SEXUAL CONFLICT AND PLASTICTY
IN THE FRUIT FLY

Sexual conflict and plasticity in the fruit fly

By

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LAY ABSTRACT

Considerable phenotypic variation exists both within and across species. Within species, one source of this variation is phenotypic plasticity, the ability for an individual to alter its phenotype based on environmental influences. When it comes to sex, both males and females in many species exhibit striking variation in their reproductive behaviour as a result of plasticity. However, the causes and consequences of this variation are not well understood. Throughout my doctoral dissertation, I used the fruit fly as a model to explore how various social experiences such as fighting, competition, sexual harassment, and mating shape the subsequent reproductive behaviour of males and females, and quantified the evolutionary consequences of this variation. The results of my studies have important implications for understanding the evolution of various behavioural strategies such as aggression and mate choice.

ABSTRACT

Animals display considerable phenotypic variation in their mating traits, and this variation can have important consequences for the evolution of dimorphic traits between the males and females within a species. In chapter 1, I outline the current state of our understanding of plasticity in reproductive phenotypes, and argue that more work needs to be done to connect the gap between plasticity in mating traits and the outcomes of sexual conflict. Across my four data chapters, I used the fruit fly as a model organism in series of experiments that attempt to better understand how plasticity in mating traits influences the fitness outcomes of both males and females. In chapter 2, I experimentally manipulated the outcome of a fighting experience, and found that males who win a previous fight have higher pre-copulatory reproductive success, but losers perform better in post-copulatory areas. In chapter 3, I manipulated the amount of competition that a male experiences and found that females mated to males who experience competition produce more early-life offspring but live shorter lives compared to females mated to males that experience no competition. In chapter 4, I manipulated the intensity of sexual conflict that a female experiences and found that males mated to females who experience high conflict have lower pre- and post-copulatory reproductive success compared to males mated to females who experience low conflict. In chapter 5. I manipulated the degree of sexual aggression that a female experiences, and found that females that experience harassment and mating from a male that displays high sexual aggression is subsequently less choosy compared to females that experience a less aggressive male. Finally, in chapter 6, I discuss the significance of my results as they relate to the evolution of reproductive traits in males and females.

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DECLARATION OF ACADEMIC ACHIEVEMENT

This dissertation is organized in a sandwich thesis format consisting of six chapters. Chapter 1 is a general introduction to the thesis and a brief overview of my dissertation objectives. Chapters 2 to 5 are complete data chapters written in manuscript format. Chapter 6 is a general discussion of the results of my dissertation, along with a brief statement on some broad takeaways.

Chapter 1: Introduction
DCSF wrote the chapter.

Chapter 2: Winners have higher pre-copulatory mating success but losers have better post-copulatory outcomes

DCSF carried out the experiments. Both authors designed the experiments, contributed to the data analysis, writing, and editing process of the manuscript.

Chapter 3: Plasticity in male mating behavior modulates female life-history in fruit flies

DCSF and RD designed the experiments. DCSF and RB carried out the experiments. DCSF wrote the first draft and performed the statistical analysis, and all authors were involved in the revision process.

Chapter 4: Female mating experience and genetic background interact to influence male mating success in fruit flies

DCSF and RD designed the experiments. DCSF and RB carried out the experiments. DCSF wrote the first draft and performed the statistical analysis, and all authors were involved in the revision process.

Chapter 5: Previous sexual aggression decreases choosiness but does not influence mating preferences in fruit flies

DCSF carried out the experiments. Both authors designed the experiments, contributed to the data analysis, writing, and editing process of the manuscript.

Chapter 6: Discussion
DCSF wrote the chapter.

CHAPTER 1

Introduction

David C.S. Filice

Introduction

The phenomenon of variation in reproductive success is so powerful and pervasive in nature that sexual selection is often viewed as an evolutionary mechanism that is unique from natural selection (Andersson, 1995). As such, biologists find it essential to investigate the causes of variation in reproductive success because of their consequences for evolution. After Darwin first described sexual selection, evolutionary biologists have often remained fixated on gene-focused views of sexual selection, largely due to the major advancements brought on by the modern synthesis of evolution in the early 1900s (Darwin, 1871; Fisher, 1930; Wright, 1930). However, conflicting results and an increasing push towards interdisciplinary studies is leading evolutionary biologists to a view that integrates dynamic elements from ecology, development, and behaviour into our understanding of variation in sexual traits (Cornwallis & Uller, 2010). Specifically, the past decade of research on sexual selection has seen a dramatic effort towards improving our understanding of how phenotypic plasticity causes sexual traits to vary in their form and magnitude (Cornwallis & Uller, 2010; Kasumovic & Brooks, 2011; Maan & Seehausen, 2011; Perry & Rowe, 2018).

Phenotypic plasticity allows individuals to modify their phenotypes across different environmental contexts and can thus be a major source of phenotypic variation (Holloway, 2002; Pigliucci, 2005; West-Eberhard, 1989). Plasticity can manifest developmentally, physiologically, and/or behaviourally, and in some cases result in irreversible outcomes based on previous environmental cues, or remain flexible in response to rapidly changing conditions (Kasumovic & Brooks, 2011; M Pigliucci, 2001; Scheiner, 1993). Since the environments that individuals inhabit can often be highly dynamic, plasticity can arise as an adaptation to allow individuals to maximize their fitness across different contexts (Price et al., 2003; Qvarnström, 2001). Given that successful reproduction is an essential component of an individual’s evolutionary fitness, it is intuitive to predict that selection would target flexibility in sexual phenotypes in order to ensure reproductive success when conditions are variable. This prediction is supported by numerous studies across many taxa that demonstrate individuals use cues in their environments to modify their traits in a way that benefits their reproductive success (Bretman et al., 2009; Kasumovic et al., 2008; Yeh & Price, 2004). Although these studies have been essential for our understanding of sexual selection, they often only focus on the evolutionary consequences of the individual exhibiting the plasticity, and ignore the consequences for the members of the opposite sex.

Reproduction overall requires cooperation and yields many mutual benefits for both the sexes. However, in many species, there is sexual conflict where the optimal reproductive interests of the males and females do not align (Arnqvist & Rowe, 2013; Parker, 1979). For example, differences in the metabolic investment required for gamete production is often much smaller for males, resulting in a higher optimal mating rate for males compared to females (Bateman, 1948). Such asymmetry can lead to the sexual coevolution of sexually antagonistic traits, where males evolve traits that are exploitative and harmful towards females, and females coevolve traits that resist this harm (Chapman et al., 2003). The expression of sexual phenotypes (which may be regulated by plasticity) can often determine the magnitude of the antagonistic costs experienced by the opposite sex. For example, traits that help males succeed in male-male competition often have a deleterious effect on female fitness, and traits that upregulate a female's mating propensity often negatively affect males (Rice, 1996; Wigby & Chapman, 2004). Thus, it should be clear that plasticity in such traits may have a significant influence on members of the opposite sex. The goal of my dissertation is to synthesize our understanding of plasticity in mating traits with our understanding of evolutionary conflicts between the sexes. Broadly, I predict that a greater expression of a sexual trait that benefits the reproductive success of one sex should have a proportionately negative effect on the fitness of members of the opposite sex. In the next section, I outline several major areas of research that demonstrate the plastic nature of sexually antagonistic phenotypes in males and females.

Plasticity in sexually antagonistic traits: The cheaper gamete (males)

In the sex that invests less into gametes (usually males), reproductive success is typically directly proportional to the number of successful matings. Thus, the most successful individuals are usually the best at outcompeting other males for access to mates, and attracting the most females (Wong & Candolin, 2005). In species where females mate more than once, male-male competition and female choice also occur after copulation in the form of sperm competition and cryptic choice (Parker & Pizzari, 2010). Males can also improve their reproductive success through other strategies such as mate guarding (Baxter et al., 2015), forced copulations (Dukas et al., 2020; Thornhill, 1980), and sneaking fertilizations (Fitzpatrick et al., 2016; Gross, 1996). Given the multitude of components that males must invest into sex, they are often limited in their expression of these traits, and cues in the social environment allow males to invest in the most appropriate strategies via phenotypic plasticity (Simmons et al., 2017).

Perhaps the most widely studied, and thus best understood source of plasticity in sexual traits involves the composition of individuals within a population. Specifically, the density of conspecific competitors and potential mates can widely vary across space and time (Kasumovic et al., 2008), and both males and females face different reproductive challenges depending on these densities. Empirical studies postulate that individuals can use a variety of tactile, acoustic, visual, and/or chemical cues to assess the density of mates and competitors in their social environment, and demonstrate that these cues can shape plasticity in reproductive traits (Kasumovic & Brooks, 2011). For example, in wild house mice, *Mus musculus domesticus*, males housed until sexual maturity with two rival males developed a thicker and more distally extended baculum bulb compared to males housed alone until maturity. The baculum bulb is part of the male genitalia in many mammals, and its shape directly covaries with sperm competitive ability, where distal extension tends to positively associate with greater post-copulatory success (André et al., 2018). In fruit flies, males reared in vials with 200 larvae developed significantly larger accessory glands compared to males reared in vials with 20 larvae (Bretman et al., 2016), and adult males housed in vials with rivals for 5 days prior to mating mate for a longer duration compared to males housed alone (Bretman et al., 2009, 2010). Similar paradigms in other taxa have found similar results, including studies on guppies (Magris et al., 2018), salamanders (Verrell & Krenz, 1998), and crickets (Lyons & Barnard, 2006).

Another important factor that shapes plasticity in male phenotypes is the outcome of social interactions with conspecifics. While the density of potential mates and/or competitors may provide an animal with information about extrinsic factors that may limit the potential number of mating opportunities, the outcomes of direct interactions with individuals may provide an animal with information about intrinsic factors such as social status or attractiveness. For example, the winners and losers of a dyadic fight experience significant changes in their physiology and behaviour, and often these changes can have an effect on future mating interactions (Hsu et al., 2005). In the blockhead cichlid fish, *ocranus casuarius*, when compared to losers, winners of a previous fight spent more time engaging in courtship behaviour, exhibited more aggressive behaviours towards their mates, and had an overall higher mating success (Lamanna & Eason, 2011). Similarly, in fruit flies, winners of a previous fight made more mating attempts and had greater mating success (Teseo et al., 2016). Another important social interaction is previous mating experience. In fruit flies, males that experience sexual deprivation are more sexually aggressive towards females, and are more likely to forcibly copulate with them compared to sexually experienced males (Baxter & Dukas, 2017). Previous agonistic and/or mating experience has also been found to influence mating traits in species such as snakes (Schuett, 1997), mosquitofish (Iglesias-Carrasco et al., 2019), and primates (Chism & Rogers, 2010).

From the perspective of male fitness, the potential benefits generated by plasticity in sexual traits are quite clear. In environments where competition is high, males may experience a net benefit by upregulating their phenotypes related to success in sperm competition, even if it physiologically costly to do so (Bretman et al., 2013). Conversely, in environments where the female to male sex ratio is high, downregulating these traits may be beneficial if males are able to secure many mating opportunities with little competition (Reuter et al., 2008). In the case of winner-loser effects, an individual that wins a previous fight may have greater success using more sexually aggressive strategies compared to a loser, and a sexually deprived male may resort to sexual aggression due to lack of opportunity. However, the fitness effects that this plasticity has on females remains quite unclear, despite intuitive predictions. In the case of the post-copulatory strategies that are plastically influenced by cues such as competition risk, traits that have evolved to help males succeed often have costs for females. For example, in the fruit fly, males have evolved a variety of accessory gland proteins (Acps) that are transferred in the ejaculate during copulation (Chapman et al., 1995). A number of these Acps aid males by displacing the sperm of rivals, and manipulating females into delaying remating and investing into short-term offspring production (Chapman, 2001). These effects are costly to females, who experience reduced lifetime fecundity and longevity as a direct result of the toxic effects of Acps (Wigby & Chapman, 2005). Furthermore, an increased expression of aggressive pre-copulatory behaviours as seen in winners and sexually deprived males can also cause physical damage to females, and having to deal with sexual harassment can leave females vulnerable to predators, or waste valuable time that could be spent foraging and raising offspring (Clutton-Brock & Parker, 1995). As such, it is essential to understand how plasticity in male mating traits may influence costly side-effects in females.

Plasticity in sexually antagonistic traits: The more expensive gamete (females)

The intense degree of competition that males face means that sexual selection acts as a powerful evolutionary mechanism and this has been hypothesized to result in a greater variability in male phenotypes compared to female phenotypes (Archer & Mehdikhani, 2003). This phenomenon has led to a greater focus on studying males when researching variation in mating strategies (Neff & Svensson, 2013). However, it is now clear that females also exhibit substantial variation in their mating strategies, including their preferences, choosiness, and frequency of mating. Since females' reproductive success typically does not hinge on the number of mates they can acquire, the most successful individuals are usually those who take their time to carefully sample males and only mate with those who provide the most direct (e.g. resources) and/or indirect (i.e. genetic) benefits, while also resisting the direct costs brought on by sexual conflict (Kokko et al., 2003). Similar to the challenges that males face, the optimal mating strategy of a female may vary depending on ecological factors such as population composition and social experience (Jennions & Petrie, 1997).

In certain environments, such as when the male to female sex ratio is drastically low, being too choosy in selecting a mate can end up being very costly if no mating occurs at all (Qvarnström, 2001). For example, in field crickets, *Gryllus lineaticeps*, females exposed to acoustic signals that indicate high male density were more choosy in their mate choice compared to females exposed to cues indicting low male density (Atwell & Wagner, 2014). Females can also shape their mating preferences based on previous experiences, which may help reduce the costs of searching for a mate by choosing easier. For example, females have been shown to prefer familiar traits that they were previously exposed to (Walling et al., 2008), and even copy the mate choice through observing other females (Galef & White, 1998). Another behaviour that females may modulate is their mating frequency. When previous males are infertile or transfer low amounts of sperm, mating multiply can increase the odds of successful fertilization and overall fecundity (Sutter et al., 2019). It has also been hypothesized that females mate multiply in order to “trade-up” in genetic quality for their offspring, and to produce offspring with more variable genotypes (Laturney et al., 2018).

Similar to plasticity in male traits, plasticity in female reproductive traits tends to solve adaptive problems for the individual that expresses them and has predictable costs for the opposite sex. When there are many prospective mates, or attractive mates are available, displaying strict choosiness can ensure that females obtain the greatest net benefit from mating (Qvarnström, 2001). Similarly, shifting mate preferences can help females reduce the costs of mate searching such as predation risk and energy expenditure (Rodríguez et al., 2013). In these cases, we can expect that future males who court females that are choosy or have specific preferences may suffer from decreased fitness in terms of reduced mating success, and increased energy investment into courtship and/or providing gifts. In terms of post-copulatory outcomes, mating multiply can benefit females directly and/or indirectly in some conditions, but males face the cost of increased sperm competition and cuckoldry. Despite these clear predictions, empirical studies have not accurately quantified these costs for males.

Dissertation objectives

In my brief literature review above, I began by highlighting the importance of integrating our understanding of phenotypic plasticity in sexual traits into sexual conflict theory. I argued that, although researchers have made great progress towards understanding how plasticity can shape sexual traits in both males and females, the consequences of this plasticity for antagonistic interactions between the sexes remains vague. To address this, I used the fruit fly, *Drosophila melanogaster*, as a model organism to study the causes and consequences of variation in sexually antagonistic traits. The fruit fly is an ideal organism for this project, as it has been extensively used as a model for studying phenotypic plasticity and sexual selection, and its mating system (particularly with regards to sexual conflict) is well understood. Furthermore, we have the ability to finely control the environment and genetics of individuals, allowing us to precisely estimate the contributions of the factors we choose to manipulate. The overarching goal of my dissertation is to explore how social experiences shape plasticity in sexual traits and investigate how this plasticity influences the fitness of not only the individuals exhibiting plasticity, but also the fitness of members of the opposite sex that they interact with. In doing so, my goal is to emphasize that the outcomes of plasticity in sexual phenotypes has both direct evolutionary consequences for the individual that expresses plasticity, and indirect consequences for individuals of the opposite sex within the same social environment. Across the four data chapters of my thesis, I manipulated a variety of ecologically relevant social experiences in males and females, and measured their subsequent behaviours and reproductive success.

In chapter 2, I studied how the outcomes of a male's previous competitive experience influences his subsequent reproductive success. I found that compared to losers, winners tend to perform better in pre-copulatory contests such as male-male competition, but losers tend to perform better in post-copulatory areas such as sperm competition.

In chapter 3, I studied how the context of a male's previous competitive environment influences the subsequent fitness of his mates. I found that males influenced the life-history strategies of their mates, where females that mated with males housed with competitors died younger and produced more offspring early in life, and females that mated with males housed alone lived longer and produced more offspring later in life.

In chapter 4, I studied how the context of a female's previous mating environment influences the subsequent fitness of her mates. I found that males who mate with females that experience a high harassment environment have lower pre- and post-copulatory reproductive success compared to males who mate with females that experience a low harassment environment.

In chapter 5, I studied how the phenotype of female's previous mating partner influences her subsequent mating behaviour. I found that females that experience harassment and mating from a male descended from a lineage of flies selected for high sexual aggression have a higher mating receptivity compared to females that experience a male from a lineage selected for low sexual aggression.

The results across these chapters highlight the importance of considering the behavioural and fitness consequences of phenotypic plasticity for all individuals involved in sexual interactions. This is important because these results will help explain previously unappreciated sources of evolution for reproductive strategies in males and females.

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CHAPTER 2

Winners have higher pre-copulatory mating success but losers have better post-copulatory outcomes

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Preface: The following chapter is a manuscript published in *Proceedings of the Royal Society B* and it is written in the style of this journal.

Abstract

In many animals, the outcomes of competitive interactions can have lasting effects that influence an individual's reproductive success and have important consequences for the strength and direction of evolution via sexual selection. In the fruit fly, *Drosophila melanogaster*, males that have won previous contests are more likely to win in subsequent conflicts and losers are more likely to lose (winner-loser effects), but the direct fitness consequences and genetic underpinnings of this plasticity are poorly understood. Here, we tested how male genotype and the outcomes of previous male-male conflicts influence male pre- and post-copulatory success. We quantified pre-copulatory success in a choice and no-choice context, and post-copulatory success by quantifying ejaculate offensive and defensive ability. We found that winners have higher reproductive success compared to losers in both pre-copulatory scenarios. However, losers consistently mated for a longer duration, boosted female fecundity, and had an increased paternity share when they were the first males to mate, suggesting increased investment into post-copulatory mechanisms. Finally, by using clonal hybrids from the *Drosophila genetic reference panel*, we quantified the proportion of phenotypic variance in the plasticity between winners and losers that was due to genetic differences. Our results place the behavioural data on winner-loser effects in an evolutionary context by documenting the potential fitness gain to males from altering their reproductive strategy based on fighting experience. Our data may also explain the presence and maintenance of trade-offs between different male reproductive strategies.

Introduction

Prior competitive interactions can have lasting effects across many species, where winners of previous contests are more likely to win in subsequent conflicts, and losers are more likely to lose [1,2]. Winning and losing also influence a variety of behaviours including those related to aggression, mating, and exploration [3,4,5] and may thus have major consequences for fitness. However, our understanding of the evolutionary biology of these behavioural effects is limited [2]. Specifically, few studies have addressed the fitness consequences and genetic underpinnings of the behavioural plasticity associated with winner-loser effects.

When thinking about winner effects, the potential fitness benefits are rather intuitive, and studies indeed have found that winners typically enjoy increased access to resources and mates [6]. These benefits may be manifested through an improved ability to deter other males from access to mates (intrasexual selection), or through increased attractiveness to potential mates (intersexual selection). For example, in the mosquitofish, *Gambusia holbrooki,* winners spent significantly more time associating with females compared to losers when physical interactions between males were allowed. Since female cooperation is not necessary for mating in mosquitofish, this result suggests that the effect was largely due to the winner preventing losers from interacting with females, or losers actively avoiding winners to reduce the potential costs of further fighting. The winners, however, did not make more copulation attempts or mate more often than the losers [7]. In other species, similar protocols have demonstrated that female choice may also influence the mating success of winners and losers. In field crickets, *Gryllus assimilis, females were more likely to mount males who won a previous fight compared to males that lost a previous fight* when presented to a single male, suggesting that the winners were more attractive [8]. Note, however, that, unlike the mosquitofish experiment, which randomly assigned males into winner and loser roles, the cricket study relied on natural male fighting outcomes, which implies selection bias [9]. Hence, in the cricket study, one cannot separate the effects of inherent male quality from the isolated effects of winning or losing.

Unlike winner effects, the evolutionary perspective of loser effects are not as clear. Some researchers suggest that individuals may benefit from the behavioural plasticity associated with losing by reducing the potential energetic costs and physical harm associated with future conflicts, but there is no clear evidence of how these modified behaviours could be maintained against the fitness costs of decreased access to resources and mating opportunities [2]. One solution to this paradox may involve the use of alternative mating tactics to maximize reproductive success. Specifically, losers may gain from strategically investing more resources into ejaculate traits if mating opportunities are few [10,11]. In the broad-horned flour beetle, *Gnatocerus cornutus*, males that lost a fight 24hrs earlier transferred significantly more sperm during copulation compared to winners [12]. In another study using the same species, males that lost a fight switched to a dispersal strategy by leaving the fighting site, compared to winners who tended to remain on the same site after winning a fight [13]. Although the flour beetle studies involved selection bias, they suggest that losers choose their mating strategies to make the best of a bad situation. To the best of our knowledge, however, no one has tested the fitness outcomes associated with the behavioural changes that accompany winning and losing.

In fruit flies, *Drosophila melanogaster*, males engage in a variety of pre- and post-copulatory contests to acquire mates and maximize paternity. Male pre-copulatory success is influenced by a combination of acoustic, visual, chemical, and tactile signals [14] and courtship interference [15], and their post-copulatory success may be influenced by the amount of sperm and accessory gland proteins (Acps) that are transferred in the seminal fluid [16,17]. Given that males can employ a variety of pre- and post-mating strategies, each involving different benefits and costs, one would expect them to choose the strategy that would maximize fitness in their current social setting [10,11]. For example, male fruit flies housed in a vial with a conspecific rival subsequently mate for longer and sire significantly more offspring (whether they are the first or second males to mate) compared to males housed alone [18]. The authors suggest that males housed with rivals perceive a greater risk for sperm competition in their social environment, and thus invest more into tactics that will improve their post-copulatory success. Given that males adaptively alter their mating strategies based on their socio-sexual environment, we expect winner-loser effects to have a significant effect on the performance of males in pre- and post-copulatory competitions.

Here, we hypothesized that the reproductive success of winners and losers would differ, with winners having higher pre-copulatory success and loser achieving higher post-copulatory success. We used fruit flies to examine the effect of winning and losing on male reproductive success in 1) a choice pre-copulatory context, 2) a no-choice pre-copulatory context, 3) a defensive post-copulatory context, and 4) an offensive post-copulatory context. We predicted that winners would have higher reproductive success in the pre-copulatory contexts (tests 1 & 2), while losers would have higher reproductive success in the post-copulatory competitions (tests 3 & 4). In addition, we used clonal hybrid lines to quantify the degree of genetic variation associated with these plastic effects. Understanding the degree to which individual genotype influences the magnitude of behavioural plasticity (and its fitness consequences) between winners and losers is of great interest to biologists because variation in male competitive and/or mating behaviours can influence the strength and direction of evolution via sexual selection [19].

