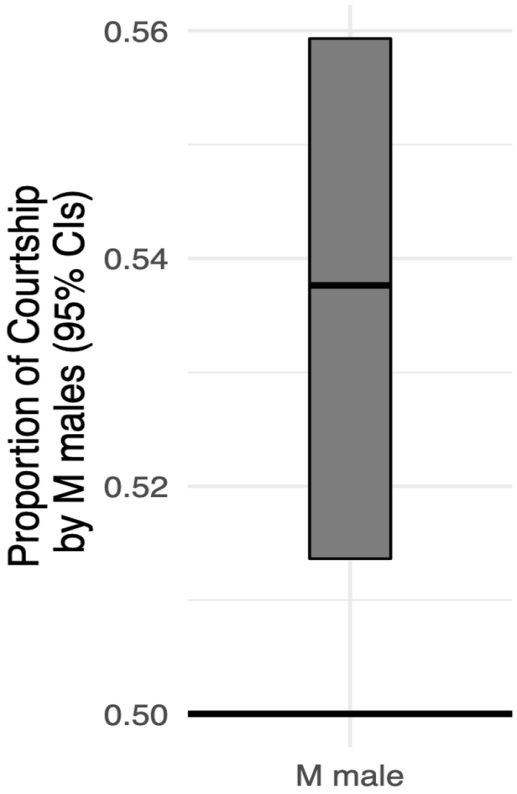

Figure 5.12: Representation of the bootstrapped one sample t-test for proportion of courtship by M males. The black line represents the null hypothesis and means with 95% bootstrapped confidence limits are plotted on the y-axis.

	Chisq	Df	Pr(>Chisq)
Intercept	503.6154	1	<2e-16 ***
Female Type	0.1303	1	0.7182

Table 5.11: Results of One way ANOVA showing the effect of "female type" on courtship frequency.

	Mating Success (F males)		Mating Success (M males)	
Predictors	*Log-Odds*	*p*	*Log-Odds*	*p*
(Intercept)	-1.18 (-1.47 – -0.89)	**<0.001**	-0.35 (-0.73 – 0.02)	0.063
Courtship proportion (F males)	-0.13 (-0.35 – 0.09)	0.257		
Courtship proportion (M males)			-0.07 (-0.26 – 0.12)	0.476

Table 5.12: Linear selection differentials of standardized courtship frequency on mating success.

	npar	**AIC**	**BIC**	**logLik**	**deviance**	**Chisq**	**Df**	**Pr(>Chisq)**
allmode	6	2050.9	2082.7	-1019.5	2038.9			
l_noint								
allmode	7	2037.0	2074.2	-1011.5	2023.0	15.851	1	$6.854e^{-05}$
l_int								

Table 5.13: Results of the likelihood ratio test to compare the slopes for the two models - mating success with virgin (M or F) females and mating success with singly mated (M or F) females.

Discussion

The results of the present study show that males evolving under higher levels of male-male competition have significantly better mating success irrespective of the female evolutionary history. When males from male-biased (M) and female-biased (F) populations were put in direct competition over mating with virgin females from LH_{st} (ancestral), M and F population, or singly mated females from M and F populations, males from M populations obtain more matings as compared to males from F populations (Figure 2). A previous study using the same populations showed no differences in body size (Chechi et al., 2017a). Thus, differential body sizes, known to influence the mating success (Baxter et al., 2018; Partridge et al., 1987a), do not contribute to the higher mating success of M males.

Males from M populations face stronger selection through male-male competition for access to females. As a result, their ability to obtain successful matings should evolve under increased competition. Our results show that M males indeed have better mating success when in direct competition with males from F populations. This result suggests that pre-copulatory selection is in play in M and F populations over mating success. However, results regarding the evolution of mating success itself have been variable. Some previous studies selecting directly for mating success have been unable to identify any response to selection (Hall et al., 2004; McGuigan et al., 2008).

In contrast, Dugand et al., (2018), selected *D. melanogaster* populations for male mating success using female preference and showed that male mating success under direct competition could evolve and that genetic variation exists for pre-copulatory traits. Our selection regime does not select directly for mating success; instead, I

manipulate the levels of male-male competition acting in the population. This can lead to divergence in investment in pre-copulatory traits that help secure matings, resulting in higher male mating success of males from M populations. Other than our study, we found two other studies which measured male mating success in populations evolving under experimentally manipulated sexual selection. Wensing et al., (2017) found that *D. melanogaster* males from the polygamous selection population get a higher proportion of matings than monogamous males in direct competition over mating success without any change in their sperm competitive ability. (2011) found that males from the male-biased regime in *Tribolium castaneum* have higher mating success than males evolved in the female-biased regime. My results and these studies show that pre-copulatory traits can diverge due to mating system manipulation.

In mating trials, it is often difficult to dissect male-male competition from the female effect (Baxter et al., 2018). I incorporated the female effect and effect of male-female interaction into the experiment design by performing the female trials with both ancestral females and co-evolving females from selection populations. I found no effect of female type or female X male interaction on mating success. The mating success of M males evolved under high intensity of male-male competition is not affected by the evolutionary history of the females they compete for. M males are better at acquiring mates when competing for both M females (which co-evolve with the M males) and the F females (which do not co-evolve with the M males but co-evolve with the F males). This indicates that the female choice and the male mating success either align in favor of M males or the fitness advantage that M males have in terms of mating success overwhelmingly masks the effect that female and male X female interaction can have on their mating success

Mating latency is sometimes used to measure female preference in *Drosophila* (Ritchie et al., 1999; Taylor, 2008). In my results, I find no significant difference in mating latency between females from M or F populations (Figure S2). These results suggest that there is no effect of female choice or at least none that we could detect through this metric. Another possible explanation is that the outcome of mating trials under direct competition is dependent only, or at least majorly, on male-male interactions. If the female is courted only by males, which chase off their competitors, females would have no choice (Jagadeeshan et al., 2015). In the assay with virgin females, the mating latency is too low to record any courtship or aggression data. Any difference in their aggressive pre-copulatory behavior is not known. The assay with the singly mated females shows that males from M populations have higher courtship frequency.

From previous studies, there are pieces of evidence of female traits influencing the outcome of male fitness. While Turiegano et al., (2013) showed that female size could affect the outcome of male mating success, Reinhart et al., (2015) showed an effect of female genotype on male mating success. Therefore, it is interesting to find that females evolving under different levels of male-male competition and having shown response to selection (Nandy et al., 2014) does not affect the male mating success of M males in any way. Reinhart et al., (2015), in their study with chromosome substitution lines of *Drosophila*, show that some males act as specialists, which perform better with some specific females; the others are "generalists" who perform better with all females genotypes. M males might have evolved to be generalists to have a fitness advantage over most female genotypes. Further experiments would help understand the male-female interaction and its effect on male mating success.

In the experimental assay with singly mated females from the selection population, M males outperformed F males in direct competition for successful mating. Mating success was not affected by the selection history of the female, nor was there any male X female interaction. Mated females are shown to be choosier than virgin females when choosing a mate. With the evolutionary history of the two female types, M and F, it is expected that the mating status could lead to differential choosiness between the females from the two selection regimes. M females that are evolving under higher male-male competition and male-biased sex ratio have more choice in terms of mates available even after obtaining a single mating.

On the other hand, F females evolving under a female-biased sex ratio have a much lower choice of available mates. Our results show that M males have significantly higher mating success with singly mated females, irrespective of the selection population the females belong to. As described in the population maintenance protocol, males from M populations have limited access to virgin females - one in three males gets to mate with a virgin female in M populations. On the other hand, in F populations, males have ample mating opportunities with virgin females. Having encountered mated females more often than virgin females, males from M populations have evolved to invest in mating with mated females. Therefore, in the assay trials with singly mated females from M and F populations, we see a much more pronounced mating success proportion in favor of males from M populations (Figure 3A). These results show that male investment in mated females has diverged due to differential male-male competition in M and F populations. Mating success has been shown to be a more significant contributor than fertilization success to the overall reproductive success of males (Pischedda and Rice, 2012). Males from the M regime are better at sperm

competitive ability than F regime males (Ali, 2018; Maggu and Prasad, 2021; Nandy et al., 2013a). Given this advantage in post-copulatory sexual selection, it is still advantageous for M males to invest in non-receptive, mated females than to avoid such matings. This is also supported by higher courtship towards single-mated females by M regime males.

In mating trials with singly mated females from the selection population, there was no significant effect of male or female on remating latency (Table S7). Crudgington et al., (2005), and Pitnick et al., (2001) have shown that remating frequency of females depends on the post-copulatory manipulation by males via the action of seminal fluids. In my setup, females from both selection populations were first mated with a common male from the LH_{st} (ancestral) population. About 60% of the total females remated from both M and F populations during the observation period. There was no significant difference in the remating rates of females from M and F populations, suggesting that differential post-copulatory manipulation by the first male does not explain our results.

Observing courtship activity in the assay with virgin females was difficult because the latency period is too short to have precise data on courtship. We recorded the courtship frequency of M and F males in mating trials with singly-mated females and found that M males court significantly more than F males irrespective of the female type (Figure 3B). Nandy et al., (2013b) have previously shown that M males have higher courtship frequency than F males towards ancestral (LH_{st}) females. My results are consistent with the previous studies (Crudgington et al., 2009; Nandy et al., 2013c), and show that M males in direct competition with F males have higher courtship frequency. Previously, studies have failed to find a correlation between courtship frequency and mating

success. Pitnick et al., (2001) do not find any correlation between courtship frequency and remating probability of females. Similarly, Bedhomme et al., (2008) show that males expressing male-limited evolved genomes acquire the same number of matings with reduced courtship frequency than control males. Although I found that males from the M population have higher mating success and courtship frequency, I did not find any correlation between courtship frequency and mating success in my assays. More courtship does not necessarily ensure mating success. Courtship is one of the pre-copulatory traits through which males can improve their competitiveness (Andersson, 1994). Furthermore, multiple cues may govern mating success (Dale and Slagsvold, 1996; Head et al., 2005; Schacht and Grote, 2015). Thus, the outcome of male mating success is a result of the overall attractiveness of the male as perceived by the female instead of a single trait (Bro-Jørgensen, 2010; Jennions and Petrie, 1997). Therefore it is likely that the M males have evolved other pre-copulatory traits that, combined with courtship frequency, might be responsible for their higher mating success.

My results thus establish that males from M populations have a pre-copulatory fitness advantage over F males in terms of mating success. Males from M populations have already been shown to have a post-copulatory fitness advantage over F males in terms of sperm competitive ability (Ali, 2018; Maggu and Prasad, 2021; Nandy et al., 2013c). The two results put together show that there is no trade-off between pre-copulatory and post-copulatory success in these populations. However, empirical studies on the correlation between pre-and post-copulatory investment are equivocal. Studies have found no trade-off or even pre-copulatory success reinforcing post-copulatory performance and vice-a-versa, in *D. melanogaster* (Bangham et al., 2002; Bretman et al., 2009; Fricke et al., 2010), *Drosophila simulans* (Hosken et al., 2008), *Tribolium*

castaneum (Lewis and Austad, 1994), and guppies (*Poecelia reticulata*) (Evans et al., 2003; Locatello et al., 2006; Pilastro et al., 2004). At the same time, some studies found a trade-off between pre-and post-copulatory traits in dung beetles (*Onthophagus taurus*)(Simmons and Emlen, 2006), dung flies (*Sepsis punctum*) (Puniamoorthy et al., 2012), fireflies (*Photinus greeni*) (Demary and Lewis, 2007), water striders (*Gerris lacustris*) (Danielsson, 2001) and guppies (*Poecelia reticulata*) (Evans, 2010). (Arnold and Wade, 1984) proposed a theoretical framework to separate the overall variance in reproductive success of a given sex into pre-copulatory and post-copulatory mechanisms. Various studies have used the approach to establish the importance of pre-copulatory and post-copulatory mechanisms in the overall reproductive success of males(Collet et al., 2012; Devigili et al., 2015; Droge-Young et al., 2012; Lüpold et al., 2014; Marie-Orleach et al., 2016; Pélissié et al., 2014; Pischedda and Rice, 2012). In *D. melanogaster*, after controlling for mating order, only a very small portion of the variance in male reproductive success is explained by differential fertilization success (Pischedda and Rice, 2012). (Lüpold et al., 2014) found that across taxa (not including *D. melanogaster*), the covariance between pre-copulatory and post-copulatory reproductive traits gradually shifts from strongly positive to strongly negative correlation with increasing male-male competition. M males have a fitness advantage over F males, both in terms of pre-copulatory (present results) and post-copulatory traits, but the relative contribution of these two types of traits to the overall reproductive fitness of M males is as yet unknown.

In conclusion, the study shows that mating success in direct competition can diverge due to male-male competition. In populations with higher male-male competition, the male mating strategy evolves to obtain more mating irrespective of the female

evolutionary history and mating status that they compete for. We did not find any effect of female type or male-female interaction on the outcome of male mating success. When competing for singly mated females, the mating success of males from a population with higher male-male competition was significantly higher than males from low levels of male-male competition. Investing to mate with the unreceptive mated females could be the by-product of higher post-copulatory fitness in terms of sperm competition. Lastly, while the courtship frequency is an important pre-copulatory trait in *Drosophila* species, my results show that courtship frequency alone cannot explain mating success.

Secondary Sexual traits: Wing morphology

Chapter 6A

Introduction

Sexual selection has led to the evolution of secondary sexual traits across various taxa (Andersson, 1994). In most species, males usually exhibit secondary sexual traits that are the target for female choice as they provide reliable information regarding the individual's quality, health, and/or social status (Andersson, 1994; Dougherty, 2021). These secondary sexual traits are often costly in terms of energy expenditure or indirect fitness cost (Mappes et al., 1996; Vehrencamp et al., 1989; Woods Jr. et al., 2007; Zuk and Kolluru, 1998). Sexual selection on these secondary sexual traits, through the mating preference of the choosing sex, can drive the evolution of more exacerbated and elaborate traits (Andersson, 1994; Lande, 1981).

Since sexual signaling through secondary sexual traits is a costly affair, only the high-quality individuals with resources can invest in them. According to the handicap principle proposed by Zahavi, sexual signal traits impose costs that high-quality individuals can more easily bear (Penn and Számadó, 2020; Zahavi, 1975). In other words, the secondary sexual traits can act as an honest signal of an individual's quality, upon which the choosier sex can base their choice (Dougherty, 2021; Grafen, 1990; Johnstone, 1995).

In *Drosophila* species, wings are often considered a secondary sexual trait as they play an essential part in the courtship process and mating success. Premating rituals in these species include a series of courtship steps involving the exchange of visual, auditory, gustatory, and olfactory stimuli between males and females and could influence the mating success (Lasbleiz et al., 2006; Markow and O'Grady, 2005; Trajković et al., 2013). A well-documented courtship ritual in *Drosophila melanogaster* includes wing

flapping, wing waving and wing semaphoring while circling the female (Greenspan and Ferveur, 2000; Spieth, 1974). Wings provide acoustic and visual signals which play an important part in the courtship ritual (Trajković et al., 2013)

The acoustic and visual signals through wings during courtship depend on the size and shape of the wings. Several studies have shown that wing morphology varies in shape and size across Drosophila species (Gidaszewski et al., 2009; Hatadani and Klaczko, 2008; Hoffmann and Shirriffs, 2002; Loh et al., 2008; Matta and Bitner-Mathé, 2010). Wing length and mating success in various Drosophila species is found to be correlated (Krishna and Hedge, 1997; Naseerulla and Hegde, 1992; Yenisetti and Hegde, 2003). Wing shape and size also influence the courtship song, the acoustic signal during courtship (Aspi and Hoikkala, 1995; Tauber and Eberl, 2003), and wing morphology affects male attractiveness and mating success. Naseerulla and Hegde (1992) reported that mating speed and wing size are correlated in *D. malerkotliana*, with longer wings giving males' an advantage in mating. Pavkovic-Luaic and Kekic (2011) reported that larger and more symmetric *Drosophila melanogaster* males are more successful in mating in nature. Trajković, Pavković-Lučić, and Savić (2013) found that *Drosophila melanogaster* males with elongated wings have higher mating success than males with rounded wings. Menezes et al. (2013) show that in *Drosophila melanogaster*, wing shape affects the courtship song quality and the mating success in competitive scenarios. From these studies, it is expected that wing morphometry could be a target for sexual selection as it influences acoustic and visual signals in the courtship ritual in *Drosophila* (Tauber and Eberl, 2003).

In this study, I examine the wing morphology of males from M and F population to investigate if the differential levels of sexual selection in these population has resulted

in the evolution of wing morphology, a secondary sexual trait in *Drosophila melanogaster*. I measured different morphology parameters including wing size, shape, and symmetry. We already know that M males have an advantage in mating success over F males and higher courtship frequency as compared to F males. I hypothesized that males from the M population, having evolved in a higher sexual selection environment, would have evolved larger, longer, and more symmetric wings, which would help them in courtship activity and higher mating success.

Material and methods

Standardization and generating experimental flies

The flies were collected from the M and F population for the experiment after one generation of standardization. In the standardization process, selection pressure is relaxed for M and F populations for one generation. It ensures that the non-genetic parental effects do not affect the outcome of the experiments.

From the standardized flies of the M and F population, the eggs are collected at the exact density of 150±2 eggs per vial in 8 ml of cornmeal molasses food media. Eggs were collected from all three replicates for the selection population M_{1-3} and F_{1-3} on the same day. Adult males were transferred into fresh food vials under light CO_2 anaesthesia on the 12^{th} day from egg collection in a group of 10 males per vial. For each selection population, seven such vials are collected per block. In total, 42 such vials are collected over three blocks of two selection populations. Males are given 1-2 hours to recover from anaesthesia effects of CO2 and then frozen at -20^0C for further dissections.

Wing dissections and imaging

From the frozen flies, both left and right wings are removed for each individual on a glass slide and sealed using nail paint and coverslip. The slides mounted with wings were imaged using a digital camera (Leica MC120HD, Leica Microsystems GmbH, Wetzlar, Germany) connected with Leica Stereo Zoom Microscope (M 205C, Leica Microsystems GmbH, Wetzlar, Germany). All the imaging is done at 4X optical zoom. Only individuals with both left and right in proper shape and without damage were used for imaging and further analysis. Three hundred and seventy four wings (both left and right) from 172 individuals, covering two selection populations and three replicate blocks, were imaged.

