PERSISTENCE AND AGGRESSION IN *DROSOPHILA MELANOGASTER*

THE ROLES OF MALE PERSISTENCE AND AGGRESSION IN MALE-MALE AND MALE-FEMALE INTERACTIONS IN *DROSOPHILA MELANOGASTER*

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LAY ABSTRACT

All animals face obstacles from their environment that they must respond to in order to survive and reproduce. In this thesis, I focused on how males use aggressive and persistent behaviours to overcome environmental obstacles. Aggression is the use of physical force against another individual, and persistence is when an individual continues in a course of action despite difficulties. I used fruit flies as a model to examine how males fight with one another over access to food and potential mates. I also studied how males persistently harass females in order to mate with them. In many experiments, I found that males use a combination of aggressive and persistent behaviours to achieve a goal. Overall, my results show that even in a simple species like fruit flies, individuals can use complex combinations of behaviours to achieve a variety of goals.

ABSTRACT

All animals face a complex environment full of obstacles that they must overcome in order to survive and reproduce. How an individual responds to its environment is essential to overcoming such obstacles in order to maximize fitness. In my thesis, I focused on the roles of persistence and aggression in achieving fitness-relevant goals. Persistence is continuing in a course of action in spite of difficulty or resistance, and aggression is any instance where an individual uses physical, and potentially damaging, force against a conspecific. I used fruit flies (*Drosophila melanogaster*) as a model system to examine the ways in which males use persistence and aggression to attain fitness-relevant goals such as defending resources, gaining access to females, and mating. I first examined how a male's age affected his persistence in courting recently mated females, who are generally unreceptive, and found that older males were more persistent than younger males (Chapter 2). Next, I showed that males of different ages differed in their courtship persistence in the presence of a competitor, and that males were able to subtly, but directly, interfere with one another's courtship attempts (Chapter 3). I then demonstrated how males were able to use aggression in a mate guarding context to reduce the likelihood that a competitor male mated with their recent mate (Chapter 4), and as a form of resource defense to defend a desirable food patch in the presence of a potential mate (Chapter 5). Finally, I considered male persistence in the pursuit of unreceptive females as a form of male sexual aggression towards recently mated and sexually immature females (Chapters 5 and 6). Overall, my thesis work demonstrates how complex, and sometimes intertwined, the roles of persistence, aggression, and sexual coercion can be even within a 'simple' model organism, such as the fruit fly.

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DECLARATION OF ACADEMIC ACHIEVEMENT

This dissertation is organized in the sandwich thesis format as approved by McMaster University. It consists of seven chapters. **Chapter 1** provides an introduction to the thesis as well as a brief overview of the data chapters. **Chapters 2** to **5** are published manuscripts and **Chapter 6** is a submitted manuscript which has been accepted for publication. **Chapter 7** discusses the results of the data chapters and how they are connected to one another through the themes of aggression and persistence.

CHAPTER 1 – Introduction
Author: Carling M. Baxter

CHAPTER 2 – Effects of age and experience on male mate choosiness
Authors: Carling M. Baxter, Rachael Barnett and Reuven Dukas
Publication: Ethology, 121: 353–363 (2015)
Comments: C.M.B. and R.D. conceived this study. C.M.B. and R.B. performed the experiments. C.M.B. analyzed the data and C.M.B and R.D. wrote the manuscript.

CHAPTER 3 – Mating success in fruit flies: courtship interference versus female choice
Authors: Carling M. Baxter, Joseph Mentlik, Ieta Shams and Reuven Dukas
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CHAPTER 4 – Aggression, mate guarding, and fitness in male fruit flies
Authors: Carling M. Baxter, Rachael Barnett and Reuven Dukas
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CHAPTER 5 – Life history of aggression: Effects of age and sexual experience on male aggression towards males and females
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CHAPTER 6 – Genetic variation in sexual aggression and the factors that determine forced copulation success
Authors: Carling M. Baxter, Janice L. Yan and Reuven Dukas
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CHAPTER 7 – Discussion
Author: Carling M. Baxter

CHAPTER 1 – INTRODUCTION

1.1 General introduction

Anyone who works closely with animals of a given species will likely notice that there is variation in the behaviour of the individuals that they work with. However, there is of course a lot of consistency in behaviour across individuals as well. This is because traits that maximize fitness are more likely to persist across generations (provided they are heritable). On the level of an individual, its optimal course of action is one that will maximize its fitness, either directly or indirectly. Even individuals of relatively 'simple' species face a variety of challenges they must work through in order to maximize their fitness. For example, in nematodes (*Caenorhabditis elegans*), individuals must be able to seek out food based on a variety of chemosensory cues in their environment. When faced with varying qualities of food individuals can even choose to stay at a food site or leave it based on its quality relative to what they have experienced before (Borisovich Shtonda & Avery, 2006). The ability of animals to modify their behaviour based on their prior experiences and current circumstances is important to being able to act optimally in a variable environment.

Behavioural patterns are flexible, and there can often be multiple ways in which an individual could face the same challenge or overcome the same obstacle. For example, meerkats (*Surikata surikatta*) consume a variety of different prey items, each with their own distinct handling needs (Doolan & Macdonald, 1996). One potentially dangerous prey type that is available to meerkats year-round is scorpions (genera *Parabuthus* and *Opistophthalamus*). Meerkats must handle scorpions carefully, and such handling can be time consuming and requires practice (Thornton & McAuliffe, 2006). If, for example, a meerkat is faced with a particularly difficult-to-dismantle scorpion it may persist in attempting to dismantle it until it is successful, whereas a different meerkat may simply abandon it and search for a simpler prey to deal with. Alternatively, a more dominant individual may forgo the hunting and handling stages entirely and simply use aggression to steal pre-handled food from others (e.g. Thornton & Samson, 2012). The end goal in each of these situations is the same: to eat. However, the behavioural patterns performed to reach that goal are distinct. In the first instance the individual persisted in a course of action despite difficulty, in the second it abandoned the course of action to pursue alternatives, and in the third it used aggression against a conspecific to bypass the difficult handling. From this example it's clear how persistence and aggression are both ways in which an individual can attempt to overcome obstacles in order to reach a goal.

It is important to note that both persistence and aggression are not behaviours themselves, but ways in which an individual can utilize behavioural patterns. There can be overlap in behaviours that are categorized as persistent or aggressive, and they can be used to reach the same types of goals, but they are distinct concepts, so I will expand on each separately below.

1.2 Persistence

Persistence can be defined as the continuation in a course of action in spite of difficulty or resistance. This can be difficulty in overcoming environmental obstacles, such as during foraging (e.g. Grieg-Smith, 1987) or in overcoming resistance by a conspecific, such as during courtship of unreceptive females (e.g. Las, 1980; Marshall, 1982). The key for an individual is to find an optimal balance between persisting in a course of action or abandoning it to pursue alternatives. This optimum can be thought of as the duration of persistence in a course of action that will maximize an individual's fitness gain per unit of effort invested; where gain is equal to the probability of success multiplied by the benefit given it succeeds, and effort invested is the time taken to encounter this item or individual plus the time and energy put into handling or persisting (combined ideas from Greig-Smith (1987) and Parker (1974)). This model assumes an individual knows something about the encounter rate in its environment as well as the variation in quality of the items it will encounter. If many alternatives are readily available, then persisting for a long duration may not be beneficial. However, if the encounter rate with such items is rare, then persisting for long durations, in spite of difficulty or resistance, is likely the optimal course of action. If the environment is constant, then an individual can use the same rule for how persistent to be in particular situations. However, if aspects of the environment change over time, then individuals who are able to adjust their persistence based on their prior experience or environmental cues will be better able to maximize their fitness. In the case of foraging, Greig-Smith (1987) found that bullfinches (*Pyrrhula pyrrhula*) seem to take into consideration the abundance and variation in quality of seeds in their environment when deciding whether to persist in handling difficult-to-open seeds versus abandoning them to search for new seeds. This implies that bullfinches are altering their persistence based on their prior experience with the seeds in the environment that they are currently foraging in. The general 'rules' of weighing the costs and benefits of persisting in a course of action can be applied across a variety of different challenges an individual may face.

In an environment that frequently changes, individuals may be faced with problems that are entirely novel to them. The ability to solve novel problems could help individuals access food in a newly urbanized environment, for example. Although one may at first expect aspects of intelligence to be fundamental to problem solving, a recent computational model predicted that persistence was more important than learning for novel problem solving (Guez & Griffin, 2016). The importance of persistence in problem solving has been supported empirically as well. When groups of wild meerkats were presented with novel 'problem boxes' to open, it was not the first individuals to approach the apparatus, nor the most dominant individuals who solved them, but simply the individuals who spent the most time attempting to open them (Thornton & Samson, 2012). In other words, the most persistent individuals were the ones most likely to solve the problem. A similar result was found in Indian mynas (*Sturnus tristis*), where the individuals who made the most attempts to open the novel problem box were more likely to succeed (Griffin, Diquelou, & Perea, 2014). Therefore, persistence may play an important role in overcoming novel problems across a variety of different species.

An important goal for any sexually reproducing individual is to reproduce with an appropriate mate. However, determining whether a potential mate is appropriate depends on the relative fitness an individual would gain from mating with them and the likelihood that mating will occur (either through acceptance, coercion or force). For males, who often have to locate and court potential mates, the decision to begin courting and persist in courting a given female depends on a variety of factors. Males seeking mating opportunities can optimize their fitness by modifying their level of persistence in the pursuit of a given female based on the encounter rate with females of varying receptivity levels. This parallels the bullfinch foraging example above, where individuals modified their persistence in attempting to open a given seed based on their encounter rate with various types of seeds (Parker, 1974). While studying male pursuit tactics in salticid spiders (*Phidippus johnsoni*), Jackson (1978) found that males modify their degree of persistence in courting females based on whether they find the female inside or outside of her nest. When a female is found inside her nest, she is more reluctant to mate and seems to require a longer duration of courtship before accepting a mate. He compares the tactics of males pursuing different 'types' of females to the way predators pursue different prey items that vary in encounter rate and handling time. More recently, Louâpre et al. (2015) have formally compared models of optimal foraging to male mate search and suggest that applying ideas from optimal foraging theory to male mate search will allow researchers to better understand male mate choice behaviours. Given the role of persistence in optimal foraging has been well established, applying these models to male mate search may shed further light on the role of persistence in male mate choosiness.

The ability of males to modify their level of persistence in pursuing a given female, versus abandoning pursuit to search for other females, has also been documented in greenhouse whiteflies (*Trialeurodes vaporariorum*, Las, 1980), orange tip butterflies (*Anthocharis cardamines*, Wiklund & Forsberg, 1986) and common pierid butterflies (*Colias philodice* and *C. eurytheme*, Marshall, 1982). In pierid butterflies, males modify their level of courtship persistence throughout the day based on the fact that there is generally a greater proportion of recently eclosed, unmated females available in the early mornings compared to later in the day (Marshall, 1982). If a male encounters an unreceptive female in the early morning, he will quickly abandon courting her and search for another female since virgin females are relatively abundant. Whereas in the afternoon, when encountering a virgin female is less likely, males will persist in courting initially unreceptive females for longer durations before either eventually succeeding in mating or abandoning courtship to search for other females. This temporal variation in a male's courtship persistence allows males to modify their reproductive efforts and maximize their potential fitness.

I previously described the 'appropriateness' of a mate as depending on the fitness gain an individual will receive by mating with them, combined with the likelihood that mating will occur. However, this appropriateness can differ for males and females, namely within a pair of individuals a male may benefit by pursuing and mating with a particular female, while she suffers a cost from being pursued by and/or mating with that male. Because the optimum for males and females often differs, it generates conflict between the sexes. Optimal mating rates are often higher for males than for females (e.g. Bateman, 1948; Norton & Uetz, 2005; Rowe, Arnqvist, Sih, & Krupa, 1994) generating conflict over the occurrence of mating, where persistence, by both males and females, can be involved. A female who has recently mated, and still has sperm from her previous mate may have relatively little to gain from mating with another male. Oftentimes, males will persistently harass mated females who resist mating, as seen in water striders (*Gerris* spp, Arnqvist, 1992; Rowe et al., 1994), fruit flies (*Drosophila melanogaster*, Byrne, Rice, & Rice, 2008; Wigby & Chapman, 2004), elephant seals (*Mirounga angustirostris*, Le Boeuf & Mesnick, 1991), and waterfowl (family: Anatidae, Mckinney, Derrickson, & Mineau, 1983; McKinney & Evarts, 1997). Females can also vary how persistently they reject males and whether they accept an unnecessary mating, as seen in water striders (*Gerris spp*, Arnqvist, 1992) and West Indian sweet potato weevils (*Euscepes postfasciatus*, Kumano, Kuriwada, Shiromoto, Haraguchi, & Kohama, 2011). If the male's persistent sexual harassment leads to direct physical harm for the female, then it becomes a form of sexual aggression, and can represent one area of overlap between the roles of persistence and aggression in overcoming resistance to achieve mating success.