Methods

Fly stocks and general

We used 28 randomly selected lines from the *Drosophila genetic reference panel* (DGRP). These lines were derived from wild flies caught in Raleigh, North Carolina, USA, and repeatedly inbred for 20 generations [20]. To alleviate the deleterious phenotypic effects associated with inbreeding, we generated hybrid lines by crossing each line to a standardized reference line, thereby creating unique hybrid clone lines (hereafter referred to as hybrids). Within lines, individuals are genetically identical, but between lines, individuals share an identical clonal haplotype inherited from their mother, and a unique clonal haplotype inherited from their father, allowing us to quantify the degree of genetic variation associated with phenotypic differences expressed from this unique haplotype.

To generate standardized competitors, we used descendants from the "bully" population obtained from the Kravitz Lab (Harvard University, Cambridge, Massachusetts, USA). These flies have been artificially selected for increased male-male aggression by choosing the winners of fights over 34-37 generations [21]. To manipulate the amount of aggression expressed by these males toward focal males, we used either 1-day or 5-day old males (hereafter referred to as young and mature bullies, respectively), because young males show little aggression compared to their older counterparts [22; see below]. One day before each test, we dusted all bully males with pink fluorescent powder to distinguish them from the focal males. We lightly tapped individual flies into vials containing sparse amounts of the powder, giving them ample time to recover for the experience phase on the following day.

To determine paternity success, we used flies derived from the *Ives* population (hereafter IV) obtained from the Long Lab (Wilfrid Laurier University, Waterloo, Ontario, Canada). Since its collection in 1975, this population has been maintained at large census size (>1000 adults/generation), on non-overlapping generations on a standardized culture protocol [*see* 23]. A sub-population of these flies carries the recessive autosomal *bw* mutation [*see* 24; hereafter referred to as IV-bw], resulting in a visible brown-eye colour phenotype in comparison to the standard red-eye wild-type individuals. As the expression of this phenotype is controlled by a single recessive allele, two individuals who possess this phenotype will always produce offspring that express it, but any offspring from a wild-type and brown-eye cross will appear wild-type.

We reared all experimental flies at a standardized density of about 100 eggs per vial containing ~5 ml of standard fly medium made of water, sucrose, cornmeal, yeast, agar and methyl paraben, and stored all flies in an incubator at 25 °C and 60% relative humidity with a 12:12h light:dark cycle. We collected newly eclosed flies within 8h of eclosion under light CO_2 anesthesia and housed males in individual vials and females in groups of 20 with a pinch of live yeast. Following their initial collection, we handled all flies using gentle aspiration. We conducted all trials in “aggression arenas” consisting of petri dishes 35mm in diameter and 8mm high. We covered the base of each arena with a circular piece of filter paper, and placed at its centre an attractive food patch 7 mm in diameter and 3 mm high consisting of standard fly medium sprinkled with live yeast [*see* 22].

Aggression tests

A major challenge in studying winner-loser effects is developing unbiased protocols that reliably generate focal winners and losers. Although this issue has been brought to attention in two major reviews [1,2], many studies continue to use self-selection protocols, which confound the effects of experience with intrinsic individual factors. To avoid self-selection biases, we randomly chose focal males of similar sizes and assigned them to either the winner or loser treatment. Focal males of the winner treatment interacted with young bullies, while focal males of the loser treatment interacted with mature bullies. As males are highly motivated to monopolize the attractive food patch in each arena by displaying aggression toward rival males [22], males that are more aggressive are consistently winners in this context [25]. In a preliminary experiment, we quantified the success of our treatments by recording aggression in matches between focal males and young bullies, and focal males and mature bullies. The focal males were derived from a recently established wild-caught population we collected in Hamilton, ON in 2015. In each trial, we placed a single 4-day old male randomly selected from our base population into an aggression arena with either a 1-day old bully (young) or a 5-day old bully (mature) and video recorded the interactions for 30 minutes using an iPod Touch. Then an observer blind to bully age used the BORIS software [26] to record from the videos the total number and duration of aggressive behaviours displayed by both flies. Aggressive behaviours were defined by the ethogram outlined by Chen et al. [27], including occurrences of wing threat, lunging, high-level fencing, charging, holding, boxing and tussling.

Experience phase

Our aggression tests indicated that mature bullies display about 18 times more aggression towards focal males on average compared to their young counterparts [*see* results and figure 1]. Hence we generated presumed winner and loser focal males by matching them with either young or mature bullies, respectively. While we were not certain that each focal male assigned to the winner treatment was indeed a winner, and that each focal male assigned to the loser treatment was indeed a loser, this merely makes our conclusions conservative as we probably included some losers with the winners and vice versa. Prior to each test, we aspirated a single 4 day old focal hybrid male into an aggression arena, followed by immediately aspirating either a young or mature bully competitor into the arena, and left the arenas undisturbed for 4h. This protocol for the experience phase was identical in each of the following four experiments.

Assay 1: Pre-copulatory choice test

At the end of the 4h experience phase, we removed the bully males from each arena and introduced a new 4 day old competitor IV male and a 4 day old IV female to each focal male [figure 2a]. The presence of a competitor meant that mating outcomes not only depended on the attractiveness of the focal male, but also on male-male interactions including aggression and courtship interference [15]. Observers blind to the focal males' experience scanned each arena until one of the males successfully mated (or for 90 min if no mating occurred). We replicated the entire protocol across 14 days of identical sessions using 28 hybrid lines, where we tested a single male from each hybrid line and treatment combination (n=56 trials per day) except in the case of missing trials. Missing trials included cases where we failed to collect sufficient numbers of males from a given line (n=103), or if mating did not occur in the test (n=12). Hence our final sample size was 333 winners and 336 losers.

Assay 2: Pre-copulatory no-choice test

At the end of the 4h experience phase, we removed the bully males from each arena and introduced a 4 day old IV female to each focal male [Figure 2b]. This allowed males to court females without the interference of a competitor, meaning that the mating outcomes primarily depended on the courtship behaviour and attractiveness of the focal male [28,29,30]. Observers blind to the focal male's experience scanned each arena until each mating concluded (or for 90 minutes if no mating occurred) and recorded all mating latencies and durations. We replicated the entire protocol across 12 days of identical sessions using 28 hybrid lines, where we tested a single male from each hybrid line and treatment combination (n=56 trials per day) except in the case of missing trials. The missing trials included insufficient numbers of males (n=132) and test trials without matings (n=6). Thus our final sample size was 258 winners and 276 losers.

Assays 3 and 4: Female fecundity after a single mating, and ejaculate competitive ability (P1 and P2 post-copulatory *success)*

We conducted two experiments to assess the post-copulatory success of winner and loser focal males that mate with a female either first (P1) or second (P2). In the first experiment, we removed the bully males from each arena after the 4h experience phase and introduced a 4 day old IV-bw female to each focal male [figure 2c]. We kept the focal males in the same arenas because transferring them to a new setting could reduce the winner and loser effects either merely due to the disturbance [31] or because a new site implies no or novel competitors [32]. An observer blind to focal males' experience recorded the latency and duration of each mating. At the end of each mating, we discarded the focal males, aspirated all females into individual food vials with live yeast, and placed them in the environmental chamber. On the following morning, we moved the females from the vials into new aggression arenas and returned the vials to the chamber. Two weeks later, we counted the number of offspring in each vial. This allowed us to compare female fecundity after a single mating with either winners or losers.

After adding the once-mated females into new aggression arenas, we introduced into each arena a 4-day old IV-bw male. An observer blind to fly treatment scanned each arena until mating concluded (or for 4 h if no mating occurred) and recorded the mating latency and duration. We discarded females that did not remate (n=86 and 77 in the winner and loser treatments respectively), placed remated females into fresh vials with live yeast and housed them in the environmental chamber for egg laying over 24h. We then discarded the females. Two weeks later, we counted the offspring fathered by focal and IV-bw males, which had red and brown eyes respectively. We tested a random subset of 6 hybrid lines (from the original 28) and conducted 3 replicates. Each replicate of 120 trials consisted of testing 10 males of each of the 6 hybrid lines and 2 treatments. The missing trials included insufficient numbers of males (n=23) and test trials without rematings (n=163). Hence our final sample size of females that remated included 89 winners and 85 losers.

In the second experiment, in which we assessed the paternity success of focal males that mate with a female second (P2), we used a similar protocol as in the previous experiment except that we reversed the mating order of the focal and IV-bw males. That is, the focal males had 4 h experience with either young or mature bullies and then were allowed to mate with females mated on the previous day to IV-bw males [figure 2d]. Here, after accounting for insufficient numbers of hybrid males (N=22) and trials without remating (79 winners 104 losers), our final sample size included 90 winners and 65 losers.

Statistical analysis

We conducted all data analyses using R v3.4.2 [33]. Data collected from the aggression test were analyzed using a generalized linear model with experience treatment as a main effect. Data collected from all other experiments were analyzed using generalized linear mixed models (GLMMs), created using the *lme4* package [34]. In the choice and paternity tests, we used a binomial response variable (the focal male did or did not mate), and data collected from the no-choice and fecundity test were analyzed using a Gaussian response variable (mating latency and mating duration). The models included focal male experience treatment as a fixed effect, and the hybrid clone line, the experience treatment crossed with hybrid line, and day of testing as random effects. Thus, variance in hybrid clone line represents genetic variation in the phenotype measured, and variance in the interaction between experience treatment and clone line represents genetic variation in the plasticity of that phenotype. In cases where our data were over-dispersed, we added an observation-level random effect [35].

We calculated the significance of the fixed effects using a log-likelihood ratio chi-square test from the *Anova* function in the *car* package [36]. For the random effects (and their interactions), we used the *bootMer* function to calculate the 95% confidence intervals based on 1000 bootstrap samples, and the significance of each variance component using a permutation test approach [37]. This involved comparing the magnitude of our models' variance components to the distribution of 10000 variance components that were determined from a randomized set of the experimental data.

Results

Aggression tests

On average, mature bully males were 17.6 times more aggressive than young bullies (χ^2 = 28.7, df = 1, p = 8.61×10^{-8}; figure 1). Overall, mature bullies were more aggressive than their focal competitors in 82.6% of the trials (19/23), while young bullies were more aggressive than their focal competitors in only 17.4% of the trials (4/23) (χ^2 = 19.6, df = 1, p = 0.0001; figure 1).

Assay 1: Pre-copulatory choice test

Fighting experience had a significant effect on the subsequent mating success of focal males competing against a novel, inexperienced male. On average, winners were successful in mating in 0.58 of the trials, and losers in 0.48 of the trials (χ^2 = 7.57, df = 1, p = 0.006; figure 3a). The effects of hybrid line, line by experience interaction, and day were not significant (figure 3a, Table 1).

Assay 2: Pre-copulatory no-choice test

Winners in the no-choice test were about 1.5 times faster to mate than losers (χ^2 = 16.464, df = 1, p = 4.959×10^{-5}; figure 3b). The effect of hybrid line was marginally significant, but the line by experience interaction and day were not significant (figure 3b, Table 1). Winners also had significantly shorter mating durations than losers (χ^2 = 32.879, df = 1, p = 9.807×10^{-9}; figure 3c). While the effects of hybrid line and day were significant, the line by experience interaction was not (figure 3c, Table 1).

Assay 3: Single-mating fecundity and sperm defensive ability (P1 paternity success)

Winners sired significantly fewer offspring than losers after a single mating with virgin females (χ^2= 9.913, df = 1, p = 0.00164; figure 4a). The hybrid line and the interaction between line and treatment were not significant, but the day of testing was significant (figure 4a, Table 2).

Fighting experience also had a significant effect on the paternity success of focal males mated to females first (P1), with winners having 8.4% less paternity on average compared to losers ($\chi^2 = 9.575$, df = 1, p = 0.00197; figure 4b). Hybrid line, the line by experience interaction, and day of testing were not significant (figure 4b, Table 2).

Assay 4: Sperm offensive ability (P2 paternity success)

Winners and losers had a similar paternity success when they were the second mating males ($\chi^2 = 0.346$, df = 1, p = 0.557; figure 4c). While the effect of clone line was significant, the effect of the interaction between clone line and treatment and day were not (figure 4c, Table 2).

Discussion

Our results indicated that, on average, 1.) Winners performed better than losers in pre-copulatory contests 2.) losers performed better than winners in post-copulatory contests, and 3.) the expression of some of this plasticity may be due to natural genetic variation present in the focal population. Thus, the outcomes of aggressive interactions can have important consequences for the evolution of sexually selected traits by influencing the reproductive success of winners and losers. Here, we used randomly assigned winners and losers and found that the outcome of a variety of reproductive competitions can be significantly affected by previous fighting experience. Previous studies have documented that the experience of winning or losing can influence the expression of pre- [7] and post-copulatory reproductive traits [12]. We add to these findings by documenting differential effects of winning and losing on pre- and post-copulatory success. We also found segregating genetic variation underlying some of our measures of male fitness. Additionally, our methodology ensures that our results are due to changes in the expression of reproductive traits (phenotypic plasticity) resulting from the outcomes of social experience, as opposed to intrinsic differences between winners and losers. This is an important difference that many previous studies have failed to distinguish due to selection bias [1,2].

When looking at the effect of fighting experience on pre-copulatory success, we found that winners significantly outperformed losers in both the choice (two males) and no-choice (one male) tests. In the choice tests, it is likely that winners experienced prototypical winner effects related to increased aggression and fighting ability [38]. When two males are placed in an arena with a single female, the mating outcome may be influenced by the aggressive interactions between the males through courtship interference [15] or via female choice [30]. If winners are more aggressive than losers, this can explain the observed difference in mating success between the two treatments through increased courtship interference. Future studies should continue to attempt and untangle the relative contribution of male-male competition and female choice in these types of interactions [15].

The increased success of winners in our no choice tests may be explained by differences in male attractiveness and courtship behaviour. Shackleton et al. [28] argued that measuring mating latency is a reliable indicator of male attractiveness, so one possibility is that this difference may be explained by winners being more attractive than losers. Previous studies have found conflicting results regarding social dominance and attractiveness. In some cases, winners may be preferred when fighting ability can signal and/or covary with good genes, where in other cases losers are preferred when fighting ability may signal a cost to females via an increased potential for sexual harassment [39,40]. However, in species with intense sexual conflict, mating with sexually coercive males can also provide indirect benefits to females via “sexy sons” and thus harmful males are thought to be preferred by females in *D. melanogaster* [41, *but see* 42]. Assuming winners are more harmful in a pre-copulatory context [39], the sexy sons hypothesis is consistent with our results. Future studies should quantify differences in male pre-copulatory harassment between winners and losers to determine the associations between attractiveness, pre-copulatory male-harm, and winner-loser effects. Another possibility is that this difference may be explained by differences in courtship behaviour between winners and losers. In *Drosophila*, Kim et al. [43] found that losers take around two times as long to initiate courtship when placed into a chamber with a virgin female, which may explain the difference that we observed in mating latency. Another interesting possibility may involve differences in cuticular hydrocarbon expression between winners and losers, which can significantly influence the outcomes of mating interactions [44]. In *Drosophila,* an individual's expression of these hydrocarbons is highly sensitive to its sociosexual environment [45,46]. Future studies should attempt to quantify traits that are known to influence attractiveness, such as courtship ability and cuticular hydrocarbon profiles, and compare them between winners and losers.

When looking at the effect of fighting experience on post-copulatory success, we found that losers significantly outperformed winners in the fecundity and sperm defense tests, but not in the sperm offense tests. These results are mostly consistent with studies that predict that dominant males trade-off by investing less resources into post-copulatory mechanisms due to a potentially greater number of future mating opportunities (and vice-versa for losers) [47]. As mating duration is positively correlated with investment into the transfer of accessory gland proteins (Acps) [17], the increased duration of mating displayed by losers in our experiments may suggest a greater transfer of Acps. In the case of fecundity, losers may transfer larger quantities of Acps in their ejaculate to maximize the number of offspring they produce, and in the case of sperm defense, to maximize their share of paternity after potential rematings [48]. In a variety of species, it is well understood that extrinsic sociosexual factors can cause rapid changes in male mating strategies [49,11,50], but we are the first to demonstrate that winner and loser effects play an important role. The fact that losers had a higher share of paternity and produced more offspring when they were first to mate offers insight into how this plasticity may be selected for. Given that the transfer of Acps have toxic-side effects that reduce the fecundity and lifespan of females [51], future studies should investigate the consequences of winner-loser effects for female fitness. Paired with the findings of our first two assays, it may be that winners are more harmful in a pre-copulatory context, while losers inflict more harm via post-copulatory mechanisms.

As sperm defensive and offensive abilities appear to be phenotypically uncorrelated in *D. melanogaster* [52], it is not surprising that we found no difference in sperm offensive ability between winners and losers, despite our detected difference in sperm defensive ability. One possible explanation is that the decreased sperm competitive ability of males carrying the *bw* mutation reduced the resolution of our results. This disadvantage has been identified by previous studies [53] and is clear in our results, as the average P2 success was 84.7% for males with the *bw* mutation vs. 92.4% for focal males. Additionally, given the strength of last male sperm precedence in fruit flies [54], it would be more advantageous for subordinate males to focus on improving their sperm defense as opposed to offense. This hypothesis is consistent with evidence for a positive correlation between sperm defense success and male-induced harm, but no correlation between sperm offence success and male-induced harm [54]. Future studies should attempt to quantify the Acp profiles and sperm characteristics of winners and losers to untangle the mechanisms underlying this plasticity.

Finally, it appears that some of the differences we observed in various metrics of male reproductive success were due to natural genetic variation present in the population. Although our statistical models only detected significant genetic variation in mating duration and sperm offensive ability, a sizeable proportion of the variances for each of the phenotypes we measured were explained by clone line (Tables 1, 2). As other experiments that focused on detecting genetic variation in traits such as sperm competitive ability have identified significant variation [56], it is likely that we did not have enough power to detect it in our models. Thus, it is still important to note the potential importance and implications of genetic variation for the expression and plasticity of these traits. Similarly, although we did not detect significant variation in the interactions between winner-loser treatment and genotype, our models indicate that a sizeable proportion of the observed variance was due to differences between lines, and the interaction between line and treatment. This means that the magnitude and direction of winner-loser effects on the phenotypes we measured depend on individual genotype [Figures 3, 4], and may explain the maintenance of genetic variation for different male reproductive strategies [57]. If some males improve their reproductive success in a given context as winners, but others improve as losers, then different reproductive strategies can be maintained, even only if one is favoured by female choice.

Taken together, our results indicate that some of the variation we observe in male mating strategies is due to the experience of winning or losing in male-male interactions. The fact that winners appear to focus on pre-copulatory strategies, while losers focus on post-copulatory strategies may represent a trade-off between reproductive strategies that is mediated by winner-loser effects. This variation in mating strategies can influence male reproductive success, and thus the strength and direction of evolution via sexual selection. If winners and losers consistently benefit from investing into pre- and post-copulatory strategies respectively, then this plasticity may be selected for in many species. Given the ubiquity of winner-loser effects throughout the animal kingdom [1], this plasticity may also explain the maintenance of variation in male reproductive strategies in many species. These results also highlight the importance of considering multiple metrics of fitness when exploring the ultimate causes of phenotypic variation. Finally, our results provide groundwork for understanding the biological bases of persisting states of moods associated with winning and losing and their fitness consequences in many animals including humans.

Ethics. The research conducted in this study did not require approval from an ethics committee.

Data accessibility. The data is available on Dryad Digital Repository and can be accessed at https://doi.org/10.5061/dryad.34jc0m7.

Authors' contributions. DCSF carried out the experiments. Both authors designed the experiments, contributed to the data analysis, writing, and editing process of the manuscript. Both authors approve of the final manuscript submission and hold accountability for the accuracy and integrity of its contents.

Competing interests. The authors declare no competing interests.

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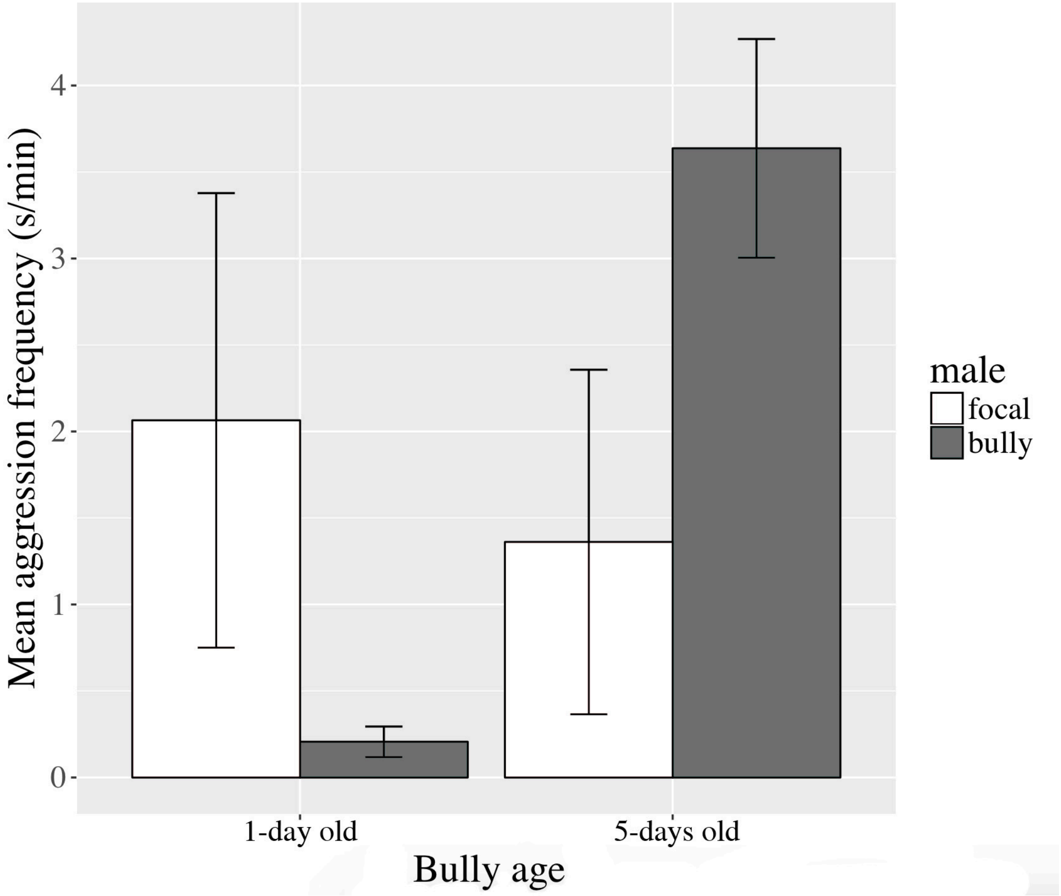


Figure 1: The mean frequency of aggressive behaviour displayed between pairs consisting of a wild-type male (focal) and either a young (1-day old) or mature (5-day old) hyperaggressive male (bully). The error bars represent 1 standard error of each sample. N=46 pairs of males (one focal and one bully).