Statistical analysis

The images (in TIFF) were listed in the tps file using tpsutil software. Eleven landmarks were selected for geometric morphometric analysis of the wings following the (Fig 6a.1) based on the previous studies by (Abbott et al., 2010). The freely available Tps Dig program, by F James Rohlf, was used for digitizing the wing landmarks (Rohlf, 2015), and the coordinates are stored in a tps file. Using tpsrelw, relative wrap analysis is performed akin to the principal component analysis of a set of thin-playte spline transformations (Zelditch et al., 2012). It produces a reference shape through the generalized Procrustes method (GPA) called the consensus shape. It is the most unbiased mean configuration, closest to the actual shape (Rohlf, 2003). The difference between the original and reference shapes is used to test intra-population and inter-population variation.

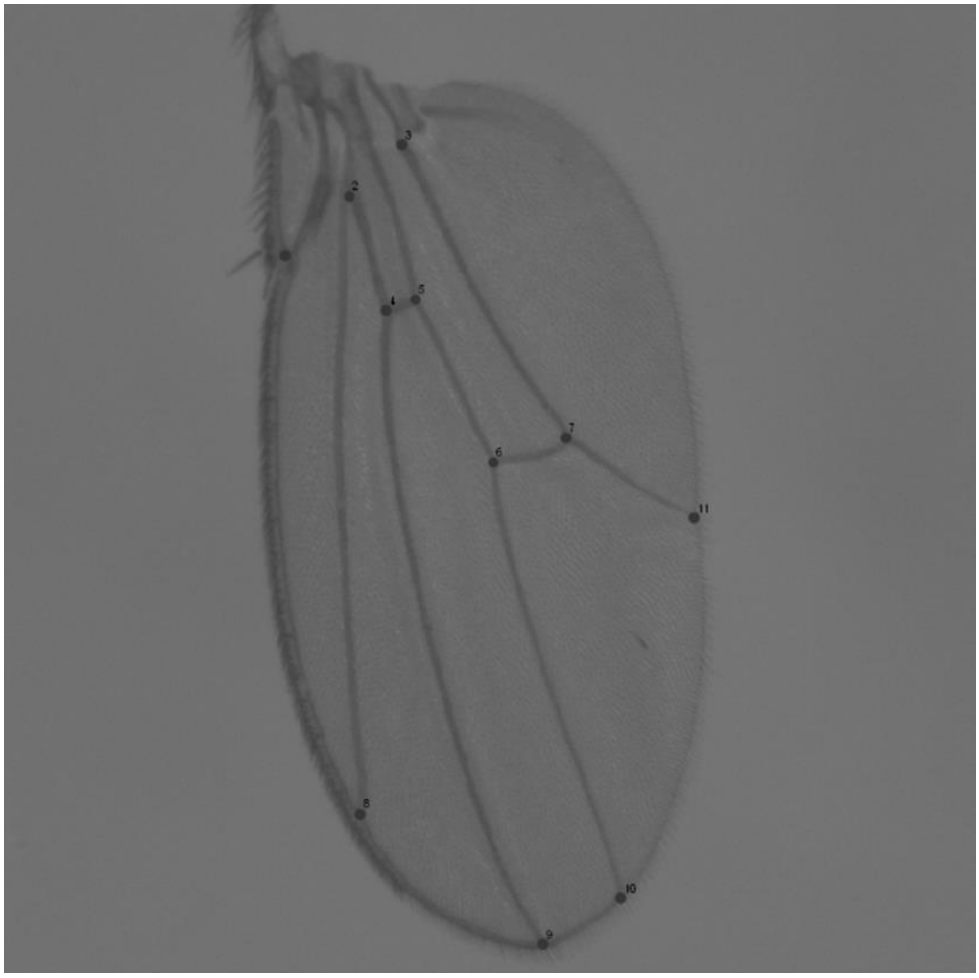

Figure 6a.1: Representing the landmarking on the wings for morphometric analysis.

The digitalization and landmark process was repeated non-sequencely four times to quantify within individual differences and the repeatability of the process. All further analysis is done in R using "geomorph" package. The Procrustes matrix generated from the relative wrap analysis was used to perform principal component analysis (PCA)

and Random Forest analysis (Dutta et al., 2018) to compare the shape and size of the wings in the sample.

Results

Principle component analysis shows no difference in the wing shape and size across the selection regimes (Figure 6a.3). The first two components of PCA analysis could explain only 52.56% of the variance., Random forest analysis could identify six groups with 87% accuracy using 18 principal component scores (Figure 6a.4). Procrustes ANOVA analysis shows that the selection regime does not affect the wing shape and size, but the interaction between the selection regime and bock was significant (Table 6a.1, Table 6a.2).

While analyzing the symmetry in the wings, I found fluctuating asymmetry and direction asymmetry, and inter-individual variation to be significant in wing shape, but there was no effect of selection regime or block (Table 6a.6). Similarly, there was fluctuating asymmetry in wing size, but it is not significantly different in the two selection regimes (Table 6a.5). When analyzing the blocks separately across the selection regimes, I do find significant interaction in some cases (Table 6a.8) but these interactions were not consistent across the selection regimes.

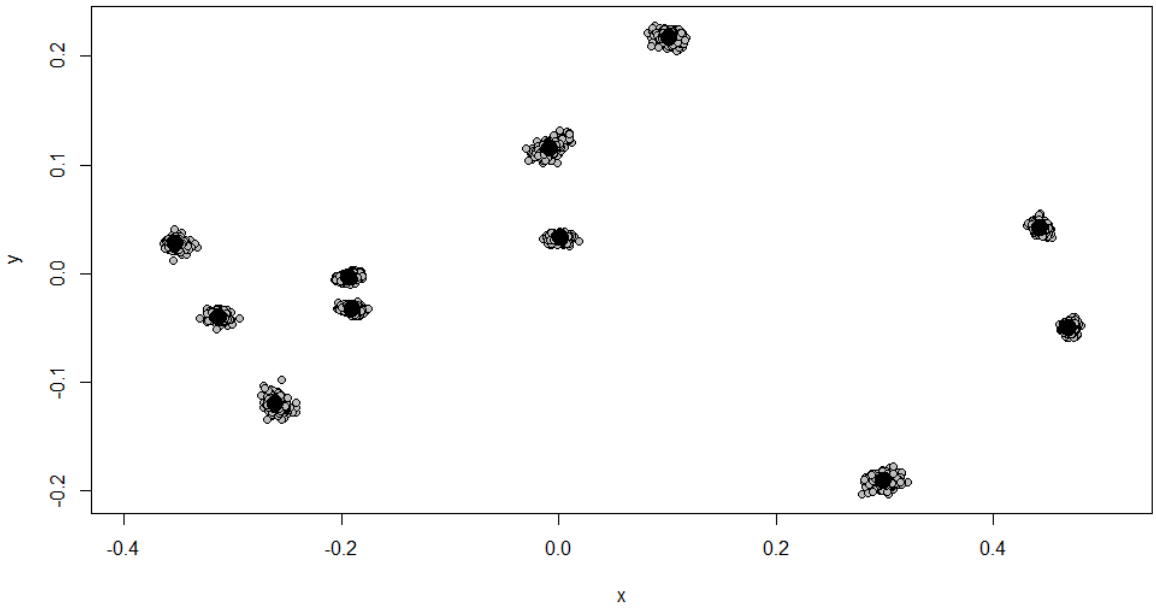

Figure 6a.2: Generalised Procrustes Superimposition showing the mean shape of the left and right wings (big black dots) of Drosophila and mapping landmarks for each specimen (small grey circle).

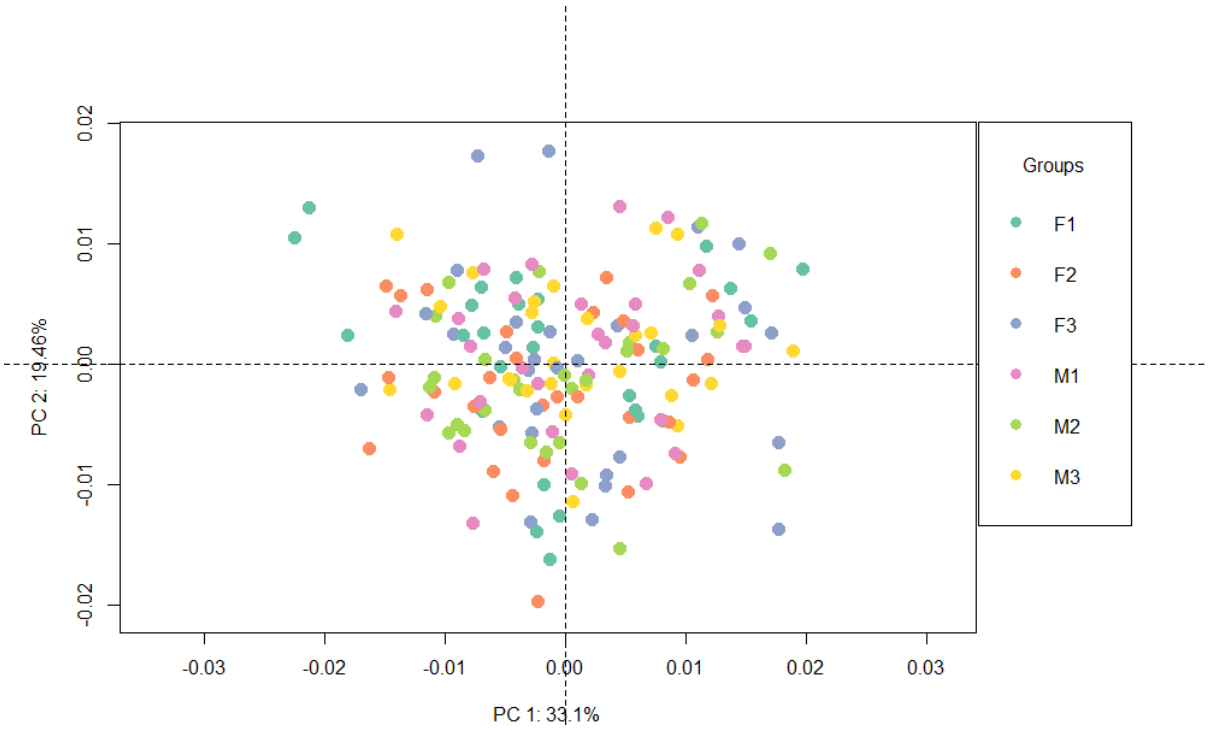

Figure 6a.3: The principal components and their contribution towards variance in the data are plotted below. NB: PC1 and PC2 only account for 52.56% of the variation in the data.

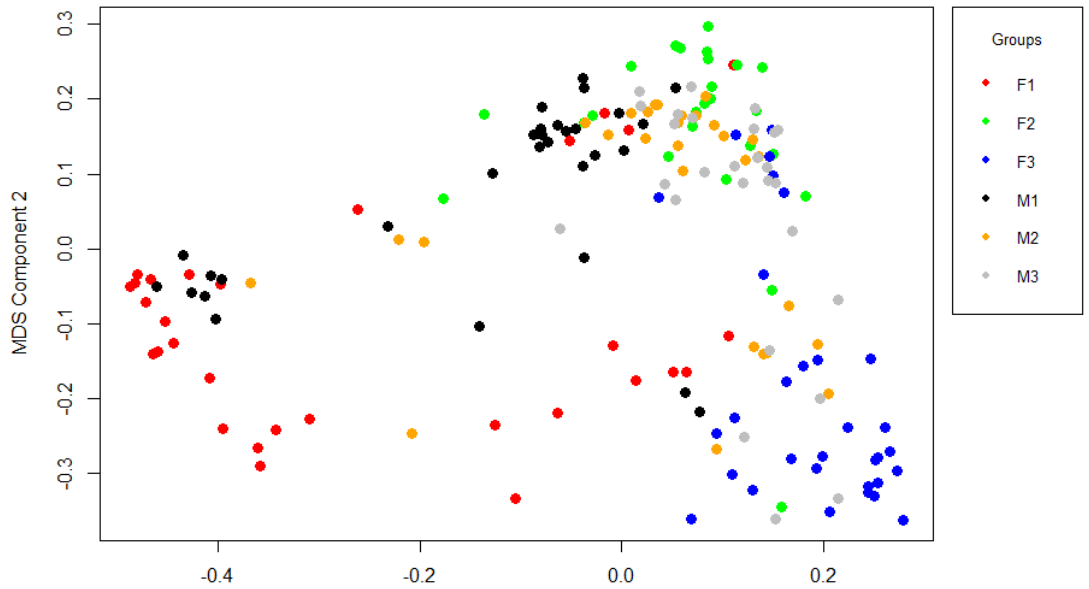

Figure 6a.4: Using 18 principal component scores of 172 individuals, Random Forest analysis could identify the 6 groups with 87% accuracy.

	Df	SS	MS	Rsq	F	Z	Pr(>F)
Selection Regime	1	0.000304	0.00030357	0.00854	1.4650	1.01200	0.150
Selection Regime:Block	4	0.000848	0.00021194	0.02385	1.0227	0.15869	0.443
Residual	166	0.034399	0.00020722	0.96761			
Total	171	0.035550					

Table 6a.1: Procrustes ANOVA for wing size using Residual Randomization Permutation procedure with selection regime as predictor variable and block as a random factor.

	Df	SS	MS	Rsq	F	Z	Pr(>F)
Selection Regime	1	0.001672	0.00167195	0.04243	0.8524	-0.1180	0.553
Selection Regime:Block	4	0.007846	0.00196147	0.19909	10.8934	7.7195	0.001**
Residual	166	0.039408	0.00018006	0.75848			
Total	171	0.035550					

Table 6a.2: Procrustes ANOVA for wing shape using Residual Randomization Permutation procedure with selection regime as predictor variable and block as a random factor.

Groups	Procrustes variances	Percentage of total disparity
F1	$5.278997e^{-05}$	22.90683
F2	$3.452880e^{-05}$	14.98287
F3	$4.481277e^{-05}$	19.44533
M1	$3.051078e^{-05}$	13.23936
M2	$2.616039e^{-05}$	11.35162
M3	$4.165245e^{-05}$	18.07399

Table 6a.3: Procrustes variances (a measure of morphological disparity) for F and M are 0.0001868078 and 0.0001607505, respectively, and there is no significant difference between them (p = 0.066)

	F1	F2	F3	M1	M2	M3
F1	1	-	-	-	-	-
F2	0.022	1	-	-	-	-
F3	0.197	0.337	1	-	-	-
M1	0.001	0.686	0.026	1	-	-
M2	0.001	0.321	0.004	0.491	1	-
M3	0. 106	0.256	0. 731	0.116	0.019	1

Table 6a.4: P-values for pairwise absolute differences between Procrustes variances

	Df	SS	MS	Rsq	F	Z	Pr(>F)
Inter-individual variation	171	$3.6471e^{-10}$	213279918	$5.5597e^{-10}$	0.9942	-0.06170	0.039*
Directional asymmetry	1	$4.2905e^{-08}$	429045729	6.5405^{e-08}	1.9999	0.74336	0.182
Fluctuating asymmetry	171	$3.6471e^{-10}$	213278421	$5.5597e^{-10}$	0.9941	-0.01825	0.670
ind:side: replicate	1032	$2.2140e^{-11}$	214533678	$3.3751e^{-11}$			
Total	1375	1.00					

Table 6a.5: Centroid Size ANOVA using Residual Randomization Permutation procedure for symmetry analysis

	Df	SS	MS	Rsq	F	Z	Pr(>F)
Inter-individual variation	171	0.31546	0.00184478	0.48089	4.4368	14.5806	0.001**
Directional asymmetry	1	0.00144	0.00144218	0.00220	3.4685	3.0193	0.001**
Fluctuating asymmetry	171	0.07110	0.00041579	0.10839	0.8730	12.3690	0.002**
ind:side:replicate	1032	0.26798	0.00025967	0.40852			
Total	1375	0.65598					

Table 6a.6: Shape ANOVA using Residual Randomization Permutation procedure for symmetry analysis.

Groups	Procrustes variances	Percentage of total disparity
F1	4.363311e-05	20.98785
F2	2.107758e-05	10.13847
F3	3.415699e-05	16.42976
M1	3.308747e-05	15.91532
M2	2.655275e-05	12.77207
M3	4.938911e-05	23.75653

Table 6a.7: Procrustes variances for fluctuating asymmetry (a measure of morphological disparity) for F and M are 0.0001932425 and 0.0002145706, respectively, and there is no significant difference between them (p = 0.392).

	F1	F2	F3	M1	M2	M3
F1	1	-	-	-	-	-
F2	0.008	1	-	-	-	-
F3	0.181	0.196	1	-	-	-
M1	0.139	0.165	0.877	1	-	-
M2	0.014	0.539	0.313	0.323	1	-
M3	0.482	0.001	0.076	0.030	0.001	1

Table 6a.8: P-values for pairwise absolute differences between Procrustes variances for fluctuating asymmetry analysis.

Discussion

In this study, I examined the wing shape and size of the males from the male-biased and female-biased populations. In *Drosophila* species, wings are considered a secondary sexual trait as their shape and size play an important role in courtship activity and affect the mating success of the individual. Previous results have shown that males from the M population have an advantage over males from the F population in terms of mating success. The M population males also have higher courtship frequency than males from the F population. Therefore, I expected that wings, an important component of the courtship activity, might be under differential selection pressure in the two populations. The results show no difference in wing shape and size of males from the M and the F populations. The differential selection pressure in the two populations does not lead to divergence in the wing shape and size of the males from the two populations.