1.3 Aggression

Aggression is the use of, or threatened use of, physical and potentially damaging force against a conspecific. As stated above, it is important to remember that aggression is not a single behaviour or even a group of specific behaviours, but rather a broad categorization of a variety of different behaviours that can be performed to achieve distinct goals both within and across species (Moyer, 1968). Although males tend to be the more aggressive sex, in many species both males and females utilize aggression to secure valuable resources.

In almost any natural environment access to resources is limited, making conflict over resources essentially unavoidable. As a result, aggressive disputes, or contests, over limited resources are common in nature and in some cases specific weaponry for aggressive contests has evolved (e.g. antlers of male deer (family: Cervidae, Clutton-Brock, 1982)). The presence of such weaponry means that the maximal costs of an aggressive encounter can be very high. Given these potentially high costs, why are aggressive contests relatively common? First, as noted by Maynard Smith and Price (1973), many aggressive contests seem to follow what they refer to as 'limited war' strategies, meaning that individuals rarely use lethal actions against their opponent even when those actions would be simple to perform. An example of a 'limited war' strategy occurs in western diamondback rattlesnakes (*Crotalus atrox*), where males attempt to wrestle one another into submission, but do not bite their opponent with their venomous fangs (Gillingham, Carpenter, & Murphy, 1983). When computationally modelling different conflict strategies, Maynard Smith and Price (1973) found that strategies where individuals rarely escalate to highly damaging aggressive acts are more evolutionarily stable than strategies where individuals are always highly aggressive or non-aggressive. Therefore, the costs associated with aggressive contests are unlikely to reach their potential maximums. Additionally, a potential benefit of engaging in aggressive contests is that they can inform an individual about the strength of their competitor. This can allow individuals to make a more informed decision about whether the value of the resource outweighs the potential cost of fighting a given competitor, based on the relative strength asymmetry between themselves and their competitor (Enquist & Leimar, 1983). This idea of mutual-assessment by fighting males has been supported by work in the bowl and doily spider (*Frontinella pyramitela*), where males who are fighting over access to females seem to adjust their persistence in fighting based on the relative strength of themselves compared to their competitor (Leimar, Austad, & Enquist, 1991). Therefore, the use of aggression in conflicts over limited resources has many potential benefits, and the costs of aggression rarely escalate to their potential maximums.

Since aggression can take on many forms, and manifest through many distinct actions, it is reasonable to categorize aggressive acts based on their function. As stated above, aggression is often used in contests over access to limited resources. However, it can also be used by both males and females to protect prior reproductive investments. Females, who typically invest more in each offspring than do males (Trivers, 1972), may protect their investments by showing maternal aggression, where a mother aggressively defends her offspring (Gammie, Hasen, Rhodes, Girard, & Garland, 2003; Maestripieri, 1992; St. John & Corning, 1973). In the absence of paternal care, males can also protect their reproductive investments by guarding their mates from other males. In Idaho ground squirrels (*Urocitellus brunneus*), after mating a male will stay close to his mate for the remainder of her oestrus stage which lasts a few hours (Sherman, 1989). During this time the guarding male will attack any intruder male who approaches the female. If no guard is present, females will often mate with, and produce offspring by, multiple males. Therefore, there is a measurable benefit to a male's paternity success from aggressively guarding his mate. Mate guarding can, of course, be costly, as demonstrated in Seychelles warblers (*Acrocephalus sechellensis*), where guarding males spend less time foraging, and as a result are in poorer body condition, than males who are not guarding their mate (Komdeur, 2001). However, these males appear to weigh the costs and benefits of guarding their mates, as they guard females less intensely (and forage more frequently) when there are fewer competitor males nearby. Therefore, there are many circumstances where both males and females can benefit from using aggression to protect their reproductive investments.

Prior to mating, males can also use aggression to increase their likelihood of mating either directly or indirectly. Male fruit flies (*Drosophila melanogaster*) are known to fight over food resources that are attractive to females (Chen, Lee, Bowens, Huber, & Kravitz, 2002; Hoffmann, 1987; Hoffmann & Cacoyianni, 1990; Lim, Eyjólfsdóttir, Shin, Perona, & Anderson, 2014) and this resource defense can lead to increased mating success (Dow & Schilcher, 1976; Hoffmann & Cacoyianni, 1989). Males can also defend large territories in order to have primary mating access to the females that live within those territories, as seen in Lake Eyre dragons (*Ctenophorus maculosus*, Olsson, 1995b). Male Lake Eyre dragons not only use aggression against other males in territorial disputes, but they also use sexual aggression directly towards females to coerce them into mating (McLean, Chan, Dickerson, Moussalli, & Stuart-Fox, 2016; Olsson, 1995a), thus demonstrating how males can utilize aggressive behaviours under a variety of situations to increase their reproductive success.

As with persistence, the key to an individual's success is finding the optimal balance between being aggressive or being complacent under a variety of circumstances. Decisions regarding an individual's aggression are not binary; one can choose to vary the intensity and duration that they will engage in aggressive contests based on many factors. Therefore, it can often be informative to measure an individual's persistence in aggressive behaviours, rather than simply the presence or absence of an aggressive response to a given situation.

1.4 Structure of the thesis

In this section I will briefly outline the chapters of my thesis and introduce how they relate to the overarching themes of persistence and aggression.

When I began my graduate studies, my initial goal was to gain a better understanding of male mate choosiness. Specifically, I was interested in how factors such as age and previous experience can affect how discriminating a male will be if presented with females of varying reproductive value. To do this I used fruit flies (*Drosophila melanogaster*) as my model organism (note: many advantages of using fruit flies as a model system are given in Chapters 2-6, so for the sake of conciseness I do not repeat them here). With this goal in mind, we designed a series of experiments comparing the courtship choosiness of males of distinct age groups (Chapter 2). We found that older males spent more time pursuing unreceptive, recently mated females, who perform a variety of rejection signals, than were younger males. Although this result can be interpreted as a lack of choosiness on the part of older males, it can also be interpreted through the lens of persistence: older males were highly persistent in courting recently mated females in spite of resistance. While performing these experiments, we also found that some males were more persistent in their courtship in the presence of a competitor and noted informally that males may have been interfering with one another's courtship attempts. It was these observations that began my shift in focus away from choosiness towards persistence and eventually to aggression.

We directly followed-up on the observation of persistence in the presence of a competitor and potential male-male interference and expanded on them in Chapter 3, where we quantified males' subtly aggressive interruptions of one another's courtship and tested whether this male-male interference affected mating success. In Chapter 4 we examined a more overt expression of aggression against competitors by measuring how males alter their aggression levels in the presence of their recent mates, and how this may affect their reproductive success. Finally, we considered male persistence in pursuit of unreceptive females as a form of male sexual aggression (via coercion and forced copulation) in Chapters 5 and 6.

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CHAPTER 2 – EFFECTS OF AGE AND EXPERIENCE ON MALE MATE CHOOSINESS

Baxter, C. M., Barnett, R., & Dukas, R. (2015) Effects of age and experience on male mate choosiness. *Ethology. 121*: 353–363.

2.1 Abstract

Mate choosiness by males has been documented in many taxa but we still do not know how it varies with age even though such variation can be important for our understanding of sexual selection on females. Theory provides conflicting predictions: young males, who are less attractive to females than older males, may be less choosy, or older males, who face fewer expected future mating opportunities, may be less choosy. In our experiments with fruit flies (*Drosophila melanogaster*), young (one-day-old) males spent relatively less time courting recently mated females than did mature (four-day-old) males. Overall, there was a gradual decline in male mate choosiness from age one to seven days. Because male age was correlated with the duration of deprivation from females, we tested for the effect of deprivation and found that same-age males previously exposed to females were more choosy than female-deprived males. We also assessed key male parameters that could affect choosiness and found that, compared to mature males, young males were less attractive to females, less competitive in intra-male interactions and less fertile. Although the lesser attractiveness and competitiveness should select for lesser mate choosiness in young males, their limited fertility and more expected future mating opportunities seem to override the other factors and lead to high mate choosiness in young males. Overall, our data indicate that young males just after reaching sexual maturity are choosy, and that subsequent exposure to females can maintain high levels of male mate choosiness with age. Hence males can contribute much more to sexual selection than previously appreciated.

Key words: male mate choice; sexual selection; courtship; fruit flies; *Drosophila melanogaster*; age; experience

2.2 Introduction

It is now well established that male mate choice is prevalent even in species where males provide only sperm (Bonduriansky 2001; Edward & Chapman 2011). Examples include water isopods (*Asellus spp.*) (Manning 1975), Fruit flies (*Drosophila melanogaster*) (Cook & Cook 1975), fowl (*Gallus gallus*) (Pizzari et al. 2003), mice (*Mus musculus domesticus*) (Ramm & Stockley 2014) and monkeys (*Macaca mulatta*) (Herbert 1968). Male mate choice in such species indicates that, even in such taxa, males are sometimes limited by factors other than access to females. Such factors may include time, sperm or seminal fluid, and the mortality costs of courtship and mating (Dewsbury 1982; Schwagmeyer & Parker 1990; Van Voorhies 1992; Wedell et al. 2002). Indeed, experiments in a variety of species indicate that courtship is associated with increased mortality rate (Cordts & Partridge 1996; Clutton-Brock & Langley 1997; Kotiaho 2000), that sperm-limited males are more choosy than males with large sperm stores (Byrne & Rice 2006; Long et al. 2009) and that males strategically allocate more sperm to mated, large, attractive and young females than to virgin, small, unattractive or old females, respectively (Wedell et al. 2002; Pizzari et al. 2003; Lüpold et al. 2011; Ramm & Stockley 2014).

The realization that even males in settings with a highly male-biased operational sex ratio can benefit from exercising choice owing to the costs of courtship and mating paves the way to analyses of the optimal allocation of males' resources to courting and mating based on the expected increments in fitness that mating with a given female can provide (Kokko & Monaghan 2001; Kokko et al. 2006). Indeed research on male mate choice indicates that males often prefer females that are more fertile as indicated by their body size, age and current mating status. Examples include male preference for larger, younger and virgin females in a wide variety of taxa (Bonduriansky 2001; Wedell et al. 2002; Edward & Chapman 2011). Another factor that should affect male mate choice is the likelihood of succeeding in mating with a given female, especially in taxa where females accept males only after an extensive period of courtship.

In addition to the female characteristics that influence male mate choice, males' optimal investment in mating effort and their level of mate choosiness may vary with their own traits, including relative quality, social status and age. While there has been significant research effort devoted to assessing age-specific allocation of resources to reproduction (Clutton-Brock 1988; Roff 1992; Stearns 1992), much of this work has focused on females. We are not aware of life history models addressing specifically the optimal allocation of resources to reproduction with age in males and it is not obvious to what degree the theory focusing on females is relevant for males. The limited experimental data on mate choice as a function of male age are inconclusive. Martel et al. (2008) reported no effect of age on choosiness in males of the parasitoid wasp *Trichogramma turkestanica* presented with virgin and inseminated females. Two related studies in fruit flies (*D. melanogaster*) documented, first, that there were slightly higher pairings (54% vs 46%) between males and the more fertile out of two available females (Edward & Chapman 2012), and that there was little variation with male age in the potential benefit from mate choice (Edward & Chapman 2013). Finally, in our own work, we found that young, one-day-old male fruit flies (*D. melanogaster*) showed higher levels of mate choosiness than mature, four-day-old males. Specifically, while the young males spent as much time as the mature males pursuing conspecific females, they devoted significantly less time to courting females of the closely related species *D. simulans*. We replicated these findings with two distinct lines of *D. melanogaster*, using a variety of protocols including no-choice and choice setups, and testing inexperienced and experienced males. We suggested that the mature males were less choosy than the young males because they had experienced a longer period of female deprivation prior to the tests (Dukas & Baxter 2014).