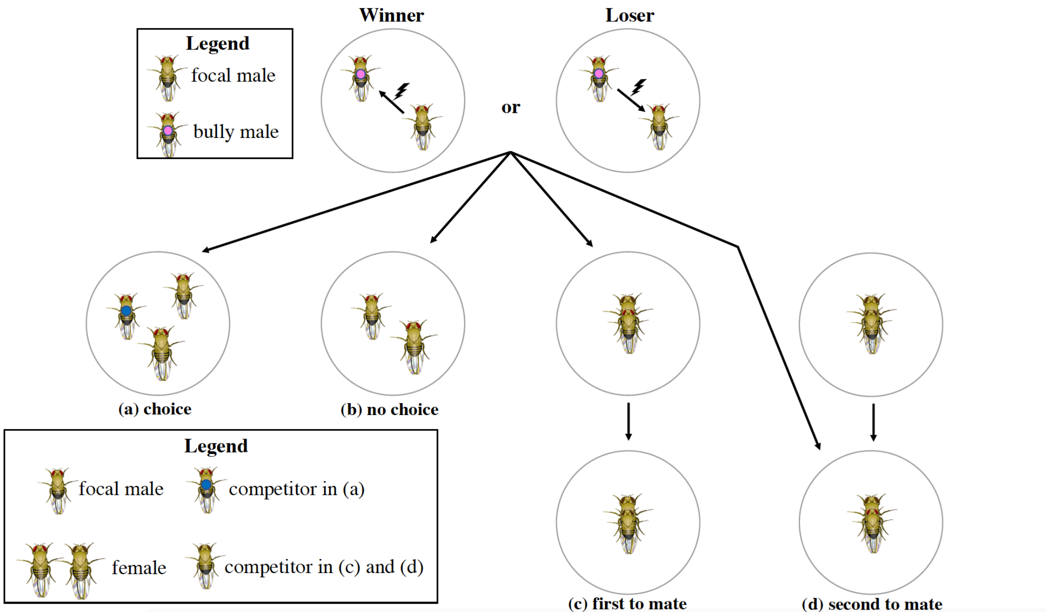
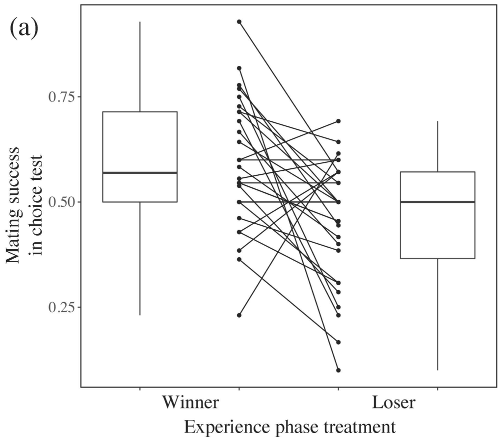
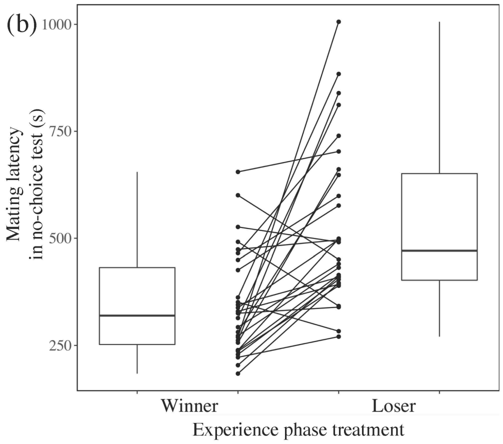


Figure 2: An illustration of the four experiments. In the first phase of all experiments, we placed each focal male inside an arena with either a docile or hyperaggressive competitor, generating winner and loser effects respectively. We then placed each winner or loser focal male in an arena (a) with a new competitor and a female (choice), (b) with only a female (no-choice), (c) as the first male to mate (P1) with a female, or (d) as the second male to mate (P2) with a female.


(a)
Mating success
in choice test
0.75
0.50
0.25
Winner
Loser
Experience phase treatment


(b)
Mating latency
in no-choice test (s)
1000
750
500
250
Winner
Loser
Experience phase treatment

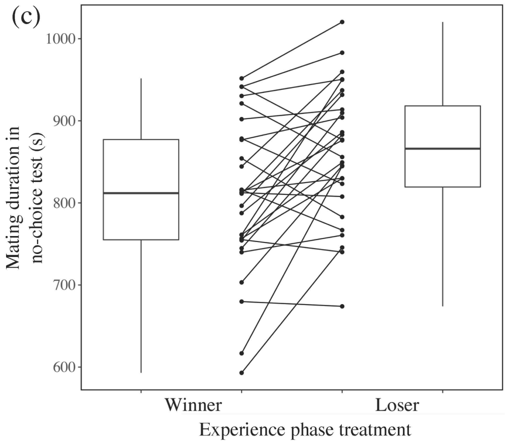
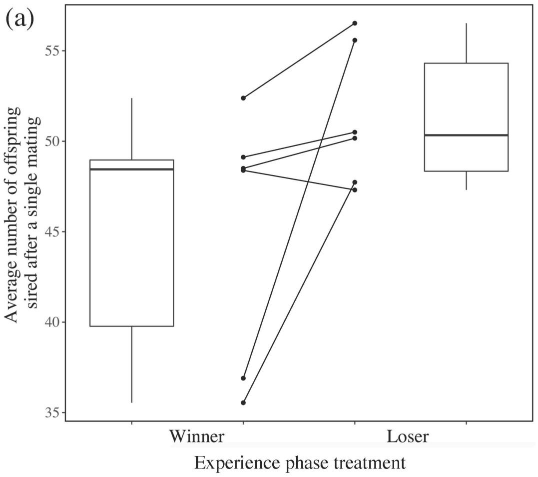
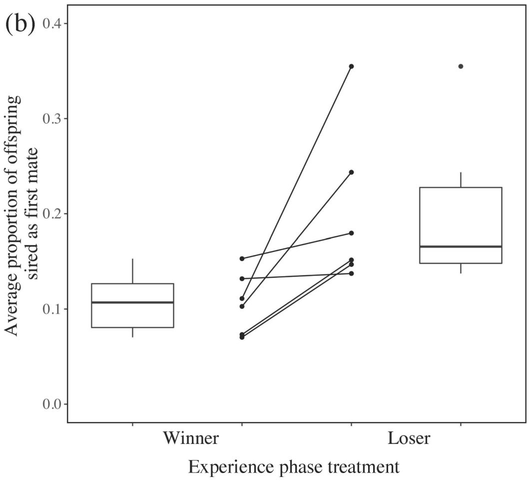

(c)
Mating duration in
no-choice test (s)
1000
900
800
700
600
Winner
Loser
Experience phase treatment

Figure 3: Effects of previous fighting experience on pre-copulatory mating success in males. The boxplots depict the distribution of the entire data set of each experience treatment. The boxes contain the middle 50% of data (interquartile range, IQR), and the horizontal lines represent the medians. The whiskers above and below each box represent values within ±1.5× the IQR. The reaction norm plot in the centre of each panel depicts the change in the calculated mean of each hybrid clone line across the two experience treatments. (a): The mating success of winner and loser focal males in mate choice trials each involving a focal male, a competitor male and a female. (b): The mating latencies of winner and loser focal males in no choice trials each involving a focal male and a female. (c): The mating durations of winner and loser focal males in the no-choice trials.


(a)
Average number of offspring sired after a single mating
55
50
45
40
35
Winner
Loser
Experience phase treatment


(b)
Average proportion of offspring sired as first mate
0.4
0.3
0.2
0.1
0.0
Winner
Loser
Experience phase treatment

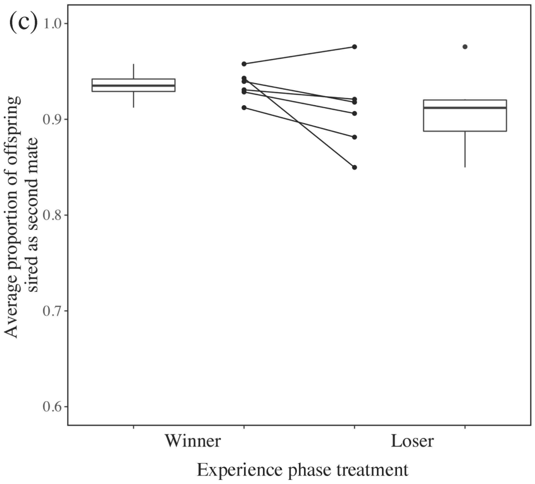
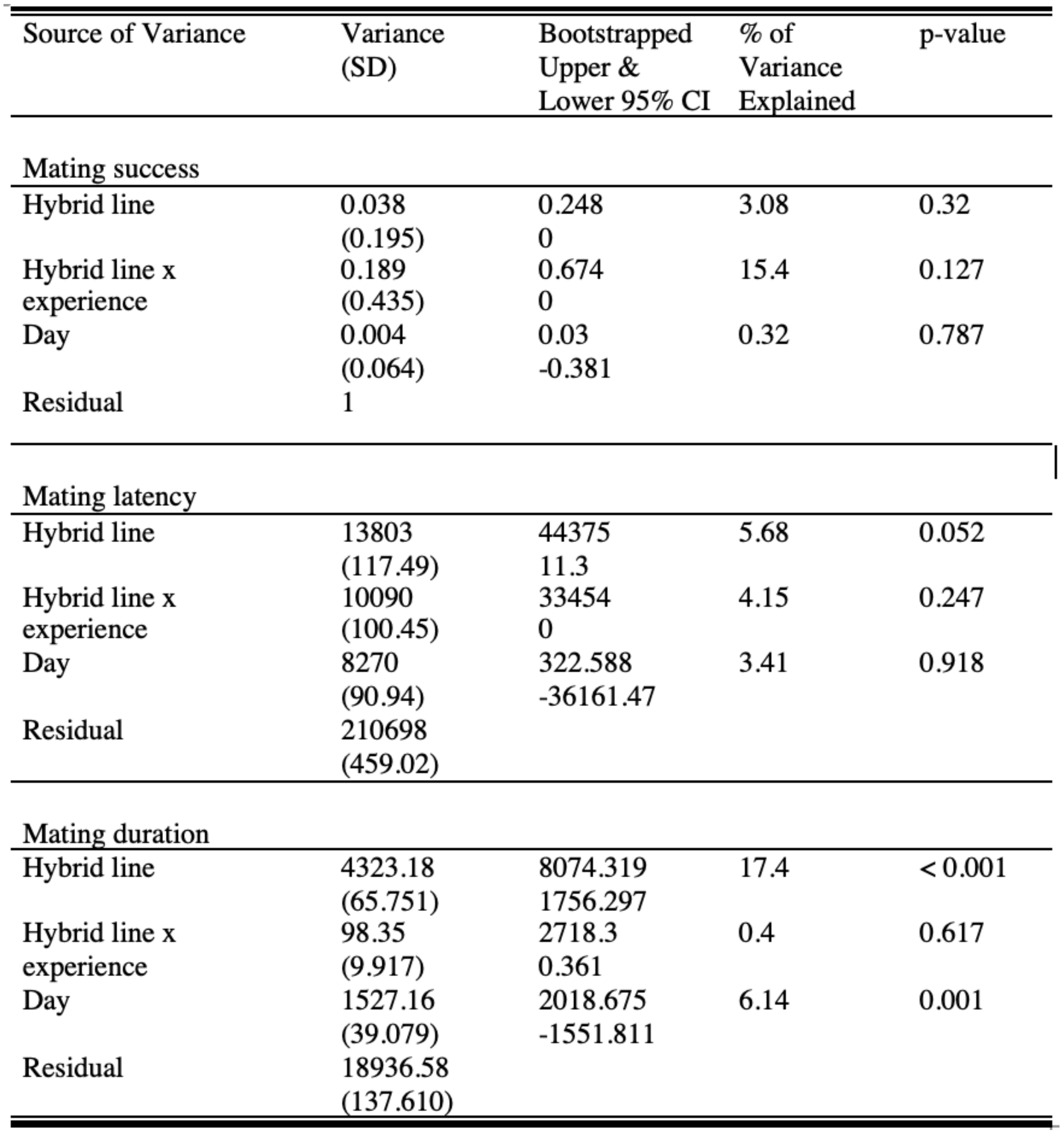

(c)
Average proportion of offspring sired as second mate
1.0
0.9
0.8
0.7
0.6
Winner
Loser
Experience phase treatment

Figure 4: Effects of previous fighting experience on post-copulatory mating success in males. The boxes contain the middle 50% of data (interquartile range, IQR), and the horizontal lines represent the medians. The whiskers above and below each box represent values within ±1.5× the IQR, and any values beyond this are outliers represented by closed circles. The reaction norm plot in the centre of each panel depicts the change in the calculated mean of each hybrid clone line across the two experience treatments. (a): Male effect on a single day of female fecundity after a single mating. (b): Male success in sperm defense assay (focal first male to mate), where success is defined as the proportion of offspring sired. (c): Male success in sperm offense assay (focal second to mate), where success is defined as the proportion of offspring sired.



Source of Variance	Variance (SD)	Bootstrapped Upper & Lower 95% CI	% of Variance Explained	p-value
Mating success				
Hybrid line	0.038 (0.195)	0.248 0	3.08	0.32
Hybrid line x experience	0.189 (0.435)	0.674 0	15.4	0.127
Day	0.004 (0.064)	0.03 -0.381	0.32	0.787
Residual	1			
Mating latency				
Hybrid line	13803 (117.49)	44375 11.3	5.68	0.052
Hybrid line x experience	10090 (100.45)	33454 0	4.15	0.247
Day	8270 (90.94)	322.588 -36161.47	3.41	0.918
Residual	210698 (459.02)			
Mating duration				
Hybrid line	4323.18 (65.751)	8074.319 1756.297	17.4	< 0.001
Hybrid line x experience	98.35 (9.917)	2718.3 0.361	0.4	0.617
Day	1527.16 (39.079)	2018.675 -1551.811	6.14	0.001
Residual	18936.58 (137.610)			

Table 1 Variance components, standard deviation, 95% confidence intervals, and p-values estimated using a generalized linear mixed model (GLMM) fit by maximum likelihood (Laplace approximation) for the reproductive success of hybrid males in pre-copulatory contexts. Males from different genetic backgrounds were randomly assigned as winners or losers.

Source of Variance	Variance (SD)	Bootstrapped Upper & Lower 95% CI	% of Variance Explained	p-value
Fecundity				
Hybrid line	1.052 (1.026)	13.949 0	0.5	0.369
Hybrid line x experience	3.139 (1.772)	24.561 $4.337x10^{-14}$	1.5	0.393
Day	13.487 (3.672)	49.971 0	6.69	0.014
Residual	183.869 (13.560)			
P1 paternity				
Individual	1.76 (1.328)	2.218 1.188	58.7	0.97
Hybrid line	0.055 (0.234)	0.298 0	1.8	0.198
Hybrid line x experience	0.181 (0.344)	0.619 $1.13x10^{-11}$	6	0.225
Day	$1.58x10^{-9}$ ($3.97x10^{-5}$)	0.004 -0.404	0	0.837
Residual	1			
P2 paternity				
Individual	0.971 (0.985)	1.399 0.668	40	0.994
Hybrid line	0.251 (0.501)	0.715 $5.885x10^{-12}$	10.3	0.007
Hybrid line x experience	0.154 (0.391)	0.69 $4.026x10^{-11}$	6.3	0.096
Day	0.051 (0.226)	0.002 -0.599	2.1	0.973
Residual	1			

Table 2 Variance components, standard deviation, 95% confidence intervals, and p-values estimated using a generalized linear mixed model (GLMM) fit by maximum likelihood (Laplace approximation) for the reproductive success of hybrid males in post-copoulatory contexts. Males from different genetic backgrounds were randomly assigned as winners or losers.

CHAPTER 3

Plasticity in male mating behavior modulates female life-history in fruit flies

David C.S. Filice, Rajat Bhargava, & Reuven Dukas

Preface: The following chapter is a manuscript published in *Evolution* and it is written in the style of this journal.

Abstract

In many species, intense male-male competition for the opportunity to sire offspring has led to the evolution of selfish reproductive traits that are harmful to the females they mate with. In the fruit fly, *Drosophila melanogaster*, males modulate their reproductive behavior based on the perceived intensity of competition in their pre-mating environment. Specifically, males housed with other males subsequently transfer a larger ejaculate during a longer mating compared to males housed alone. While the potential fitness benefits to males from such plasticity are clear, its effects on females are mostly unknown. Hence we tested the long-term consequences to females from mating with males with distinct social experiences. First, we verified that competitive experience influences male mating behavior and found that males housed with rivals subsequently have shorter mating latencies and longer mating durations. Then, we exposed females every other day for 20 days to males that were either housed alone or with rivals and subsequently measured their fitness. We found that females mated to males housed with rivals produce more offspring early in life but fewer offspring later in life and have shorter lifespans but similar intrinsic population growth rates. These results indicate that plasticity in male mating behavior can influence female life-histories by altering females' relative allocation to early vs late investment in reproduction and survival.

Introduction

Sexual conflict occurs when the reproductive interests of males and females differ (Parker 1979; Chapman et al. 2003a; Arnqvist and Rowe 2005). This is predicted to result in the evolution of selfish male traits that are harmful to females, and in response, the coevolution of female traits that resist this harm (Rice 1996; Wigby and Chapman 2004). Empirical and theoretical studies have demonstrated the importance of sexual conflict in driving the evolution of dimorphism between the sexes, variation in mating tactics, and even speciation (Arnqvist 1998; Parker and Partridge 1998; Gavrilets and Waxman 2002; Martin and Hosken 2003). Thus, understanding the ecological and genetic factors that influence the expression of sexually antagonistic traits is of great interest to evolutionary biologists.

Theory predicts that the intensity of conflict between the sexes increases with the degree of promiscuity in a mating system, where the magnitude of promiscuity may be regulated by the optimal mating rate of females or by the amount of male-male competition (Chapman et al. 2003). This theory has been tested by experimentally evolving populations under manipulated levels of sexual selection (Holland and Rice 1999; Hosken et al. 2001; Crudgington et al. 2009). Holland and Rice (1999) generated divergent populations of fruit flies, *Drosophila melanogaster*, by either enforcing monogamy or maintaining promiscuity for 47 generations. Their results suggest that females mated to males descended from populations evolved under enforced monogamy live longer and have a greater reproductive rate compared to their counterparts from populations that evolved under promiscuity. This makes sense, because when monogamy is enforced, selection does not act on harmful male traits integral for success in male-male competition, such as persistent courtship or large investment into the transfer of accessory gland proteins (Acps), resulting in the evolution of male mating phenotypes that are less harmful to females (Chapman et al. 1995; Friberg and Arnqvist 2003; Wigby and Chapman 2005; Hollis et al. 2019). Furthermore, analysis of the natural genetic variation in male competitive ability reveals a similar trend. Civetta and Clark (2000) compared the relationship between success in male-male competition and male-induced harm across 51 distinct genetic backgrounds, and found that males from genetic backgrounds with higher sperm defensive ability also tended to be more harmful to their mates. In sum, these studies provide evidence that there is a direct relationship between the magnitude of the expression of male traits that influence intra-sexual competitive success and the amount of harm inflicted on females via mating.

Although the studies that have explored the relationship between mating system dynamics and male harm are critical for our understanding of the expression of male-induced harm and their consequences for female fitness, they were typically conducted in uniform environments, leaving out the important roles of variation in social and ecological factors (Arbuthnott et al. 2014). Recent theoretical work, however, predicts that phenotypic plasticity in sexually antagonistic traits can either strengthen or weaken the intensity of sexual conflict (McLeod and Day 2017; Day and McLeod 2018). In the past few years, several studies have highlighted the importance of considering these socio-ecological effects when quantifying the intensity of sexual conflict (Perry and Rowe 2018). These studies suggest that environmental factors such as space availability and complexity, predation risk, and population density can modulate the expression and/or evolution of sexually-antagonistic traits and thus the magnitude of male-induced harm (Yun et al. 2017; Gomez-Llano et al. 2018; García-Roa et al. 2019). For example, García-Roa et al. (2019) demonstrated that plasticity in male-induced harm can be modulated by temperature, whereby females exposed to males at 29°C had shorter lifespans and fewer lifetime offspring compared to females that mated with males at 25°C or 21°C. Although these studies highlight the importance of considering a broad range of socio-ecological factors when measuring the intensity of sexual conflict, to the best of our knowledge, no experimental studies have tested how the modulation by social experience of male reproductive traits influences the magnitude of male-induced harmful effects on females.

In species where males mate multiply, males should be prudent with their degree of investment into mating opportunities because of the costs associated with the production of sperm and other features that aid in sperm competition (Parker et al. 1997; Parker and Pizzari 2010). Thus, males are highly sensitive to cues in their socio-sexual environment that indicate the likely number of mating opportunities and/or the intensity of competition (Bretman et al. 2011a). In particular, the presence of rivals in the social environment has proven to increase the expression of traits involved in intrasexual competition (Aragón 2009; Bailey et al. 2010; Bretman et al. 2011b; Kelly and Jennions 2011). These responses are phylogenetically widespread, as species across many taxa (insects, reptiles, birds, mammals) alter the size and composition of their ejaculates in the presence of a single rival (Kelly and Jennions 2011). In the fruit fly, a model species extensively used to study both behavioral plasticity and sexual conflict, males that are housed with rivals prior to a mating opportunity mate for longer and transfer larger volumes of ejaculate containing more sperm and some accessory gland proteins (Acps) during copulation compared to males housed alone (Bretman et al. 2009; Wigby et al. 2009; Fedorka et al. 2011; Moatt et al. 2014). In general, the Acps transferred during mating have positive effects for males and can have both positive and negative effects on female fitness. This plasticity has fitness benefits for males as increased sperm and Acp transfer can result in increased number of offspring produced (Bretman et al. 2009, 2013), and sometimes also paternity share (in Bretman et al. 2009 but not in Bretman et al. 2013). Although the increased number of absolute offspring represents a short-term benefit for females, the long-term repercussions of this plasticity for female fitness remain unclear. In a short-term context, certain Acps such as sex peptide and ovulin stimulate offspring production and delay remating rate (Fricke et al. 2009; Wigby et al. 2009). However, exposure to the same Acps reduces female long-term fecundity and lifespan (Johnstone and Keller 2000; Wigby and Chapman 2005), and it is unclear if plasticity in male behaviors can influence these long-term fitness costs.

To address this issue, we tested whether plasticity in male traits that lead to increased siring success under intra-sexual competition have a negative long-term influence on the females that they mate with in terms of lifetime offspring production and survival. First, we replicated previous work (Bretman et al. 2009, 2013) to verify that males alter their expression of sexually-antagonistic traits in response to perceived sperm competition. Specifically, we predicted that males under perceived competition would be quicker to mate, mate for longer durations, and delay the remating interval of their mates for longer than males kept alone. Second, we tested whether perceived sperm competition influences the magnitude of harmful effects on the females that males mate with. We predicted that males housed with rivals would reduce their mates' lifetime offspring production and longevity more than males housed alone. Additionally, we predicted that females mated to males that perceive sperm competition risk would have lower intrinsic population growth rates, a rate-sensitive fitness measure that takes into account both lifetime reproductive success and lifespan. In order to test the robustness of any effects detected, we replicated our test in two distinct populations of fruit flies. Finally, we tested males from multiple genotypes in order to quantify the degree of genetic variation associated with the plasticity of male response to intra-sexual competition and the potential variation in subsequent effects on female fitness.