The males from M and F populations are under differential selection pressure in terms of male-male competition and female choice. Though wing shape and size affect the courtship activity and mating success, it does not seem to be a trait under sexual selection in the populations. Baur et al. (2020), in a similar study, also found that in populations with increased male-male competition, wing shape and size are not under direct sexual selection. They found that selection acts on the overall body size and not individual trait morphology. Wing size in *Drosophila melanogaster* is often used as a proxy for body size. Various studies have shown a correlation between body size and mating success, with larger males gaining an advantage in mating success as they can deliver more courtship (Bangham et al., 2002; Partridge et al., 1987a, 1987b; Pavković Lučić and Kekić, 2009; Taylor and Kekic, 1988). Moreover, the wing size does not evolve in populations under differential levels of sexual selection, indicating that wing

size might not be a trait under selection. The advantage of larger body size in mating success is not universal as there are studies that have failed to find a correlation between the two (Menezes et al. 2013; Steele and Partridge 1988; Zamudio, Huey, and Crill 1995; Santos et al. 1992; Markow, Bustoz, and Pitnick 1996). The advantage in mating success of the M males is not related to body size. No body size difference has been observed between the males in the M and the F populations. Therefore, if the selection acts on the overall body size and not an individual trait, like in (Baur et al., 2020) study, we would not find any difference in wing size. Mating success is a complex trait and has multiple factors contributing. In the M and the F population itself, previously, we have failed to find a correlation between courtship activity and mating success, although M males are found to have higher courtship activity.

Wing shape is also known to evolve and affect courtship activity (Trotta et al. 2011; Menezes et al. 2013). How the wing shape might influence the courtship activity and/or mating success other than courtship song is still not well studied. Wing shape can influence the courtship song produced by the drosophila males during the courtship activity. The courtship song produced through wing vibrations are species-specific(Hoikkala et al., 1982; Tomaru et al., 1995; Williams et al., 2001) and is thought to be under female choice(Hoikkala et al., 1998; Klappert et al., 2007; Ritchie et al., 1999; Routtu et al., 2007; Tauber and Eberl, 2003). The wing shape of the males of the M and the F populations is not significantly different. The differential selection pressure in the M and F populations does not affect the wing shape. There is variation in wing shape in the population as block and selection interaction is significant, but the variation in wing shape between blocks is not consistent across the selection regime.

For insects, wings are essential for flight performance as they are important for dispersal are thought to be under natural selection (Harrison, 1980; Hill et al., 1999; Roff and Fairbairn, 1991; Stevens et al., 2010). At the same time, they are also thought to be under sexual selection, playing a role in courtship activity and influencing mating success. Therefore, potentially under both natural selection and sexual selection, these two selection forces interact and shape the evolution of wing morphology. The M and the F population differ only in their levels of sexual selection, while natural selection acting in the two selected populations is expected to be the same. How the two selection forces- natural selection and sexual selection- interact would define how the trait evolves. The present result, where we did not find any difference in wing morphology in the M and the F population males, points towards the fact that sexual selection alone might not be enough to drive the divergence in the wing morphology. How wing morphology would evolve with both the natural selection and sexual selection acting on the trait and their interaction is yet unknown and would be an interesting question to investigate further.

Another explanation for not finding any difference in shape and size of the wings in males from the M and the F populations lies in their diet. Studies have shown that diet is another crucial aspect influencing wing morphology. Trajković et al. 2021 shows that nutrition is an important factor that affects male attractiveness via change in wing morphology. Similarly, Pajač Živković et al. 2018 demonstrated plasticity in wing shape in *Drosophila suzukkii* when reared on different diets. In my study, the M and the F populations are maintained on the same food medium (cornmeal molasses food) and the same amount of food. These populations have been reared on the same food for

over 200 generations. So any difference in the food medium is not expected in these populations and thus can result in a lack of difference in wing morphology.

Along with morphometry, I also examined symmetry in the wings. Asymmetry is often correlated with development instability because of environmental or genetic stress (Costa et al., 2015; Møller and Pomiankowski, 1993; Watson and Thornhill, 1994). In secondary sexual traits, it is often considered to be under sexual selection as studies have found that symmetric males have an advantage in male-male competition and female choice (Costa et al., 2015; Møller and Pomiankowski, 1993; Pavkovic-Luaic and Kekic, 2011). Symmetry indicates the genetic quality of the individual and its ability to handle stress. While fluctuating asymmetry is usually more closely associated with developmental instability, directional asymmetry is more difficult to associate with genetic or environmental factors (Costa et al., 2015).

I found inter-individual variation in wing size in the M and F populations, but there was no directional or fluctuating asymmetry. In terms of wing shape, I again found inter-individual variation, and significant directional asymmetry and fluctuating asymmetry were also present. Comparing the asymmetry across the selection regimes, I did not find a significant effect of selection on either of the asymmetry components. The presence of asymmetry suggests that environmental or genetic stress is present in the populations; however selection is not acting on the asymmetry present as I found no difference in the symmetry between the two selection populations, which face differential levels of male-male competition and female choice.

In conclusion, I did not find any difference in the wing morphology, both size, and shape, across the M and the F populations. There was inter-individual variation and

asymmetry in wing shape, but again, no difference was recorded across the selection regimes. The results suggest that sexual selection, as a result of the differential selection in the M and the F populations, does not influence wing morphology in the males. Even the symmetry is not under sexual selection in these populations. Wing morphology, the secondary sexual traits that lead to a difference in mating success, are not under sexual selection and do not diverge across the populations under different sexual selection. Along with sexual selection, other factors like natural selection and environmental stress might be as important or even more important in deciding the trait values.

Wing Interference Pattern

Chapter 6B

Introduction

Visual signals, in context of animal communication have been well studied across taxa (Barraclough et al., 1995; Bell et al., 2017; Dale et al., 2015; Gerlach et al., 2014; Girard et al., 2011; McDiarmid et al., 2017; White et al., 2020, 2015). Colour pattern and pigmentation can serve as multi-functional signal for, predator avoidance (Janzen et al., 2010), mimicry or camouflage (Skelhorn et al., 2010), and mate choice (Houde, 2019; Katayama et al., 2014; Oliver et al., 2009; Siefferman and Hill, 2005). Males often use visual clues as secondary sexual traits, upon which the females base their choice. These clues serve as honest signals upon which sexual selection can act, as provide honest representation of the reproductive fitness of the males. Female choice for plumage and beak colour in birds, bright body colour in guppies and sticklebacks, colour patterns and pigmentation in butterflies, and melanin spots on wings of *Drosophila* are a few examples. Recently, wing interference patterns have been described in many insect species as part of the visual communication and is suggested to be under sexual selection.

Wing interference pattern (WIP) is a form of vivid coloration pattern on the otherwise transparent wings of the insects. WIPs are formed because of thin film interference from the wings (Shevtsova et al., 2011). When light strikes the thin insect wings, a part of the light is reflected from the chitinous membrane layer, while the rest is refracted into the membrane of the wings. Because of the thin film interference this result in wings reflecting colourful interference patterns. These colour sequences reflecting from transparent insect wings have been reported even before Darwin's time. But they were discarded as unstable soap bubble iridescence effect. But recent results on WIPs have demonstrated that these are stable, non-iridescent structural colour pattern with a wide

viewing angle. WIPs are found to be stable over the lifetime of the individual, species specific, and heritable (Katayama et al., 2014; Shevtsova et al., 2011). Originally, WIPs were expected to play a role in species identification as species specific WIPs were reported across Diptera, Hymenoptera (Buffington et al., 2012; Shevtsova and Hansson, 2011) and Hemiptera (Simon, 2013). In insects, for purpose of optimal flight, wings have decreased size of wing membrane enforced with wing corrugation, presence and placement of microtrichia and venation. Wing interference patterns (WIPs) are also found to be dependent on the wing morphology, membrane thickness, wing corrugation, and presence and placement of microtrichia (Shevtsova et al., 2011). Therefore while on one hand wings are under selection for optimal aerodynamic function, on the other hand they are under selection to serve as visual clue and a sexual trait.

Insects are known to have evolved their signal-receiver architecture of thin membranous wings and colour vision a very long time. Recent work on WIPs has focused on the intraspecies variation and them being a potential target for sexual selection. Like most other species in *Drosophila melanogaster*, male attractiveness as expected would be a complex combination of all traits and sensory signals. But visual cues during courtship ritual is well documented in *Drosophila melanogaster*, where wings play an important role in the whole courtship ritual includes courtship dance and song. Therefore WIPs being under sexual selection is a very likely possibility. Katayama *et al.*, (2014) using isogenic *Drosophila melanogaster* lines shows that WIPs are a target of female mate choice and can evolve under sexual selection. They show that females find males with more vivid WIPs to be more attractive than males with dull WIPs. Similarly, using experimental evolution technique Hawkes *et al.*, (2019)

reports that in *Drosophila simulans* sexual selection drives the evolution of WIPs and is correlated with male attractiveness. Males from polygamous population have brighter, higher contrast WIPs as compared to males from monogamous population.

In the present study using experimental evolution technique, I investigated how wing interference pattern is impacted by sexual selection. I used the males from the M and the F populations that have evolved in terms of pre-copulatory sexual traits, such as increased courtship and mating success advantage of M males over F males. I predicted that WIPs as a target for sexual selection would diverge in the two populations with M males, evolving under higher levels of male-male competition and stronger female choice, showcasing WIP more attractive to females and thus helping in pre-copulatory mating success.

Material and methods

Standardizing and generating experimental flies

Experimental flies are subjected to a standardization process to avoid non-genetic parental effects on the experiments' outcome. The flies are maintained for one generation without selection, as in the ancestral population, i.e not subjected to virgin collection and biased sex-ratios. For the experiments in this study, eggs were collected from these standardized flies at the exact density of 150 ±2 per vial in cornmeal molasses food. On day 9-10 post egg collection, virgin flies were collected in a single-sex vial at the density of 8 flies per vial. For all the experimental assays, 12 days (post egg collection) old flies from M_{1-3}, F_{1-3}, and LH_{st} population were used.

Male mating latency assay

Virgin males from M population and F population was combined with virgin female from ancestral LHst stock population as single pair per vial. Male mating latency was measured as the time taken from introducing the mating pair of male and female in a vial, to start of mating. It is expected that females would initiate mating faster with more attractive males. Time from combining the male and female to the start of mating is recorded to nearest of second. 30 vials for each $M_{(1-3)}$ and $F_{(1-3)}$ male, per replicate line were setup. Over three replicate lines, data from 90 M males and 90 F males was used for the analysis.

Wing dissections and imaging

Wings from males and females from both M and F population were dissected out. Digital photos of the wings were obtained under uniform illumination and magnification using a digital camera (Leica MC120HD, Leica Microsystems GmbH, Wetzlar, Germany) connected with Leica Stereo Zoom Microscope (M 205C, Leica Microsystems GmbH, Wetzlar, Germany). We followed the image processing protocol similar to (Katayama et al., 2014). In brief, all the digital imaged were transferred to image J for processing. The largest panel of wing, clearly demarcated by veins, and corresponding to the M-sector distal to cross vein dM-Cu was selected as area for measurement. Using the Lpixel plugin, the lpixel colour function was used to convert RGB values from each pixel to HSV values. Values of average hue, average standard deviation in hue, average saturation, average brightness value and average standard deviation in brightness value were recorded for each image.

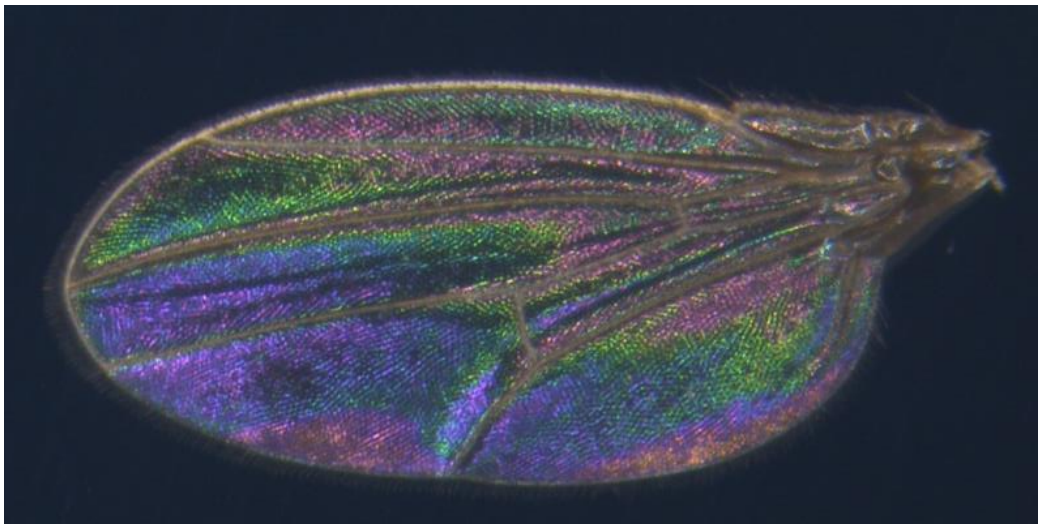

Figure 6b.1: Representing the Wing interference pattern as observed for the imaging.

Quantifying Wing Interference Pattern

Wing interference patterns are quantified through the HSV colour (Hue, Saturation and Value) model. HSV model is alternative to the RGB colour model, used to quantifying colour. HSV is a cylindrical colour model that remaps the RGB primary colours into dimensions that are easier for humans to understand. HSV model is more aligned to the way the human vision perceive colour making attributes. It has three components; hue, saturation and value. This colour space describes colour (hue) in terms of their shade (saturation) and their brightness value.

- Hue: Hue is a quantitative value given to every colour corresponding to the angle of the colour on the RGB colour circle. Hue refers to the dominant wavelength of the light being reflected or produced. The values for hue range from 0 to 360, corresponding to the universally accepted location of that colour on the RGB colour circle.

- Saturation: Saturation refers to the purity or the intensity of the hue. Its value ranges from 0 to 1. Saturation value of 1 refers to the most saturated colour while a value of 0 corresponds to the absence of colour, giving it a duller appearance. More saturated the colour is, more vivid, bright it appears.
- Value: Value refers to the lightness or darkness of the colour present. It indicates the quantity of the white light reflected from the object. Value ranges from 0 to 1, with 0 corresponding to black, while 1 corresponding to the brightest colour based on the hue.

Statistical analysis

All statistical tests were performed in the R statistical environment (v4.0.2, R Core Team 2020), using "lmerTest" package and "lsmeans" package.

Using linear mixed effects model, hue, saturation and brightness values were analysed. ANOVA test was performed on these variables independently, with sex and selection as fixed factors, and blocks as random factor. Similarly, male mating latency was analysed using linear mixed model, performing ANOVA on mating latency, with selection as fixed factor and block as random factor.

Results

Hue

There was a significant effect of sex and selection population on the average hue values, without any interaction between sex and selection population. Males on average have higher hue values than females (Table 6b.1, Figure 6b.2). Comparing across the

selection regimes, M population has higher average hue values than the F population. There was no significant interaction between sex and selection regime (Figure 6b.2, 6b.3).

Saturation

There was a significant effect of selection and sex on the saturation values (Table 6b.2, Figure 6b.4). Males have higher saturation values in WIPs as compared to the females. Across the selection regimes, M population has higher saturation value in WIPs as compared to the F population. There was no significant interaction between selection and sex. The block effects were significant, but the overall trend for all the three blocks is similar (Figure 6b.5).

Value

There was a significant effect of sex and population on brightness value as well (Table 6b.3, Figure 6b.6). Males have higher brightness values as compared to the females. Across the selection population, M population have higher brightness for WIPs as compared to the F population. The interaction between sex and selection was not significant. There was no block effect (Figure 6b.7).

When looking at the standard deviation for average brightness values, there was a significant effect of selection population, with M population having higher standard deviation in average brightness values (Table 6b.4, Figure 6b.8). The effect of sex and its interaction with selection population was not found to be significant. The block effects were significant, but the trend was similar in all the three blocks (Figure 6b.9).

Mating latency

There was no significant effect of male identity on the mating latency, suggesting that there is no difference in mating latency of males from both M and F populations (Table 6b.5, Figure 6b.10). There was no block effect as well in the mating latency analysis (Figure 6b.11).

	Sum Sq	Mean Sq	NumDF	DenDF	F value	Pr(>F)
Population	4231.4	4231.4	1	283.05	29.2579	0.000000135***
Sex	15008.9	15008.9	1	283.02	103.7781	< 2.2e-16***
Population:Sex	57.8	57.8	1	283.02	0.3994	0.5279

Table 6b.1: Results of ANOVA for Hue value analysis. Here population describes the M and F population.

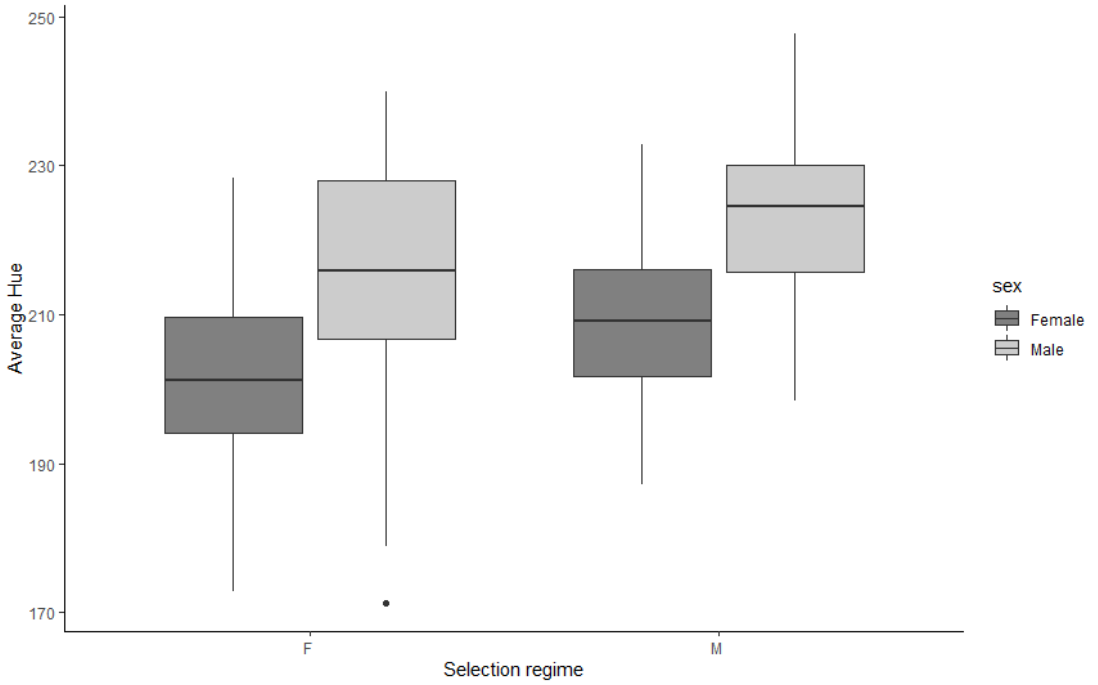

Figure 6b.2: Representing average hue (y-axis) of females (black boxes) and males (grey boxes) from the two selection regimes (x-axis) M and F. Boxplots indicate

median and interquartile range. Whiskers indicate variability outside the upper and lower quartiles. Dots represent outliers.