Our data indicating variation in male mate choosiness with age called for a few follow up experiments. First, the fact that young males were rather selective and mostly avoided courting heterospecific females is highly relevant for research on incipient speciation (Peterson et al. 2005; Servedio & Dukas 2013). To assess the pertinence of our findings for work on sexual selection on females, we wished to quantify male mate choosiness when encountering distinct categories of conspecific females. To this end, we tested how male age influences mate choosiness when encountering either small versus large females, or virgin versus recently mated females. Second, to refine our analysis of male age and mate choosiness, we wished to assess male mate choosiness with age over males' first 7 days of life. This period corresponds to males' realistic expected life span in the field (Rosewell & Shorrocks 1987). Third, we wished to test our deprivation hypothesis, which states that males deprived of females are less choosy than males that encounter females prior to the test (Dukas & Baxter 2014). Fourth, mate choice typically involves two-way interactions between prospective mates and their chooser. In order to focus on male mate choice, we always used females that consistently rejected males. Still there was a slight chance that females responded differently to young and mature males. To test this possibility, we conducted detailed observations quantifying females' responses to courting young and mature males. Finally, to help us explain the observed variation in mate choosiness with age, we wished to quantify relevant parameters in young and mature males. These included attractiveness to females, competitive ability and fertility.

2.3 General methods

We used wild-type *Drosophila melanogaster* housed in population cages with several hundred flies per cage. The cages were kept in an environmental chamber at 25°C and 60% relative humidity with a 12:12 hour light:dark cycle, with the lights turning on at 10:00 am. Unless stated otherwise, we reared the experimental flies in 240-mL bottles with 50 mL of food and approximately 300 eggs per bottle. The food was a standard fly medium consisting of sucrose, cornmeal, yeast, agar and methyl paraben.

We sexed flies within 4 hours of eclosion to ensure virginity. We used gentle aspiration to sex and transfer males individually into food vials, whereas we used CO_2 to sex and place females in groups of 20 per food vial. We did not use CO_2 with the focal males in order to avoid a possible confound owing to different recovery times for young than mature males. Each 40-mL vial contained 5mL of the standard fly medium and the females' vials also contained a dash of live yeast. At the time of testing, mature males and females were 4 days old, young males were 1 day old and virgin females were less than 20 hours old and hence sexually immature. Immature virgin females are as sexually attractive to males as are sexually mature virgin females, but they always reject males' advances (Dukas and Dukas 2012). By using immature virgins, we ensured that all females persistently provided males with rejection signals so we could focus on measuring males' willingness to court a given female based on his motivational level and his subjective estimate of his likelihood of mating with that female. Recently mated females were 4-days old and mated with 4-day old males 1–4 hours before being used in the observation phases. In our laboratory, such females rarely remate (Dukas and Dukas 2012). We used small amounts of pink fluorescent powder to mark females in order to allow us to distinguish between the two female types when placed simultaneously in vials. Such marking has no effect on either fly behaviour or attractiveness (Dukas and Baxter 2014). Courtship observation phases were 15 min long, during which observers blind to male age recorded all courtship behaviours.

We used generalized estimating equations with gamma distributions and log link functions to analyze the data for which there were repeated measures for each male, and generalized linear models with either gamma or Tweedie distributions with log link functions to analyze the data for which there was only one measure per individual using IBM SPSS (IBM-Corp. 2011). We applied sequential Bonferroni corrections to all post-hoc comparisons.

2.4 Effects of male age on conspecific mate choosiness

First, we wished to broaden our investigation by examining the effect of male age on choosiness when encountering two conspecific females of distinct qualities. We tested two female qualities, body size (small versus large), which is highly correlated with expected fertility in fruit flies as well as other insects (Robertson 1957; Honěk 1993), and mating status (recently mated versus virgin), which informs males about their likelihood of mating (lower in mated than in virgin females, Dukas (2005a)).

2.4.1 Methods

In the first experiment, we tested whether there were age-related differences in males' mate choosiness between small and large females. We placed each male (young or mature) in a vial with one large and one small virgin female and recorded the proportion of time he spent courting each female (n=94 males, half of each age).

We obtained small and large females by rearing flies under different densities. We reared small females in high density food vials with approximately 200 eggs per vial containing 1.5mL of the standard fly medium, whereas we reared large females in low density food bottles with about 75 eggs per bottle containing 50mL of the standard fly medium. After testing, we measured the wing length of a random sample of 12 small and 12 large females. We removed the right wing of each female, mounted it on a microscope slide and measured the linear distance between the humeral-costal break and the end of the third longitudinal vein (Gilchrist & Partridge 1999). The wings of large females were about 45% longer than those of the small females, 2.18 $\pm$ 0.02 vs 1.5 $\pm$ 0.028 mm, respectively (Wald χ^2_1=328, n=24, *P*<0.001).

In the second experiment, we tested for age-related differences in males' choosiness between virgin and recently mated females. We placed males in vials with one female of each type and recorded the proportion of time that males spent courting each female (n=88 males, half of each age).

2.4.2 Results

When each male encountered one small and one large female simultaneously, there was no interaction between male age and female type (Wald χ^2_1=0.05, n=94, *P*=0.8; Fig. 2.1). Both male ages spent more time courting large than small females (P<0.001).

Males that were presented with both a virgin and recently mated female spent more time courting the former, but there was a significant interaction between male age and female category (Wald $\chi^2_1=6.9$, n=88, $P<0.01$; Fig. 2.2). While the young males spent less time courting the recently mated female than did the mature males ($P<0.05$), both male categories spent similar, large proportions of time courting the virgin females ($P=0.6$).

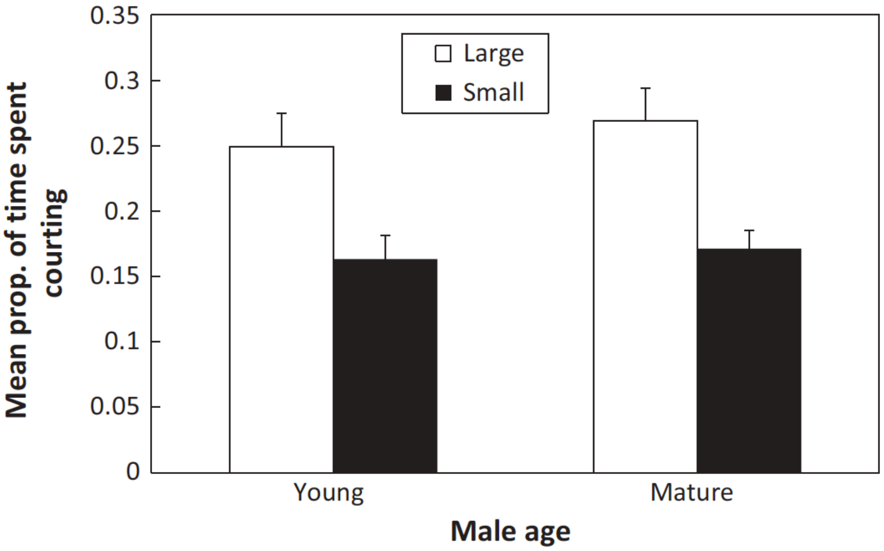

Figure 2.1

The proportion of time (mean ± 1 SE) that young (1-day-old) and mature (4-day-old) males spent courting large and small females (n=47 young and 47 mature males) when presented with one female of each type simultaneously. There was no significant (*P*=0.8) male age by female size interaction.

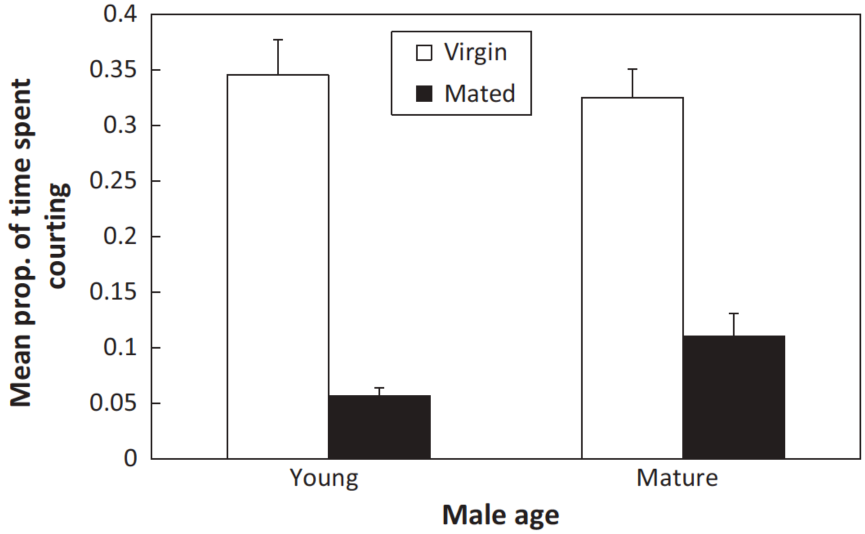

Figure 2.2

The proportion of time (mean ± 1 SE) that young (1-day-old) and mature (4-day-old) males spent courting virgin and mated females (n=44 young and 44 mature males) when presented with one female of each type simultaneously. There was a significant interaction (*P*<0.01) between male age and female mating status.

2.5 Males' mate choosiness from age one to seven days

Our previous experiment as well as all the experiments in Dukas and Baxter (2014) compared mate choosiness in one day old versus four day old males. Here we wished to broaden our analysis in order to examine whether there is further decline in mate choosiness in males older than four days but within a realistic age range encountered in the field (Rosewell & Shorrocks 1987). Owing to enormous day and time of day variation, we have to conduct all our comparisons simultaneously and thus focused on males that were 1, 3, 5 and 7 days old.

2.5.1 Methods

We tested males that were 1, 3, 5 and 7 days old by placing them with one virgin and one recently mated female (*n*=120 males, 30 of each age) and recorded the proportion of time males spent courting each female.

2.5.2 Results

While males of all ages spent more time courting virgin than recently mated females, there was a significant interaction between male age and female type. This was caused by both an increase with male age in the time spent courting recently mated females and a decrease with male age in the time spent courting virgin females (Wald χ^2_2=66, n=120, *P*<0.001; Fig. 2.3).

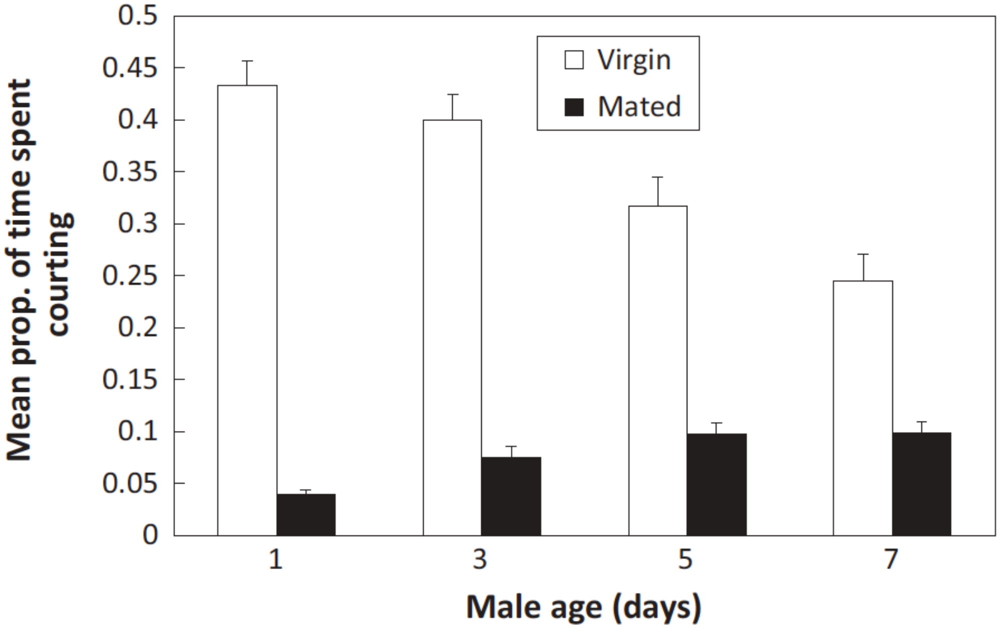

Figure 2.3

The proportion of time (mean ± 1 SE) that males aged 1, 3, 5 and 7 days spent courting virgin and recently mated females when presented with one female of each type simultaneously (n=120 males, 30 of each age). There was a significant interaction (*P*<0.001) between male age and female mating status.

2.6 Effects of female deprivation on male mate choosiness

The standard protocol in mate choice experiments is to sex eclosing adults and keep them in single-sex vials until the test in order to control for their experience and ensure female virginity. Males that encounter no females for a longer period might show lower mate-acceptance criteria and this could explain our observed differences in mate choosiness between young and mature males (Dukas & Baxter 2014). To test for this possibility while controlling for male age, we compared mate choosiness of same-age, mature males that had either encountered and mated with females or encountered no females prior to the test.