Methods

Fly stocks and general

All focal males descended from 28 lines of the *Drosophila genetic reference panel* (DGRP) (Mackay et al. 2012). These lines were derived from wild flies caught in Raleigh, North Carolina, USA, and repeatedly inbred for 20 generations. In order to alleviate the deleterious phenotypic effects associated with inbreeding, we generated hybrid genotypes by crossing each line to a randomly selected standardized reference line, thereby creating unique hybrids (hereafter referred to as hybrids). Within each hybrid, individuals are genetically identical, but between hybrids, individuals share an identical clonal haplotype inherited from their mother, and a unique clonal haplotype inherited from their father, allowing us to quantify the degree of genetic variation associated with phenotypic differences expressed from this unique haplotype.

Focal females tested in the remating assay (part 1) and the first replicate of the fitness assay (part 2) were descendants of a wild-caught population of flies collected from multiple locations throughout southern Ontario in August 2014 (hereafter ON) (Baxter and Dukas 2017). Since its establishment, we housed this population in two cages each measuring 20 x 20 x 35 cm and containing several hundred flies maintained with overlapping generations, meaning that each fly lived in the cage until natural death, and had the opportunity to produce multiple generations of offspring.

In the second replicate of the fitness assay, we used females derived from the *Ives* population (hereafter IV) obtained from the Long Lab (Wilfrid Laurier University, Waterloo, Ontario, Canada). The IV population was originally collected in South Amherst, MA, USA in 1975. In 1980, Michael Rose created a lineage of this population that has been maintained at large census size (>1000 adults/generation) and on a standardized culture protocol with non-overlapping generations (Rose 1984). Since then, this same lineage of IV has been maintained under identical conditions and used extensively as a model for studying longevity and sexual conflict (Rose 1984; Martin and Long 2015; Filice and Long 2016). Unlike the ON population, when the IV females are 4 days post-eclosion, they have a single 24h window to lay their eggs for the next generation.

Competitor males for the focal males in both parts 1 and 2 were descendants of a sub-population of the IV line that had the *e* (hereafter, ebony) mutation introgressed. This mutation results in a darker body colour that is clearly visible with the naked eye, allowing us to easily identify the focal hybrid male during our trials. Although these males tend to be at a competitive disadvantage to wild-type flies, the phenotype is naturally-occurring and confers a selective advantage in some contexts (Pool and Aquadro 2007; Takahashi et al. 2007). Regardless, given that these flies were standard competitors, any competitive disadvantages would be consistently realized across trials and should not impact our results.

We reared all experimental flies at a standardized density of 100 eggs per vial containing ~5 ml of standard fly medium made of water, sucrose, cornmeal, yeast, agar and methyl paraben, and stored all flies in an incubator at 25 °C and 60% relative humidity with a 12:12h light:dark cycle. We collected newly eclosed virgin flies within 8h of eclosion under light CO_2 anesthesia. Following their initial collection, we handled all flies using gentle aspiration.

Part 1: Effects of perceived competition on mating latencies and durations, and females' remating rates

We started each replicate by collecting 4 focal males from each of the 28 hybrid genotypes. We placed 2 of these males individually into a vial alone, and 2 individually into a vial each containing 2 competitor ebony males. Simultaneously, we collected females and housed them in groups of 20 with a dash (~10 mg) of live yeast. Three days later, which is a sufficient amount of time to induce a strong response to rivals (Bretman et al. 2011b), we paired each focal male with a single virgin female in fresh vials containing a dash of live yeast. Observers blind to treatment scored the mating latency and duration to the nearest second. We discarded and replaced pairs that did not mate within 90 minutes. To prevent multiple matings, we removed males immediately after each mating concluded. We kept females in these vials and returned them to their environmental chamber.

The following morning, we introduced a new wild-type male to each female, and observers blind to treatment measured the latency and duration of all matings. We observed the flies for 4h and classified the females that did not mate by this point as "not remated". We repeated the entire above procedure in 6 identical replicates over 12 days, resulting in 12 replicates/male hybrid/treatment, except in the case of missing trials. Missing trials included cases in which we were unable to collect a sufficient number of hybrid males, and instances of female escape or death, resulting in a final sample size of N = 542 trials.

Part 2: Fitness assay

We collected 120 wild-type females and housed them in groups of 20 in vials that contained a dash of live yeast. Simultaneously, we collected 20 focal males from 6 hybrid backgrounds (a random subset from the original 28 used in part 1). We placed 10 of these males into vials alone, and 10 into vials each containing 2 competitor ebony males (Fig. 2). Three days later, we placed each female into an individual food vial with a dash of live yeast and paired her with a single male from one of the two experience treatments. Observers blind to male hybrid identity and experience treatment scanned the pairs of flies for 3h and recorded the latency and duration of each mating. To prevent multiple matings, we removed the males from each vial after the first mating concluded or after 3h if no mating occurred. We placed the females in the environmental chamber and allowed them to lay their eggs undisturbed for 45h. Following this period, we moved each female into a fresh food vial with live yeast, and paired her with a new 3-day old male from the same treatment and line combination as before. Again, observers blind to male hybrid identity and treatment recorded the latency and duration of any matings, and we removed males following a mating or after 3h had passed. We repeated this procedure every other day over 20 days, meaning that each female had ten 3h opportunities to mate with a male (Fig. 2). After the tenth mating opportunity, we transferred females into fresh vials with live yeast every 5 days until they died. We checked for mortality every morning at the same time, until all females died.

Two weeks following each testing day, observers blind to treatment counted the number of offspring in each vial. Overall then, for each female alive through age 24 days, we had 10 offspring vials. The offspring of females that died before day 24 were only counted up until the day that the females died. In other words, we dropped females from analysis after mortality, rather than counting their offspring production as zero. We performed two replicates of this procedure, one with ON females (N=120) and the other with IV females (N=120).

Statistical analysis

We conducted all data analysis using R version 3.5.2 (R Core Team 2013). To analyze the effect of perceived competition on male mating behavior, we constructed generalized linear mixed models (GLMMs) using the *lme4* package (Bates et al. 2014). We analyzed mating latency and duration using a gaussian distribution, and rematings using a binomial distribution. Our maximal models included male treatment as a fixed effect, and hybrid genotype, day of testing, and all possible interactions as random effects. However, we simplified our models until we had no singular fits. In all three cases, our simplified models excluded the interactions with day effect. We calculated the 95% confidence intervals of our random effects by using the *bootMer* function to re-simulate our models 1000 times.

In our fitness analysis, we analyzed female mating frequency using a GLMM with a binomial response variable defined by the total number of matings weighed by the number of mating opportunities. We included male treatment and female population as fixed effects, and male genotype as a random effect. To analyze female offspring production, we constructed a GLMM with a negative binomial response in order to deal with overdispersion. We included male treatment, female population, and female age as fixed effects, and hybrid genotype with all possible crossed interactions as random effects. To account for repeated measures, we also included individual female identity as a random effect. Similar to our other GLMMs, we simplified our models until there were no singular fits. In this case, our simplified model excluded the interactions with hybrid genotype. To analyze female lifespan, we constructed a mixed effects Cox survival model. This included lifespan as a survival term, male treatment and female population as fixed effects, and male hybrid genotype as a random effect. Finally, we calculated a measure of fitness for each individual female, intrinsic population growth rate (λ). This is a rate-sensitive measure that gives more weight to offspring produced earlier in life (McGraw and Caswell 1996) and is most relevant in expanding populations (Gilbert and Charlesworth 1981). λ is calculated by placing individual life-history data (offspring production and survival) into a Leslie matrix, and calculating the dominant eigenvalue of each matrix (McGraw and Caswell 1996). To analyze differences in λ, we constructed a GLMM with male treatment and female population as fixed effects, and hybrid genotype with all possible crossed interactions as random effects. We calculated the p-values of the fixed effects in all of our above models using the *Anova* function from the *car* package (Healy 2005).

Results

Part 1: Effects of perceived competition on mating latencies and durations, and females' remating rates

Males housed with rivals had shorter mating latencies than males housed alone (an average of 7.83 minutes faster; $\chi^2 = 11.3$ df = 1 $p = 0.0007$; Fig. 1a). There was no significant variation in mating latency between different hybrid backgrounds (SD = 111.9), but the interaction between hybrid genotype and experience treatment (SD = 236.7) was significant (Fig. 1a, Table 1). Males housed with rivals mated for significantly longer compared to males housed alone (an average of 2.04 minutes longer; $\chi^2 = 33.3$, df = 1, $p < 0.0001$; Fig. 1b). Males from different hybrid backgrounds (SD = 57.1) varied significantly in their mating duration, but the interaction between genotype and experience (SD = 0) was not significant (Fig. 1b, Table 1). There was a marginally non-significant trend whereby females mated to males housed with rivals were less likely to remate a day later (an average of 7.2% less frequently; $\chi^2 = 3.2$ df =1, $p = 0.0727$; Fig. 1c). The effects related to hybrid genotype (SD = 0.342) and the interaction between genotype and experience treatment (SD = 0.204) were both significant in influencing females' likelihood to remate (Fig. 1c, Table 1).

Part 2: Fitness assay

Male treatment did not have a significant effect on the proportion of times each female remated ($\chi^2 = 0.0057$, df = 1, p = 0.94), but females from the IV population mated significantly more frequently than females from the ON population ($\chi^2 = 79.7$, df = 1, $p < 0.0001$; Figure 3). The interaction between these two factors was not significant ($\chi^2 = 0.043$, df = 1, p = 0.837), nor was the random effect of male genotype (SD = 0).

Male treatment did not have a significant effect on the number of offspring females produced over the 10 egg laying periods ($\chi^2 = 0.444$, df = 1, p = 0.505). However, the interaction between male treatment and time period was significant ($\chi^2 = 7.84$, df = 1, p = 0.005). Over time, the slope in the rival treatment is more negative compared to the slope in single treatment, indicating that females mated and remated to males previously housed with rivals had more offspring early in life and fewer offspring later in life than females mated and remated to males previously housed alone (Fig. 4). The effects of day of egg laying ($\chi^2= 111.7$, df = 1, $p < 0.0001$) and the population of females being tested ($\chi^2= 38.9$, df = 1, p = < 0.0001) both had a strong effect. The interactions between day and female population ($\chi^2= 292.5$, df = 1, $p < 0.0001$) was also significant, but the interactions between male treatment and population ($\chi^2= 2.48$, df = 1, p = 0.116) and male treatment x female population x day: $\chi^2= 0.584$, df= 1, p = 0.445) were not. The effect of male genotype on female offspring production was small and not significant (SD = $3.8x10^{-5}$).

Females mated to males housed with rivals lived significantly shorter than females mated to males housed alone ($\chi^2 = 4.5$, df = 1, p = 0.034; Fig. 5a). While females from the ON population lived much longer than females from the IV population ($\chi^2 = 78.4$, df = 1, $p < 0.0001$), the interaction between experience and population was not significant ($\chi^2 = 0.137$, df = 1, p = 0.711). The random effect of male genotype represented a small, non-significant proportion of the variance in female lifespan (SD = 0.098).

Finally, females mated to males from different social treatments did not significantly differ in their fitness when measured in terms of intrinsic population growth rate (λ) ($\chi^2 = 1.17$, df = 1, $p = 0.277$; Fig. 5b), but females from the IV population had significantly higher fitness compared to the ON population ($\chi^2 = 279.6$, df = 1, $p < 0.0001$). The interaction between the two was not significant ($\chi^2 = 0.007$, df = 1, $p = 0.933$), and the amount of variance explained by male genotype was negligible (SD $= 9.7 \times 10^{-7}$). When looking at the relationship between lifespan and population growth rate, there was a strong negative correlation between the two metrics ($\rho = -0.283$, S = 2882300, $p < 0.0001$; Fig. 5c)

Discussion

Here, we replicated the results of previous studies, first by documenting that male fruit flies exposed to rivals mate for longer compared to males housed alone (Fig. 1b; Bretman et al. 2009, 2010, 2011b, 2013; Wigby et al. 2009) and second, by finding significant genetic variation in mating duration (Fig. 1b; Fiumera et al. 2007). Our study is the first to report that the changes induced by a male's experience with rivals have a significant effect on the life-history of his mates by 1.) stimulating early-life reproduction at a cost of decreased late-life reproduction (Fig. 4), and 2.) reducing their lifespan (Fig. 5a). However, our estimates of intrinsic population growth rates suggest that the later life costs imposed on females of multiply mating with males that perceive sperm competition risk are balanced out by the early life benefits, contrary to our prediction (Fig 5b). The results from other studies have demonstrated that the context in which mating interactions take place can influence the magnitude of male-induced effects on female fitness (Arbuthnott et al. 2014; Yun et al. 2017; García-Roa et al. 2019). We add to these findings by documenting that the perception of sperm competition risk in a male's social environment can elicit phenotypic changes in his mating behavior that have significant consequences for their mates' life-histories.

When looking at the effect of competitive experience on male mating behavior, our results were mostly consistent in direction with those of similar studies. We found that, on average, males exposed to rivals had shorter mating latencies (Fig. 1a), longer mating durations (Fig. 1b), and tended to reduce the remating rate of their mates compared to males that were not exposed to rivals (Fig. 1c). In fruit flies, mating duration is positively associated with the total amount of seminal fluid transferred (Wigby et al. 2009), and studies have consistently found that increased sperm competition risk results in a greater investment into mating duration (Bretman et al. 2009, 2013; Wigby et al. 2009). This result is intuitive, as males that invest more into the donation of Acps may increase the short-term fecundity of their mates, and have increased success in securing paternity via sperm competition (Hollis et al. 2019). Here, we also identified significant genetic variation in male mating duration, but not in the interaction between genotype and male treatment. In other words, different male genotypes varied in their mating duration, but the change in duration between experience treatments was relatively consistent across genotypes. This is an interesting result, as it suggests that males vary in their investment into the transfer of Acps during mating, but are consistent in adjusting their investment in response to the presence of rivals. Given the evidence for genetic trade-offs in different male reproductive strategies such as between male-male competition and the ability to simulate oviposition (Filice and Long 2018; Nguyen and Moehring 2019), future studies should continue to explore how investment into the production and transfer of Acps are genetically correlated with other male traits.

Unlike the persistent findings about mating duration, the documented effects of perceived competition on mating latency have been mixed. Bretman et al. (2009) found no significant difference in mating latency between males housed alone or with rivals, but Bretman et al. (2013) found that males housed alone were quicker to mate. Here, we reported that males housed with rivals were quicker to mate. It is possible that the mixed results reflect variation in the dominance hierarchies (which are rapidly formed when multiple males are placed in a vial) and the subsequent types of male-male interactions that occur during the male experience phase (Filice & Dukas, 2019). In fruit flies, males who have won a previous fight tend to have shorter mating latencies compared to losers (Filice and Dukas 2019). In previous experiments where no difference in latency was found, males housed with rivals were randomly selected out of each vial and then tested (Bretman et al. 2009). Here, we selected from each vial a predetermined focal male that was housed with standard ebony competitors. Thus, assuming that each vial has a single dominant male, the variance in male status is higher when there are 3 or more possible focal males in a vial, whereas in our protocol, each focal male was probably of more similar status when housed with two standard males. It is also possible that the ebony competitors are at a general competitive disadvantage to the focal males, resulting in more frequent winner-effects among our focal flies (Takahashi et al. 2007). This mechanism could also explain the significant interaction that we observed between hybrid genotype and male treatment, if males of some genotypes are more likely to be the dominant males than males of other genotypes. Finally, although it was not significant, males exposed to competitors decreased the sexual receptivity of their mates slightly more than males housed alone, and we add to previous findings by identifying significant genetic variation in this effect (Fiumera et al. 2007). Similar to the genetic variation we identified in mating duration, this result suggests that males vary in the quality and/or quantity of Acps transferred during mating depending on their genetic background (Fiumera et al. 2007). Furthermore, the significant interaction between male treatment and genotype indicates genetic variation in the plasticity associated with experience-dependent manipulative male tactics like the delaying of remating. In other words, some males may switch to strategies that involve delaying remating when there is a risk of sperm competition, while males from a different genetic backgrounds may not.

When looking at the effect of male-competitive experience on female fitness, we found that females mated to males previously exposed to competitors invested more into early life reproduction at a cost of decreased later life reproduction and shorter lifespans. Specifically, females from different experience treatments varied in the amount of offspring they produced over time, characterized by females mated to competitor males having higher production early in life, but lower production later in life (Fig. 4). This result is consistent with the finding that females mated to males housed with rivals tend to lay more eggs in the 24h following a single mating (Bretman et al., 2009), but offers new insight into a potential late-life reproductive cost associated with this effect. Similar studies to our own have found that females trade-off their lifetime reproduction for early-life reproduction in response to different experiences with males, but we are the first to show that male perception of sperm competition risk can induce this effect (Crudgington et al. 2010; Edward et al. 2011). For example, Edward et al. (2010) found a consistent trend, whereby females with a high exposure to males produced more offspring in the first 8 days of their life but produced fewer offspring throughout the remainder of their lives, compared to females that had a low exposure to males (Edward et al. 2011). In both cases, it is likely that the females received more sex peptide and ovulin, Acps that stimulate egg production. In our study, males exposed to rivals probably transferred a greater volume of Acps (Wigby et al. 2009), and in Edward et al. (2010), females with a high exposure to males mated more often and thus received more Acps, which is consistent with a study that shows males from populations that evolved under high competition deplete their ejaculates faster (Linklater et al. 2007). In our study, males that perceive the risk of sperm competition can benefit from stimulating their mates to produce as many offspring as they can in the short-term because of the high likelihood that they will lose paternity to males from subsequent matings.

Although this increase in early-life reproduction appears to be costly for a female's reproductive potential later in life, these later life costs appear to balance out the early-life benefits as females mated to rival-exposed and single males have similar intrinsic population growth rates. This result suggests that females may greatly benefit from a single mating with a male that transfers more egg-stimulating Acps, but repeated exposure to these males may result in long-term costs. As short-term reproduction is highly important for fitness in species with life-histories similar to *D. melanogaster*, this may have major implications for female mate choice. In *D. melanogaster*, males that are detrimental to long-term female fitness also tend to be preferred (Friberg and Arnqvist 2003). However, in the study just mentioned and many others, females are consistently housed in small vials, which may result in more harassment and matings than would naturally occur (Pitnick and García-González 2002; Crudgington et al. 2010; Edward et al. 2011). Thus, it may be that in natural settings, the costs associated with prolonged exposure to males that stimulate short-term offspring production may never be realized and males that are deemed as "harmful" in laboratory settings would actually have a net-benefit for females in nature. It is therefore important that future studies consider their methodologies when making predictions about the fitness effects of sexually antagonistic interactions (Yun et al. 2017).

It is also interesting to note the large differences in the average number of offspring produced over time and in intrinsic population growth rate between the ON and IV populations, effects that are almost certainly due to the maintenance protocols and consequent evolved life-histories of these populations. Our IV population has been maintained with non-overlapping generations for hundreds of generations, and females of this population only have a single 24h window when they are 4-days old to lay their eggs for the next generation. Therefore, these flies have been selected to invest as much as they can into early-life reproduction. On the other hand, the ON was recently caught (2014) and has been maintained with overlapping generations, meaning that flies can produce offspring throughout their life. The consequences of this are clear when looking at the average number of offspring produced over time (Fig. 4) and is represented by the significant interaction between population and day. Similar to the interaction between male treatment and day, the large investment into early-life reproduction of the IV population is associated with decreased later life reproduction, compared to the ON population.

The shorter lifespan of females mated to males exposed to rivals may be explained by a larger donation of Acps during matings from these males, as it is well known that exposure to Acps is associated with reduced longevity in female fruit flies (Chapman et al. 1995; Wigby and Chapman 2005). A potential mechanism is that the increased investment into early reproduction results in an increased rate of senescence (Bretman and Fricke 2019). In fruit flies, females that have a genetic propensity to mate more produce more offspring early in life and die younger (Chapman et al. 1995; Travers et al. 2015), and lineages of the IV population that were artificially selected for increased longevity displayed a decreased investment into early-life offspring production and increased late-life production compared to the base population (i.e. a trend similar to the IV population in Fig. 4) (Rose 1984). Furthermore, the negative correlation we identified between lifespan and population growth rate indicates that females who produced the most offspring early on tended to die younger (Fig. 5c). Bretman and Fricke (2019) reported that female longevity and the onset of senescence is not influenced by the receipt of sex peptide, but females with more exposure to males (i.e. more matings) have reduced longevity and an accelerated expression of senescent decline in traits such as climbing speed and starvation resistance. Although sex peptide on its own appeared to have no detectable effect on female longevity, it may be that other Acps transferred during mating mediate the harmful effects of mating such as those related to stimulating reproduction (Bretman and Fricke 2019). Given that, in our trials, we detected no significant difference in the average number of matings between females exposed to males housed either alone or with rivals, it is likely that the differences we observed in female longevity are due to variation in the total amount of some Acps donated during each mating, or due to variation in other factors such as increased behavioral harassment, risk of transmitting infection, or weaker immune response to infection (Schwenke and Lazzaro 2017). Future studies should continue to investigate the individual and cumulative effects of different Acps to determine their effects on female postmating phenotypes.

Similar to offspring production, we also reported a large difference in lifespan between the two populations we tested, whereby ON females lived much longer than IV females (Fig. 5a). Again, this difference is expected given the maintenance schedule and consequent life-histories of these populations. Since the IV population has been maintained with non-overlapping generations, females have been selected to maximize early life reproduction, and traits associated with survivorship past 4 days of adulthood are less important. Specifically, the difference in female survivorship and early-life offspring production between the populations may be manifested due to a 50% higher number of matings in the IV than ON females (Fig. 3). Multiple matings in the IV population increase early-life fecundity (Filice & Dukas, unpublished data), so females likely have a high propensity to mate multiply despite the potential long-term costs that this population has not experienced. In some regard, the survivorship difference we observed between the populations can simply be viewed as a version of the differences that we observed between females mated to males from different experience treatments (Fig. 5a), as both types of differences are probably driven by “live-fast, die-young” life history strategies.

Taken together, our results have important implications for our understanding of how the context of a social environment influences the life-history strategies of males and females, and more broadly the sexual interactions that occur between males and females within a population. Specifically, we found that males that perceive high levels of sperm competition selfishly alter their mating behavior in a way that influences the amount of harm to their mates expressed as reduced survival and late-life reproduction. In terms of lifetime fitness, however, the consequence of this harm appears to balance out due to increased early-life benefits associated with offspring production. We propose that much of the identified male harm to females in sexual conflict research is due to this "produce-my-offspring-fast, die-young" manipulation, which varies in intensity depending on a male's experience. Future studies should continue to explore how environmental variation such as the sociosexual landscape influences the expression of mating behavior in order to improve our understanding of how sexual selection shapes the evolution of behavioral phenotypes as well as how plasticity in sexual behavior affects sexual selection and evolution.