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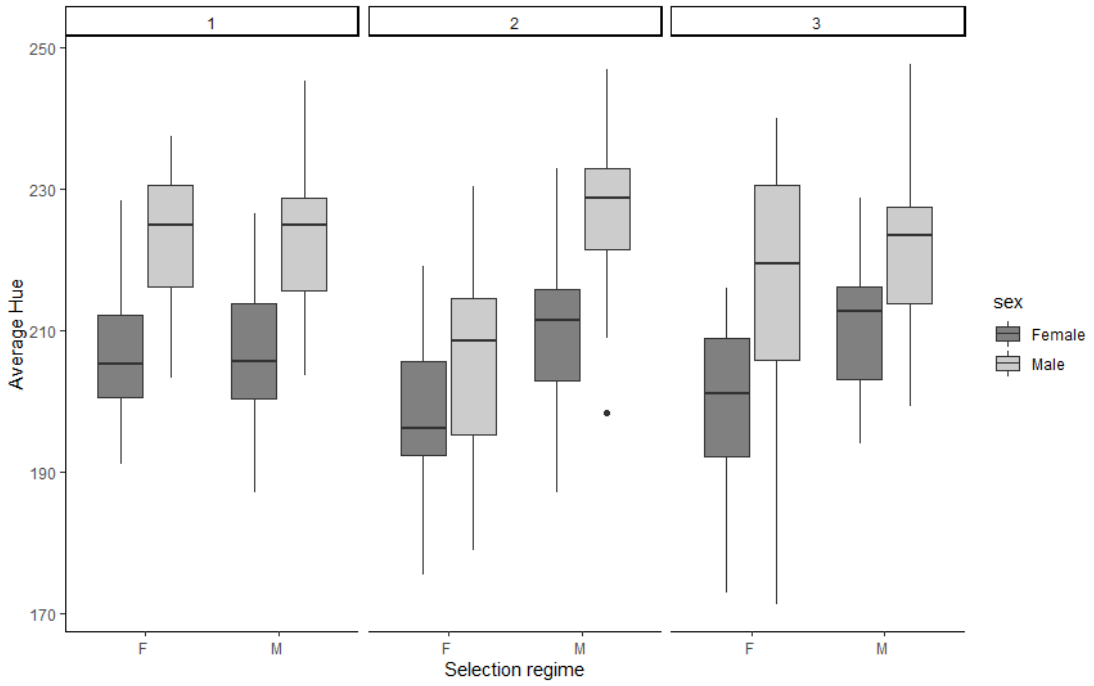

Figure 6b.3: Representing average hue (y-axis) of females (black boxes) and males (grey boxes) from the two selection regimes (x-axis) M and F across the three blocks (grids). Boxplots indicate median and interquartile range. Whiskers indicate variability outside the upper and lower quartiles. Dots represent outliers.

	Sum Sq	Mean Sq	Num DF	Den DF	F value	Pr(>F)
Population	0.007996	0.007996	1	283.01	21.3557	0.000005803***
Sex	0.004864	0.004864	1	283	12.9898	0.0003699***
Population:Sex	0.000145	0.000145	1	283	0.3869	0.534434

Table 6b.2: Results of ANOVA for saturation value. Here population describes the M and F population.

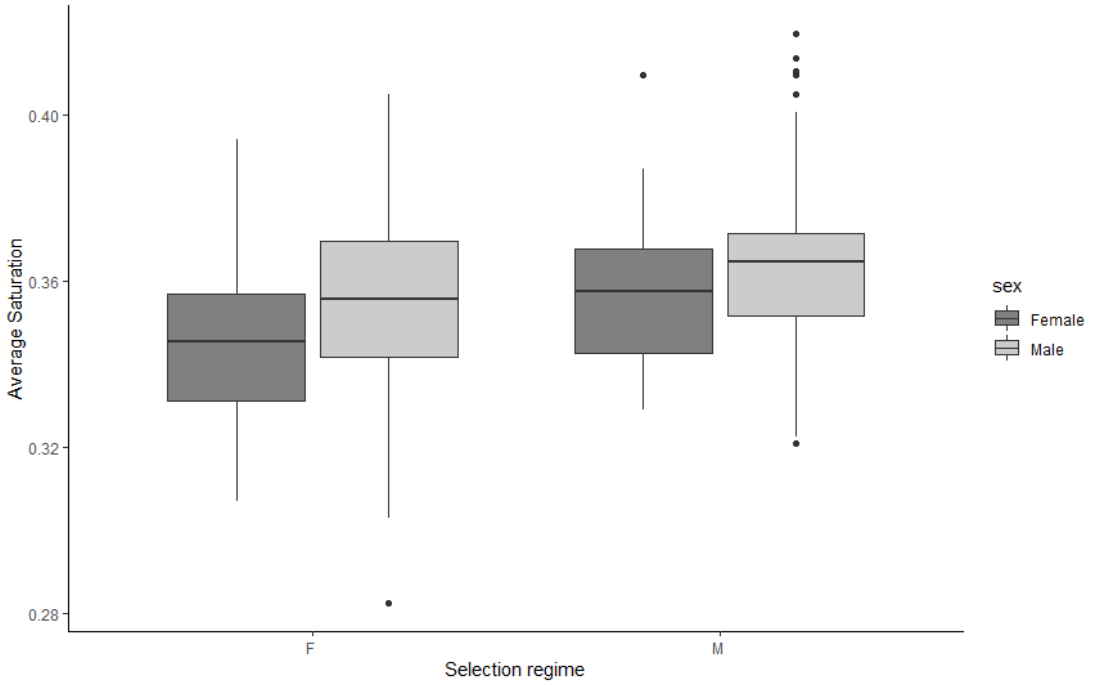

Figure 6b.4: Representing average saturation (y-axis) of females (black boxes) and males (grey boxes) from the two selection regimes (x-axis) M and F. Boxplots indicate median and interquartile range. Whiskers indicate variability outside the upper and lower quartiles. Dots represent outliers.

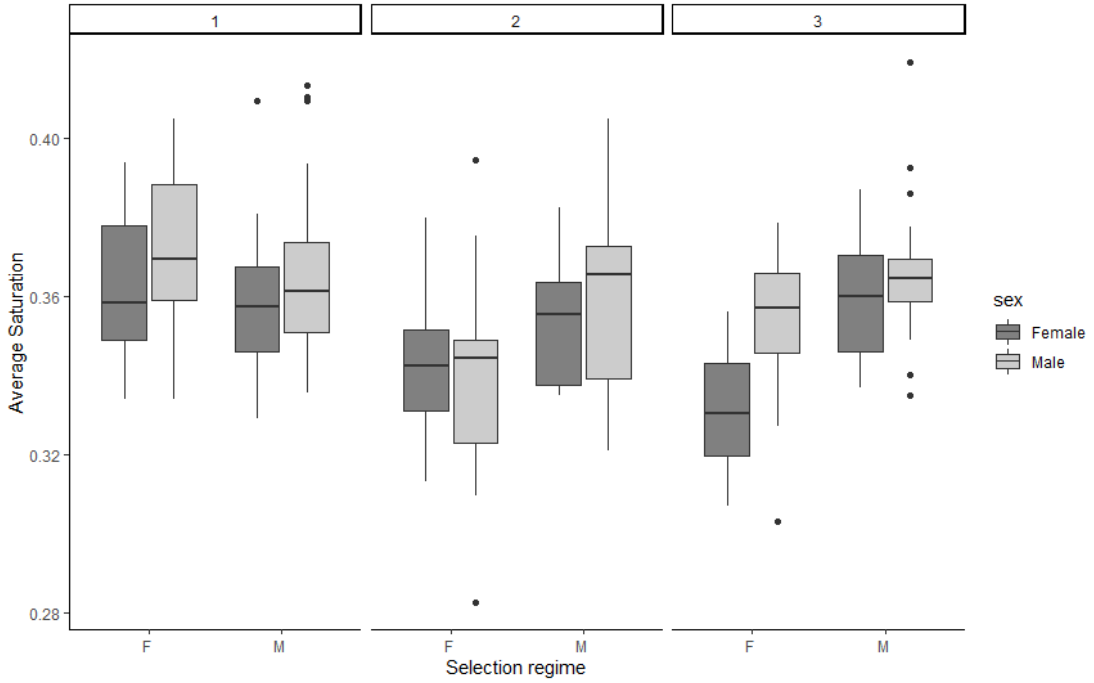

Figure 6b.5: Representing average saturation (y-axis) of females (black boxes) and males (grey boxes) from the two selection regimes (x-axis) M and F across the three blocks (grids). Boxplots indicate median and interquartile range. Whiskers indicate variability outside the upper and lower quartiles. Dots represent outliers.

	Sum Sq	Mean Sq	NumDF	DenDF	F value	Pr(>F)
Population	0.024977	0.024977	1	285	24.649	1.19e-06***
Sex	0.046926	0.046926	1	285	46.311	5.95e-11***
Population:Sex	0.000184	0.000184	1	285	0.182	0.6699

Table 6b.3: Result of ANOVA for average brightness values. Here population describes the M and F population.

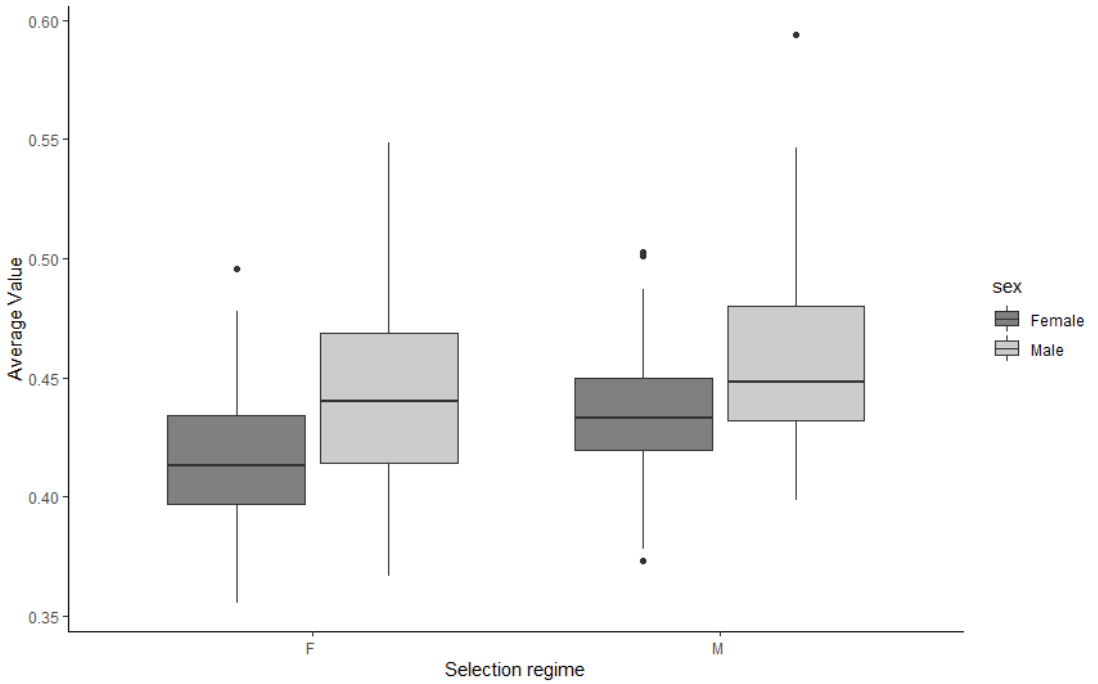

Figure 6b.6: Representing average brightness value (y-axis) of females (black boxes) and males (grey boxes) from the two selection regimes (x-axis) M and F. Boxplots indicate median and interquartile range. Whiskers indicate variability outside the upper and lower quartiles. Dots represent outliers.

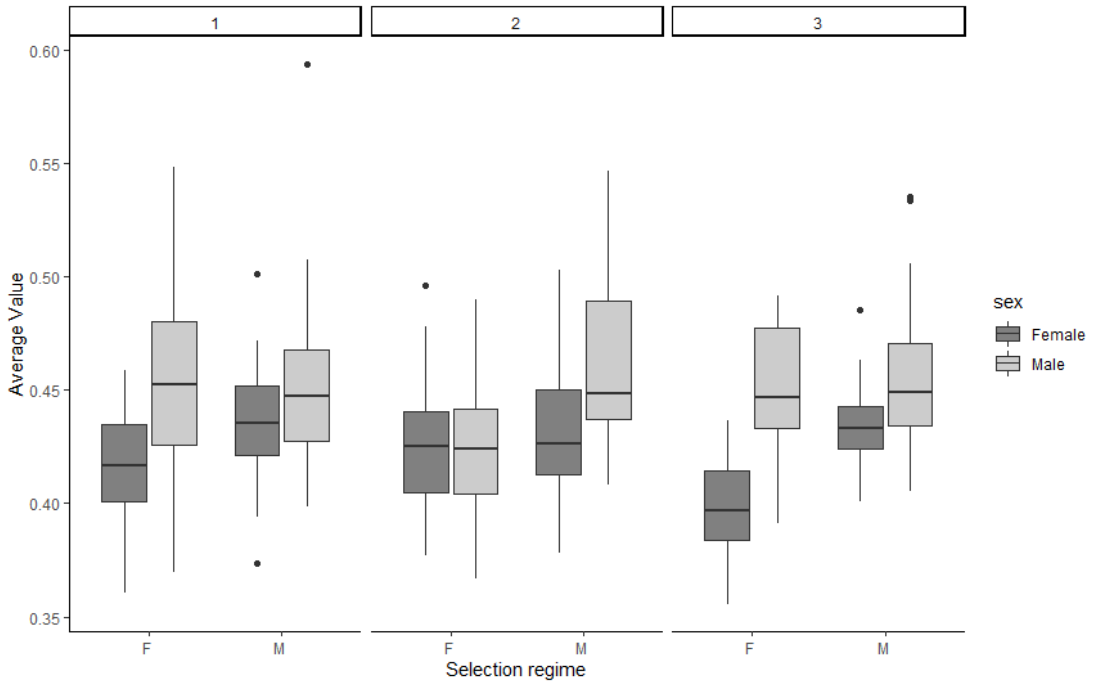

Figure6b.7: Representing average brightness value (y-axis) of females (black boxes) and males (grey boxes) from the two selection regimes (x-axis) M and F across the three blocks (grids). Boxplots indicate median and interquartile range. Whiskers indicate variability outside the upper and lower quartiles. Dots represent outliers.

	Sum Sq	Mean Sq	NumDF	DenDF	F value	Pr(>F)
Population	0.00175	0.00175	1	283.03	13.2779	3.19e-04***
Sex	0.0000229	0.0000229	1	223	0.1737	0.6771
Population:Sex	0.0000281	0.0000281	1	283.01	0.2132	0.644646

Table 6b.4: Results of ANOVA for standard deviation in average brightness values. Here population describes the M and F population.

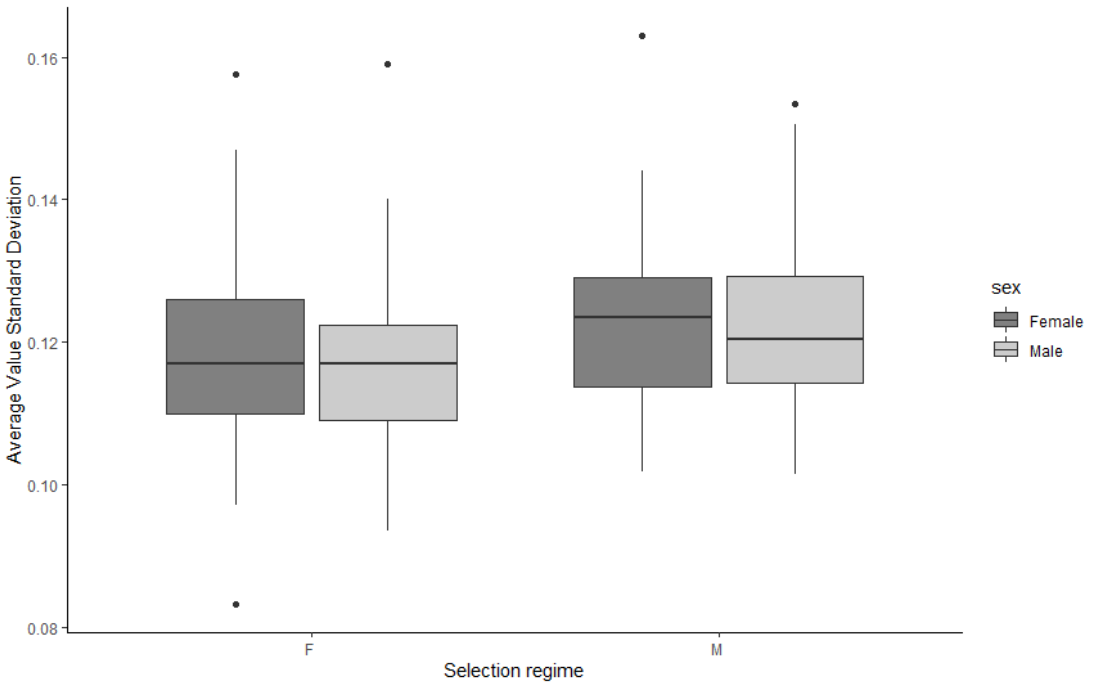

Figure 6b.8: Representing average brightness value standard deviation (y-axis) of females (black boxes) and males (grey boxes) from the two selection regimes (x-axis) M and F. Boxplots indicate median and interquartile range. Whiskers indicate variability outside the upper and lower quartiles. Dots represent outliers.