2.6.1 Methods

We randomly assigned males to either a deprived or an experienced treatment group. Deprived males encountered no females prior to the test while experienced males had a mature virgin female added to their vial on days 1, 2 and 3. To simulate realistic settings in which males experience both mature virgin and mated and females, we kept all added females in the vials. We also moved all flies into new food vials on day 3 to ensure the availability of fresh food. On Day 4, we moved the males into test vials with one virgin female and one recently mated female and recorded the proportion of time that the males spent courting each female (n=72 males, half from each treatment).

2.6.2 Results

There was a significant interaction between male treatment and female type (Wald χ^2_2=21, n=72, P<0.001; Fig. 2.4). Both experienced and deprived males courted virgin females for similar proportions of time (P=0.75). However, experienced males courted mated females for significantly less time than deprived males did (P<0.001).

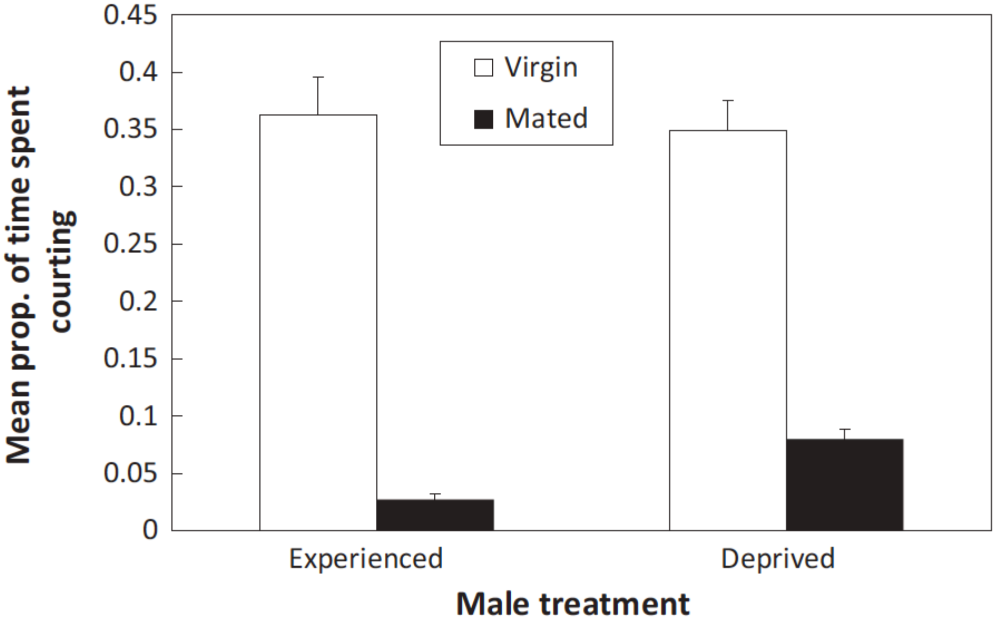

Figure 2.4

The proportion of time (mean ± 1 SE) that mature (4-day-old) males experienced with females (n=36) and mature males deprived of females (n=36) spent courting virgin and recently mated females when presented with one female of each type simultaneously. There was a significant interaction (*P*<0.001) between male age and female mating status.

2.7 Females responses to courtship by young versus mature males

Male fruit flies that encounter a female have a variety of cues that can inform them about their likelihood of mating with that female. First, as in many insects, the female's blend of cuticular hydrocarbons contains species- and age-specific information (Jallon 1984; Ferveur 2005; Howard & Blomquist 2005; Everaerts et al. 2010). Second, at least two volatiles provided by males during copulation, cis vaccenyl acetate (cVA) and CH503, mark the female as mated and dramatically reduce that female's attractiveness to males (Brieger & Butterworth 1970; Zawistowski & Richmond 1986; Yew et al. 2009; Ng et al. 2014). Finally, if the male initiates courtship, the female's behaviour might provide further information about her receptivity (Spieth 1952; Lasbleiz et al. 2006). Although we used females that rejected males, one might argue that females could vary in their responses towards young and mature males and that this in turn determined the observed difference in male mate choosiness. To assess this possibility, we compared the behavioural responses of mature virgin females to young and mature males.

2.7.1 Methods

Our methods were identical to those used in a project dedicated to quantifying female behavioural responses to males, in which we have found significant variation in the behaviour of different female categories towards conspecific and heterospecific males (R. Dukas, unpublished data). We placed 4 sexually mature virgin females about 25 h old inside a 10x10x10 cm Plexiglas cage. The cage contained a cylinder of 5 ml regular food medium with a dash of live yeast illuminated with light from an LED lamp. We then added a single male, either 1-day old or 4-days old. We recorded male and female behaviour for 5 min starting with the first courtship, which usually occurred within a few min. We recorded all starts and ends of male courtship bouts and the female's behaviour while being courted. Females either were non-responsive and appeared to continue with their pre-courtship activity, most often feeding, or clearly responded to males with wing fluttering, raising their abdomen or decamping (Spieth 1952). While we also recorded whether females were feeding, walking or idling on the cage wall, we had decided a priori to focus on the three explicit female rejection behaviours. We calculated for each female the proportion of time spent wing fluttering and abdomen raising, and the decamping rate. We calculated the proportions of times and decamping rates based on the trial duration for each female, which was 5 min in trials with no matings and the mating latency in trials with matings. We tested 20 males of each age category.

2.7.2 Results

Females' rates of abdomen raising (1.1 ± 0.5 vs 0.65 ± .37 per min, Wald χ^2_2=1.1, n=40, *P*=0.3), wing fluttering (4 ± 1.2 vs 3.4 ± 1 per min, Wald χ^2_2=0.9, n=40, *P*=0.6) and decamping (0.26 ± 0.08 vs 0.37 ± 0.15 per min, Wald χ^2_2=1.5, n=40, *P*=0.2) were similar towards young and mature males. Sixty percent of the young and 60% of the mature males mated during the 5-min trials and their mating latencies were not significantly different (69±12 vs 100±25 for young and mature males respectively; Wald χ^2_2=1.1, n=24, *P*=0.3).

2.8 Attractiveness to females of young and mature males

A variety of female and male parameters could influence the observed age-specific variation in male mate choosiness. We began by assessing the perceived attractiveness of males to females. Because young and mature males courted with similar intensities and mated at similar frequencies in no-choice trials with mature virgin females (Dukas & Baxter 2014), we focused on the mating latencies, which we expected to be negatively correlated with male attractiveness.

2.8.1 Methods

We tested whether young and mature males differed in their mating latencies across three successive matings. We placed each male inside a food vial with a mature female and allowed them to mate (*n*=32 males, half of each age). If males did not mate with the female they were originally presented with in each trial, we replaced the female with a new one every 20 minutes until mating occurred, or until the 60-minute trial was over. Observers blind to male age recorded each male's mating latency. After each mating, we gave the males a 15 minute break before placing them with a new female. All of the mature males mated in each of the three trials. In the first trial, all of the young males also mated. In the second trial, two young males did not mate within the hour and were given extra time to mate. In the third trial, 4 of the 16 young males did not mate and these matings were excluded from the analysis. In a follow-up experiment, we compared the mating latencies of young and mature males only in their first mating (n=42 males, half of each age).

2.8.2 Results

There was a significant male age by trial interaction for mating latencies (Wald χ^2_1= 22, n=32, *P*<0.001; Fig. 2.5, results shown to the left of the black line). The mating latencies of young and mature males were similar in the first mating trial (*P*=0.9) but mating latencies were shorter in mature than young males in the subsequent trials (Wald χ^2_1= 23, n=32, *P*<0.001). In the follow-up experiment, mature males had shorter mating latencies than young males in their first mating (Wald χ^2_1=6.6, n=42, *P*=0.01; Fig. 2.5, results shown to the right of the black line).

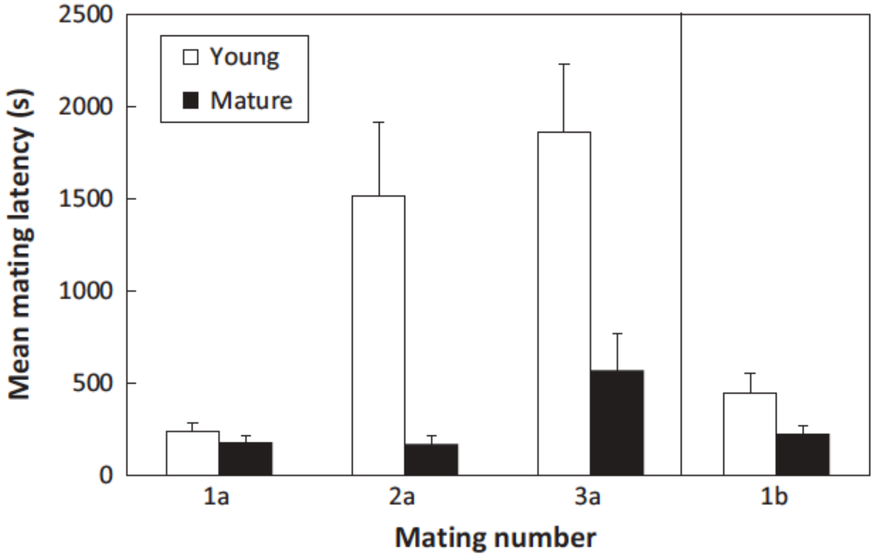

Figure 2.5

The mating latencies (mean ± 1 SE) of young (1-day-old) and mature (4-day-old) males across three successive mating trials. Mating numbers 1a, 2a and 2a correspond to mating latencies in the first experiment (n=16 young and 16 mature males; significant male age by trial interaction (*P*<0.001), whereas mating number 1b corresponds to results from the follow-up experiment (n=21 young and 21 mature males with shorter mating latencies by mature than young males, *P*=0.01).

2.9 Competitive ability of young and mature males

Because the operational sex ratio in fruit flies is strongly male biased (Bateman 1948; Spieth 1974), one can readily observe two males pursuing the same female simultaneously. We thus wished to measure the competitive ability of young and mature males placed together with a single female. Specifically, we wanted to see whether males of a given age could monopolize the female and thus reduce the mating probability of males of the other age.

2.9.1 Methods

We conducted two types of trials. In the individual trials, we placed one male (young or mature) in a vial with one immature virgin female (n=54 males). In the competitive trials, we placed both a young and mature male in a vial with an immature virgin female (n=50 males). We coloured one male per vial in the competitive trials with a pink fluorescent powder to allow the observers to differentiate between them. Male colouring was counterbalanced across trials and did not affect male courtship (Wald χ^2_1=0.1, n=50, P=0.7).

2.9.2 Results

In the individual trials, when each male was alone with a virgin female, both young and mature males spent similar proportions of time courting (Wald χ^2_1= 0.001, n=54, P=1; Fig. 2.6). However, in the competitive trials, when a young and mature male were together with a virgin female, mature males spent significantly greater proportions of time courting than did young males (Wald χ^2_1 = 9.6, n=50, P<0.005; Fig. 2.6).

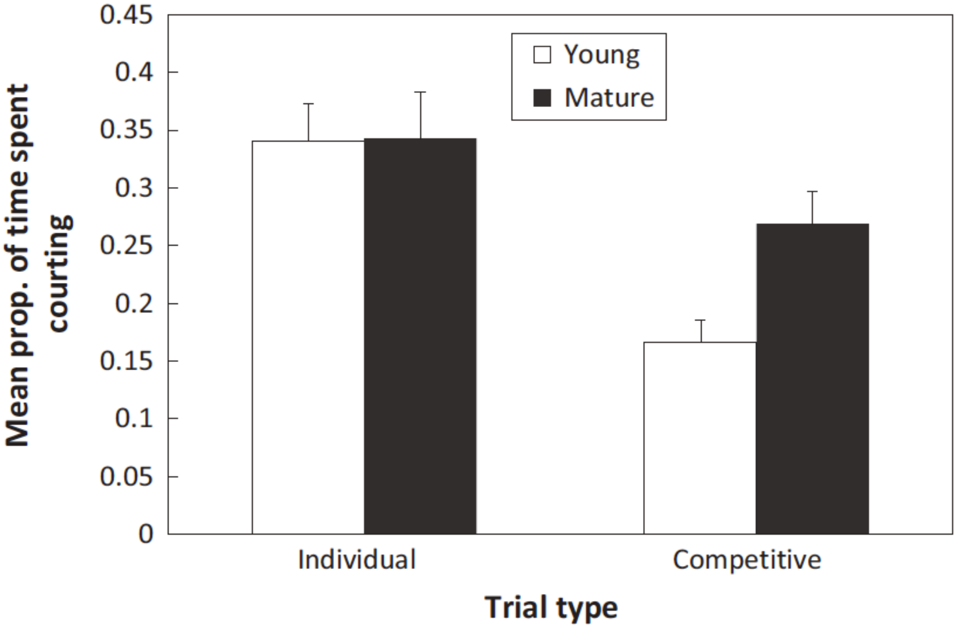

Figure 2.6

The proportion of time (mean ± 1 SE) that young (1-day-old) and mature (4-day-old) males spent courting virgin females under individual (1 male per vial, n=54 males) and competitive (1 young and 1 mature male per vial, n=50 males) conditions. Mature males spent more time than young males courting females under the competitive than individual condition, $P<0.005$).