Authorship Contributions:

DCSF and RD designed the experiments. DCSF and RB carried out the experiments. DCSF wrote the first draft and performed the statistical analysis, and all authors were involved in the revision process.

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Conflict of Interest Statement

The authors declare no conflicts of interest.

Data accessibility:

Data available from the Dryad Digital Repository: doi:10.5061/dryad.0k6djh9wn

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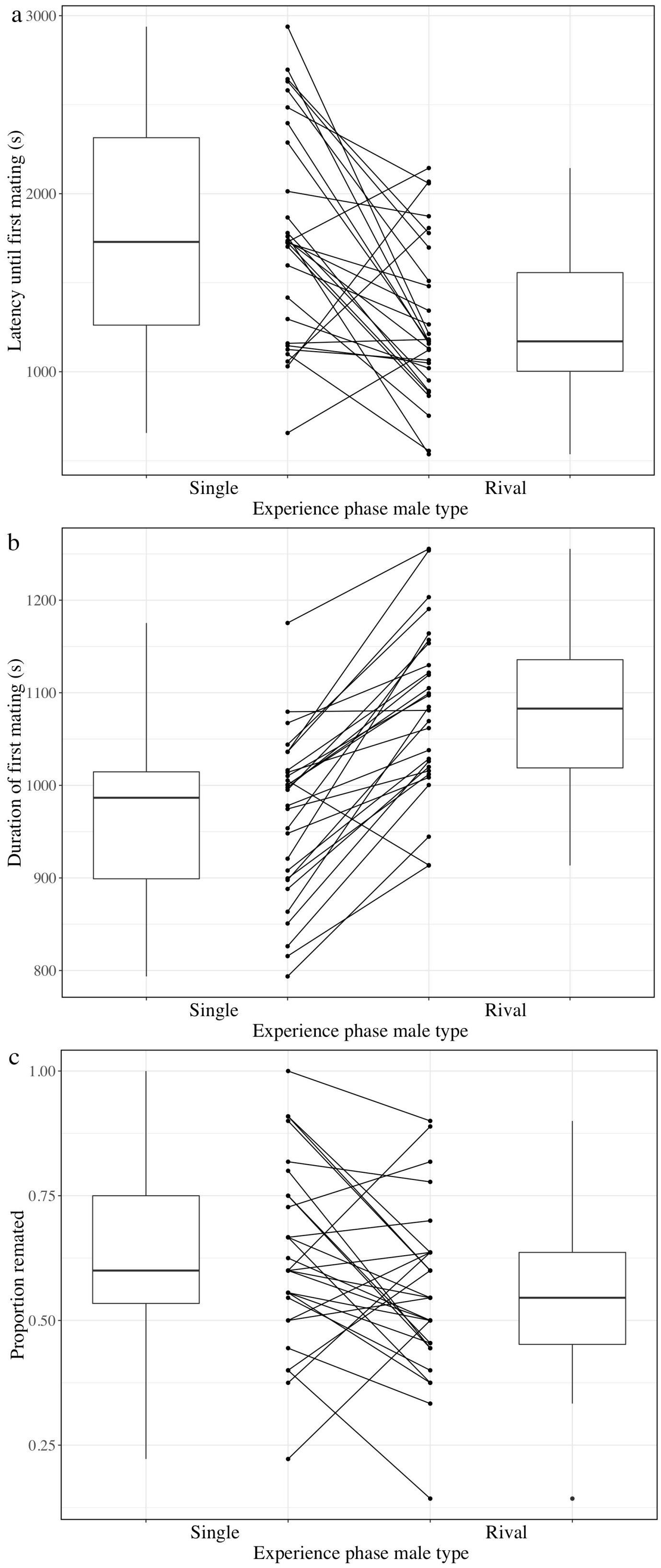
a
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Latency until first mating (s)
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Figure 1: Effects of previous exposure to rivals on male mating phenotypes. The boxes contain the middle 50% of data (interquartile-range, IQR), and the horizontal lines represent the medians. The whiskers above and below each box represent values within ±1.5 IQR, and any values beyond this are outliers represented by closed circles. The reaction norm plot in the centre of each panel depicts the change in the mean of each hybrid genotype across the two experience treatments. a.) Mating latency with virgin females. b.) Mating duration with virgin females. c.) Mating rate of second male with mated females (i.e. effect of first mating on subsequent female receptivity).

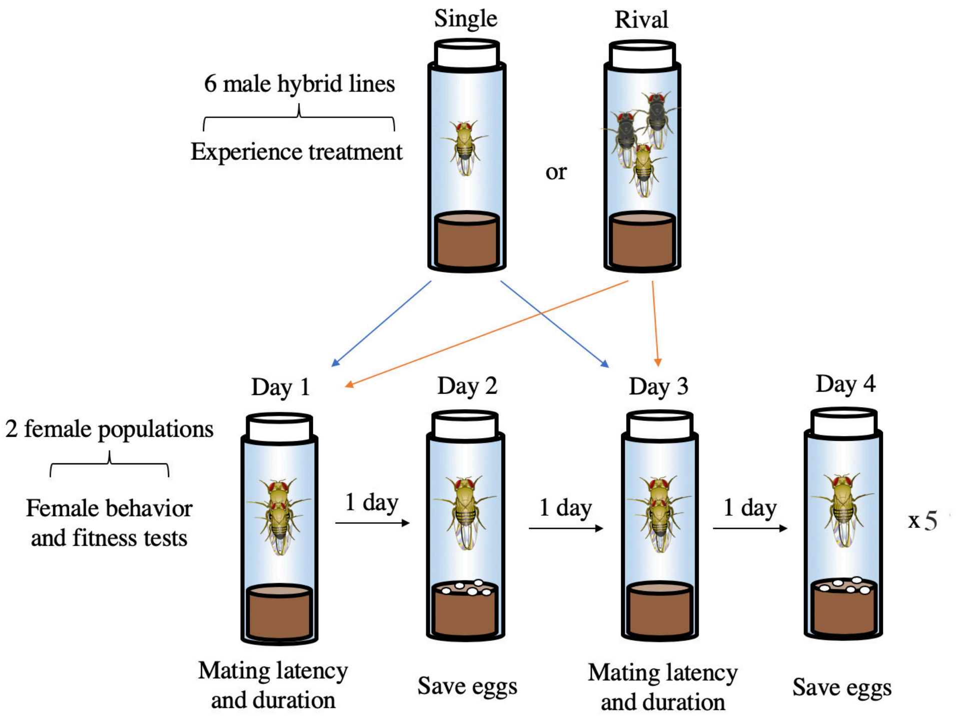


Figure 2: An illustration of female experience in the fitness experiment. Females from one of two different populations were exposed to a focal male that was either previously housed alone or with two competitors. After three hours, males were removed and females were left alone for a day and then exposed to another male from the same initial treatment. This was repeated a total of 5 times, resulting in a total of 10 brief exposure periods to males from one of the two treatments.

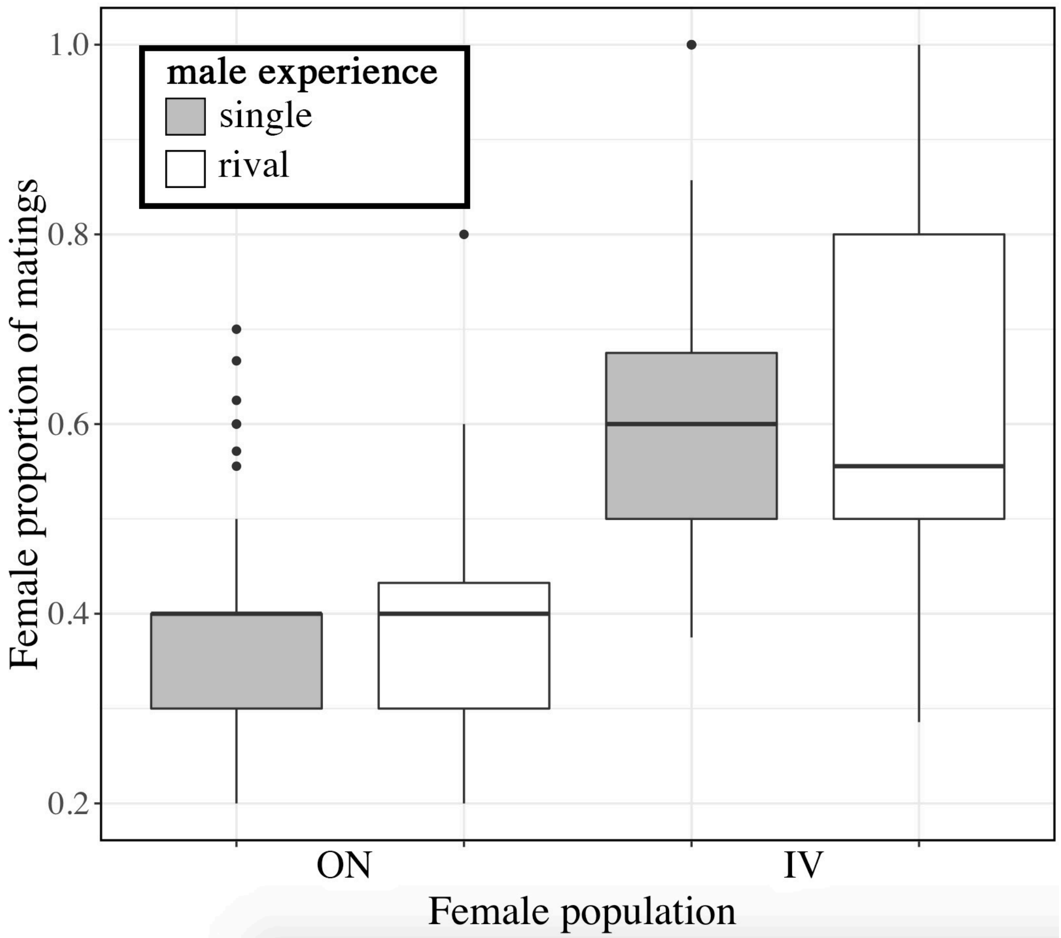


Figure 3: Proportion of times each female from either the ON or IV populations mated out of her number of mating opportunities. The boxes contain the middle 50% of data (IQR), and the horizontal lines represent the medians. The whiskers above and below each box represent values within ±1.5 IQR, and any values beyond this are outliers represented by closed circles. The shaded boxes represent females mated to males held alone and the white boxes represent females mated to males exposed to rivals.

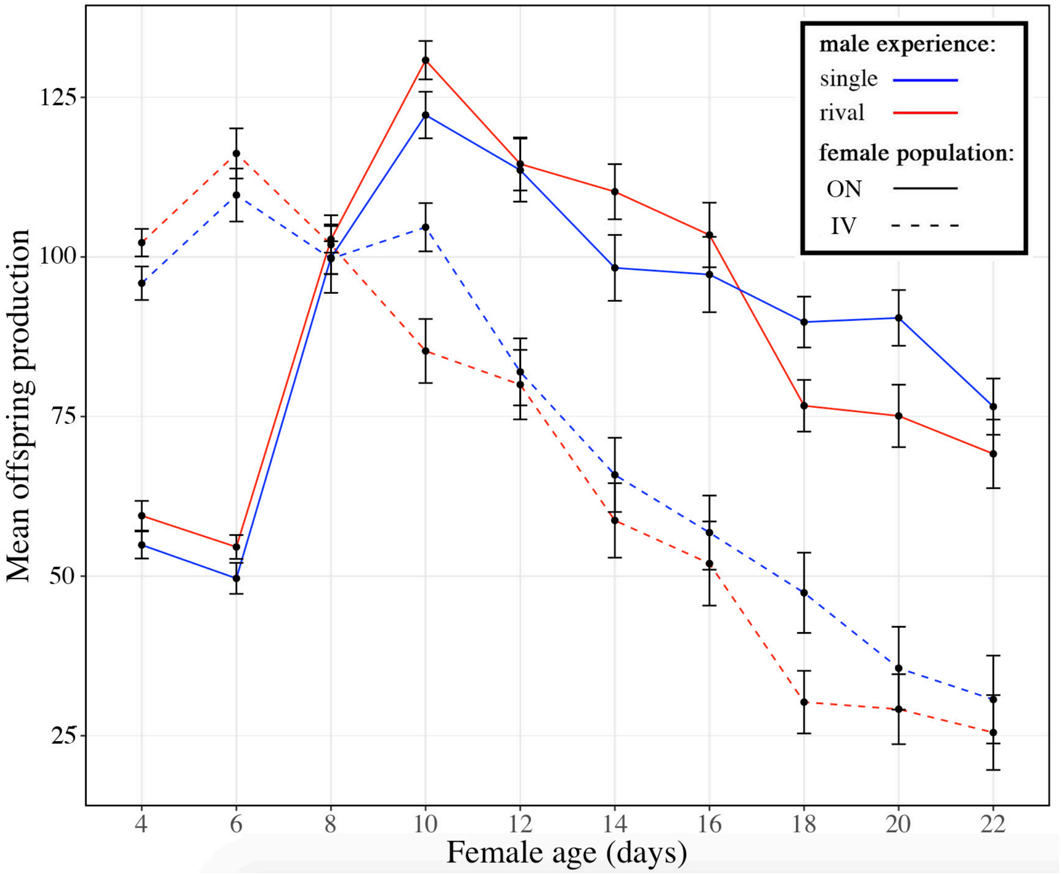


Figure 4: Effect of males' previous exposure to rivals on their mates' offspring production over the first 22 days of the mates' lives. Each point represents the mean number of offspring produced by all the females within each treatment, and the bars above and below each point represent the standard error. The red curves represent females mated to males exposed to rivals, and the blue curves are females mated to males held alone. The solid curves represent females descended from the ON population, and dashed curves are females descended from the IV population.

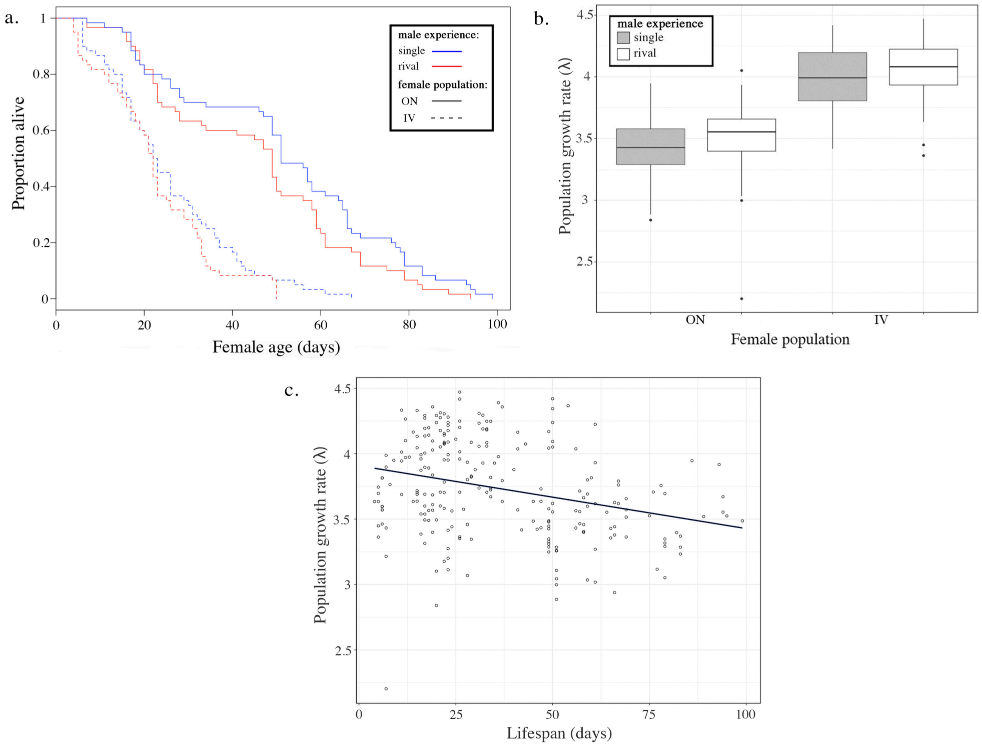
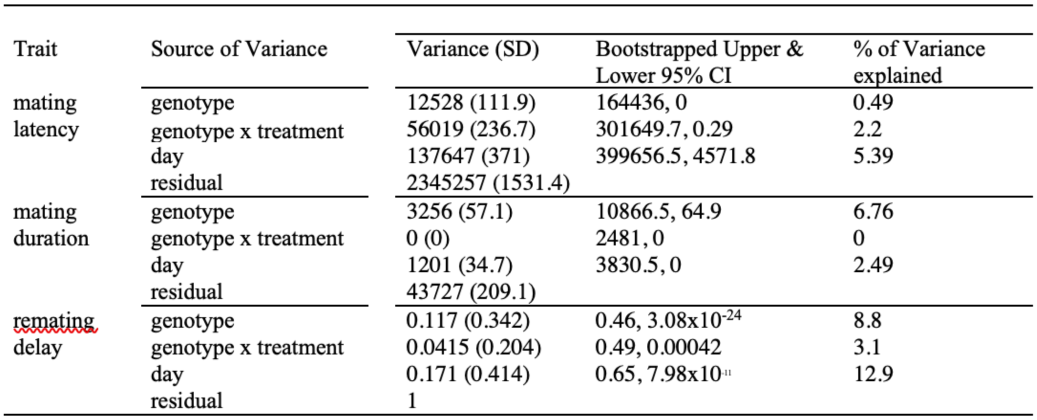


Figure 5: a. Effect of males' previous exposure to rivals on their mates' lifespan. Each survival curve represents the proportion of females alive over time. The red curves represent females mate to males exposed to rivals, and the blue curves are females mated to males held alone. The solid curves represent females descended from the ON population, and dashed curves are females descended from the IV population. b. Effect of males' previous experience on their mates' fitness measured in terms of population growth rate (λ). The boxes contain the middle 50% of data (IQR), and the horizontal lines represent the medians. The whiskers above and below each box represent values within ±1.5 IQR, and any values beyond this are outliers represented by closed circles. The shaded boxes represent females mated to males held alone, and white boxes are females mated to males exposed to rivals. c. The relationship between lifespan and population growth rate. Each dot represents a single female tested. The black line represents Spearman's rank correlation, and the shaded region is the 95% confidence interval.



Trait	Source of Variance	Variance (SD)	Bootstrapped Upper & Lower 95% CI	% of Variance explained
mating latency	genotype	12528 (111.9)	164436, 0	0.49
	genotype x treatment	56019 (236.7)	301649.7, 0.29	2.2
	day	137647 (371)	399656.5, 4571.8	5.39
	residual	2345257 (1531.4)		
mating duration	genotype	3256 (57.1)	10866.5, 64.9	6.76
	genotype x treatment	0 (0)	2481, 0	0
	day	1201 (34.7)	3830.5, 0	2.49
	residual	43727 (209.1)		
remating delay	genotype	0.117 (0.342)	0.46, $3.08x10^{-24}$	8.8
	genotype x treatment	0.0415 (0.204)	0.49, 0.00042	3.1
	day	0.171 (0.414)	0.65, $7.98x10^{-11}$	12.9
	residual	1		

Table 1: Variance components, standard deviation, 95% confidence intervals estimated using GLMMs fit by maximum-likelihood (Laplace approximation) for the mating phenotypes of hybrid males. Males from different genetic backgrounds were randomly assigned as experiencing two rivals or no rivals in the premating environment.

CHAPTER 4

Female mating experience and genetic background interact to influence male mating success in fruit flies

David C.S. Filice, Rajat Bhargava, & Reuven Dukas

Preface: The following chapter is a manuscript submitted to *Journal of Evolutionary Biology* and it is written in the style of this journal. The manuscript is currently under major revisions for resubmission.

Abstract

When the reproductive interests of males and females conflict, males can evolve traits that are harmful to females, and females can coevolve traits to resist this harm. In the fruit fly, *Drosophila melanogaster*, there is genetic variation in female resistance traits, which can affect the pre- and post-mating success of males that try to mate with them. However, it is not clear to what extent the expression of these phenotypes can be modified by environmental factors such as sociosexual experience. Here, we tested how the genetic background of a female and her previous mating experience interact to affect the mating success of focal males. In the experience phase, we placed females from 28 distinct genetic backgrounds individually either with a single male (low conflict) or with three males (high conflict) for 48 hours. In the subsequent test phase, we measured the mating and post-mating fertilization success of focal males paired individually with each female. We found that focal males paired with females from the high conflict treatment were less successful at mating, took longer to mate when they were successful, and had a lower proportion of paternity share. Furthermore, we identified significant female genetic variation associated with male mating success. These results indicate that female experience, along with intrinsic genetic factors, can independently influence different fitness components of her subsequent mates and has implications for our understanding of plastic female mating strategies and the evolution of sexually antagonistic traits in males and females.

Introduction

In many species, the reproductive interests of males and females conflict, resulting in the evolution of sexually antagonistic traits that, when expressed, increase the individual fitness of one sex at the expense of the other (Chapman et al. 2003; Arnqvist and Rowe 2005). Understanding the genetic and ecological underpinnings of sexually antagonistic traits is a key question for evolutionary biologists, as these traits can exaggerate the evolution of dimorphisms between the sexes and even lead to speciation (Arnqvist 1998; Parker and Partridge 1998; Gavrilets and Waxman 2002; Martin and Hosken 2003). In mating systems where males have evolved harmful traits, females are expected to coevolve traits that resist these traits. Although recent studies have quantified the impact of ecological factors on male induced harm, few have focused on the potential consequences for female resistance (Rostant et al. 2020).

Many early studies of sexual conflict have been conducted in uniform environments, and although evolutionary biologists are beginning to recognize the importance of environmental influences when quantifying the outcomes of sexual interactions, much of our understanding of flexibility in sexually-antagonistic traits is limited to the water strider system (Rowe et al. 1994; Fricke et al. 2009; Arbuthnott et al. 2014; Perry and Rowe 2018). In the fruit fly, *Drosophila melanogaster*, factors such as temperature (García-Roa et al. 2019), spatial complexity (Yun et al. 2017), and degree of male-male competition (Filice et al. 2020) have all been shown to influence the magnitude of male-induced harm and thus female fitness. Given this, we expect that socioecological effects would similarly influence female resistance strategies and consequently, the reproductive success of males as it is now well known that females play an active role in determining the outcomes of sexual interactions (Clark and Begun 1998; Kokko et al. 2003; Travers et al. 2015; Laturney et al. 2018). Recent theoretical work predicts that plasticity in response to socioecological factors should improve female resistance and thus decrease the effect of sexual conflict on the evolution of sexually antagonistic traits (McLeod and Day 2017). For example, in fruit flies, mated females upregulate proteases that degrade male accessory gland proteins (Acps) (Pilpel et al. 2008). The transfer and activation of these proteins are essential for success in male sperm competition and fertilization, and yet the fitness consequences of this upregulation for subsequent male mating partners is unknown. To the best of our knowledge, no one has directly tested if the changes brought on by differences in a female's social experience influence the reproductive success of her subsequent prospective mates.