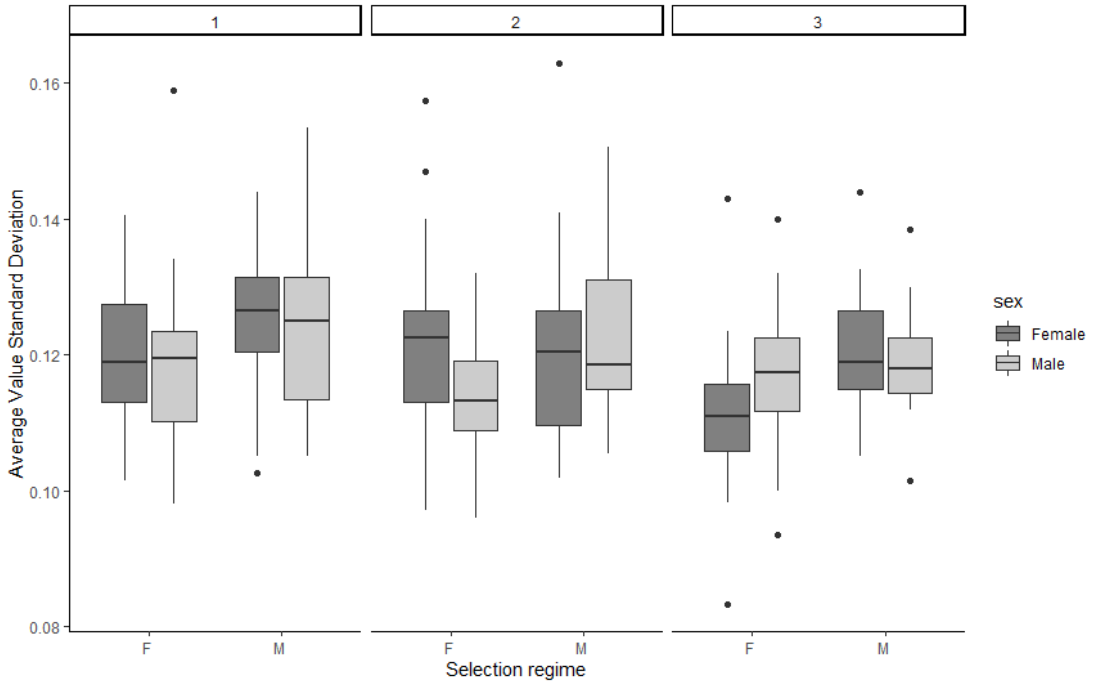

Figure 6b.9: Representing average brightness value standard deviation (y-axis) of females (black boxes) and males (grey boxes) from the two selection regimes (x-axis) M and F across the three blocks (grids). Boxplots indicate median and interquartile range. Whiskers indicate variability outside the upper and lower quartiles. Dots represent outliers.

	Sum Sq	Mean Sq	NumDF	DenDF	F value	Pr(>F)
Selection	0.000498	0.000498	1	2.02	0.0127	0.9205

Table 6b.5: Results of ANOVA for mating latency. Here population describes the M and F population.

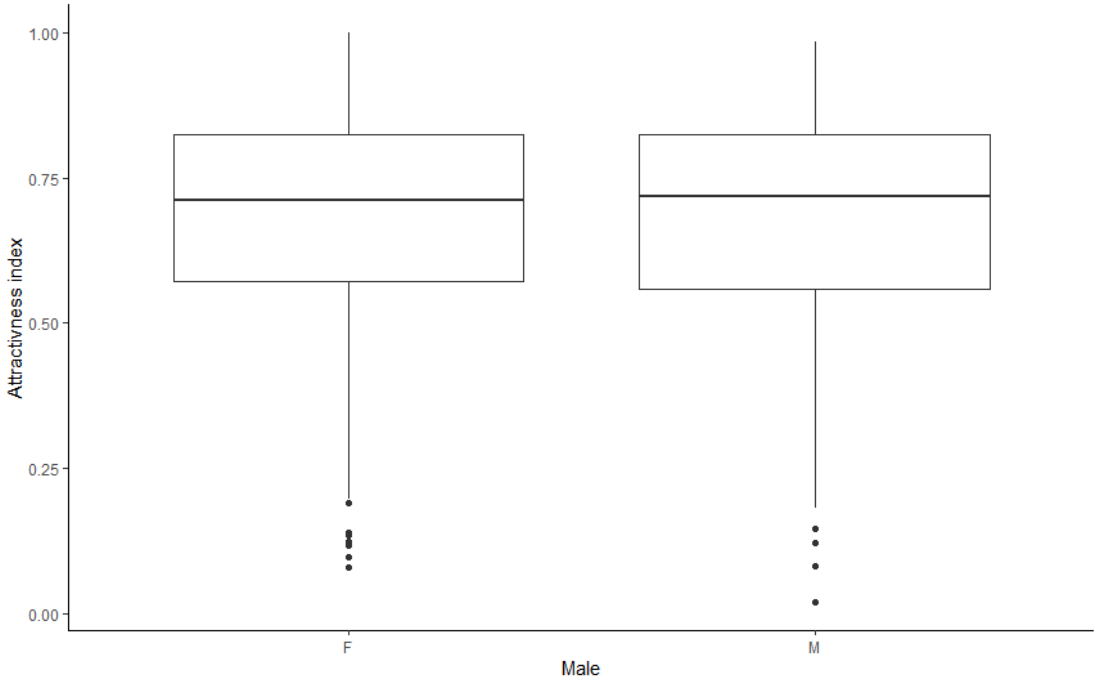

Figure 6b.10: Representing attractiveness index, quantified as mating latency of males from the two selection regimes (x-axis) M and F, with common females. Boxplots indicate median and interquartile range. Whiskers indicate variability outside the upper and lower quartiles. Dots represent outliers.

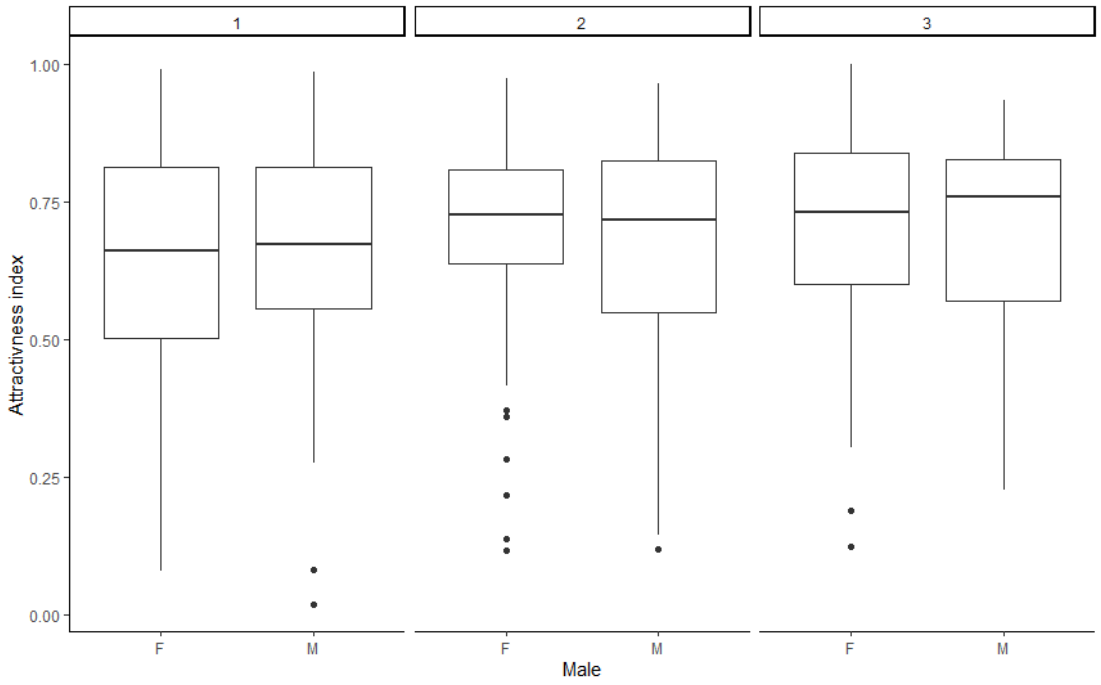

Figure 6b.11: Representing attractiveness index (y-axis) of males from the two selection regimes (x-axis) M and F across the three blocks (grids). Boxplots indicate median and interquartile range. Whiskers indicate variability outside the upper and lower quartiles. Dots represent outliers.

	M male	F male	M female	F female
Average Hue	223.9108	215.3057	208.5707	201.811055
Average Saturation	0.36393	0.35465	0.357053	0.34517123
Average brigthness value	0.458423	0.441421	0.434527	0.41432877
Standard devation in Average brightness value	0.122282	0.11675	0.122227	0.11798767

Figure 6b.6: Average values for Hue, saturation, brightness and standard deviation in brightness value, for the M and F population males and females.

Discussion

In this study, I quantified the wing interference pattern (WIP) of the males and females from M and F populations, evolved under differential levels of sexual selection. I took the largest area panel of the wing which was used to extract the hue, saturation and brightness value (HSV) values. Along with that, I measured the attractiveness of the males from M and F population, in terms of mating latency. I found that sexual dimorphism exists in WIPs in these populations, and differential levels of sexual selection in M and F population leads to significantly different wing interference patterns. WIPs are found to under sexual selection and have diverged for the males from M and F population. At the same time, I did not find any significant difference in the attractiveness of males (measured as mating latency to mating) from the two populations.

Wing interference pattern are quantified using the hue, saturation and brightness values. These parameters allow to compare the colour intensity and saturation similar to the way human eyes interpret the colours. I found that sex and selection has significant effect on all these three parameters. There is sexual dimorphism in WIPs with males having WIPs with higher hue values, saturation and brightness values. Comparing across the selection regimes, M populations have higher hue, more saturation and brightness as compared to the F population.

When comparing the hue values, all the hue values for M and F population falls in the green to blue colour pallet. The Drosophila eye contains 5 different photoreceptors (Schnaitmann et al., 2013). Out of these 5 photoreceptors, two have a narrow peak sensitivities in the human-visible spectrum roughly corresponding to green (Rh6) and

blue (Rh5) light (Rister et al., 2013; Schnaitmann et al., 2020, 2013). Across the selection regimes M population has higher hue values (more closer to blue) as compared to the F population (closer to green). Previous studies have also found the similar range for the hue values in *Drosophila melanogaster* (Katayama et al., 2014) and *Drosophila simulans* (Hawkes et al., 2019). Within populations, in both the M and the F populations, males have significantly higher hue values as compared to females.

Higher saturation signifies pure and more vibrant colour. M males have higher saturation as compared to the F males. In females, M females have higher saturation as compared to the F females. More vibrant colours in WIPs have been shown to be preferred by females and males with more vibrant WIPs are more attractive (Hawkes et al., 2019; Katayama et al., 2014). Similarly, M population has higher brightness values than F population. Higher brightness value adds to the colour of WIP and makes it more attractive.

Sexual dimorphism in secondary sexual traits is a common trend as sexual selection is often stronger in males than in females (Hosken and Stockley, 2004; Shuster and Wade, 2019). Sexual dimorphism in WIPs have been reported across taxa in previous studies as well (Butterworth et al., 2021; Hawkes et al., 2019). The M males have brighter, vivid and more colourful WIPs. Previous studies have found that males with WIPs similar to M population males in terms of hue saturation and brightness values, have higher attractiveness index (Hawkes et al., 2019; Katayama et al., 2014). (Hawkes et al., 2019) shows that males from polygamous selection population of *Drosophila simulans* have more vibrant and colourful WIPs, and are more attractive as compared to the males from monogamous populations.

The divergence in WIPs across the selection regimes, indicate that the WIPs are under sexual selection as in my selected populations M and F, the only difference is in levels of sexual selection in the two populations. To further investigate how the WIPs contribute to the male reproductive fitness, I chose male attractiveness, quantified as the mating latency to mating, as a focal trait. The decision was based on the previous studies which have also looked at male attractiveness as a trait which would be affected by WIPs because WIPs are supposed to play a role in courtship activity (Hawkes et al., 2019; Katayama et al., 2014). Any advantage in courtship, would lead to faster mating via female choice and could be captured in mating latency. While those studies found a difference in male attractiveness based on the WIPs, I did not find any difference in male attractiveness across the M and the F populations even when there WIPs have diverged. My results on WIPs being under selection are similar to the two studies mentioned above, but differ from these two studies when looking at the reproductive advantage that WIPs provide in terms of male attractiveness.

Capturing quantitative differences in mating latency with virgin *Drosophila melanogaster* females turned out to be difficult, as the latency is very low (~2 min in this assay). (Hawkes et al., 2019) used selection populations of *Drosophila simulans* in their study where the mating latency time is around 60 to 90 mins. (Katayama et al., 2014) used male attempts to copulate before mating success to score attractiveness. With a very short latency time, quantifying attempts is also not a feasible method to adopt in my assay. Previously, comparing the competitive mating success results of M and F males, I have found that the mating success advantage of M males over F males is much more amplified with mated females than the virgin females. A male

attractiveness assay, in terms of mating latency, with mated females might capture the difference in male attractiveness of M and F males as well if it exists.

Another reasoning for not finding difference in male attractiveness even after significant difference in WIPs of M and F population males could lie in the fact that male attractiveness and reproductive fitness cannot be defined based on one trait itself. For example in the M and F populations, difference in mating latency has never been recorded, but there is a clear difference in mating success of the males from the M and F population, with M males having an advantage over F males. Therefore, though I did not find a difference in male attractiveness in the M and F population, M males have an advantage in mating success over F males, and WIPs might be playing a more important role in mating success which might not be captured in the attractiveness measured in terms of mating latency.

Another possible explanation for the results could be that WIPs are not under direct selection, and evolve as a by-product of selection acting on another trait. WIPs are influenced by the wing morphology and thickness. While we already know that wing morphology of M and F population males is not different, wing thickness has not been investigated in these populations. WIPs are dependent on wing thickness as the thickness of the wing determines the colour of the refracted light from the membrane (Shevtsova et al., 2011). Along with that wing corrugation, presence and placement of microtrichia and venation also affect the WIPs (Shevtsova et al., 2011; Shevtsova and Hansson, 2011). Wing thickness, corrugation and placement of microtrichia could also play a role in acoustic traits like courtship song and courtship activity, as well as in flight optimisation. Therefore, selection pressure on any of the above traits would indirectly put select WIPs.

In conclusion, the study shows that in populations evolved under differential levels of sexual selection, males from the male biased population evolve more vibrant, colourful and bright WIPs as compared to the males from the female biased populations. Although the WIPs have diverged, I did not find any difference in male attractiveness measured as mating latency of the males. Therefore, while WIPs are under sexual selection in these populations, they can either be the target for sexual selection or a by-product of selection on another trait.

Male - Female interaction in sperm competitive ability

Chapter 7

Introduction

The optimal reproductive strategies of males and females rarely align and leads to the evolutionary conflict between the two. This results in sexually antagonistic coevolution, which is defined as the evolutionary arms race between the two sexes of the same species due to the evolutionary conflict over reproduction (Arnqvist and Rowe, 2002; Rice and Holland, 1997). Typically, the male-male competition to increase their reproductive fitness gives rise to the traits which are harmful to female fitness. Females, in turn, evolve resistance to male-induced harm and decrease male fitness. This kind of antagonistic interaction takes place over control of fertilization success. In promiscuous species, females mate multiple times and sire progeny with multiple males (Arnqvist and Rowe, 2013; Taylor et al., 2014). As a result, sexual selection can continue beyond mating in terms of sperm competition and cryptic female choice (Eberhard, 1996; Parker, 1970b).

When female mates with multiple males, it gives rise to sperm competition, where ejaculate from multiple males compete within the female reproductive tract to fertilize the egg (Parker, 1970b). The evolution of sperm competitive ability in males has been well studied to its genetic basis and variation (Civetta and Ranz, 2019; Clark et al., 1995; Friberg et al., 2005; Hughes and Leips, 2006) and through experimental evolution by altering levels of sexual selection (Firman and Simmons, 2011; Hosken and Ward, 2001; Nandy et al., 2013a; Rice, 1996a; Simmons, 2019; Simmons and García-González, 2008). Further studies have investigated molecular mechanisms of male ejaculates proteins that manipulate female behavior and physiology to maximize male competitive fitness (Ravi Ram and Wolfner, 2007; Sirot et al., 2011; Wolfner, 1997a)

and sperm traits that influence male fertilization success (Lüpold et al., 2020, 2012; Lüpold and Pitnick, 2018; Simmons and Fitzpatrick, 2012). While most studies have focused on the evolution of male traits and male manipulation of the competitive fertilization process, very few studies have looked at the female effect on fertilization success in sperm competition. Female genotypes have been shown to affect the outcome of competitive fertilization success (Ala-Honkola and Manier, 2016; Chen et al., 2019; Clark and Begun, 1998; Lüpold et al., 2013). (Clark et al., 2000) showed that the sperm competitive ability in males is non-transitive in *Drosophila melanogaster*. (Birkhead et al., 2004) found similar results in birds, supporting the argument that females play a role in the outcome of sperm competitive ability. In *Drosophila melanogaster* species, females eject sperms after copulations, and the timing of sperm ejaculation also plays a role in the outcome of sperm competition (Manier et al., 2010b). (Lüpold et al., 2012), using fluorescent-tagged sperms, showed that heritable variation exists in female traits like remating latency, sperm ejection time, and sperm storage. All these studies together show that females play an active role in deciding the outcome of sperm competition.

As males and females both have a role in sperm competition, the outcome of sperm competition would be decided by the sperm-female interactions and post-ejaculate modifications in sperms with respect to the female reproductive tract (Lüpold et al., 2020; Miller and Pitnick, 2002). (Clark et al., 1999) showed male X female genotype effect on the outcome of sperm competition. (Miller and Pitnick, 2002), using *Drosophila melanogaster* populations selected experimentally for sperm length or seminal receptacle length (female-sperm storage organ), showed that fertilization success is determined by an interaction between sperm and female reproductive morphology, and interestingly sperm length evolution occurred as a correlated response

to selection on the seminal receptacle length. (Bjork et al., 2007), in their study using selected populations of *Drosophila melanogaster,* found that the sperm competitive abilities (both sperm defense and sperm offense) were repeatable only across mating pairs involving the same pair of competing males with the same female. The repeatability decreased when the rival males were the same, but the female changed. The complex interaction between the two sexes that defines the outcome of sperm competitive ability is still far from being understood and how it affects the evolution of the traits of both the two sexes in the interaction still needs to be worked out (Civetta and Ranz, 2019; Lüpold et al., 2020; Reinhart et al., 2015).