2.10 Fertility of young and mature males

Our previous data (Dukas & Baxter 2014) indicated similar fertility of young and mature males mated once with virgin females. The extensive literature on males' sophisticated sperm allocation strategies (Wedell et al. 2002; Lüpold et al. 2011) suggests, however, that mate choosiness can be higher in males with more limited supplies of sperm and seminal fluids than in less limited males. We thus wished to quantify the total fertility of males over three successive matings. Because male fertility is determined by both sperm and seminal fluid, we preferred to count male offspring rather than sperm. Furthermore, we counted adult offspring because they are a more relevant measure of male fitness than fertilized eggs.

2.10.1 Methods

We allowed young (n=16) and mature (n=16) males to mate three times in succession with mature virgin females. We reported the mating latencies for these males above (Fig. 2.5, left side). We transferred the mated females into fresh food vials with live yeast each day until they no longer laid fertilized eggs. Observers blind to male treatment counted all adult offspring. We excluded from the analysis 13 females (14%) that died on or before Day 5 of egg laying.

In a follow-up experiment, we allowed young and mature males to mate three times in succession with 3 virgin 3-day-old females and counted the offspring that the males fathered in their third mating. Although we started with 22 males of each age category, 50% of the young males and 5% of the mature males failed to mate for the third time (Pearson χ^2_1=11.5, n=44, P<0.001), leaving us with sample sizes of 11 and 21 respectively.

2.10.2 Results

There was a significant male age by mating number interaction for fertility (Wald χ^2_2=58, n=32 males, P<0.001; Fig. 2.7, results shown to the left of the black vertical line). Young and mature males fathered similar numbers of offspring in their first mating (P=1).

Mature males, however, fathered significantly more offspring than young males in their second and third matings (both $P<0.001$). Overall, mature males fathered more than twice as many offspring as did young males ($P<0.001$).

In the follow up experiment, mature males again fathered more offspring in their third successive mating than did young males (Wald $\chi^2_1=17$, n=32, $P<0.001$; Fig. 2.7, results shown to the right of the black vertical line).

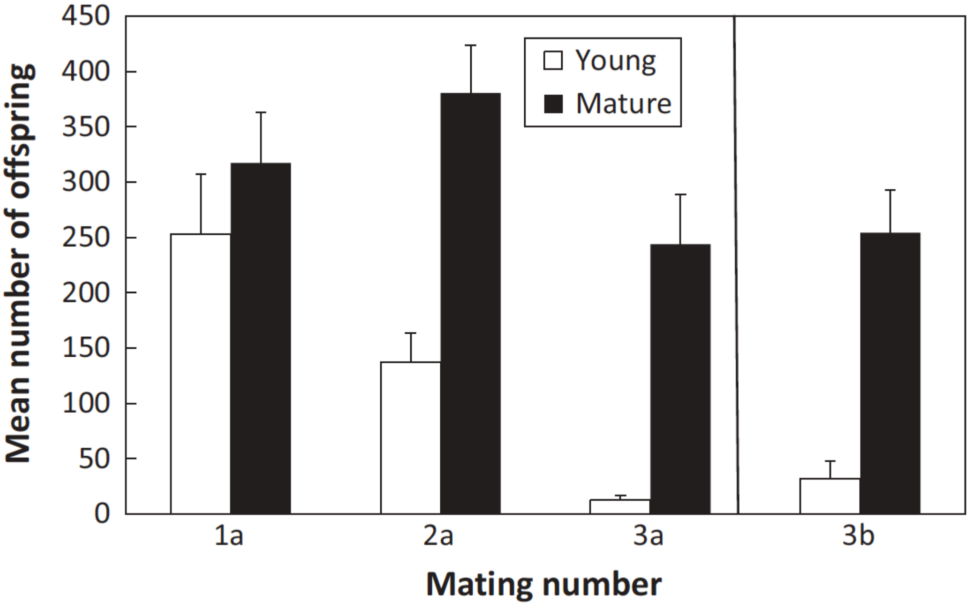

Figure 2.7

The number of offspring (mean ± 1 SE) fathered by young (1-day-old) and mature (4-day-old) males across three successive mating trials. Mating numbers 1a, 2a and 3a correspond to offspring produced from the first experiment (n=16 young and 16 mature males), whereas mating number 3b corresponds to results from the follow-up experiment (n=11 young and 21 mature males). Mature males fathered more offspring in all matings (*P*<0.001) except for 1a (*P*=1).

2.11 Fertility of mature males that have either encountered or been deprived of females

Because we documented higher mate choosiness in males that encountered females than in males deprived of females, we wished to examine whether this difference could be attributed to lower sperm and seminal fluid supplies in the former males. We thus compared their fertility over three successive matings.

2.11.1 Methods

The mated treatment (n=20) consisted of males that had mated once each day on days 1–3. The unmated males (n=20) had not mated prior to the test. On day 4, we allowed all males to mate successively with three females and kept females from the third matings for progeny count as described above. We excluded from the analysis one female that died on Day 4 of egg laying.

2.11.2 Results

Previously unmated mature males produced significantly more offspring in their third consecutive mating than did previously mated males (Wald χ^2_1= 6, n=39, *P* = 0.014; Fig. 2.8).

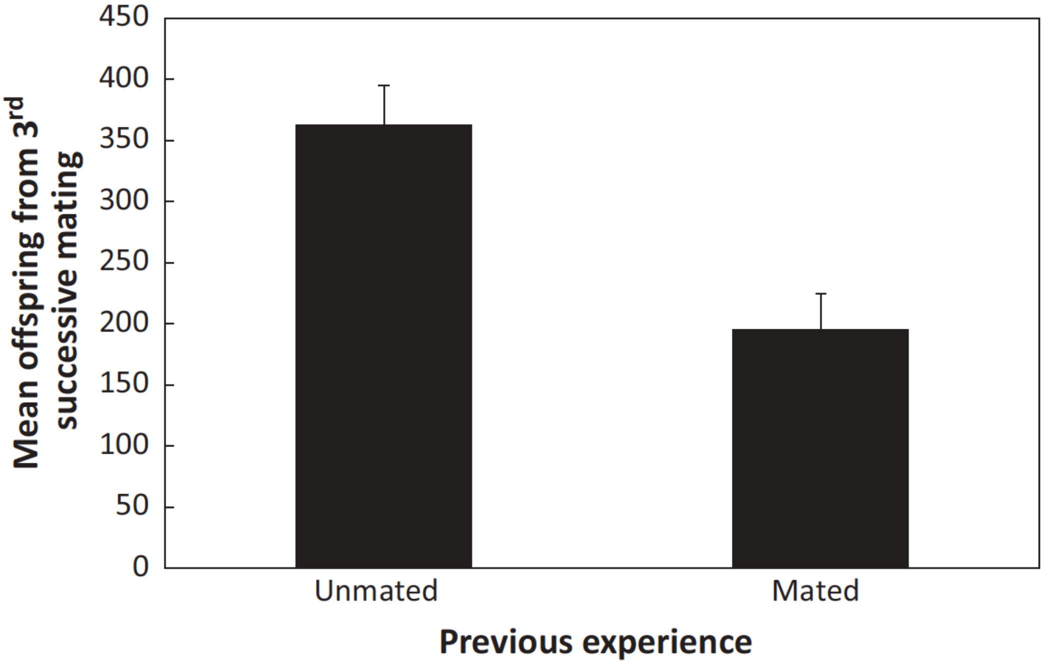

Figure 2.8

The number of offspring (mean ± 1 SE) fathered by previously unmated (n=19) and previously mated (n=20) mature (4-day old) males as a result of their third consecutive mating. Unmated males fathered more offspring than previously mated males (*P*<0.02).

2.12 Discussion

Our two tests of the effects of male age on conspecific mate choosiness showed no effect of age on males' preference to court large over small females (Fig. 2.1) but higher choosiness by young than mature males encountering virgin and mated females (Fig. 2.2). Together with our results showing higher mate choosiness in young than mature males encountering conspecific and closely related heterospecific females (Dukas & Baxter 2014), we believe that the overall pattern is of young males being more reluctant than mature males to persist in courting females with whom the probability of mating is low. That is, males have some knowledge about their relatively low likelihood of mating with mated conspecific females and virgin heterospecific females (Brieger & Butterworth 1970; Jallon 1984; Ferveur 2005; Ng et al. 2014) as indicated by the fact that, regardless of age, males spend less time courting such females (Figs 2.1-2.4 and Dukas and Baxter, 2014). The virgin conspecific females, however, are highly attractive and even small females appear to be above a threshold attractiveness that elicits as much courtship from young as from mature males.

Refining our analysis of male age and mate choosiness, we found a steady pattern of reduction in mate choosiness with males' age (Fig. 2.3). This pattern is consistent with the two non-mutually exclusive hypotheses on the effect of an individual's age on mate choosiness. First, from a life history perspective, one would expect a positive correlation between the current mate choosiness and the expected future mating opportunities. That is, older males face a shorter time horizon due to senescence and thus should be less choosy. Field data indicate expected life span of about seven days in fruit flies (*D. melanogaster*) (Rosewell & Shorrocks 1987) and male fertility shows dramatic decline in males over seven days old (Edward & Chapman 2013) so the reduced mate choosiness with male age strongly agrees with life history theory, which has traditionally focused on offspring production by females (Stearns 1992). Indeed, data from distinct taxa indicate reduced mate choosiness with age in females (Kodric-Brown & Nicoletto 2001; Moore & Moore 2001; Uetz & Norton 2007; Wilgers & Hebets 2012). While we focus here on the ultimate mechanism for the reduction in mate choosiness with age, a possible related proximate mechanism could be a reduction in males' perceptual ability to discriminate between female types with age. That is, the mechanism for the age-specific reduction in mate choosiness may be based on differences in either perception or decision.

The other hypothesis linking age and mate choosiness involves the fact that, often, age is positively correlated with the length of deprivation from the other sex. Both intuition and optimality models predict a positive correlation between the encounter rate with preferred prospective mates and mate choosiness (Wilson & Hedrick 1982) and data for females in a variety of species agree with this prediction (Shelly & Bailey 1992; Berglund 1995; Dukas 2005b; Willis et al. 2011). Hence, in our protocols, encountering no females at all for longer durations may have decreased male mate choosiness. We critically tested a prediction derived from this hypothesis, that mate choosiness would be higher in mature males previously exposed to females than in same-age, mature males previously deprived of females. Our results indeed agree with this prediction (Fig. 2.4), but we cannot conclude whether mate choosiness increases due to the daily matings by the experienced males or their learning to focus courtship on females that are more likely to accept them as mates. Males' learning in the context of courtship has been well examined in this system so we know that, even among mature males deprived of matings, males that have experienced rejections by either heterospecific or mated females are more choosy than inexperienced males (Dukas 2005a; Dukas & Dukas 2012). It is likely though that both the effects of deprivation and learning play a role in male mate choosiness.

In addition to the two hypotheses linking age and mate choosiness discussed above, one can readily think of other age related factors that can influence male mate choosiness. These include a positive correlation between males' mate choosiness and both their own age-specific attractiveness to females and ability to outcompete other males, and a negative correlation between males' age-specific sperm and seminal fluid stores and mate choosiness (Fawcett & Johnstone 2003; Byrne & Rice 2006). Our experiments addressing these factors revealed that young males are less attractive to females than mature males as indicated by the longer time it took females under no-choice conditions to accept young than mature males as mates. We found such differences even in males' first encounters with females, and the gap in attractiveness widened dramatically in males previously mated once or twice (Fig. 2.5). When we allowed one young and one mature male to compete for a single female, the mature males monopolized the female, indicating that they can outcompete young males for access to females (Fig. 2.6). Finally, our fertility tests indicated that mature males are much more fertile than young males (Fig. 2.7).