In fruit flies, the optimal mating frequency and competition for access to mates for males is greater than it is for females, resulting in the evolution of harmful male traits expressed during courtship and copulation and the coevolution of female traits that attempt to minimize this harm. The genetic basis of female resistance is well documented. There is standing genetic variation associated with a female's ability to resist male harm (Friberg 2005; Linder and Rice 2005), and researchers starting with a baseline population could experimentally evolve increased female resistance by manipulating the intensity of sexual conflict over many generations (Wigby and Chapman 2004). Furthermore, the genetic background of female fruit flies influences male reproductive success in the form of females' mating propensity (Travers et al. 2015) and males' fertilization success (Clark and Begun 1998). While the current knowledge about heritable variation in female resistance traits is highly pertinent, it is equally important that we elucidate how socioecological factors such as females' experience with males influence their subsequent resistance to males in order to understand the relative contribution of genetic and environmental factors toward female post-mating phenotypes.

Here, we tested how the intensity of early life sexual conflict that females of distinct genetic backgrounds experience influences the reproductive success of subsequent male suitors. Specifically, we wanted to test how female experience and genetics influence a focal male's success in (1) a pre-mating context where reproductive success was determined by successful mating and the latency of these successful matings, and (2) a post-mating context where reproductive success was determined by measuring the paternity success of the focal males. We predicted that, in both contexts, focal males paired with females that previously experienced high intensity sexual conflict would have lower reproductive success compared to focal males paired with females that previously experienced low intensity sexual conflict. We were also interested in quantifying the effect of female genetic background on focal males' pre- and post mating success and predicted that male mating success would significantly vary with female genotype, given the documented variation in female mating propensity and resistance traits (Clark and Begun 1998; Linder and Rice 2005; Travers et al. 2015). Finally, we were interested in quantifying any potential interactions between female experience and genetic background, as this would indicate that females respond differently to the same experiences depending on their genotype (i.e. genetic variation in phenotypic plasticity).

Methods

Fly stocks and general

All the females in this experiment were derived from 28 randomly selected lines from the *Drosophila* Genetic Reference Panel (DGRP) (Mackay et al. 2012). These lines were derived from wild flies caught in Raleigh, North Carolina, USA, and repeatedly inbred for 20 generations. To alleviate the deleterious phenotypic effects associated with inbreeding, we generated hybrids by crossing each line to a single standardized reference line, thereby creating unique hybrid clones (hereafter referred to as hybrid genotypes) (Scott et al. 2018; Filice and Dukas 2019). Within hybrids, individuals are genetically identical, but between hybrids, individuals share an identical clonal haplotype inherited from their mother, and a unique clonal haplotype inherited from their father, allowing us to quantify the degree of genetic variation associated with phenotypic differences expressed from this unique haplotype.

All males were derived from the *Ives* population (hereafter IV) obtained from the Long Lab (Wilfrid Laurier University, Waterloo, Ontario, Canada). The IV population was originally collected in South Amherst, MA, USA in 1975. In 1980, a lineage of these flies was established at large census size (>1000 adults/generation) on a standardized culture protocol with non-overlapping generations (Rose 1984). Since then, this same lineage of IV has been maintained under identical conditions and used extensively as a model for studying evolutionary fitness and sexual conflict (Rose 1984; Martin and Long 2015; Filice and Long 2016). The males used in the sexual conflict experience phase were descendants from the standard wild-type IV population. Focal males in the testing phases were descendants of a sub-population of the IV line that had the *bwD* (hereafter, brown-eye) mutation introgressed via repeated backcrossing for 10 generations (Long et al. 2006). This mutation results in a brown-eye phenotype (as opposed to the wild-type red-eye phenotype). This mutation is an autosomal dominant marker, allowing us to determine the paternity of all offspring produced by focal brown-eye males that mated with females previously inseminated by red-eye males during the experience phase.

We reared all experimental flies at a standardized density of 100 eggs per vial containing ~5 ml of standard fly medium made of water, sucrose, cornmeal, yeast, agar and methyl paraben, and stored all flies in an incubator at 25 °C and 60% relative humidity with a 12:12h light:dark cycle. We collected all flies as virgins (within 8h of eclosion, as females are not sexually receptive prior to 18 hours in this population) under light CO_2 anesthesia. Following their initial collection, we handled all flies using gentle aspiration.

Experiment 1: Mating success of focal males

We started each replicate by collecting 4 virgin females from each of the 28 hybrid genotypes and placing each into a food vial with a dash of live yeast (~5 mg). Immediately after being placed into vials, we randomly assigned half the females of each hybrid genotype into a low-conflict treatment and half into a high-conflict treatment. Each female vial contained a single male in the low-conflict treatment and 3 males in the high-conflict treatment. These males belonged to the IV population and were virgins collected within 8h of eclosion.

Manipulating the sex ratio is a standard way to generate variability in the intensity of sexual conflict (due to both more harassment and/or matings, and increased male-male competition in more male-biased environments)(Holland and Rice 1999; Wigby and Chapman 2004). For females housed in individual vials, exposure to three males results in a significantly reduced lifespan and lifetime reproductive success compared to females exposed to a single male, so we chose to manipulate this number of males to generate high and low sexual conflict experiences respectively (García-Roa et al. 2019). After 48h of male exposure, we removed all males from the vials and allowed females to remain isolated for 24h prior to testing. In each replicate, we aimed to have 2 females from each of the 56 hybrid x treatment combinations for a total sample size of 112 trials.

On the morning following the experience phase, we added a focal brown-eye male to each female vial and measured the latency and duration of any matings that occurred to the closest second (Fig. 1). Any pairs that did not mate within four hours were considered to have not remated. We conducted 7 identical replicates that each took place on an independent day. While we aimed to have 784 trials, our actual sample size was 727. The 57 missing trials included cases where we were unable to collect enough hybrid females, and cases where females escaped or died within the three day experience phase. Overall, our sample sizes ranged between 10 to 14 for the 28 hybrid genotypes and treatment combinations.

Experiment 2: Paternity success of focal males

Testing the paternity success of focal males required a replication of the steps conducted in experiment 1. Hence we took the opportunity to test again the mating success of males as we did in experiment 1. We started each replicate by collecting 10 virgin females from each of the 28 hybrid genotypes and randomly placed half into a low-conflict treatment and half into a high-conflict treatment as detailed above. Simultaneously, we collected 280 brown-eye males and placed them in individual vials.

On the day following the 72h experience phase (48h with males, 24h alone), we placed each female into a fresh vial containing a focal brown-eye male and recorded all matings. Since our paternity analysis required that the females remate, recording the matings ensured that the focal male had a chance to inseminate the experienced females, and also provided an additional block of mating success data that could be compared with the data from experiment 1. Females that did not remate within four hours were excluded from further analysis. Two weeks later, we counted all the adult offspring from the female vials and quantified paternity based on the proportion of brown-eye offspring in each vial (Fig. 1). We conducted 3 replicates but had only 558 trials owing to cases where we were unable to collect enough hybrid females, and cases where females escaped or died within the three day experience phase. Out of the 558 trials, 224 females remated during the test for the paternity analysis. Overall, our sample sizes of remated females ranged between 1 to 11 for the 28 hybrid genotypes and treatment combinations. The large variation in sample sizes per genotype is consistent with the large genetic variation in remating rates documented in experiment 1 (Fig. 2b).

Statistical analysis

We conducted all data analysis using R version 3.5.2 (R Core Team 2013). For the mating success test, we constructed a Cox proportional hazard model using the *Surv* and *coxme* functions from the *survival* and *coxme* packages (Therneau and Grambsch 2000), which took into account the binomial outcome of mating success and the latency of successful matings as a survival term. Our model included experience treatment as fixed factor, and hybrid genotype and replicate as random factors. We also analyzed the binomial outcome of mating success on its own by constructing a generalized linear mixed-effects model (GLMM) using the *glmer* function from the *lme4* package (Bates et al. 2014) for the data obtained in both experiment 1 and experiment 2. In both models, we included experience treatment as a fixed effect, and hybrid genotype, the crossed interaction between treatment and genotype, and replicate as random factors. To analyze male paternity success, we constructed a GLMM with a binomial response variable defined by the number of brown-eye offspring weighed by the number of red-eye offspring. We included experience treatment as a fixed effect, and hybrid genotype crossed with experience treatment and replicate as random effects. However, this initial model was overdispersed, so we added an observer-level random factor that assigns each observation a unique ID to our final model (Harrison 2015). We determined the significance of all the fixed effects in our models by calculating p-values using a Wald χ^2 test with the *Anova* function from the *car* package (Fox et al. 2014). For the random effects in our cox model, we determined statistical significance by performing a likelihood ratio test. This involved comparing the fit of two nested models: one that contained the random effect of interest, and one that did not (Bolker et al. 2009). For the random effects in our GLMMs, we tested the significance of each variance component using a non-parametric bootstrapping approach, which involved comparing the magnitude of our models' variance components to the distribution of 10000 variance components that were determined from a randomized set of the experimental data (Ziegel and Manly 1998).

Results

Experiment 1: Mating success of focal males

Focal males paired with females from the high conflict treatment were both slower and less likely to mate compared to males paired with females from the low conflict treatment ($\chi^2 = 8.5$, df = 1, $p = 0.0035$, Fig. 2A). The female hybrid genotype had a significant effect on the mating success of focal males ($p < 0.0001$, Fig. 2B, Table 1) and the effect of experimental replicate was not significant ($p = 0.5$, Table 1). When looking at focal male success and only taking into account the binomial outcome of mating success, males paired with females from the high conflict treatment were still less likely to mate ($\chi^2 = 4.06$, df = 1, p = 0.044). Similarly, female hybrid genotype had a significant effect on mating outcome ($p < 0001$, Table 1), but the interaction between female treatment and genotype was not significant (p = 0.65, Table 1), nor was the effect of experimental replicate (p = 0.64, Table 1).

Experiment 2: Mating success of focal males

Females from the high conflict treatment were significantly less likely to remate than females from the low conflict treatment ($\chi^2 = 6.69$, df = 1, p = 0.0097, Fig 3A). The effect of hybrid genotype was significant (p = 0.002, Table 2, Fig 3A), as was the effect of experimental replicate ($p < 0.0001$, Table 2), but the interaction between experience and genotype was not significant (p = 0.084, Table 2). The correlation between the binary outcome of male mating success when mating with females from the same hybrid genotype in experiments 1 and 2 was strongly positive (t = 3.3, df = 26, r = 0.54, p = 0.0029; Fig 3B). In other words, males had a similar mean mating success when paired with a female from a particular genetic background in both experiments 1 and 2.

Experiment 2:Paternity success of focal males

Focal males paired with females from the high conflict treatment also had significantly lower paternity success compared to focal males paired with females from the low conflict treatment ($\chi^2 = 33$, df = 1, $p < 0.0001$, Fig. 4). Both female hybrid genotype and the interaction between experience treatment and hybrid genotype did not significantly effect paternity success of the focal males (hybrid: p = 0.76, Table 2; experience x hybrid: p = 0.55, Table 2; Fig. 4), nor did experimental replicate (p = 0.12, Table 2).

Discussion

In this study, we set out to test how a female's previous social experience and her genotype influence the subsequent reproductive success of her suitors. In both pre- and post-mating scenarios, focal males had lower reproductive success when paired with females that previously experienced high than low sexual conflict. Specifically, focal males paired with females that had experienced high conflict mated at a lower frequency, took longer to mate, and had lower paternity success (Figs 2-4). Furthermore, the genetic background of females was an important factor determining male mating success in both our pre-mating tests (Figs 2B, 3A), and the positive correlation between the pre-mating test results in experiments 1 and 2 (Fig. 3B) suggests some of these genetic effects produce replicable outcomes over time. Previous studies have documented that the genetic identity of a female influences the reproductive success of her mates (Clark and Begun 1998; Clark et al. 1999), and we add to this by finding that the magnitude of this heritable effect can change depending on the socioecological experience of a female.

In our pre- and post-mating tests, the lower reproductive success of focal males paired with females from the high conflict treatment likely represents a combination of male and female responses to increased sexual conflict. A key problem in the current sexual conflict literature is disentangling the degree to which female post-mating responses represent male manipulation and/or mutually beneficial responses that females play some part in (i.e. via phenotypic plasticity). In the case of pre-mating outcomes, the delaying of a female's remating interval has clear benefits from a male standpoint as it can reduce the risk of sperm competition, and is driven by the transfer of Acps in the ejaculate that are shaped by natural selection, as males that strategically invest into the transfer of Acps tend to have higher reproductive success (Johnstone and Keller 2000; Wolfner 2002; Alonzo and Pizzari 2013; Hopkins et al. 2019). During the experience phase, females in the high conflict treatment likely mated more (García-Roa et al. 2019), and the males they mated with likely upregulated the expression of competitive traits such as seminal fluid transfer and harassment due to the presence of male-male competition (Bretman et al. 2009; Hopkins et al. 2019).

This means the females in the high conflict treatment were likely subjected to more male manipulation and may have more to lose from a subsequent remating, which could explain the lower mating success of the focal males. On the other hand, we do not know the conditions for which it is in a female's best interest to strategically increase her own resistance to multiply mating. This is because polyandry can sometimes increase female reproductive success due to an increased short-term reproductive output from either nuptial gifts (Arnqvist and Nilsson 2000) or other male effects (Rubinstein and Wolfner 2013). However, matings past the optimal degree of polyandry can have deleterious effects and reduce the longevity and lifetime reproductive output of females (Chapman et al. 1995; Stewart et al. 2005). Therefore, mated females can potentially gain from either accepting future prospective mates or modulating their mate choice in order to avoid exploitative males that may decrease their fitness (Holland and Rice 1998; Filice and Long 2017). Given that the direct and indirect benefits of polyandry in fruit flies vary across time and with body condition (Long et al. 2010a,b), we should expect that females may regulate their mating rate based on previous mating experience. Furthermore, it may be that the perception of male density in a previous environment influences mating propensity (Rowe et al. 1994). When a female experiences a high male-density environment, it may make sense to increase mating resistance in order to adequately sample all available males before making a choice (Atwell and Wagner 2014), but on the other hand it may be beneficial to reduce receptivity in order to avoid the costs of high male harassment (i.e. convenience polyandry) (Rowe 1992). The fact that females in our study appear to increase their mating resistance in response to increased male density may suggest a lack of convenience polyandry in this species. Overall, in our tests, it is likely that male manipulation and female-driven remating behaviours are both in part responsible for the lower mating success of focal males paired with females that had experienced high-conflict. In order to further disentangle the relative contributions of male-induced effects and female volition toward various female post-mating responses such as remating delay, future studies should continue to systemically determine how the volume and constitution of male Acps influence female remating propensity.

In our post-mating tests, the lower paternity success of males paired with females that had experienced high conflict may also be explained by a combination of factors driven by both males and females. In many mating systems, the last male to mate typically has an advantage in securing the most paternity, a pattern known as last male sperm precedence. However, the strength of this effect can break down when a female mates multiply, which could potentially be explained by increased male sperm competition (Zeh and Zeh 1994) or female driven effects that modulate male paternity success (Laturney et al. 2018). Specifically, Laturney et al. (2018) identified a positive relationship between the penultimate to last mating interval and the paternity success of the last male, suggesting that by modulating remating latency, females have some control over the outcomes of last male sperm precedence. This lends to the argument that polyandry can be adaptive if females gain direct benefits in the form of increased short-term offspring production, or indirect benefits in the form of increased genetic quality and/or variety (Arnqvist and Nilsson 2000). It could be that in our study, females from the high conflict treatment that mated more frequently during the experience phase could balance any direct costs of multiple mating by reducing the paternity share of their last mate and thus increase the genetic diversity of her offspring. A potential mechanism of this may be related to the fact that mated females upregulate proteases that degrade male Acps, which are important for success in sperm competition (Pilpel et al. 2008). However, it may also simply be that the upregulation of Acp degrading proteases is a response to mitigate the direct harm associated with the receipt of some Acps (Chapman et al. 1995). As such, it is critical that future studies should investigate the relationship between the expression of Acp degrading proteases, the number of times a female has mated, and her fitness. If, for example, females that positively upregulate these proteases in response to more matings have higher fitness than those who express less in response to the same number of matings, this could suggest that the degradation of Acps is an adaptive response to gain indirect offspring benefits and/or to reduce the direct harm associated with the receipt of these Acps.

Finally, our results, which indicated that the pre-mating success of focal males was affected by female genotypes (Fig. 2B, Fig. 3A), confirm that some of the decision to remate is due to female-specific effects. This also agrees with previous studies with similar outcomes (Simmons 2003; Travers et al. 2015; Filice and Long 2017). Genetic variation in remating rate may represent adaptive variation in female reproductive strategies associated with trade-offs between survival and maximizing early-life reproductive output (Travers et al. 2015; Filice et al. 2020). Additionally, such genetic variation may represent variation in females' choices to either remate with or reject the single focal male type we presented to them, which may operate as a strategy to resist the harm of subsequent matings (Linder and Rice 2005). However, contrary to previous studies that have found significant female genetic variation in the effect of last male paternity success (Clark and Begun 1998; Clark et al. 1999), we failed to identify a similar outcome. One possibility is that a small sample size in some of our experience x hybrid groups resulted in insufficient statistical power to detect differences attributable to female genotype. It is also possible that the outcomes of post-copulatory male-male interactions such as sperm competition largely drown out female-specific effects in determining last-male paternity success. Such female specific effects include cryptic choice, sperm storage and upregulation of proteases (Birkhead 1998; Pilpel et al. 2008; Avila and Wolfner 2017). In this case, females can still rely on the pre-copulatory rejection of males to modulate their reproductive outcomes. Future studies should continue to investigate this by identifying female genotypes that vary in their post-mating responses, and test the mechanisms that underlie such differential responses.

Taken together, our results have important implications for our understanding of how social experience can determine the expression of sexually antagonistic traits. Specifically, we found that females who experience high levels of sexual conflict can modify their phenotypes in a way that reduces the pre- and post-mating success of their future suitors, and that these outcomes also depend on females' genetic background. We propose that these effects represent adaptive mechanisms to offset the costs of male-induced harm by allowing a female to modulate her remating rate in a way that is best for her own fitness, and thus have important consequences for our understanding of how socioecological factors can influence the evolution of sexually antagonistic traits. Future studies should continue to untangle the relative contribution of female driven effects in mating interactions in order to improve our understanding of adaptive female mating behaviours, which may have major consequences for the outcomes of sexual selection and evolution.

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Authorship Contributions:

DCSF and RD designed the experiments. DCSF and RB carried out the experiments. DCSF wrote the first draft and performed the statistical analysis, and all authors were involved in the revision process.

Data accessibility:

Data will be submitted to Dryad Digital Repository if accepted.

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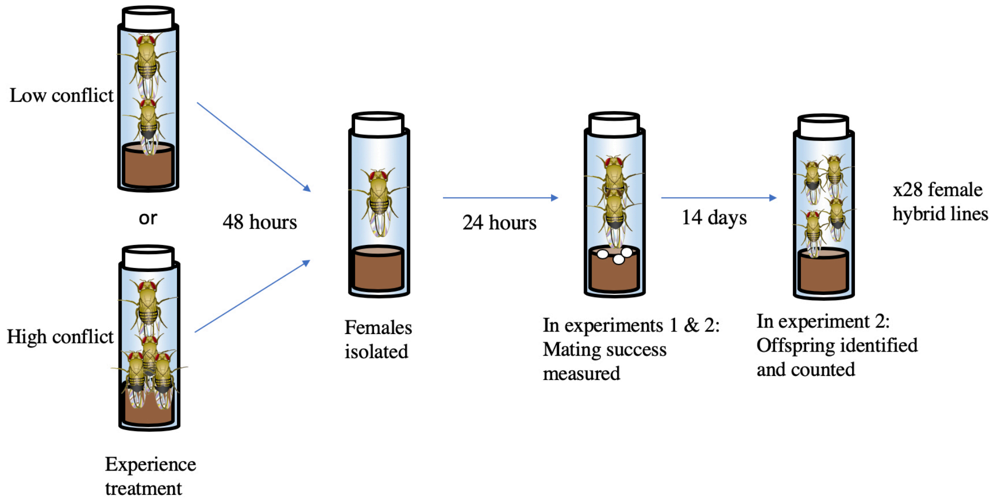

Figure 1

An illustration of the experimental design for both our experiments. Females were exposed to either a single male or three males for 48 hours, and then housed in isolation for an additional 24 hours. After this experience phase, each female was paired with a brown-eye focal male for 4 hours and mating behaviours were scored. In experiment 2, females remained in these vials for 24 hours to lay their eggs, and the resulting offspring were counted two weeks later.

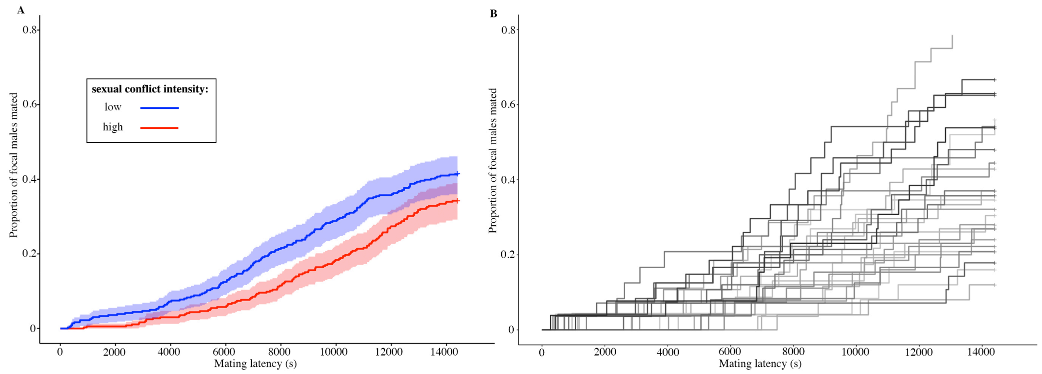

Figure 2

A: Effect of female sexual conflict experience on the subsequent mating success of focal males in experiment 1. Each cox-regression curve represents the cumulative proportion of matings by focal males paired with females that previously experienced either low (blue) or high (red) sexual conflict.

B: Effect of female genetic background on the subsequent mating success of focal males in experiment 1. Each cox-regression curve represents the cumulative proportion of males that mated over time. The varying shades of grey represent the 28 different hybrid female genotypes that were tested.