Here, I attempt to explore the male x female interaction, in the outcome of sperm competition, in the M and F populations selected for differential levels of sexual selection. (Nandy et al., 2013a) has shown that males from the M population have a significant advantage over males from the F population in terms of sperm competition when mated to a common ancestral female. (Ali, 2018) found that the last male precedence is lower for females from the M population than F. The time to sperm ejection after copulation was lower in M females than in F females. Thus, the sperm competitive ability of males and the influence of females on sperm competitive ability have evolved in M and F populations. In this study, I planned to investigate the male X female interaction in the outcome of the sperm competition in the M and F populations. In particular, I examined whether the co-evolutionary history of male-female from a selected population leads to differential male X female interaction affecting the outcome of sperm competition. For this, a full factorial sperm competition assay was conducted using males and females from M, F, and ancestral LH_{st} populations.

Material and methods

Standardization and generating experimental flies

Flies are maintained as per baseline population maintenance without selecting one generation before the experiment. This is done to avoid the non-genetic parental effects from affecting the experiments' results (Ref). These standardized flies are used to generate the experimental flies.

For the experiment, the eggs the collected at an exact density of 150±2 eggs per vial from standardized M, F $LH_{st,}$ and LH populations. On days 9^{th}-10^{th} from the egg collection, the virgin flies are collected in single-sex vials as required for the experiment. The sperm competition assays were conducted with 12 day old flies (from the day of egg collection).

Sperm defense (P1) assay

For the sperm defense assay, females from each population were divided into three treatments to mate with males from three populations. For example, females from the M population were randomly divided into three treatments. Females from the first treatment were combined with M males, from the second treatment were combined with F males, and from the third treatment, females were combined with LH_{st} males. Three treatments were set up for F females and LH_{st} females like M females. For each such treatment, 30 vials were set up per female population. Each vial was observed to record the mating latency and copulation duration. After the first mating is observed, the males are discarded from the vials. After 1-2 hours, the second mating assay is set up. In all the vials with singly mated females, LH males are introduced. The second mating

observations last for 48 hours, during which remating latency and copulation duration for each mating is recorded. Females which did not mate at the end of 48 hours of observations were not included in the experiment further. As soon as the second mating is observed and recorded, the female from the vial is transferred into a test tube containing cornmeal molasses food media for egg-laying. After 48 hours, females are discarded from the test tubes, and the eggs in the test tube are allowed to develop under laboratory conditions. On day 15^{th} from the experiment, when all the pupae have hatched, the test tubes are frozen at -20^0C. Since all the flies involved in the first mating have recessive scarlet eye color markers, the progeny from the first mating will be scarlet-eyed. Males in the second mating were from the LH population, which has a dominant red eye color marker; therefore, all the progeny from the second mating will be red-eyed. The progeny from the test tubes is scored based on the eye color marker.

Sperm offense (P2) assay

The experimental setup for the sperm offense assay was the same as the sperm defense assay, except that the order of males mating is reversed. Females from all three populations were first mated with LH males. The first mating was observed to record the mating latency and copulation duration. After the first mating, the males are discarded from the vials. Singly mated females from each population are divided into three treatments. For second mating, females from the first treatment were mated with M males, second treatment with F males, and third treatment females with LH_{st} males. The remating observations lasted for 48 hours, where remating latency and copulation duration of second mating. Females which did not mate at the end of 48 hours were not included in the experiment further. As soon as the second mating is observed and recorded, the female is transferred into a test tube containing cornmeal molasses food

media for egg-laying. The females are allowed to lay eggs for 48 hours. After 48 hours, the females are discarded, and the test tubes are kept in the standard laboratory environment for eggs to develop. On day 15^{th} from the experiment, when all the pupae have hatched, the test tubes are frozen at -20^0C. Males in the first mating were from the LH population, which has a dominant red eye color marker; therefore, all the progeny from the first mating will be red-eyed. Since all the flies involved in the second mating have recessive scarlet eye color markers, the progeny from the second mating will be scarlet-eyed. The progeny from the test tubes is scored based on the eye color marker.

Statistical analysis

All the analyses are performed in R (3.2.1), using generalized linear models (GLM) in R package lme4. Mating latency, remating latency, copulation duration for both first and second mating, and proportion of progeny for both P1 and P2 were analyzed using the GLM model. The proportion of progeny for both the P1 and P2 assay was arcsine transformed to better fit the normal distribution. For each of the above data sets, the GLM model was set up, and ANOVA was performed. Further posthoc pairwise comparisons were done using the R package "lsmeans" via Tukey's method.

Results

There was a significant effect of the selection regime on sperm precedence. For sperm defense, the male and female effects were significant, but there was no male X female interaction (Table 7.1, Figure 7.1). As expected, M males have higher sperm defense ability than the males from F and LH_{st} populations. When looking at females, the sperm defense ability of males was higher with LH_{st} females as compared to the F and M

females (Figure 7.1). In mating behavior, there was no difference in mating latency across the selection regimes (Table 7.2 Figure 7.3) for the first mating, nor in the remating latency of females for second mating with the common LH males (Table 7.4, Figure 7.5). In copulation duration, females had a significant effect for the first mating (Table 7.3, Figure 7.4). Overall copulation duration of males with virgin LH_{st} females was lower than with females from M and F populations. In the case of the remating with LH males, there was a significant male X female effect (Table 7.5, Figure 7.6). LH males have a higher copulation duration with M females previously mated with M males and lower copulation duration with F females previously mated to M males (Figure 7.6).

In sperm offense (P2), there was a significant effect of selection regimes with a significant male X female interaction. M males have significantly higher P2 as compared to F and LHst males (Table 7.6, Figure 7.7). When comparing females, the P2 proportion was lower with M females as compared to F and LHst females (Figure 7.8). Interestingly, when the pair was M male and M females, the P2 proportion was significantly higher. While overall P2 proportion was lower with M females, when the male is from the M population, the P2 proportion even with M females is higher (Figure 7.7). There was no difference in mating behavior, the copulation duration for both first mating (Table 7.8, Figure 7.10) and second mating (Table 7.10, Figure 7.12). In terms of mating latency, LH_{st} females had lower latency than M and F females for first mating (Table 7.7, Figure 7.9). There was no effect of male or male X female interaction in mating latency for either first or the second mating (Table 7.9, Figure 7.11).

	Sum Sq	Mean Sq	NumDF	DenDF	F value	Pr(>F)
Male	3.1791	1.58953	2	817.41	22.1811	4.16100e-10***
Female	0.764	0.382	2	817.87	5.3307	0.00501**
Male:Female	0.4758	0.11895	4	817.29	1.6598	0.15735

Table 7.1: Results of ANOVA for sperm defense in terms of proportion of progeny from first mating. Here, Male describes the males from M, F and LHst population in first mating; Female describes the females from M, F and LHst populations used in the assay.

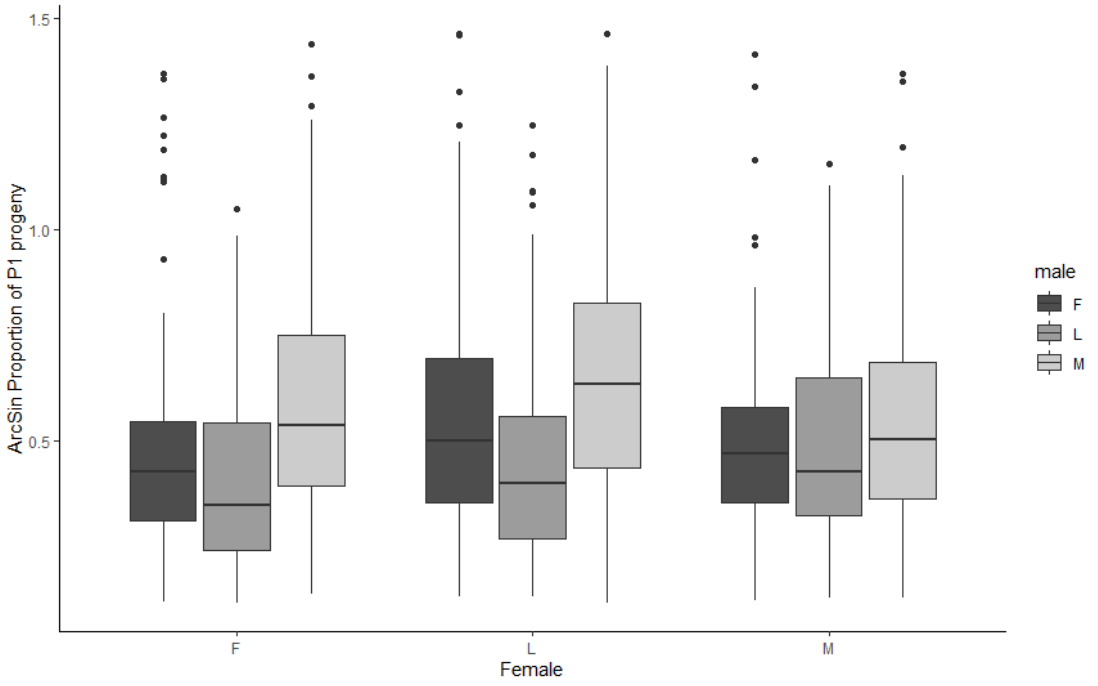

Figure 7.1: Representing Proportion of progeny from first mating in sperm defense assay (y-axis) for males from F (black box) LHst (dark grey box) and M (grey box) population, when paired to females from F, LHst and M populations (x-axis). Boxplots indicate median and interquartile range. Whiskers indicate variability outside the upper and lower quartiles. Dots represent outliers.

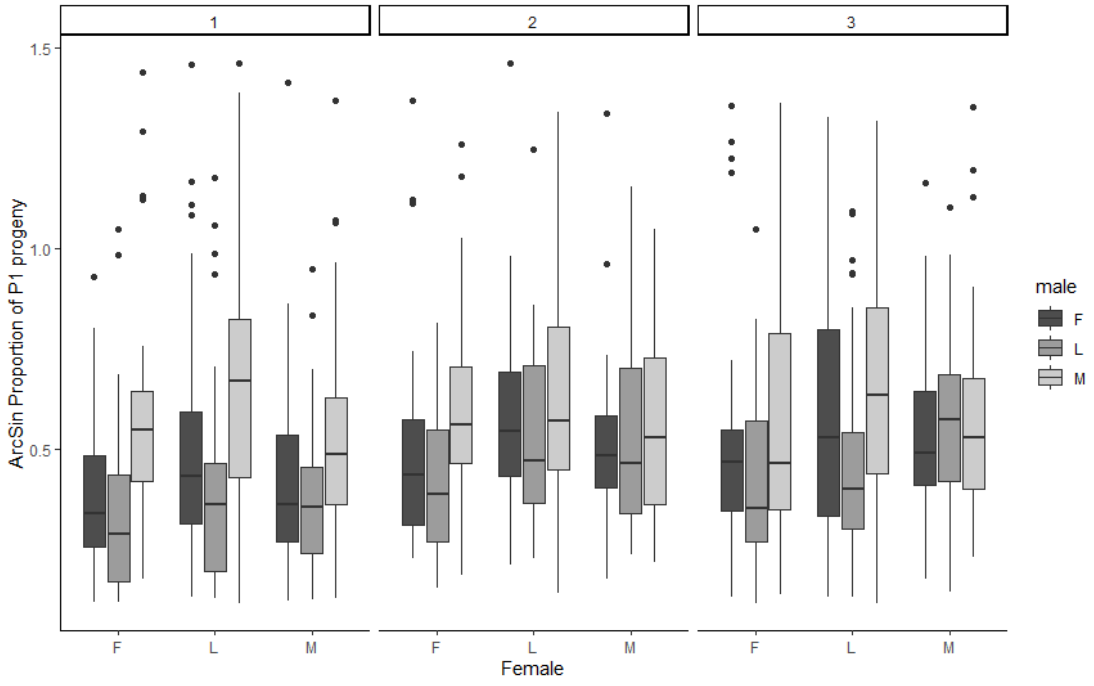

Figure 7.2: Representing Proportion of progeny from first mating in sperm defense assay (y-axis), across the three blocks (grids), for males from F (black box) LHst (dark grey box) and M (grey box) population, when paired to females from F, LHst and M populations (x-axis). Boxplots indicate median and interquartile range. Whiskers indicate variability outside the upper and lower quartiles. Dots represent outliers.

	Sum Sq	Mean Sq	NumDF	DenDF	F value	Pr(>F)
Male	5.851	2.926	2	4.05	0.1719	0.84787
Female	198.278	99.139	2	3.75	5.826	0.07081
Male:Female	79.444	19.861	4	1051.62	1.1672	0.32368

Table 7.2: Results of ANOVA for mating latency for the first mating of sperm defense assay with focal males. Here, Male describes the males from M, F and LHst population in first mating; Female describes the females from M, F and LHst populations used in the assay.

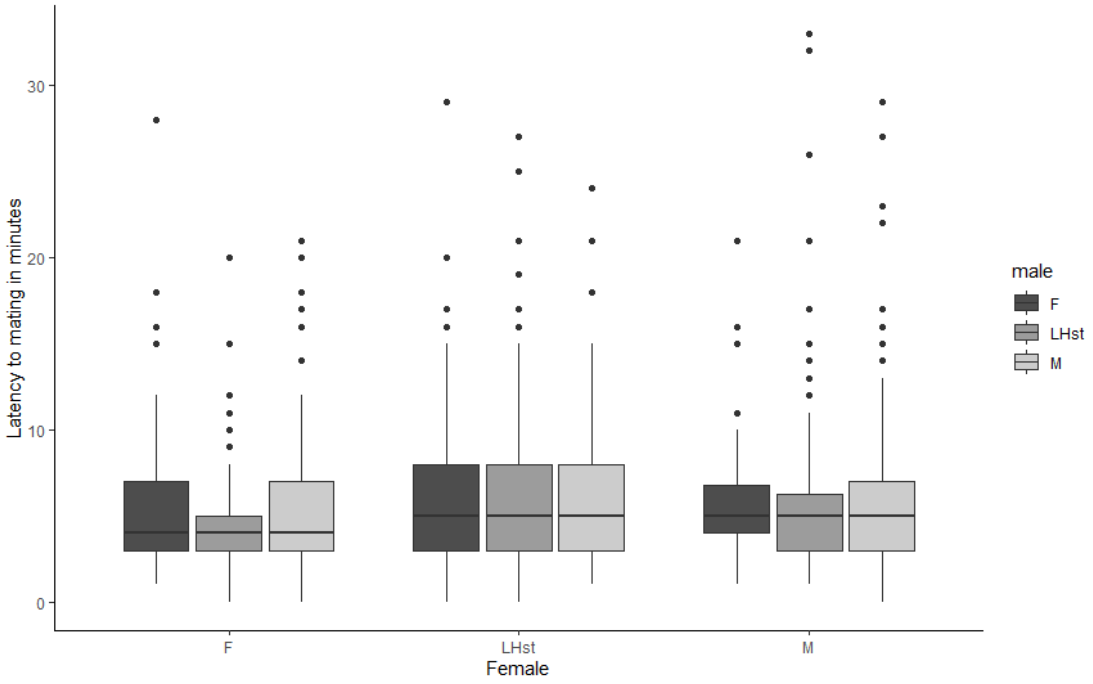

Figure7.3: Representing mating latency for first mating in sperm defense assay (y-axis) for males from F (black box) LHst (dark grey box) and M (grey box) population, when paired to females from F, LHst and M populations (x-axis). Boxplots indicate median and interquartile range. Whiskers indicate variability outside the upper and lower quartiles. Dots represent outliers.

	Sum Sq	Mean Sq	NumDF	DenDF	F value	Pr(>F)
Male	83.392	41.696	2	4.04	3.7112	0.12166
Female	293.491	146.745	2	1055.17	13.0611	2.49e-06***
Male:Female	127.252	31.813	4	1055.18	2.8315	0.2363

Table 7.3: Results of ANOVA for copulation for the first mating of sperm defense assay with focal males. Here, Male describes the males from M, F and LHst population in first mating; Female describes the females from M, F and LHst populations used in the assay.

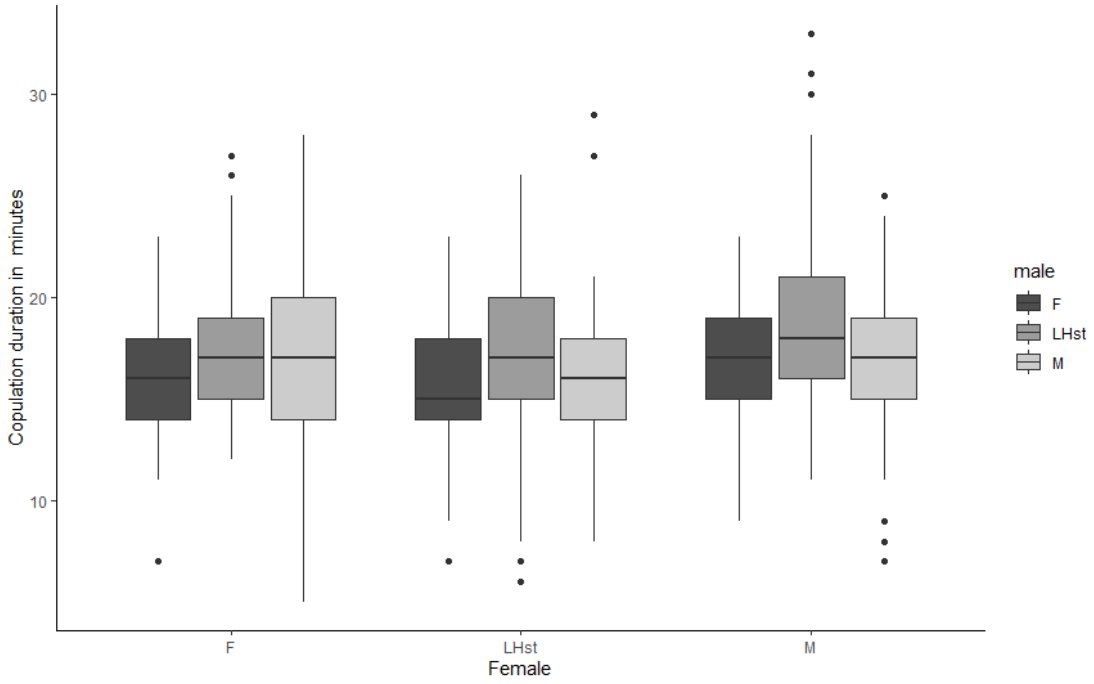

Figure 7.4: Representing copulation duration for first mating in sperm defense assay (y-axis) for males from F (black box) LHst (dark grey box) and M (grey box) population, when paired to females from F, LHst and M populations (x-axis). Boxplots indicate median and interquartile range. Whiskers indicate variability outside the upper and lower quartiles. Dots represent outliers.