Previous studies also documented higher mating success of older over younger males. In one set of experiments involving two males of distinct ages and a single female (Long et al. 1980), males' mating success was highest when 8 days old, intermediate when 4 days old and lowest when 2 days old. Male fertility in that study was low when males were 2 days old and equally higher when they were 4 and 8 days old. In our previous study (Dukas & Baxter 2014), we found a higher mating success of mature, 4-day-old males than of young, 1-day old males when we placed one male of each age with a single female. Our new data indicate that this outcome could reflect both the higher competitive ability of the mature males (Fig. 2.6) and females' preference for mature over young males (Fig. 2.5). While our data agree with those of Long et al (1980) indicating higher fertility of mature than young males, we found no difference in males' fertility in their initial mating (Dukas & Baxter 2014) but much lower fertility in subsequent matings (Fig. 2.7). Intriguingly, males' fertility, as measured in their third successive mating, was lower in males exposed to females prior to the test (Fig. 2.8). Such males could mate with virgin females only once per day prior to the test day. The fact that this was sufficient to reduce male fertility indicates that the males are rather limited in their ability to mate successively and that such limitation could drive high male mate choosiness.

Surprisingly, two of the three male characteristics that we measured suggest that young males should actually be less choosy than mature males because we expect less attractive and less competitive males to be more willing to court less attractive females. There are two non-mutually exclusive explanations to this apparent contradiction. First, it is possible that the males' relatively low sperm and seminal fluid stores override the other factors and reduce their willingness to persist in courting less attractive females. Second, the young males' higher expected probability of encountering receptive females, perhaps combined with multiple costs of courtship and mating, may make them more choosy than mature males.

Overall, our data show that, in males with no previous exposure to females, there is a gradual decrease in male mate choosiness with age (Fig. 2.3). Prior exposure to conspecific females, however, significantly increases male mate choosiness (Fig. 2.4). Furthermore, low expected fertility, most likely due to ejaculate limitation (Figs. 2.7 and 2.8), is associated with high levels of male mate choosiness. Finally, previous work indicates that male learning, based on either encounters with conspecific or heterospecific females, increases male mate choosiness (Dukas 2004; Dukas 2005a; Dukas & Dukas 2012). Combining all this information, we expect that, in nature, young males will be choosy, and that they will maintain high levels of choosiness once they gain further experience with age. We still do not know whether conflicting factors, most notably, decreasing probability of future matings with increasing age, would ultimately reduce male mate choosiness. Our data thus suggest that male mate choosiness may be an important factor influencing both sexual selection and incipient speciation.

2.13 Acknowledgements

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CHAPTER 3 – MATING SUCCESS IN FRUIT FLIES: COURTSHIP INTERFERENCE VERSUS FEMALE CHOICE

Baxter, C. M., Mentlik, J., Shams, I. & Dukas, R. (2018) Mating success in fruit flies: Courtship interference versus female choice. *Animal Behaviour*. *138*: 101–108.

3.1 Abstract

The outcomes of sexual selection often differ when mating success is determined by male contest rather than female choice. Many studies, however, inferred sexual selection driven by female choice without carefully assessing the role of subtle male aggression. Relying on close-up video analyses, we documented novel courtship interference between male fruit flies, a key model system in research on sexual selection, sexual conflict and speciation. In experiments comparing male mating success under choice (2 males + 1 female) and no-choice (1 male + 1 female) conditions, we found that, in some cases, courtship interference altered male mating success. Both choice and no-choice protocols have known weaknesses. Choice protocols do not control for male-male interactions while no-choice protocols do not allow females to compare and choose between males. To overcome these weaknesses, we developed a new protocol (true-choice), which allows females to freely visit and assess each of two males while preventing direct male-male interactions. Results from the true-choice protocol suggest that traits enhancing male aggression have a greater role in determining mating success in fruit flies. Furthermore, it is possible that the mating system of scramble competition observed in many species should be reclassified as subtle male contest, which can drive sexual selection for aggressive male features.

Key words: aggression; competition; courtship; *Drosophila melanogaster*; fruit flies; mate choice

3.2 Introduction

In many animals, the evolution of sexually selected traits is driven solely by either contests among males for access to females or females' choices of mates among the males that they encounter. In such clear cases, one can safely focus on either intra- or inter-sexual selection. Often, however, sexual selection is determined by a combination of male-male contests and female choice (Andersson 1994; Qvarnström & Forsgren 1998; Hunt et al. 2009; Shuker & Simmons 2014). If the same traits, such as body size or dominance, are favoured by both male-male contests and female choice, then the distinction between the two mechanisms may be negligible. If different features, however, determine which males have access to females and which males are preferred by females, then it is crucial that we assess the separate contributions to sexual selection of male-male interactions and female choice.

Quantifying the distinct effects of male contest and pure female choice is challenging and attempts at doing so have had variable success determined by species' natural histories and the experimental protocols employed. An example of a successful attempt involves the scarlet-tufted malachite sunbird (*Nectarinia johnstoni*), in which field observations supplemented by experiments indicate that the males' scarlet pectoral tufts are important in male contests over territories whereas the males' tail lengths affect female choice (Evans & Hatchwell 1992a, b). Often, attempts to separate female choice from male contest involve species in which interactions are assumed to be primarily visual or auditory. In the visual species, partitions between males and females and tests for female proximity to each male might indicate female choice while controlling for male contest (e.g. Bischoff et al. 1985; Zuk et al. 1990). Similarly, use of speakers in species with acoustic courtship eliminates male contest (e.g. Ryan 1980; Eriksson & Wallin 1986). However, in many animals including the species that rely on visual or auditory features, physical contact is an integral component of courtship. Such contact allows for the exchange of olfactory, gustatory and somatosensory information (e.g. Lack 1940; Johnston 2003; Hughes et al. 2007; Ferveur 2010; Wlodarski & Dunbar 2014). In such species, it is challenging to provide females with choice while simultaneously eliminating male-male interactions.

The most common method for addressing female choice in species in which contact is part of the courtship ritual is to simply assume negligible effects of male contest. This indeed has been the standard protocol in numerous experiments on fruit flies (*Drosophila melanogaster*), a key model system in research on mate choice, sexual conflict and speciation (Coyne & Orr 2004; Rice et al. 2006). In such choice studies, experimenters simultaneously present to each focal female two males belonging to distinct categories and record which male succeeds in mating. An alternative protocol is to present each focal female with only a single male at a time and record mating rates and latencies. This protocol, often referred to as 'no-choice', prevents females from comparing males with distinct features before deciding whether to mate or not with the only male they have encountered. Choice protocols reveal stronger mate preferences than do no-choice tests perhaps owing to the ease of comparison between prospective mates and lower cost of rejecting a potential mate when females encounter two rather than one male (Dougherty & Shuker 2015). The choice protocol, however, cannot rule out male-male interactions, which could affect access to females even in species with no overt aggression.

The recent adoption of fruit flies as a model system for mechanistic research on aggression (Chen et al. 2002; Asahina 2017) has sensitized us to the possibility that apparent mate choice under the prevalent choice protocol is influenced by subtle male aggression. As with many other species (Emlen & Oring 1977), fruit flies' mating system varies as a function of density and resource distribution. The recent work on fruit fly aggression has appropriately focused on the mating system scenario characterizing low fly density and small, defendable fruits. In such settings, fruit flies show resource defence polygyny, where capable males rely on aggression to defend attractive fruits where females gather to feed and lay eggs (Markow 1988; Hoffmann & Cacoyianni 1990; Baxter et al. 2015b). Under the prevalent conditions of high fly density and large food clumps, however, the predominant assertion has been that male fruit flies show scramble competition for mates (Spieth 1974), a mating system that is rather common in insects (Thornhill & Alcock 1983). Scramble competition is consistent with choice protocols, because it assumes that multiple males court females and females choose their mates.

Because past observations in many laboratories including ours indicated no overt aggression during mate choice trials, we wished to test whether subtle aggression could bias male access to females. Based on previous work, we chose two realistic choice scenarios, one involving males that varied in age and the other involving males that varied in size. For both scenarios, our own and others' research suggested female preference for older over younger males (Long et al. 1980; Dukas & Baxter 2014; Baxter et al. 2015a) and for large over small males (Partridge et al. 1987; Dukas 2005). These studies, however, did not assess the possible influence of subtle male aggression. We thus asked whether males rely on subtle aggression to monopolize access to females, and whether such interactions bias male mating success. To this end, we first conducted video recording trials, each with two males and an immature female to quantify subtle aggression. Second, we simultaneously assessed male mating success in choice and no-choice trials. Finally, to resolve the weaknesses of the no-choice and choice trials, we developed a new protocol in which a female can freely assess sequentially two males housed in distinct compartments and then choose her mate. We refer to this set up as 'true-choice'. We then compared male mating success under the classical choice protocol, which cannot separate effects of male-male interactions and female choice, and under the true-choice setup, which eliminates male-male interactions.

3.3 General methods

We used descendants of wild-caught *D. melanogaster* collected in several southern Ontario localities in August 2014. We housed the flies in population cages containing several hundred flies per cage. We kept the cages in an environmental chamber at 25°C and 60% relative humidity with a 12:12 h light:dark cycle, with the lights turning on at 10 am. Unless stated otherwise, we reared the experimental flies at a low density of about 300 eggs per 240 ml bottle containing 50 ml of standard fly medium made of water, sucrose, cornmeal, yeast, agar and methyl paraben. We sexed flies within 4 hours of eclosion to ensure virginity and minimal experience with other flies. We used gentle aspiration to live-sex and transfer males into individual 40 ml vials each containing 5 ml of fly medium. We anaesthetized females with CO_2 to sex and place them in groups of 20 per vial, which contained the same amount of fly medium plus a dash of live yeast.

Unless stated otherwise, we conducted all tests in cylindrical arenas made of Plexiglas (3 cm in diameter and 2.5 cm high). We covered the floor of each arena with a piece of filter paper. To deter flies from climbing on the arenas' walls and ceilings, we coated the walls with Insect-a-Slip (Fluon; BioQuip, Gardena, CA, U.S.A.) and the ceilings with Surfasil (Sigma Aldrich, Oakville, ON, Canada). When dry, Fluon and Surfasil form an odorless slippery film, which flies cannot walk on. Fluon and Surfasil have been used in insect studies for a long time and do not appear to have negative effects (Radinovsky & Krantz 1962; Asahina et al. 2014).

3.4 Male size, courtship interference and mating success

We first wished to test whether males relied on subtle aggression for monopolizing access to females. To this end, we relied on close-up video recordings to quantify subtle aggression between small and large males in choice trials. Based on preliminary observations, we focused on male takeovers, defined as a male positioning himself between the courting male and the female and taking over the courtship (Fig. 3.1). Previous data indicated that large males outcompete small males for access to attractive food patches (Hoffmann 1987; Asahina 2017). We thus predicted higher takeover rates by large than small males. To assess the effects of courtship interference on male courtship, we compared the courtship duration of large and small males under both no-choice and choice protocols (Fig. 3.2). We predicted that large males would spend more time courting females than small males in the choice than in the no-choice trials. Finally, we predicted a higher relative mating success by large than small males in the choice than no-choice trials.

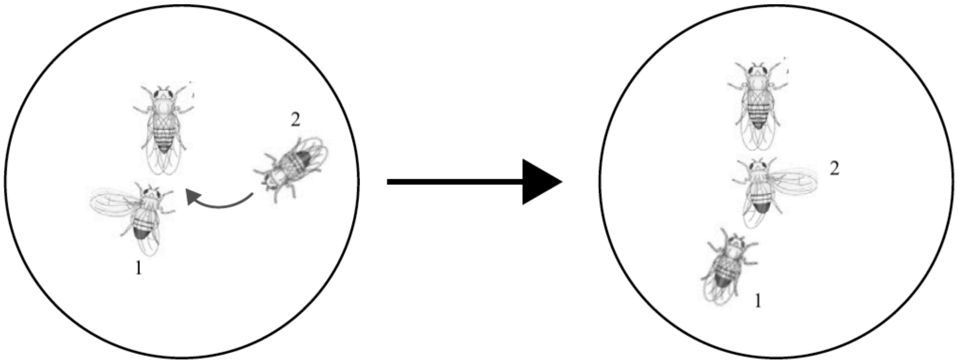

Figure 3.1

Depiction of a courtship takeover. In the left circle, male 1 is courting the female and male 2 pushes in between them. In the right circle, male 2 has displaced male 1 and is courting the female.