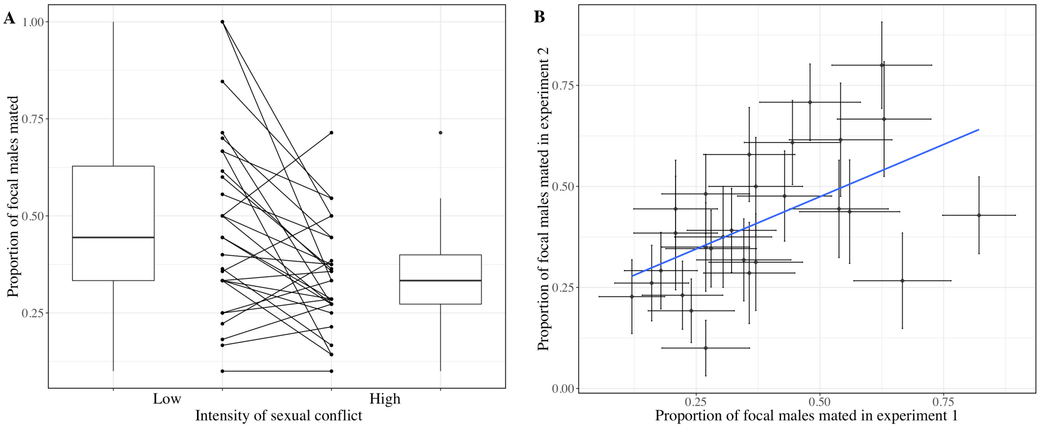

Figure 3

A: Effect of female sexual conflict experience and genotype on male mating success in experiment 2. The boxes contain the middle 50% of data (interquartile range [IQR]), and the horizontal lines represent the medians. The whiskers above and below each box represent values within±1.5 IQR, and any values beyond this are outliers represented by closed circles. The reaction norm plot in the center of the plot depicts the change in the mean of each female hybrid genotype across the two experience treatments.

B: Female genetic correlation between male mating success in experiments 1 and 2. Each open circle represents the mean mating success of males with a particular female genotype, and the horizontal and vertical bars represent standard errors. The blue slope represents the regression line.

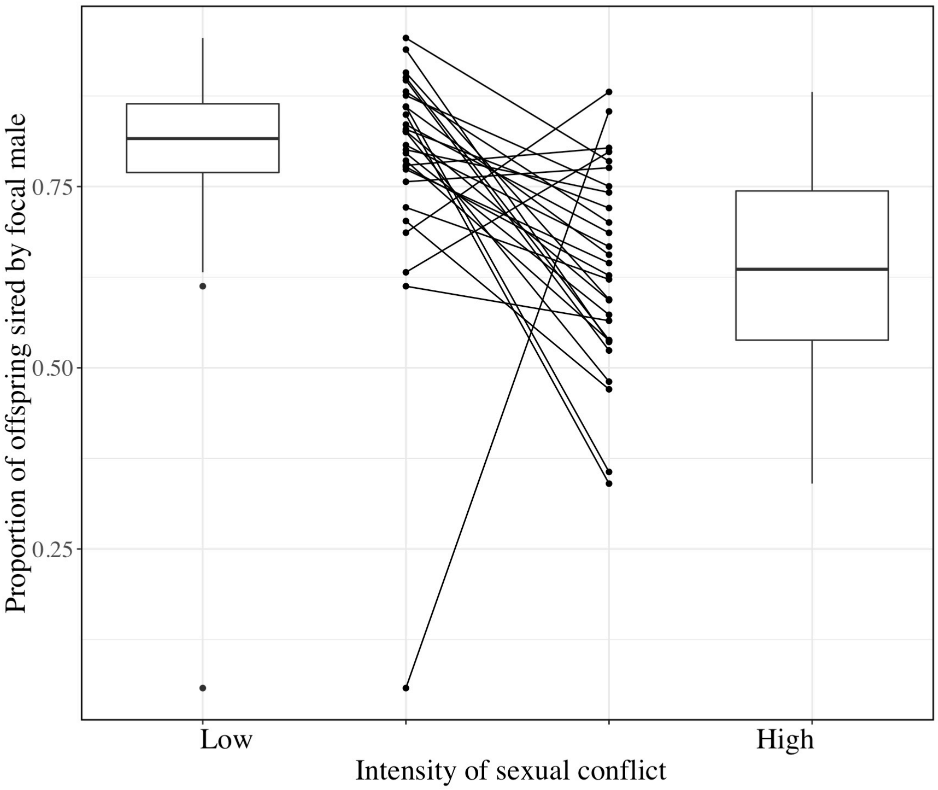

Figure 4

Effect of female sexual conflict experience and genotype on subsequent male post-mating success. The boxes contain the middle 50% of data (interquartile range [IQR]), and the horizontal lines represent the medians. The whiskers above and below each box represent values within±1.5 IQR, and any values beyond this are outliers represented by closed circles. The reaction norm plot in the center of the plot depicts the change in the mean of each female hybrid genotype across the two experience treatments.

Table 1: Variance components, standard deviation, and p-values estimated using a GLMM fit by maximum-likelihood (Laplace approximation) for the reproductive success of males paired with females from one of 28 genetic hybrid backgrounds from the experiment 1 data set.

Response	Source of Variance	Variance	Standard Deviation	% of Variance Explained	p-value
Cox hazard mating success (mating latency, proportion mated)	hybrid	0.26	0.51	20.4	4.9×10^{-9}
	replicate	0.012	0.11	0.94	0.5
Proportion mated	hybrid	0.32	0.57	23.4	4.2×10^{-7}
	hybrid x experience	0.033	0.18	2.4	0.65
	replicate	0.013	0.12	0.95	0.64

Table 2: Variance components, standard deviation, and p-values estimated using a GLMM fit by maximum-likelihood (Laplace approximation) for the reproductive success of males paired with females from one of 28 genetic hybrid backgrounds from the experiment 2 data set.

Response	Source of Variance	Variance	Standard Deviation	% of Variance Explained	p-value
Proportion mated	hybrid	0.27	0.52	17	0.002
	hybrid x experience	0.08	0.29	5	0.084
	replicate	0.26	0.51	16.1	1×10^{-7}
Paternity success	individual	1.77	1.33	62	0.99
	hybrid	8.6×10^{-9}	9.3×10^{-5}	<0.0001	0.76
	hybrid x experience	0.04	0.2	1.4	0.55
	replicate	0.045	0.21	1.6	0.12

CHAPTER 5

Previous sexual aggression decreases choosiness but does not influence mating preferences in fruit flies

David C.S. Filice & Reuven Dukas

Preface: The following chapter is a manuscript in preparation for submission to *Animal Behaviour* and it is written in the style of this journal.

Abstract

Female mate choice is a complex decision making process that involves many context-dependent factors, and understanding the factors that shape variation in female mate choice has important consequences for evolution via sexual selection. In fruit flies, *Drosophila melanogaster*, males often use aggressive mating strategies to coerce females into mating, but it is not clear if a female's experience with sexual aggression shapes her future behaviours. Here, we used males derived from lineages that were either artificially selected to display low or high sexual aggression toward females to determine how experience with these males shapes subsequent female mate choice. First, we verified that males from these lineages differed in their mating behaviours, and found that males from high aggression backgrounds spent more time harassing virgin females, and had a quicker mating latency but shorter mating duration compared to males from a low aggression background. Next, we tested how either a harassment or mating experience with males from either a high or low aggression background influenced subsequent female mate choice behaviours, and found that in both scenarios, females mated quicker and at a higher proportion with a second partner, regardless of his identity. We discuss the significance of these results in the context of the evolution of trade-offs in male strategies, and the evolutionary consequences of flexible female mate choice.

Introduction

Animals respond to experiences in their environment by altering their physiological and behavioural traits, a process known as phenotypic plasticity (West-Eberhard 1989). In the case of behavioural plasticity, animals tend to exhibit striking variation in their mating behaviours in response to environmental cues (Mery and Burns 2010; Dingemanse and Wolf 2013; Dukas 2013). Given the importance of mating behaviours for successful reproduction to occur, plasticity in mating behaviours is thought to represent adaptations that help animals make decisions that maximize their evolutionary fitness across changing environmental contexts (Rodríguez et al. 2013). For example, females can benefit from having flexible mate choice thresholds that allow them to accommodate variation in factors including predation risk, time and energetic costs of mate choice, and abundance of potential mates (Qvarnström 2001). Mate choice encompasses two major components: choosiness (the investment into discriminating between different mates), and mating preference (the rank function of preferred stimuli exhibited by potential mates) (Widemo and Sæther 1999). Given that the outcomes of female mate choice can have a major influence on the evolution of male traits via sexual selection, understanding the factors that shape flexible mate choice is an essential task for biologists (Andersson 1995; Kokko et al. 2003).

Recently, the study of behavioural plasticity in female mate choice has made swift progress using invertebrate models, due to precise and effective environmental and genetic control techniques (Kelly 2018). For example, in the field cricket, *Teleogryllus oceanicus*, a species where females rely on male acoustic signals to locate and choose mates, females reared in silent environments are less choosy of male song calling compared to females reared in environments with acoustic songs (Bailey and Zuk 2008). The authors suggest that this may be an adaptive tactic to compensate for the reduced availability of male sexual signals. Similar studies have shown that females can modulate their mate choice based on ecological factors such as cues of mate availability (Scott et al. 2020), intrinsic condition (Hunt et al. 2005), and previous courtship and/or mating experience (Dukas 2005; Rebar et al. 2011; Travers et al. 2016; Filice and Long 2017). Despite our growing understanding of the importance of mating experience in shaping flexible mate choice, there have been few attempts to incorporate the importance of sexual conflict theory when generating hypotheses and interpreting results.

In many species, the optimal reproductive interests of males and females conflict, resulting in the evolution of traits that are antagonistic towards the opposite sex (Parker 1979; Arnqvist and Rowe 2013). In the fruit fly, *Drosophila melanogaster*, intense male-male competition has led to the evolution of male phenotypes that inflict harm on females. Some examples of male-induced harm include genital damage during copulation (Kamimura 2007), wing damage during courtship and harassment (Dukas and Jongsma 2012), and the toxic side effects of accessory gland proteins (Acps) transferred in the ejaculate during insemination (Chapman et al. 1995). Often, this harm directly translates into reduced fitness in terms of both longevity and lifetime fecundity (Chapman et al. 2003). Arguably, the most extreme form of sexual conflict is forced copulation, which essentially allows males to bypass female mate choice (Thornhill 1980; Mckinney et al. 1983; Smuts and Smuts 1993; Clutton-Brock and Parker 1995). Dukas et al. (2020) demonstrated a wide natural phenotypic range in male sexual aggression by artificially selecting for males that were the least and the most successful in forced copulation with sexually immature females (0.14 proportion in sample of males selected for low forced copulation, 0.31 in sample of males selected for high forced copulation, after 20 generations). Given the potential costs for females that experience sexual harm, we predict that selection should favour females that utilize flexible mate choice based on the levels of sexual aggression in their environments in order to resist these costs. Although others have predicted that the avoidance of harmful male phenotypes can act as a mechanism to offset the direct costs of sexual harm (Holland and Rice 1998; Gavrilets et al. 2001; McLeod and Day 2017), empirical studies that test this hypothesis are lacking (Filice and Long 2017).

To address this, we tested how previous experience with males that vary in their expression of sexual aggression influences subsequent female mating choice. Specifically, we were interested in comparing the effect of exposure to males selected for either high or low sexual aggression on a female's choosiness and mating preferences in 1) a scenario where immature females experience prior harassment from males and 2) a scenario where mature females experience prior mating with males. First, we needed to verify that our distinct male lineages varied in their expression of sexual aggression in both these scenarios. Based on previous results using these lineages (Dukas et al. 2020), we predicted that males from lineages selected for high sexual aggression would spend more time harassing immature females to mate, and would coerce mature females into mating quicker than males from lineages selected for low sexual aggression. Next, we tested subsequent female behaviour to these scenarios and predicted that in both contexts, experience with a high sexually aggressive male would result in a lower mating receptivity in order to offset the costs induced by a sexually aggressive partner. In terms of mate choice, we predicted that females would develop a preference for the type of male they did not previously have experience with. In other words, females that were previously paired to a low aggression male would mate more frequently with a high aggression male, and vice versa. This prediction is empirically based on previous results which demonstrate female preference to mate with unfamiliar individuals (Ödeen and Moray 2008; Filice and Long 2017), and theoretically based on the potential for trade-offs in female mate choice decisions related to maximizing offspring production at the cost of longevity (Arbuthnott 2018; Filice et al. 2020).

Methods

Fly stocks and general

All focal females were derived from the *Ives* population (hereafter IV) obtained from the Long Lab (Wilfrid Laurier University, Waterloo, Ontario, Canada). The IV population was originally collected in South Amherst, MA, USA in 1975. In 1980, a lineage of these flies was established at large census size (>1000 adults/generation) on a standardized culture protocol with non-overlapping generations (Rose 1984). Since then, this same lineage of IV has been maintained under identical conditions and used extensively as a model for studying evolutionary fitness and sexual conflict (Rose 1984; Martin and Long 2015; Filice and Long 2016).

All the males in our experiments were derived from six artificially selected lineages descended from 500 wild-caught females collected in Hamilton, ON in 2018. In three of these lineages, Dukas et al. (2020) selected for males that did not forcibly copulate with teneral females within a 2h period. In the other three lineages, they selected for males that did forcibly copulate within a 2h period. Hence, the former three lineages selected for males low in sexual aggression (hereafter low lineages) and the latter three selected for males high in sexual aggression (hereafter high lineages). Owing to the high demands on time and space, Dukas et al. (2020) always conducted selection on one low and one high lineages per day over three successive days. This generated 3 sets, each including one low and one high lineage. Artificial selection lasted for 20 generations.

We reared all experimental flies at a standardized density of 100 eggs per vial containing ~5 ml of standard fly medium made of water, sucrose, cornmeal, yeast, agar and methyl paraben, and stored all flies in an incubator at 25 °C and 60% relative humidity with a 12:12h light:dark cycle. We collected all flies as virgins (within 8h of eclosion) under light CO_2 anesthesia. Following their initial collection, we handled all flies using gentle aspiration.

Experiment 1: Harassment and post-harassment tests

On the first morning of each replicate, we collected 100 virgin females from the IV population within 1 hour of eclosion and placed them into individual vials. Four hours later, we placed a single male from a high lineage into half of these female vials, and a single male from a low lineage into the other half. Within each replicate, all males came from one of the three sets each consisting of one low and one high lineage (hence, replicate and lineage set were confounded by design). During the first 10 minutes of each pairing, an observer blind to male treatment recorded to the nearest second the duration of female harassment using the *Drosophila Assay Assistant* app on an iPod Touch. Behaviours that counted as harassment included chasing, courtship, and mounting attempts. Following this initial observation period, each pair of flies was left undisturbed for an additional four hours. On the following morning, 17 hours after being isolated, each female was introduced to a new male from either a high or low lineage in a fully reciprocal design. In other words, half of the original females from each treatment were paired with a new male from a low lineage, and the other half were paired with a male from a high lineage. In this post-harassment test, two blind observers systematically scanned all 100 vials and recorded the latency and duration of all matings to the nearest second. Pairs that did not mate within 2 hours were considered to have not mated. We replicated this procedure across the three sets of low and high lineages twice each. Hence, our total sample size for the harassment tests was N = 600. However, 15 females died or escaped in between the harassment and post-harassment test, resulting in a total N of 585.

Experiment 2: Mating and post-mating tests

On the first morning of each replicate, we collected 80 newly-eclosed IV females and placed them in individual vials with a dash (~5mg) of live yeast. Simultaneously, we collected 40 newly-eclosed males from a low lineage and placed them into groups of three, and did the same with 40 males from a high lineage. On the following morning, we collected an additional 40 males from each of the high and low lineages and placed them into vials in groups of three. Similar to experiment 1, within each replicate, all males belonged to one of the three sets, each including one low and one high lineage. Two days later, we placed a single male collected on the first day from either a low or high lineage into each female vial. Two blind observers systematically scanned each pair for 90 minutes and noted the latency and duration of each mating. Trials where the pair did not mate within 90 minutes were excluded from further analysis (N = 53 from low treatment, N = 18 from high treatment). Following this 90 minute period, we removed and discarded all males, and placed the females back into the chamber for 24h. On the following morning, we individually placed all females into fresh food vials, and then added a single male collected on the second day from either a high or low lineage. Similar to experiment 1, our design was fully reciprocal, so half of the females from each initial treatment were paired with a low lineage male, and the other half to a high lineage male. Two blind observers systematically scanned the vials for 3 hours and recorded the latency and duration of each mating. Pairs that did not mate within 3 hours were considered to have not mated. We replicated this procedure across each of the six selection lineages two times each, resulting in a total sample size of N=409, after accounting for the females that did not mate during the first mating experience.

Statistical analysis

We conducted all data analysis using R version 3.5.2 (R Core Team 2013). For our data collected during the experience treatments (harassment in experiment 1 and mating in experiment 2), we constructed generalized linear mixed models using the lmer function from the lme4 package (Bates et al. 2014). We treated all our dependent factors (harassment, mating latency, and mating duration) as gaussian response variables and verified all model fits by visually inspecting plots of model residuals. We included the selection treatment identity of the experience male (low or high sexual aggression) as a fixed effect, and the lineage set as a random effect in these models. For our data collected during the post-experience tests (post-harassment and post-mating tests), we constructed Cox proportional hazard mixed models, using the coxme function from the coxme package (Therneau and Grambsch 2000). For both the post-harassment and posting-mating results, we constructed a model that took into account the binomial outcome of mating success and the latency of successful matings as a survival term. These models included the selection treatment identity of the experience male, the selection treatment identity of the post-experience male, and the interaction between these two factors as fixed effects, and the lineage set as a random effect. We calculated the significance of the fixed effects using a log-likelihood ratio χ^2 test using the Anova function from the car package (Fox et al. 2014).

Results

Experiment 1: Harassment tests and post-harassment experience tests

Males descended from high sexual aggression lineages displayed significantly more harassment toward sexually immature females compared to males descended from low lineages (Wald $\chi^2 = 62.7$, df = 1p < 0.0001; Fig. 1). The random effect of lineage had a standard deviation of 25.3. On the day following harassment experience, females that were previously exposed to males from one of the three high lineages mated quicker and had a higher overall proportion of matings compared to females previously exposed to males from one of the low lineages (Wald $\chi^2 = 6.9$, df = 1, $p = 0.0085$; Fig. 2a). However, when looking at the identity of the second male, females had a similar mating latency and proportion whether their second partner was from a low or high lineage (Wald $\chi^2 = 2.6$, df = 1, $p = 0.1$; Fig. 2b). The interaction between the identity of the first and second male was not significant (Wald $\chi^2 = 0.41$, df = 1, $p = 0.52$). The random effect of lineage had a standard deviation of $9.7\text{x}10^{-3}$.

Experiment 2: Mating and post-mating tests

Males descended from high sexual aggression lineages displayed significantly faster mating latencies (Wald $\chi^2 = 17.8$, df = 1, $p < 0.0001$; Fig. 3a), but mated for shorter durations (Wald $\chi^2 = 8.9$, df = 1, $p = 0.0028$; Fig. 3b). The random effect of lineage had standard deviations of 155.1 and 41.6 for mating latency and duration respectively. On the day following mating experience, females that previously mated with males from one of the high lineages remated quicker and had a higher overall proportion of rematings compared to females previously exposed to males from one of the low lineages (Wald $\chi^2 = 5.1$, df = 1, p = 0.024; Fig 4a). When looking at the identity of the second male, females mated significantly faster and at a greater proportion with males from a high lineage compared to females paired with males from a low lineage (Wald $\chi^2 = 4.2$, df = , p = 0.04; Fig. 4b). The interaction between the identity of the first and second male was not significant (Wald $\chi^2 = 2.5$, df = 1, p = 0.11). The random effect of lineage had a standard deviation of 0.36.

Discussion

Previous courtship and/or mating experience is well known to shape subsequent female mate choice behaviour (Rodríguez et al. 2013). Here, we add to our understanding by demonstrating that male phenotypes associated with the artificial selection of sexual aggression are one source that shape this plasticity. Firstly, we verified that males derived from lineages selected for high and low sexual aggression differ in their mating behaviours. As expected, males from high aggression lineages display more harassment, mate quicker, and mate for shorter durations compared to males from low aggression lineages. Next, we tested how experience with males from either a low or high aggression background influences subsequent female mating behaviour, and found that females that experience harassment and/or mating from a male descended from a high sexual aggression lineage display less choosiness and have shorter mating latencies and higher mating propensities when paired with a subsequent male. Overall, these results advance our understanding of how sexually antagonistic traits can shape plasticity in mating behaviours.

In terms of behavioural differences between males from low and high sexual aggression lineages, it was not surprising to see that males from lineages selected for high sexual aggression tended to display a greater frequency of harassment behaviours and tended to display faster mating latencies. During the artificial selection regime, males from high aggression lineages were always sired by males that were successful in forceful copulation with a sexually immature female. One major consequence of artificial selection is the unexpected selection of phenotypes that correlate with the target phenotype, such as the various domesticated physical features that emerged in foxes, *Vulpes vulpes*, when selected for behavioural tameness (Trut 1999). It is possible that Dukas et al. (2020) indirectly selected for other traits related to forced copulation success, such as persistence and time spent pursuing females. Although Dukas et al. (2020) found no differences between the high and low male lineages in their time spent pursuing females, it is important to note a key difference was they looked at mature females, while we used immature females similar to the conditions during the artificial selection. Thus, a possibility is that males may specifically modulate their pursuit behaviours towards females of a particular age and is a topic that warrants future investigation.

When looking at mating duration, we made no explicit prediction about differences between male lineages, but it is possible that the longer durations observed in males selected for low sexual aggression represents a trade-off between pre- and post-copulatory mating strategies (Simmons et al. 2017; Filice and Dukas 2019). Given that the males still had to compete amongst each other for matings after artificial selection occurred (Dukas et al. 2020), it would make a lot of sense that traits targeting post-copulatory success would be selected for in order to compensate for the pre-copulatory traits that were being selected against. In the horned beetle, *Onthophagus nigriventris*, males that were prevented from developing horns, a characteristic important for pre-copulatory competition and mate choice, tended to develop larger testes. In fruit flies, males from genetic backgrounds that have high mating success in pre-copulatory scramble competition tended to have lower success in stimulating egg production in their mates (Filice and Long 2018). Although these are example of developmental and genetic trade-offs respectively, future studies should continue to utilize artificial selection techniques to investigate if selection can act as a source of trade-offs between pre- and post-copulatory mechanisms.

The main motivation of this study was to investigate how the differences in the male phenotypes described above influence female mate choice in terms of choosiness and preference. Surprisingly, opposite to what we predicted, we found that females who experience harassment and mating from a high sexual aggression male were less choosy when presented with a mating opportunity on the following day. There are several possible explanations for these unexpected outcomes. First, in the case of the harassment tests, the increased harassment experienced by females may have caused them to develop sexual maturity quicker than females that experienced less harassment. It is well known that environmental factors can influence the development of sexual maturity in many species including humans (DeLamater and Friedrich 2002), but to the best of our knowledge, no one has directly tested the effects of early life harassment on development. In nature, female fruit flies will experience harassment from males immediately after they eclose as adults (Markow 2000). Since these fruit flies were derived from a population that selected for early-life reproduction as a consequence of lab maintenance (and fruit flies generally benefit from a life-history strategy that focuses on early reproduction in expanding populations (Edward et al. 2011)), it would be ecologically relevant to invest into maturity as quickly as possible if mating opportunities are available (*see* Filice et al. 2020). Furthermore, female fruit flies tend to become sexually receptive somewhere in between 1 and 2 days following eclosion (Manning 1967). In our study, the harassment phase took place on the morning of day 0 when females were recently eclosed, and the post-harassment mating test took place on the following morning of day 1, meaning that some aspect of the experience phase is causing earlier sexual receptivity. Future studies should continue to investigate this by looking at the developmental and physiological consequences of early-life exposure to sexual harassment.