	Sum Sq	Mean Sq	NumDF	DenDF	F value	Pr(>F)
Male	1176091	588046	2	3.9	6.9439	0.05187
Female	143727	71864	2	856.03	0.8486	0.42837
Male:Female	269537	67384	4	857.38	0.7957	0.52807

Table 7.4: Results of ANOVA for remating latency for the second mating of sperm defense assay with common males. Here, Male describes the males from M, F and LHst population in first mating; Female describes the females from M, F and LHst populations used in the assay.

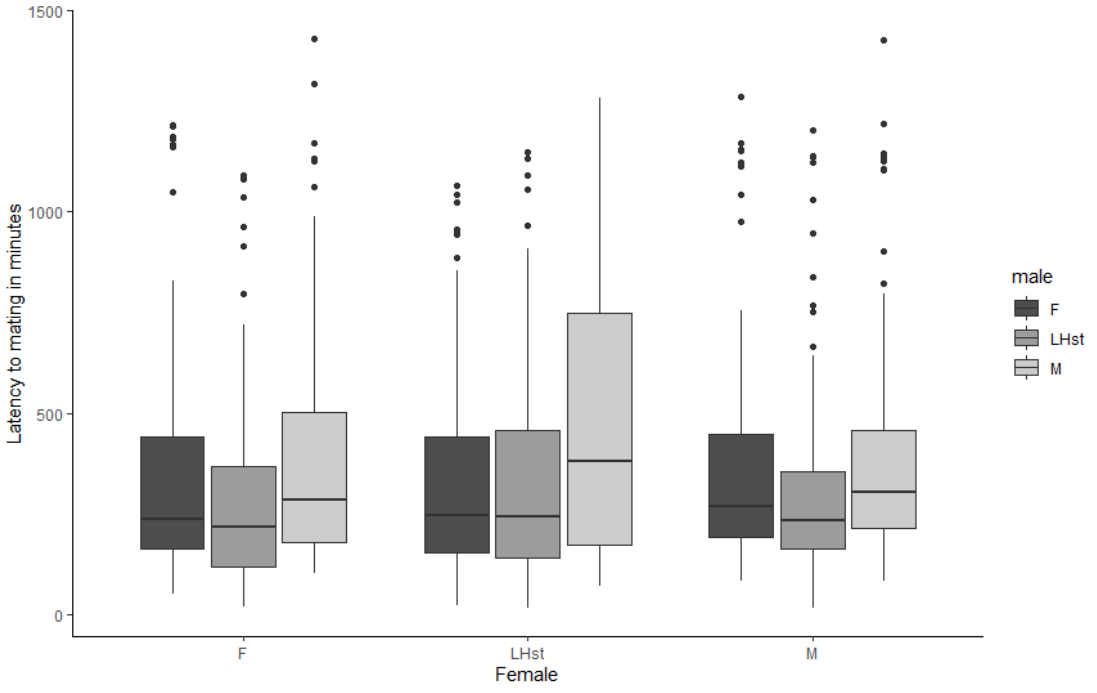

Figure 7.5: Representing remating latency for second mating in sperm defense assay (y-axis) when females from F, LHst and M populations (x-axis), mated to males from F (black box) LHst (dark grey box) and M (grey box) population in first mating, are paired with common LH males. Boxplots indicate median and interquartile range. Whiskers indicate variability outside the upper and lower quartiles. Dots represent outliers.

	Sum Sq	Mean Sq	NumDF	DenDF	F value	Pr(>F)
Male	50.21	25.103	2	859.08	0.6807	0.50660
Female	15.51	7.756	2	858.8	0.2103	0.81040
Male:Female	456.67	114.168	4	858.37	3.0957	0.01520*

Table 7.5: Results of ANOVA for copulation duration for the second mating of sperm defense assay with common males. Here, Male describes the males from M, F and LHst population in first mating; Female describes the females from M, F and LHst populations used in the assay.

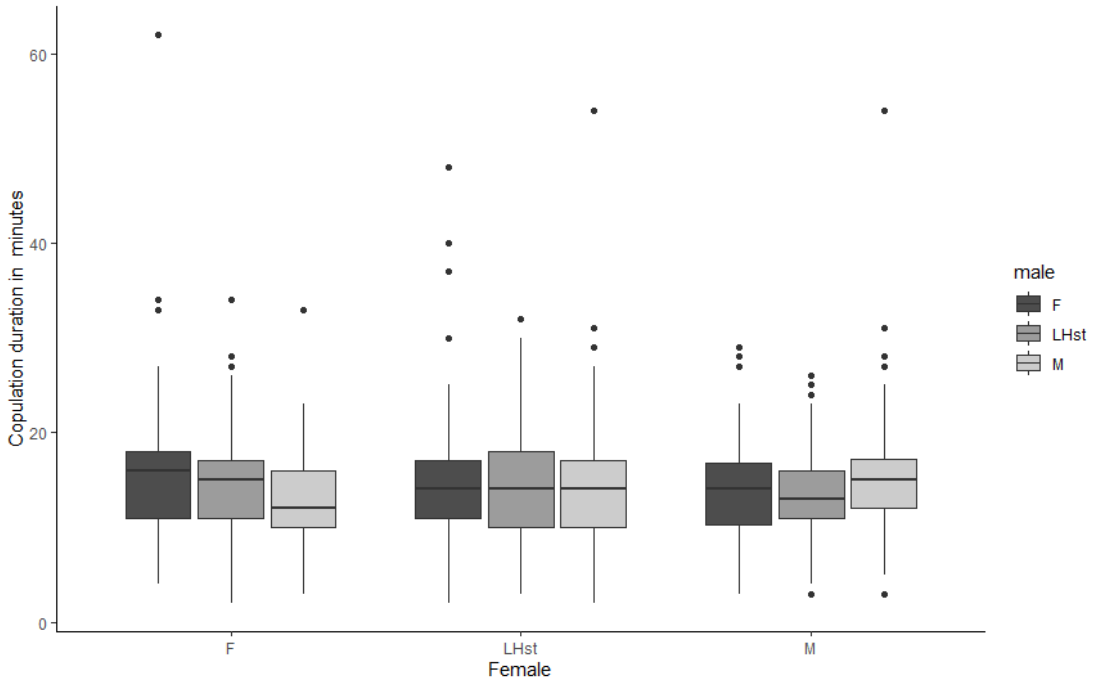

Figure 7.6: Representing copulation duration for second mating in sperm defense assay (y-axis) when females from F, LHst and M populations (x-axis), mated to males from F (black box) LHst (dark grey box) and M (grey box) population in first mating, are paired with common LH males. Boxplots indicate median and interquartile range. Whiskers indicate variability outside the upper and lower quartiles. Dots represent outliers.

	Sum Sq	Mean Sq	NumDF	DenDF	F value	Pr(>F)
Male	2.44506	1.22253	2	758.47	21.7042	6.82e-10***
Female	0.53283	0.26642	2	758.78	4.7298	0.00909**
Male:Female	0.96753	0.24188	4	758.3	4.2943	0.001926**

Table 7.6: Results of ANOVA for sperm offense in terms of proportion of progeny from second mating. Here, Male describes the males from M, F and LHst population in second mating; Female describes the females from M, F and LHst populations used in the assay.

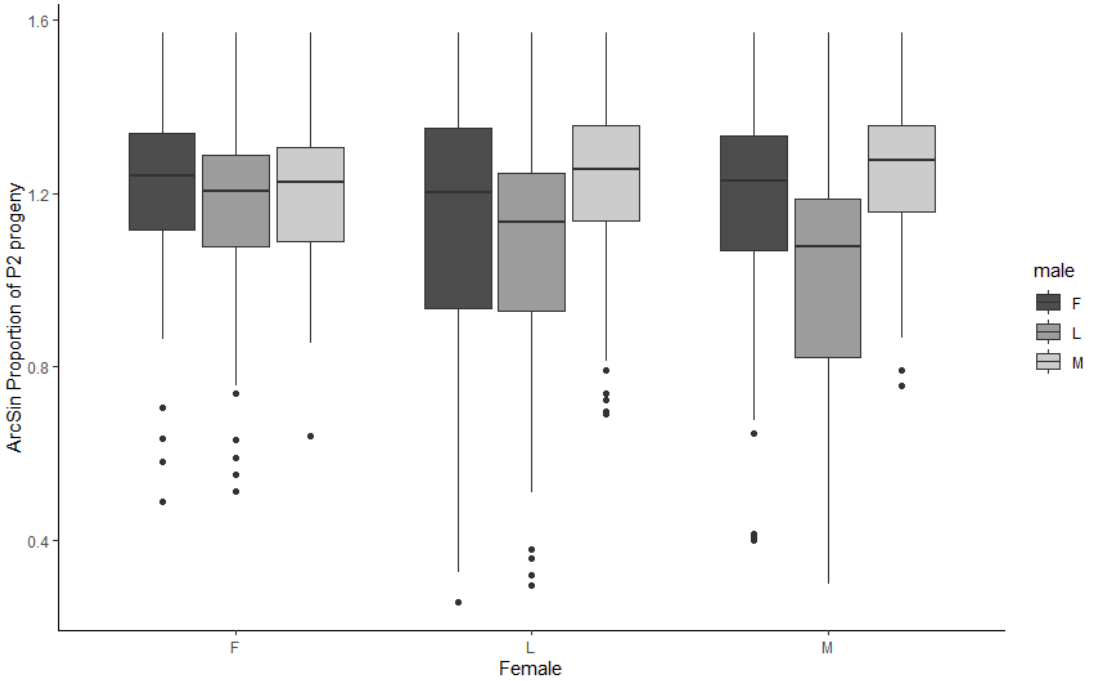

Figure 7.7: Representing Proportion of progeny from second mating (P2) in sperm offense assay (y-axis) for males from F (black box) LHst (dark grey box) and M (grey box) population, when paired to females from F, LHst and M populations (x-axis). Boxplots indicate median and interquartile range. Whiskers indicate variability outside the upper and lower quartiles. Dots represent outliers.

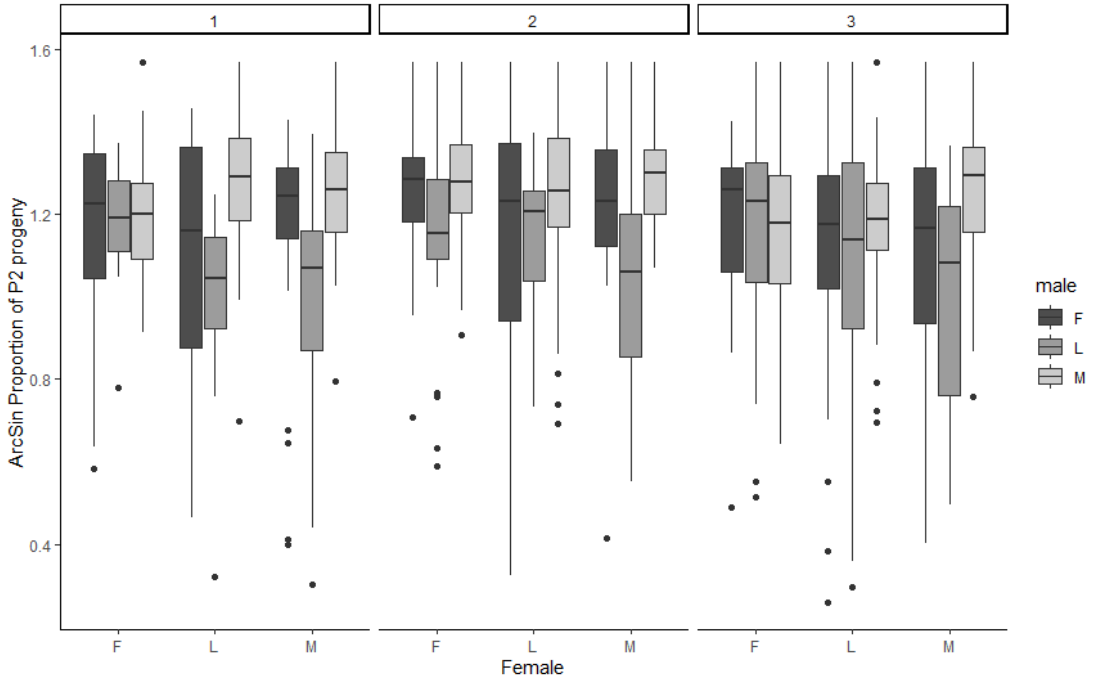

Figure 7.8: Representing Proportion of progeny from second mating (P2) in sperm offense assay (y-axis) across the three selection regime blocks (grids), for males from F (black box) LHst (dark grey box) and M (grey box) population, when paired to females from F, LHst and M populations (x-axis). Boxplots indicate median and interquartile range. Whiskers indicate variability outside the upper and lower quartiles. Dots represent outliers.

	Sum Sq	Mean Sq	NumDF	DenDF	F value	Pr(>F)
male	46.684	23.342	2	3.98	0.686	0.55460
Female	124.86	62.43	2	1092.95	1.8347	0.16020
Male:Female	136.685	34.171	4	1092.92	1.0042	0.40420

Table 7.7: Results of ANOVA for mating latency for the first mating of sperm offense assay with common males. Here, Male describes the males from LH population in first mating; Female describes the females from M, F and LHst populations used in the assay.

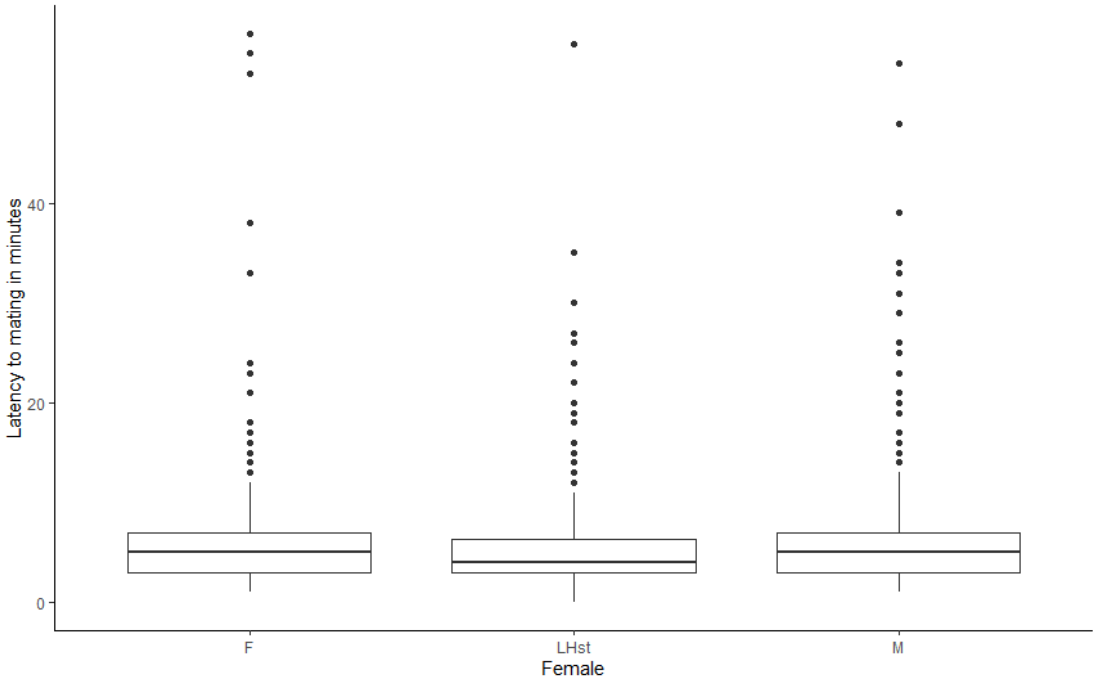

Figure 7.9: Representing mating latency for first mating in sperm offense assay (y-axis) for females from F, LHst and M populations (x-axis) when paired with common LH males. Boxplots indicate median and interquartile range. Whiskers indicate variability outside the upper and lower quartiles. Dots represent outliers.

	Sum Sq	Mean Sq	NumDF	DenDF	F value	Pr(>F)
Male	8.176	4.088	2	1096	0.4333	0.6485
Female	230.167	115.084	2	1096	12.1973	5.77e-06***
Male:Female	5.581	1.395	4	1096	0.1479	0.964

Table 7.8: Results of ANOVA for copulation for the first mating of sperm offense assay with common males. Here, Male describes the males from LH population in first mating; Female describes the females from M, F and LHst populations used in the assay.