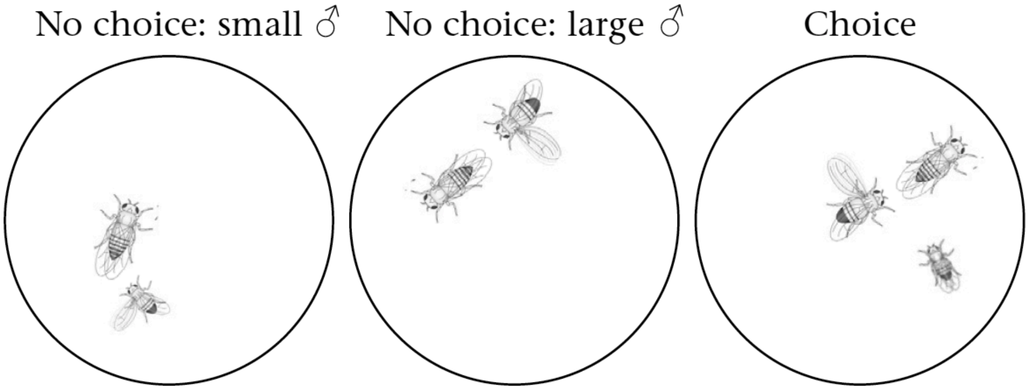

Figure 3.2

Diagram of the protocols for courtship interference and mating success in small vs. large males. Females were sexually immature in the courtship experiment and sexually mature in the competitive mating experiment. Note that males are smaller than females and have a dark posterior.

3.4.1 Courtship interference - Methods

We conducted two types of trials: no-choice and choice (Fig. 3.2). In no-choice trials, we placed one male (either a small or large male) with one immature virgin female (<18 hours post eclosion) in an arena (N_{small}=30, N_{large}=30). In the choice trials, we placed two males (one small and one large male) with one immature female in an arena simultaneously (N_{choice}=30). We used immature virgin females because they are as sexually attractive to males as mature virgin females, but they always reject males (Dukas & Dukas 2012). By using females who consistently reject males we could accurately assess male courtship and competitive ability, without female preference affecting each male's courtship opportunities.

We obtained small and large males by rearing flies under different densities. While we refer to the males by their obvious size differences, they probably differed in a variety of other traits influenced by larval density (Bangham et al. 2002; Lüpold et al. 2011; Wigby et al. 2016). We reared small males in high density food vials with approximately 200 eggs per vial containing 1.5 ml of the standard fly medium, whereas we reared large males in low-density food bottles with approximately 100 eggs per bottle containing 50 ml of the standard fly medium. Within 4 hours of eclosion, we transferred adult males to individual vials with ample food until the time of testing (4 days later). Females were reared in food bottles with 50mL of food and 300-400 flies per bottle. After testing, we measured the wing length of a random sample of 12 small and 12 large males. We removed the right wing of each male, mounted it on a microscope slide and measured the linear distance between the humeral–costal break and the end of the third longitudinal vein (Gilchrist & Partridge 1999). The wings of large males were approximately 25% longer than those of small males, 1.85 ± 0.017 vs. 1.48 ± 0.020 mm, respectively (t-test: t_{22}=14.14, N=24, p<0.001).

After placing the flies in the arena, we began video recording for 15 minutes using Logitech HD Pro C920 and iPad Air cameras. Observers scored the videos using the Observer software (Noldus Information Technology, Wageningen, The Netherlands). Due to the males' visible size differences, it was not possible for observers to be blind to the male size manipulation. However, the observers were not aware of our predictions about male size and competitive courtship ability.

In the no-choice condition, where there was just one male and one female per arena, observers recorded the duration of courtship, and who terminated each courtship bout. If the male turned or flew away from the female, or simply stopped chasing her, we counted it as the male terminating the courtship bout. If the female decamped (jumped or flew away from the male) we counted it as the female terminating the courtship bout (Dukas & Scott 2015). In the choice condition, where there were two males and one female per arena, observers recorded the duration of courtship and the number of courtship takeovers performed by each male. Because courtship typically involves the male closely following the female, only one male can court at any given time. Furthermore, only the non-courting male can interfere with the courtship of the other male. That is, courtship and courtship interference are mutually exclusive.

We used linear and generalized linear mixed-effects models (LMM and GLMM) in R version 3.3.3 (R-Core-Team 2014) with the package lme4 version 1.1-12 (Bates et al. 2014) to analyze the takeover frequency, courtship duration and courtship termination data. For tests of the fixed effects, we report Wald χ^2 values generated with the Anova function from the car package version 2.1-4 (Fox & Weisberg 2011). For the takeover data, we used a GLMM with a negative binomial distribution with the total number of takeovers each male performed as the dependent measure, male size as a fixed factor and arena ID as a random factor (to account for the non-independence of two males in the same arena). We also included the log duration of each male's competitor's courtship as an offset in the model to account for the fact that a male's takeover opportunities are constrained by his competitor's courtship (for example, a male whose competitor barely courts has fewer takeover opportunities than a male whose competitor courts frequently). For the courtship duration data, we used a LMM with the proportion of time spent courting as the dependent measure, male size and trial type as fixed factors and arena ID as a random factor. We analyzed the female courtship termination data similarly to the takeover data (with total female courtship terminations as the dependent measure and the log duration of courtship as an offset).

3.4.2 Courtship interference - Results

In the choice trials, large males performed significantly more courtship takeovers than small males (GLMM with negative binomial distribution, χ^2_1=14.9, N=60, p<0.001; Fig 3.3). Across both trial types, large males spent significantly more time courting than small males (LMM, χ^2_1= 7.67, N=90, p<0.01; Fig. 3.4). However, contrary to our prediction, the effect of the interaction between male size and trial type on the duration of time males spent courting was not significant (χ^2_1=0.42, N=90, p=0.52; Fig. 3.4). In the no-choice trials, we recorded who terminated each courtship bout (i.e. the female or the male). There was no effect of male size on the frequency with which females terminated courtship (GLMM with negative binomial distribution, χ^2_1=0.045, N=60, p=0.83).

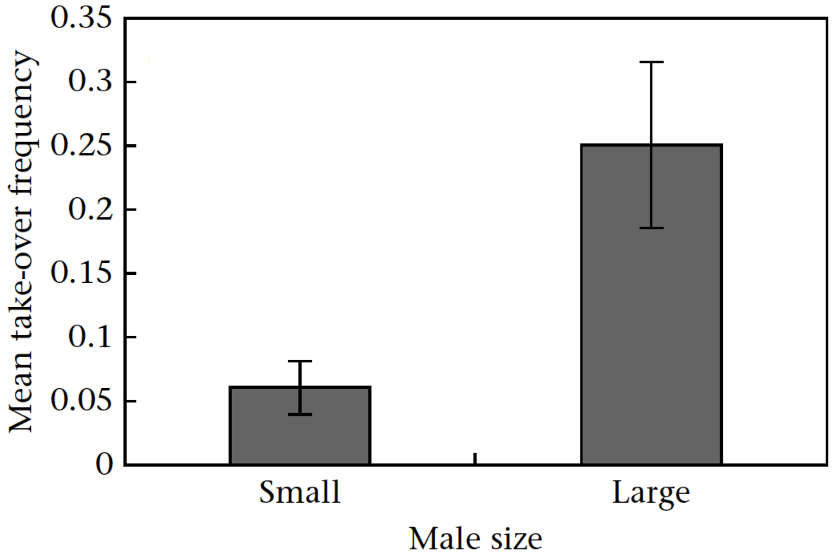

Figure 3.3

Mean ±SE takeover frequency for small and large males in the choice trials (N_{choice}=30).

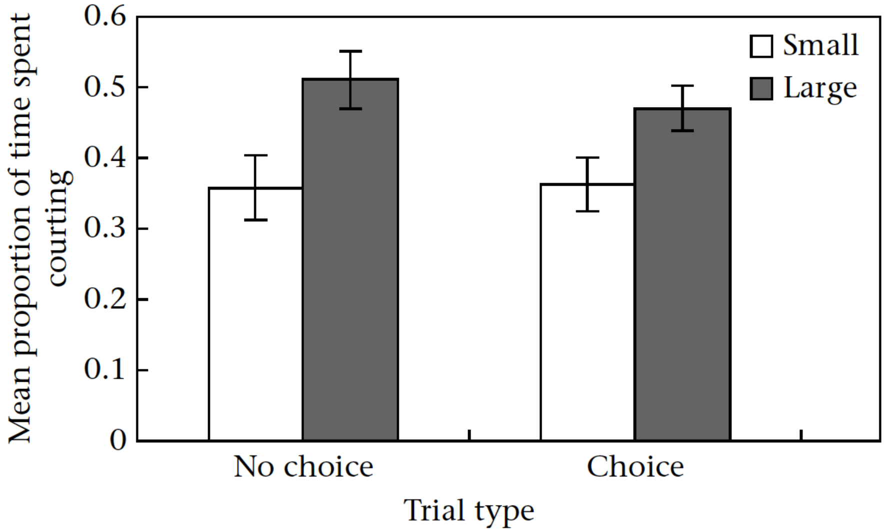

Figure 3.4

Mean ±SE proportion of time males spent courting a female in no-choice trials (1 male + 1 female (N_{small}=30, N_{large}=30)) and in choice trials (small male + large male + female simultaneously (N_{choice}=30)).

3.4.3 Mating success in choice vs. no-choice trials - Methods

In the previous experiment, we used sexually immature females so that we could assess male courtship effort and competitive ability under controlled conditions of consistent rejection from females. To assess the differential mating success of males with and without competition, we performed a similar experiment to the previous one, but used mature virgin females.

We again conducted no-choice and choice trials (Fig. 3.2). In the no-choice trials, we placed one male (small or large) with a mature, 4-day-old, virgin female in the arena (N_{small}=123, N_{large}=122). In the choice trials, we placed two males (one small and one large) with a mature, 4-day-old, virgin female in the arena (N_{choice}=122). Observers who were naïve to our predictions scanned the arenas for mating. Trials ended once mating occurred, or after 30 minutes had passed.

As in the previous experiment, after testing, we measured the wing length of a random sample of 13 small and 13 large males. The wings of large males were approximately 24% longer than those of small males, 1.80 ± 0.014 vs. 1.45 ± 0.014 mm, respectively (T-test: t_{24}=17.31, N=26, p<0.001)

We performed a binomial test in IBM SPSS (IBM Corp., 2011) to compare the observed proportions of matings in the choice treatment to the expected proportions, which were calculated based on the mating success in the no-choice treatment by small and large males. In this analysis, we treated each vial containing two males and a female as the experimental unit.

3.4.4 Mating success in choice vs. no-choice trials - Results

The proportion of large males mated in the choice trials was nearly identical to what was expected based on the proportion mated in the no-choice trials (binomial test, $N_{no\text{-}choice}$=245, N_{choice}=122 p=0.24; Fig. 3.5).

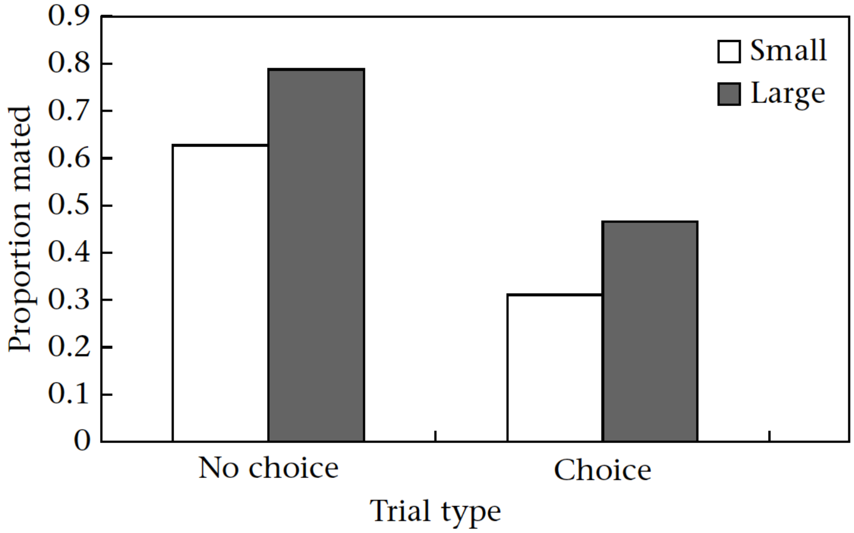

Figure 3.5

Proportion of small and large males that mated in no-choice (N_{small}=123, N_{large}=122) and choice trials (N_{choice}=122).

3.5 Male age, courtship interference and mating success

This set of experiments was analogous to the set of experiments above except that males varied by age rather than size (Fig. 3.6). We used males that were 1 and 4 days old, as males of both of these ages are sexually mature and readily court and mate with females (Dukas & Baxter 2014; Baxter et al. 2015a; Baxter & Dukas 2017). First, we predicted that 4-day-old males (hereafter mature males) would spend more time courting females than 1-day-old males (hereafter young males) in the choice than in the no-choice trials. Second, we predicted higher takeover rates by mature than young males. Finally, we predicted a higher relative mating success by mature than young males in the choice than no-choice trials.