In the case of the mating tests, the difference in female choosiness may be explained by differences in the post-copulatory male manipulation expressed between the two male treatments. In other words, it could be that males from the low sexual aggression treatment transfer different volumes and/or compositions of accessory gland proteins in their ejaculate that results in their mates having a described mating receptivity (Wigby et al. 2009). This is consistent with our above hypothesis regarding the evolution of trade-offs in pre- and post-copulatory traits, as these males may have evolved different strategies to compensate for the traits selected against during artificial selection. It is also consistent with evidence that suggests longer mating durations tend to positively associate with reduced female remating receptivity (Bretman et al. 2009). However, another and more intriguing possibility is that females are flexibly controlling their own mating rate to gain direct and/or indirect benefits (Arnqvist and Nilsson 2000; Parker and Birkhead 2013). If, for example, males from the high aggression treatment stimulated less oviposition or were of poorer genetic quality compared to males from the low aggression treatment, females could benefit via remating to increase her fecundity, or to “trade-up” genetic quality respectively (Long et al. 2010; Sutter et al. 2019). Future studies should continue to link the outcomes of flexible female mating behaviour to her fitness outcomes in order to disentangle when this plasticity is a female adaptation and when it may simply be manipulation that benefits males.

Finally, in terms of mating preferences, we found that female mate preferences did not change between females exposed to high or low sexual aggression males (as characterized by the insignificant interactions) in both the harassment and mating tests. In both experiments, females mated quicker with males from a high aggression background regardless of the identity of their previous partner. This is consistent with a result from a similar test in Dukas et al. (2020), and suggests that in general, males from the high treatment have higher pre-copulatory mating success. This increased success is likely due to some sort of behavioural component that was inadvertently selected for during the artificial selection (e.g. persistence and/or pursuit), but it also may be due to increased attractiveness. Nonetheless, it may be that in this particular case, the effects of the second male consistently overrode the effects of the first male on female preferences.

Taken together, our results are important for our understanding of variation in the expression of male reproductive behaviours, and how a female's experience with these different behaviours may influence her own subsequent behaviour. Specifically, we found that females that experience harassment and mating from a male artificially selected for high sexual aggression are more willing to mate on the following day compared to females that experience a male selected for low sexual aggression. We propose that this may represent an adaptive mechanism to gain direct and/or indirect benefits, but future studies should continue to investigate the link between female plasticity and fitness in order to determine the specific conditions for flexibility in polyandrous behaviour to be adaptive.

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Authorship Contributions

DCSF carried out the experiments, wrote the first draft of the manuscript and performed the statistical analysis. Both authors designed the experiments and were involved in the revision process.

Data accessibility

Data will be submitted to Dryad Digital Repository if accepted.

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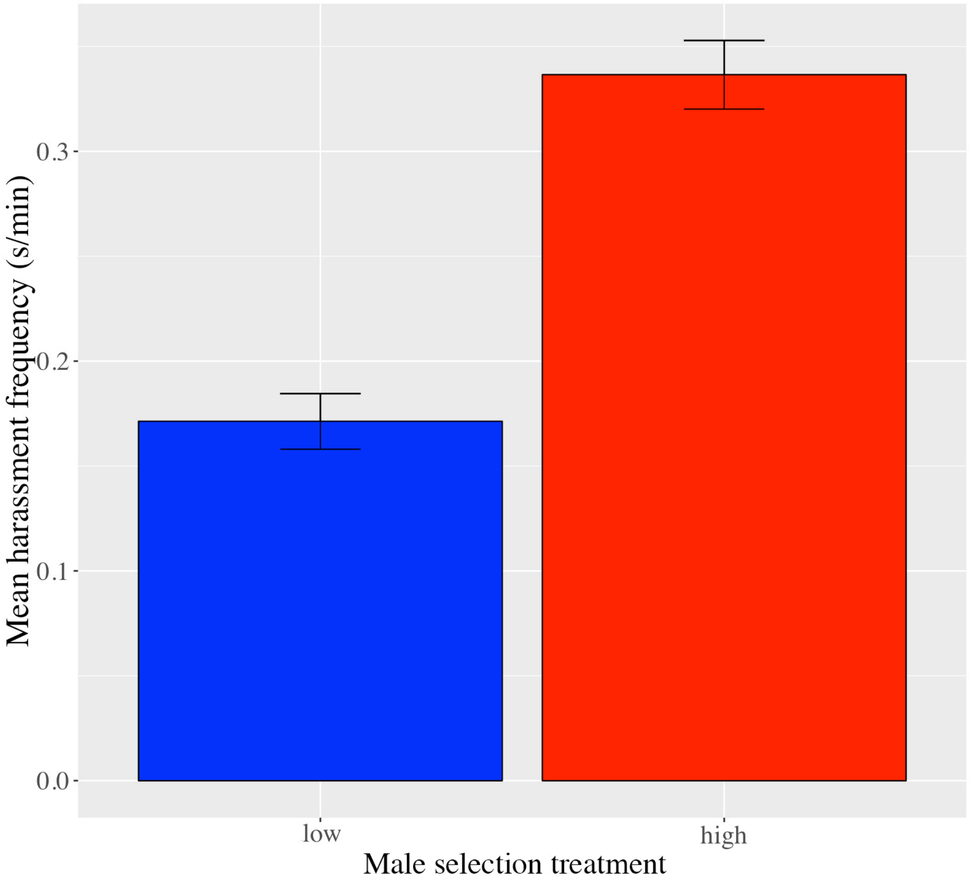

Figure 1

The mean harassment frequency displayed by males during a ten minute observation period for males derived from a lineage selected for low (blue bar) and high (red bar) sexual aggression. The error bars represent 1 standard error of each sample.

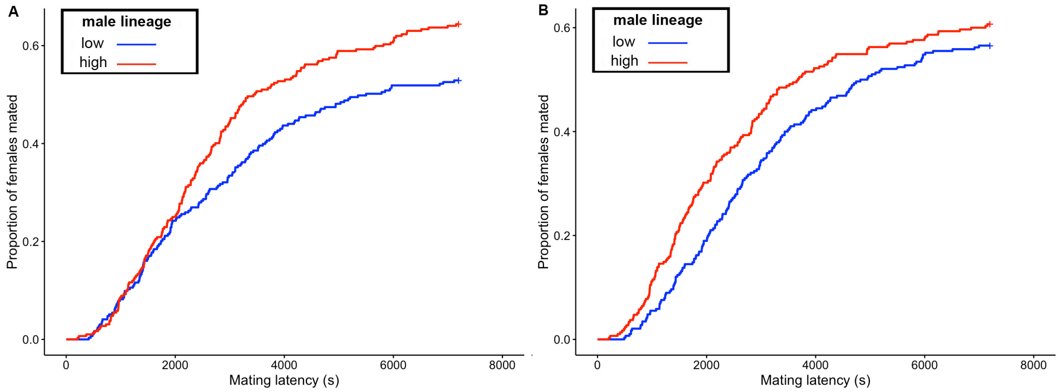

Figure 2

A: Effect of male selection lineage of the harassment experience male on subsequent female mating propensity. Each cox-regression curve represents the proportion of females mate over time. The blue curve represents females paired with a low sexual aggression male and the red represents females paired with a high sexual aggression male during the harassment experience

B: Effect of male selection lineage of the mating test male following previous harassment on female mating propensity. Each cox-regression curve represents the proportion of females mate over time. The blue curve represents females paired with a low sexual aggression male and the red represents females paired with a high sexual aggression male during the mating test

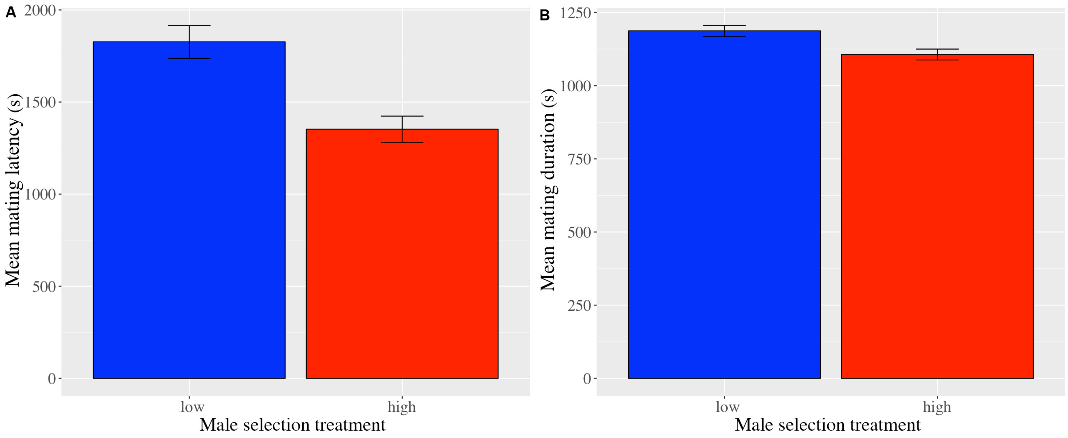

Figure 3

The mean mating latency (A) and duration (B) displayed by males during a 90 minute observation period for males derived from a lineage selected for low (blue bars) and high (red bars) sexual aggression. The error bars represent 1 standard error of each sample.

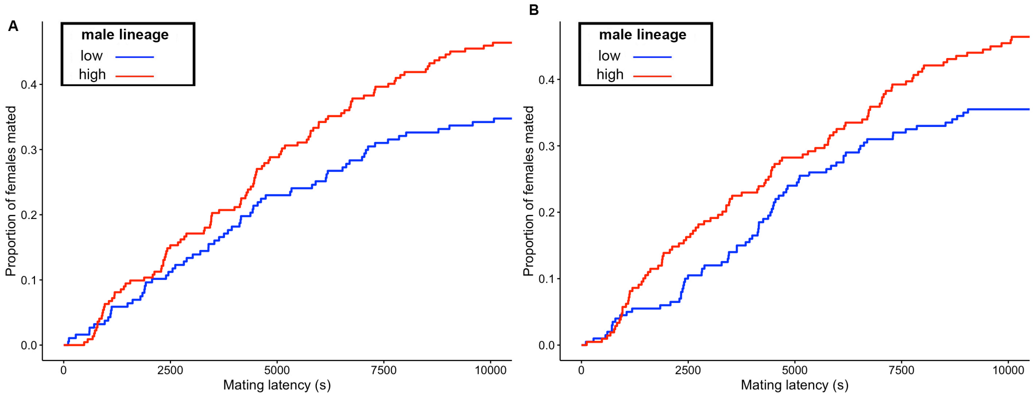

Figure 4

A: Effect of male selection lineage of the mating experience male on subsequent female mating behaviour. Each cox-regression curve represents the proportion of females mate over time. The blue curve represents females paired with a low sexual aggression male and the red represents females paired with a high sexual aggression male during the first mating experience

B: Effect of male selection lineage of the mating test male following previous mating on female remating propensity. Each cox-regression curve represents the proportion of females mate over time. The blue curve represents females paired with a low sexual aggression male and the red represents females paired with a high sexual aggression male during the remating test

CHAPTER 6

Discussion

David C.S. Filice

Discussion

In my general introduction, I reviewed a variety of promising areas that are continuing to disentangle how environmental heterogeneity can shape phenotypic plasticity in sexual phenotypes, and provided examples to argue that plasticity may have important consequences for the outcomes of conflicts between the sexes. In my four data chapters, I supported this argument by experimentally manipulating the social experiences of males and females and measuring their subsequent reproductive phenotypes and evolutionary fitness. In chapter 2, I found that winners of a previous fight tend to fare better in pre-copulatory areas, but losers tend to perform better in post-copulatory areas. In chapter 3, I found that females mated to males previously housed with rivals tend to die younger but invest more into early-life offspring production compared to females mated to males housed alone. In chapter 4, I found that males mated to females that previously experienced a high male density environment have lower pre- and post-copulatory success compared to males mated to females from a low male density environment. Finally, in chapter 5, I found that females that were previously harassed and/or mated to a male artificially selected for high sexual aggression were less choosy compared to females previously exposed to a male selected for low sexual aggression. Here, I consider these results to discuss how plasticity in sexual traits may explain the evolution of flexible strategies in males and females, how such flexible strategies may have evolutionary consequences for the opposite sex, and offer future directions to test my hypotheses.

Evolutionary consequences of plasticity in sexually antagonistic traits: The cheaper gamete (males)

Given the intense competition that males experience, males must invest into many different traits in order to be reproductively successful (Cornwallis & Birkhead, 2008). Sometimes, aspects of mating traits are fixed and expressed by all the males of a particular species, such as grasping behaviour in water striders

(Arnqvist & Rowe, 2002), and traumatic insemination in bedbugs (Morrow & Arnqvist, 2003). Although the absolute expression of these strategies is fixed, males can still vary in their magnitude of expression of these phenotypes. For example, in water striders, *Gerris incognitus,* males have evolved complex grasping structures in order to latch onto females during mating attempts, which females aggressively try to resist due to the physical damage they cause. Although all males using this grasping strategy, they vary in their morphologies and behaviours related to grasping across different ecological populations (Perry & Rowe, 2012). In some cases, this variation may be due to different selective pressures from different environments resulting in the evolution of micro-variation in grasping traits (Rowe et al., 1994). But some of this variation is also likely due to plasticity, where the optimal expression of grasping traits may vary depending on factors such as population density and predation risk (Arnqvist, 1994). In the case of variation generated by plasticity, the consistent expression of plasticity in a certain direction could generate stable selection pressures for females. For example, if more extreme grasping traits are expressed in the absence of predation, then selection may favour more effective anti-grasping traits in females. If then, the ecology changes to high predation where plasticity causes a reduction in the expression of male grasping traits, males may fall behind in the sexually antagonistic arms race and traits that further enhance grasping ability will be selected for (Perry & Rowe, 2018).

Consider this system in comparison to my results in chapter 3, where I found that plasticity in male mating traits influences the life-history of females where males under competition elicit a “live fast, die young” strategy in their mates. In environments where high competition is stable and slower life history strategies are favoured (i.e. maintained on overlapping generations), females may be selected to increase their resistance to males via mechanisms such as reduced mating receptivity. Future studies should test this prediction by experimentally evolving females under two conditions: one where females mate with males reared in competitive environments every generation, and one where females mate with males reared in isolation. If this hypothesis is accurate, we should expect females to evolve greater resistance when evolving in environments with high male-male competition.

In other cases, males of the same species may use radically different reproductive strategies altogether (Gross, 1996). In nature, where resources are limited, males may only be able to invest into the expression of certain phenotypes that are constrained by condition dependence (Rowe & Houle, 1996). It has recently been hypothesized that plasticity can act as an adaptation to help individuals make informed "trade-offs" between their pre- and post-copulatory traits by strategically allocating investment into phenotypes that will maximize their fitness based on their current condition (Simmons et al., 2017). Given the vast landscape of competition that occurs both pre- and post-copulation, these trade-offs may typically occur between secondary sex characteristics such as male weaponry, and ejaculate traits such as testes size (Simmons & Emlen, 2006). In other words, when multiple male strategies are viable in a stable environment, plasticity may allow males to take up different sexual "niches". For example, in guppies, *Poecilia reticulate*, males that experience constant interaction with a female for 7 days prior to testing produce significantly more sperm and had more forced copulations compared to males that experience isolation, while the isolated males relied more on courtship behaviours (Cattelan et al., 2016). This strategy may be adaptive, because when sperm production is upregulated in the presence of mates, coercive strategies may result in a higher number of copulations.

In chapter 2, my results suggest that winner-loser effects may be one of the social factors that can modulate trade-offs in pre- and post-copulatory traits. Overall, these results have important implications for the evolution of male reproductive strategies. If different strategies rely on multiple traits (some of which may not be plastic), then selection may favour traits that complement the most successful strategies within a population. Over short evolutionary timeframes, this hypothesis may offer a solution to the lek paradox, which postulates how genetic variation in male reproductive traits is maintained in the face of directional selection (Kokko & Heubel, 2008), and over long evolutionary time frames, it may lead to the evolution to distinct strategies and promote speciation altogether (Pfennig et al., 2010). Similar to suggestion in the previous paragraph, this hypothesis could be tested by utilizing experimental evolution techniques. After multiple lineages of males have evolved under different contexts that influence plasticity in mating traits (such as winner-loser experiences), we can test the lineages in equivalent conditions to see if other traits have evolved to support pre- or post-copulatory strategies. In the context of the results of chapter 2, we may expect individuals evolving under a losing strategy to evolve traits such as increased testes and/or accessory gland size, and individuals evolving under a winning strategy to evolve traits such as increased body size. This hypothesis may even explain the longer mating duration (a trait which positively correlates with post-copulatory success in the fruit fly (Bretman et al., 2009)) displayed by males selected for low sexual aggression in chapter 5. In general, future studies should continue to utilize experimental evolution along with modern genetic techniques such as genomics in order to better understand how phenotypic plasticity can influence the evolution of populations.

Evolutionary consequences of plasticity in sexually antagonistic traits: The more expensive gamete (females)

A long standing question in evolutionary biology postulates the conditions under which mating multiply in females (polyandry) is beneficial. Although mating multiply can sometimes yield direct benefits such as increased fecundity or nuptial gifts, or indirect benefits such as higher quality genes and/or greater genetic diversity in offspring, females have to balance these potential benefits with the costs associated with mating (Arnqvist & Nilsson, 2000). Given the range of costs and benefits, we might expect that selection may act on traits that enable the flexible expression of polyandry when it best suits the female (Gowaty, 2013). For example, in the fruit fly, females that mate with males that were experimentally manipulated to be infertile are more likely to remate compared to females that mate with fertile males (Sutter et al., 2019). Given that in the same study, females that had a lower reproductive output also tended to remate more frequently, is seems that the flexible modulation of remating rate may be an adaptive mechanism to recuperate infertile and/or genetically incompatible matings.

It is interesting to note how this hypothesis relates to life-history strategies in the fruit fly. When flies are adapted to an environment that favours investment into early-life reproduction, such as from the *Ives* population in chapters 3 and 5, we should predict females to be more polyandrous early in life because of the short-term fitness benefits of multiple matings. This is exactly what we saw in chapter 3, where females from the *Ives* population mated significantly more frequently compared to the population that was more recently caught in the wild and maintained on overlapping generations. With regards to plasticity, it may be that females can use cues in their social environment to determine when maximizing investment into early-life reproduction is appropriate, and respond by upregulating their mating rate. For example, in chapter 5, we saw that females paired with males that display high amounts of harassment tended to have a higher mating receptivity on the following day. It could be that frequent harassment is a signal females would typically experience in high density populations, which is a context in which investment into early-reproduction tends to be beneficial due to higher population growth rates (Edward et al., 2011), and the potential risk of earlier mortality. Future studies should continue to test this hypothesis by exploring other ecologically relevant conditions that favour an early-reproducing life history strategy to determine what cues females can respond to when expressing flexible polyandry.

Finally, it is worth considering how this plasticity in female mating traits may enable the evolution of male harm. In the case of polyandry, the more a female mates, the more post-copulatory competition males have to face, as seen in chapter 4 (Simmons, 2003). Thus, in environments where frequent polyandry is a stable strategy, selection will favour male phenotypes that excel in sperm competition, and as a result, traits that may be more harmful to females. On a scale that enforces polygamy or monogamy, it has been demonstrated numerous times that enforced monogamy tends to result in the evolution of decreased male harm and investment into some post-mating traits (Crudgington et al., 2009; Holland & Rice, 1999; Martin & Hosken, 2003). However, to the best of my knowledge, no one has tested if experimentally manipulating female remating rate rather than enforcing it, can yield similar outcomes. In order to fully appreciate the coevolutionary arms race between the sexes, it is essential to continue to study the impact of flexible phenotypes on evolutionary trajectories.

Final thoughts

The original goal of this dissertation was to be an expansion of my Master's thesis, which described how female experience with males that differ in their magnitude of harmfulness shapes variation in female mate choice behaviours (similar to the objectives of chapter 5). Upon completing my Master's, it was clear to me that more work on this subject needed to be done. Firstly, I saw it was essential to also consider how plasticity in male phenotypes may influence the outcomes of sexual conflict. In general, when it comes to sexual conflict, a lot of research tends to be one sided in focusing on either males or females alone. In nature, male and female phenotypes are interacting in a complex interplay that determines the reproductive outcomes for both the sexes, so I think it is important to keep that in mind when studying any question related to sexual selection. After finishing my Master's, I also thought it was important to connect the outcomes of behavioural plasticity to evolutionary fitness, and the *Drosophila* model system is a perfect system to investigate this. Although, as I describe below, my results related to fitness ended up being quite convoluted, that just gives me a greater motivation to continue studying the tricky relationship between adaptations and rapidly changing environments. To conclude, there are three major academic themes that extend beyond the scope of my dissertation that I would like to discuss.

Firstly, my graduate studies have exposed me to the vast nuances that underlie scientific inquiry. As an undergraduate, you are exposed to a variety of foundational concepts in natural concepts that are often presented as absolute facts. Although I like to think that I had developed the capacity to critically think during my undergraduate education, it wasn't until graduate school that I realized that the actual data that underlie scientific theories can't be interpreted on their own, and have to be carefully considered before being placed in the bigger picture. Graduate school has really taught me the importance of approaching scientific questions with an open mind, to expect that results might not turn out the way we expect them to, and even when they don't, understand that the outcome is just one tiny piece in the grand scientific process. One study doesn't change the world, but it's the culmination of work of many people over many years that has allowed us to develop such a sophisticated understanding of the world.

Next, my studies taught me the importance of considering multiple metrics of evolutionary fitness when trying to describe the ultimate explanations of phenotypes. Earlier on in my graduate career, I was pitfall to the idea that if a study could show that a phenotype increases fitness in some way, it was certainly an adaptation (and even more dangerously, if we couldn't find at fitness benefit, it was not). The results of chapters 2 and 3 reminded me how important it is to view fitness in a life-history framework, as a trait that appears to be deleterious in one context might actually be advantageous in another. A hot topic in the biological sciences is trying to understand if species will be able to adapt to the rapidly changing climates on Earth, but I think in general, we often ignore how radically we have changed our own environments in the last century. Humans tend to view conditions related to well-being such as depression, phobias, and fevers as disorders, but we can't truly understand these conditions unless we view from an evolutionary lens that considers their function in the environments we evolved in. As the world continues to change at a rapid rate, especially with regards to how we socially interact, I believe we have to be very cautious in understanding how these changes affect our behaviours in light of the conditions they are adapted for.

Finally, my time at McMaster University opened my eyes to the importance of integration across all scientific disciplines. As someone who is more interested in the ultimate causes of phenotypes as opposed to their mechanisms, I always found it impressive when biologists are able to draw from areas across multiple disciplines to explore their topics. Given the wide array of open ended questions I left throughout my dissertation, from genomics to CHC and Acp analyses, I hope that I inspire other researchers to tackle these questions with their expertise, or perhaps even learn some of these skills on my own. After completing my degree in a psychology department I also hope that this type of interdisciplinary thinking can be used to help bridge the gap between the natural and social sciences. After all, we are animals, and it is essential to be biologically informed when attempting to understand human behaviour.

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