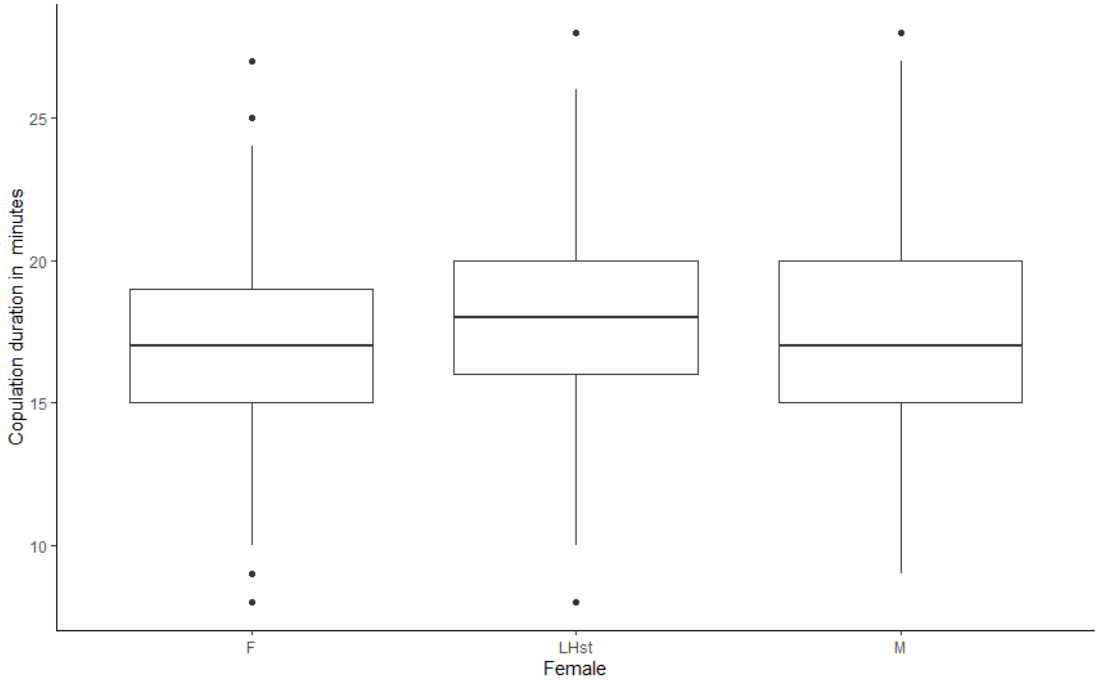

Figure 7.10: Representing copulation duration for first mating in sperm offense assay (y-axis) for females from F, LHst and M populations (x-axis) when paired with common LH males. Boxplots indicate median and interquartile range. Whiskers indicate variability outside the upper and lower quartiles. Dots represent outliers.

	Sum Sq	Mean Sq	NumDF	DenDF	F value	Pr(>F)
Male	124782	62391	2	5.78	0.7622	5.08E-01
Female	1585497	792749	2	5.41	9.6851	0.01633*
Male:Female	69666	17417	4	844.26	0.2128	0.93139

Table 7.9: Results of ANOVA for remating latency for the second mating of sperm offense assay with focal males. Here, Male describes the males from M, F and LHst population in second mating; Female describes the females from M, F and LHst populations used in the assay.

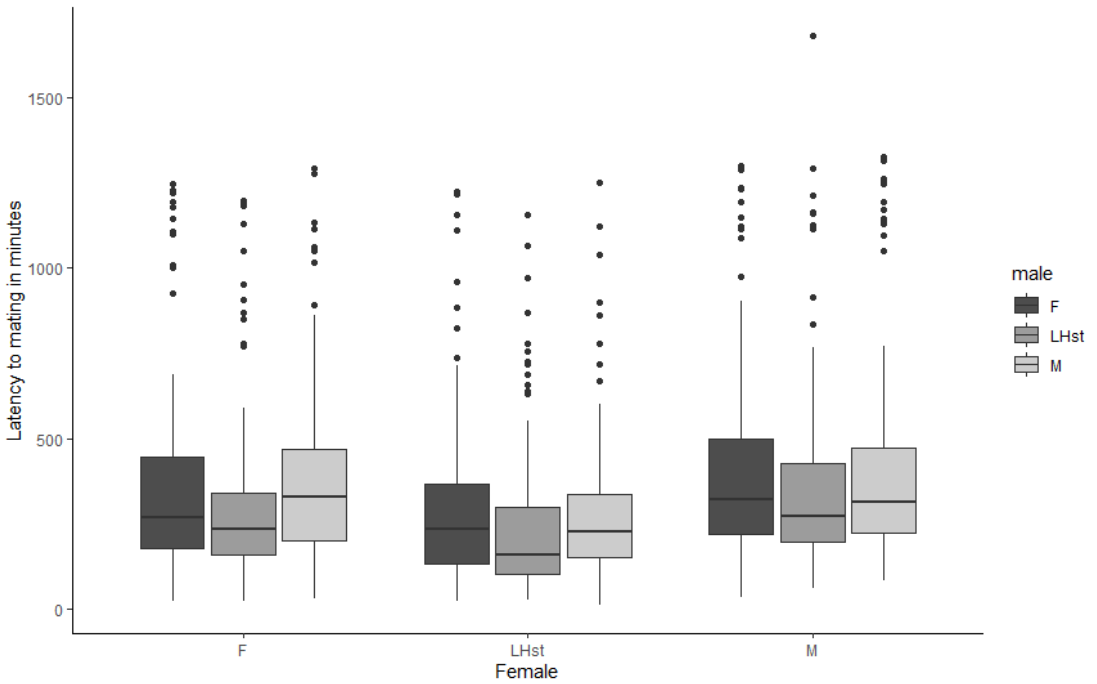

Figure 7.11: Representing remating latency for second mating in sperm offense assay (y-axis) when females from F, LHst and M populations (x-axis), are paired with males from F (black box) LHst (dark grey box) and M (grey box) population. Boxplots indicate median and interquartile range. Whiskers indicate variability outside the upper and lower quartiles. Dots represent outliers.

	Sum Sq	Mean Sq	NumDF	DenDF	F value	Pr(>F)
Male	30.847	15.4235	2	4.04	0.5728	0.60390
Female	62.858	31.429	2	4.26	1.1673	0.3943
Male:Female	36.051	9.0127	4	844.62	0.3347	0.8546

Table 7.10: Results of ANOVA for copulation duration for the second mating of sperm offense assay with focal males. Here, Male describes the males from M, F and LHst population in second mating; Female describes the females from M, F and LHst populations used in the assay.

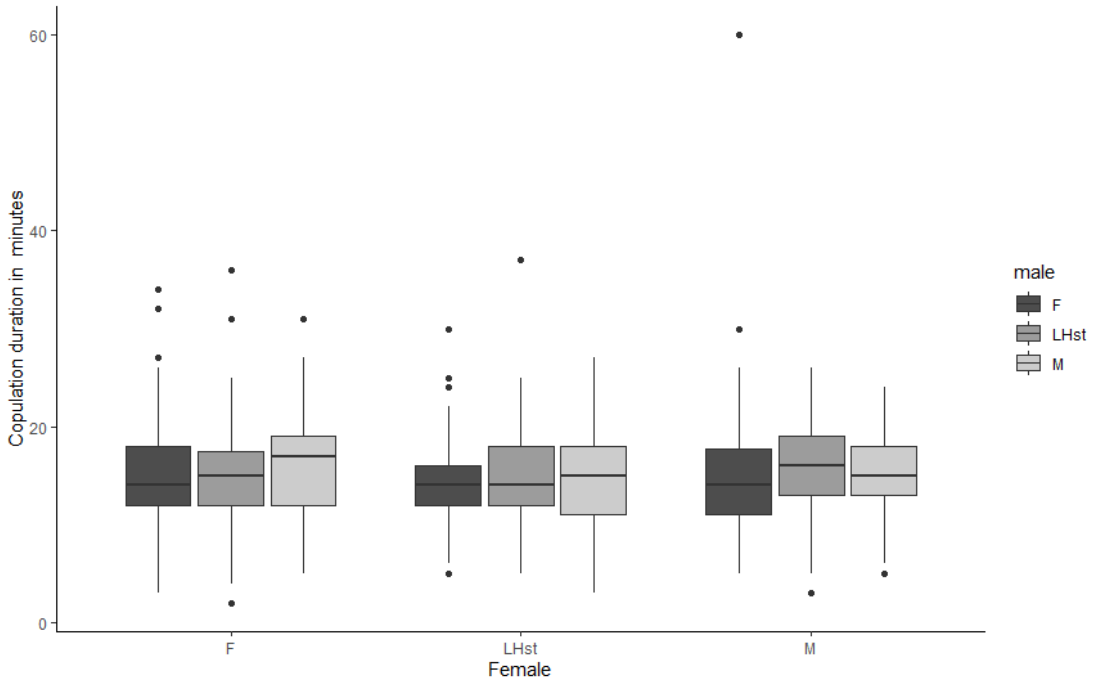

Figure 7.12: Representing copulation duration for second mating in sperm offense assay (y-axis) when females from F, LHst and M populations (x-axis), are paired with males from F (black box) LHst (dark grey box) and M (grey box) population. Boxplots indicate median and interquartile range. Whiskers indicate variability outside the upper and lower quartiles. Dots represent outliers.

Discussion

This study shows the influence of male X female interaction in the populations that evolved under differential levels of sexual selection. The sperm competitive ability of males and the influence of females on sperm competitive ability have evolved under sexual selection. In sperm offense ability, there is a clear male X female interaction in the M populations. When comparing sperm offense (P2), M females that otherwise have significantly lower P2 proportions tend to have a significantly higher P2 proportion with M males compared to other males. Male ability and female influence in sperm defense ability evolve under sexual selection, but I did not find any male X female interaction in sperm defense ability in terms of proportion of progeny from first mating.

My results are consistent with previous results on sperm competitive ability in M and F populations. Using M and F populations, (Nandy et al., 2013a) have shown the evolution of sperm competitive ability of males as a result of sexual selection, with males from the M population having a significant advantage over F males. (Ali, 2018) has also shown the evolution of female influence on sperm competitive ability, with last male precedence being lower with M females as compared to F females. Along with these previous studies, my results show male X female interaction in M populations. I did not find any male X female interaction in the F population or the LH_{st} ancestral population pairs, which points to the fact that while sexual selection leads to the evolution of male and female traits that influence the sperm competitive ability and the interaction of these evolved traits in both males and females decide the outcome of sperm competitive ability.

Females in the M population eject sperms significantly faster, leaving the last male sperms, with less time to replace the first male's sperms from the female's seminal receptacle. Thus M females have lower last male precedence or, in other words, P2 proportion (Ali, 2018). However, my results show that when the last male to mate is M males, the P2 proportion of M females is significantly higher. One of the explanations for this could lie in the co-evolutionary history of the males and females in selected populations. Previously in experimentally selected *Drosophila melanogaster* populations, (Miller and Pitnick, 2002) showed that fertilization success is determined by an interaction between sperm and female reproductive morphology. Interestingly, sperm length evolution occurred as a correlated response to selection on the seminal receptacle length. This could be the case leading to the male X female interaction in the M population. Evolving under high levels of sexual selection, the sperm length in males or the seminal receptacle length in females might be under post-copulatory sexual selection, triggering a correlated response in the opposite sex. Though there is no difference in testes length in the males from M and the F populations, the evolution of sperm length cannot be ruled out. Using *Drosophila melanogaster* (Pattarini et al., 2006) have shown that sperm quality, in terms of sperm length, has a more significant impact on the outcome of the sperm offense than the sperm quantity. At the same time, they also found that longer sperms are more difficult to replace in SR and therefore have a higher proportion of P1 in sperm defense. The sperm length trait does fit into the explanation of the M males' increased sperm competitive ability. Investigating the sperm traits and seminal receptacle length in the M and the F population could likely explain the mechanism of male X female interaction in sperm competitive ability.

In my results, I do find a significant effect of selection in the mating behavior of males and females in the assay. In the sperm defense (P1) assay, there is no difference in the mating latency of the males and females and nor their interaction. There was a significant female effect on copulation duration in the first mating, but the male-female interaction factor was non-significant. Copulation duration of males from all the three M, F, and LH_{st} populations was significantly lower with LH_{st} females than with M and F females. In *Drosophila melanogaster*, the copulation duration of the first male is positively correlated to the progeny sired by the first male (Nandy and Prasad, 2011). However, the proportion of P1 progeny for LH_{st} females was significantly higher than F females and similar to M females. Further, when looking at the copulation duration of females remating with the second male, there was a significant male X female effect, with F females mated to M males first having lower copulation duration in remating, whereas there was no such effect in M and LH_{st} females mated to M males first. An explanation for this could lie in the ejaculate of the M males. M males evolving under high levels post-copulatory sexual selection are expected to have evolved ejaculate quality, including seminal proteins. These seminal proteins affect female physiology (Ravi Ram and Wolfner, 2007; Wolfner, 1997a). While studies have shown an effect of seminal fluids on remating latency of the females, these proteins could also affect the copulation duration of the mating to follow. Having co-evolved with M males, M females might be more resistant to manipulation via seminal proteins in the ejaculate of M males. This does raise the question concerning LH_{st} females, which would be expected to respond in a similar way as the F females to the M male ejaculate. This male X female interaction and lower copulation duration in remating of F females mated to M males first would add to the sperm defense advantage of the M males.

In the sperm offense (P2) assay, I did not find any difference in the mating latency of females in the first mating with a common male. In copulation duration for the first mating, LH_{st} females had significantly higher copulation duration than females from the M and F population with common males. Interestingly, with the focal males, LHst females have the lowest remating latency for the second mating, with males from all the three M, F, and LH_{st} populations. (Lüpold et al., 2013) found heritable variation in females in traits such as remating latency, which have been shown to impact the outcome of sperm competition, but there was no male X female interaction in remating latency. Therefore, the mating behavior is unlikely to affect the inference of male X female interaction I found in the progeny production in sperm offense assay.

In conclusion, I found that in populations evolving under sexual selection, the outcome of sperm competitive ability is governed by male X female interaction. This could possibly result from the coevolution of male ejaculate and female reproductive tract morphology as a result of sexual selection. Further investigation of ejaculate quality and female reproductive tract morphology would shed light on the mechanism and evolutionary process resulting in male X female interaction in sperm competitive ability.

Conclusion

Chapter 8

The mismatch between male and female evolutionary interests lays the ground for the evolution of sexual conflict. These conflicts might arise due to differences in parental effort, mating frequency, female remating behavior, fertilization, female reproductive rate, clutch size, and other factors. Both sexes are expected to develop a variety of sexually antagonistic adaptations that favor their own interests in the end (Holland and Rice, 1998b). The importance of sexual conflict in the development of a variety of life history and reproductive features in both sexes is well established. Despite our remarkable progress thus far, there is still much to be done in the field of sexual selection and conflict study, and many problems remain unanswered.

In this thesis, I have tried to investigate the reproductive traits that are likely to be the target of sexual selection and how their evolution impacts both sexes' fitness components. I employed a laboratory experimental evolution technique to address these questions. The population's operational sex ratio was changed to achieve varying levels of sexual selection and hence change the level of male-male competitiveness. Male-biased (M) regimes are subjected to higher levels of sexual selection, whereas female-biased (F) regimes are subjected to decreased sexual selection. Here, I outline the unique results of this thesis describing how sexual selection and conflict plays a role in the evolution of male and female reproductive features (such as mating rates, mate preference, success, and male-female interaction in sperm competitive ability).

Major findings from my thesis are summarized as follows:

Sexual conflict over mating rates controlled by females.

According to the theory of sexually antagonistic coevolution, the two sexes try to optimize their own fitness at the cost of the other sex. This leads to a constant sexual conflict between the two sexes over mating rates and parental care, among other traits. My results show that in populations that have evolved under higher levels of sexual selection, males evolve to maximize their mating capacity, and females evolve to minimize their remating. As a result, there exists sexual conflict over mating in populations with increased levels of sexual selection. In my study, I found that females control this conflict over mating rates. While the M males can maximize their mating to a much higher level, the mating rates of the M population are defined by the M females' mating rates.

Male mating preference is based on the fitness of females available.

In populations with traditional sex roles, males are expected to show mating preference when they are resource-limited, and there exists a difference in the fitness of available mates. My results provide empirical evidence for the same. While the males evolved under different levels of sexual selection do not differ in their preference, the females with lower resistance to mating and higher fecundity are preferred by the sperm-depleted males.

Evolution of competitive male mating success.

Results from this study show that competitive male mating success evolves as a result of higher levels of sexual selection and male-male competition. The competitive mating success of the males from the population with higher levels of male-male competition

comes irrespective of the female selection history. I also found that while the courtship frequency is an important pre-copulatory trait in *Drosophila*, courtship frequency alone cannot explain mating success.

Wing interference pattern but not wing morphology under sexual selection.

In *Drosophila* species, wings are often considered a secondary sexual trait as they play an essential part in the courtship process and mating success. In the M and F populations, I did not find any difference in the wing morphology in terms of both size and shape. There was inter-individual variation and asymmetry in wing shape, but not across the selection regimes. The results suggest that sexual selection, due to the differential selection in the M and the F populations, does not influence wing morphology in the males.

In terms of Wing interference pattern, I found that sexual dimorphism exists in WIPS and in populations that evolved under differential levels of sexual selection, males from the male-biased population evolve more vibrant, colorful, and bright WIPs as compared to the males from the female-biased populations. Although the WIPs have diverged, I did not find any difference in male attractiveness measured as the mating latency of the males. Therefore, while WIPs are under sexual selection in these populations, they can either be the target for sexual selection or a by-product of selection on another trait.

Overall, there are mixed results of wings as secondary sexual traits, being the target of sexual selection in the M and the F populations. While there was no difference in wing morphology, the wing interference pattern does show response to differential levels of sexual selection in the M and F populations.

Male X female interaction decides the outcome of sperm competitive ability.

In promiscuous species like *Drosophila*, sperm competitive ability is a vital fitness trait influencing post-copulatory fertilization success. For quite a while, post-copulatory sexual selection studies looking at sperm competitive ability considered females as passive observers in the whole process. However, recent studies have shown that females play an active role in the outcome of the sperm competitive ability via cryptic female choice or by male-female interactions. I found that in populations evolving under sexual selection, the outcome of sperm competitive ability is governed by male X female interaction. This could result from the coevolution of male ejaculate and female reproductive tract morphology as a result of sexual selection.

In conclusion, through this thesis, I attempted to uncover the implications of sexual selection and conflict on the reproductive traits of males and females, which have not been previously addressed. I sincerely hope my thesis will contribute a fresh insight into the sexual selection and conflict research area and will be helpful in understanding the complexity of this exciting subject.

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