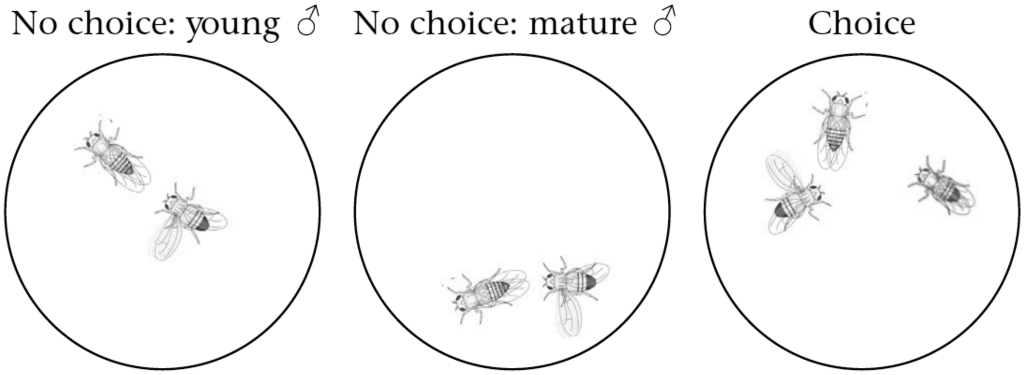

Figure 3.6

Diagram of the protocols for young vs. mature male courtship interference and mating success. In the courtship experiment, the females were sexually immature, whereas in the competitive mating experiment, they were sexually mature.

3.5.1 Courtship interference - Methods

In no-choice trials, we placed either a young or mature male with an immature virgin female (N_{young}=28, N_{mature}=30). In the choice trials, we placed one young and one mature male with an immature female in the arena simultaneously (N_{choice}=58). In order to differentiate between males in the choice treatment, we coloured males with pink and blue fluorescent powder while counterbalancing male colour with male age.

After placing the flies in the arena, we began video recording for 15 minutes. Later, observers blind to male age scored the videos. In the no-choice condition, observers recorded the duration of courtship, and who terminated each courtship bout. In the choice condition, observers recorded the duration of courtship and the number of courtship takeovers performed by each male.

3.5.2 Courtship interference – Results

In the choice trials, mature males performed significantly more courtship takeovers than young males (GLMM with negative binomial distribution, χ_1^2=15.6, N=58, p<0.001; Fig. 3.7. Across both trial types, mature males spent significantly more time courting than young males (GLMM with gamma distribution and inverse link function, χ_1^2=12.4, N=116, p<0.001; Fig. 3.8). There was a significant interaction between male age and trial type, meaning that there was a larger difference between the courtship duration of mature vs. young males in the choice than no-choice trials (χ_1^2=6.06, N=116, p<0.05; Fig. 3.8). That is, mature males had a competitive courtship advantage over young males.

In the no-choice trials, females terminated courtship with mature males more frequently than they terminated courtship with young males, but this difference did not reach significance (GLMM with negative binomial distribution, χ_1^2= 2.41, N=58, p=0.12).

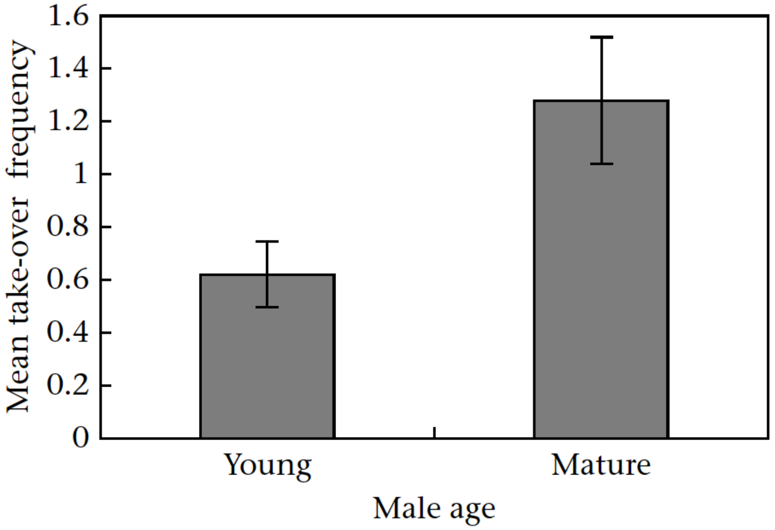

Figure 3.7

Mean ±SE takeover frequency for young and mature males in the choice trials ($N_{\text{choice}}=58$).

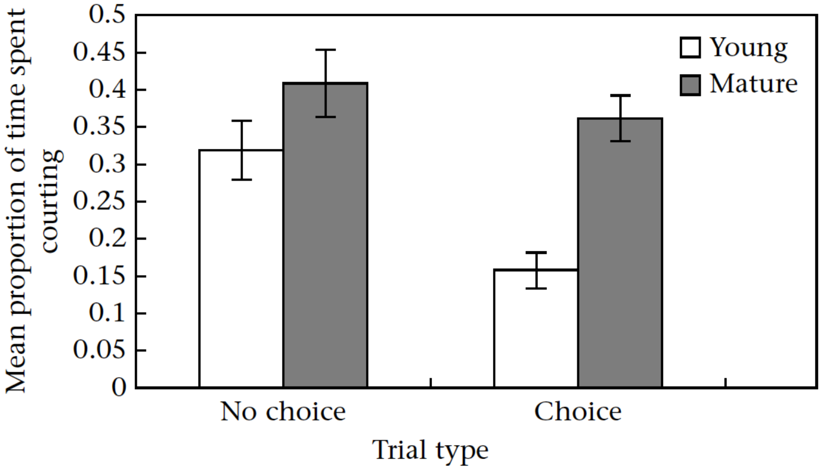

Figure 3.8

Mean ±SE proportion of time males spent courting a female in no-choice ($N_{\text{1-day-old}}=28$, $N_{\text{4-day-old}}=30$) and choice trials ($N_{\text{choice}}=58$).

3.5.3 Mating success in choice vs. no-choice trials - Methods

In the previous experiment, we used sexually immature females to assess male courtship effort and competitive ability. In order to assess how competitive courtship ability translates into mating success, we repeated the same experiment with mature virgin females, again conducting no-choice and choice trials (Fig. 3.6). In the no-choice trials, we placed one male (young or mature) with a mature virgin female (4 days old) in the arena (N_{young}=120, N_{mature}=120). In the choice trials we placed two males (one young and one mature) with a mature virgin female in the arena (N_{choice}=120). Observers blind to male age scanned the arenas for matings. Trials ended once mating occurred, or after 30 minutes had passed.

We used a binomial test to compare the observed proportions of matings in the choice treatment to the expected proportions, which were calculated based on mating success in the no-choice treatment by young and mature males (IBM Corp., 2011).

3.5.4 Mating success in choice vs. no-choice trials - Results

The proportion of mature males mated in the choice trials was significantly larger than that expected from the no-choice trials (binomial test, $N_{no\text{-}choice}$=240, N_{choice}=120 $p<0.001$; Fig. 3.9).

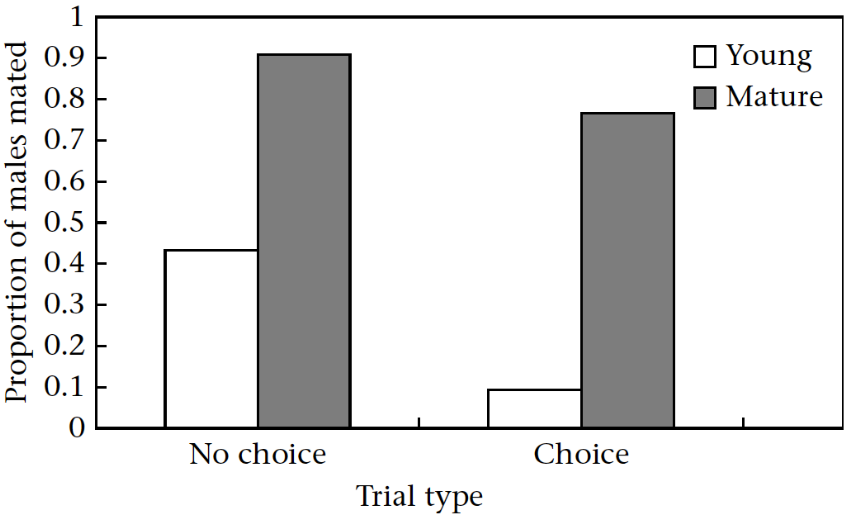

Figure 3.9

Proportion of young and mature males that mated in no-choice ($N_{\text{1-day-old}}=120$, $N_{\text{4-day-old}}=120$) and choice trials ($N_{\text{choice}}=120$).

3.6 True-choice vs. classical choice

The experiments above indicated that subtle interactions between males could determine the outcomes of mate choice tests. That is, data attributed to female mate choice under the classical choice protocol can actually be driven by male-male competition for access to females. There is thus an obvious need for an additional protocol that eliminates male-male competition from the choice protocol. To address this requirement, we developed a new apparatus that allows a female to freely travel between two compartments, each housing one male, and then choose her mate. We refer to this set up as ‘true-choice’. We then compared the mating success of young and mature males under the choice protocol and under the true-choice setup. Based on the data above (Figs. 3.6 – 3.9), we expected higher relative mating success by mature than young males in the choice than true-choice trials.

3.6.1 Methods

We constructed two types of arenas for the true-choice and choice trials (Fig. 3.10). The true-choice arena was a novel apparatus inspired by Byrne et al. (2008). It had two adjacent rectangular compartments that isolated the males from each other. Each compartment was 1 x 1 x 4 cm in size. In the shared wall of the two compartments were 24 holes approximately 0.95 mm in diameter. The holes were small enough to prevent large flies (in this case males) from passing between compartments, but at the same time large enough to allow small flies (in this case small females) to pass through and visit both compartments. The choice arena, which allowed for simultaneous female choice and male-male competition, consisted of a single compartment 1 x 1 x 6.5 cm in size. Both arenas contained standard food to the 1.5 cm mark from one end and sealed with a foam plug at the other. After adding food, the space in the choice arena was twice the length of the true-choice arena so that the total space the female could explore in both arenas was equivalent.

In fruit flies, females are typically slightly larger than males. To develop small females, we reared them at a density of approximately 250 eggs per 1 ml of standard fly medium (Byrne et al. 2008; Baxter et al. 2015a). Although small females are less attractive than large females as shown by less courtship from males, young and mature males court small females with equal intensity (Baxter et al. 2015a). To develop large males, we reared them at a density of 100 eggs per 50 ml of medium.

We wanted females to have ample time to interact with each male before choosing to mate with one of them. To this end, we placed sexually immature, recently eclosed females in the arenas with a recently eclosed male and a 3-day-old male. This allowed the females to have an extended period of interaction with both males before becoming sexually mature, approximately 12 hours later. Once females became sexually mature, they were able to make informed choices of whether to mate with the now 1-day-old (young) or 4-day-old (mature) male. In the true-choice arena, females started half of the trials with the mature, and half with the young males, and this was counterbalanced with her starting on the left or right side of the arena.

We ran trials in humidified chambers at 25°C and 80 ± 10% relative humidity with bright ambient light. Trials lasted 48 hours beginning when we placed flies into the arenas. We recorded the arenas with iPods using the time-lapse application OhSnap! to record a single frame every three minutes. Observers blind to male age scored the first mating of each female from the resulting time-lapse photos. A mating was counted if the same male was mounting a female for 3-5 consecutive frames. This was an adequate measure as matings in *D. melanogaster* typically last for about 15 minutes (Ashburner 1989).

We excluded from the data two types of trials. First, we removed true-choice trials where females did not receive courtship by both males before mating (N=16) as this did not constitute a choice by the female. Second, we excluded all trials that did not result in mating ($N_{\text{choice}}=11$, $N_{\text{true-choice}}=7$). This left us with a sample size of 157 choice trials and 124 true-choice trials. We split the trials into six 8-hour blocks to assess the proportion of matings in each trial type across time. We analyzed the data in R (R-Core-Team 2014) using the gee package version 4.13-19 (Carey et al. 2015). We used a generalized estimating equation (GEE) with a binomial distribution to assess the cumulative proportion of matings attained by mature males across the 8-hour blocks, with arena ID as the subject ID (for repeated samplings of the same arena across the 8-hour blocks) and 8-hour block as an ordered factor. We predicted that the opportunity for male-male interactions would result in a larger proportion of matings attained by the mature males in the choice than in the true-choice trials.

3.6.2 Results

The mature males had a higher proportion of matings in the choice treatment than in the true-choice treatment (GEE with binomial distribution, robust $z=2.15$, $N=281$, $p<0.05$; Fig. 3.11).

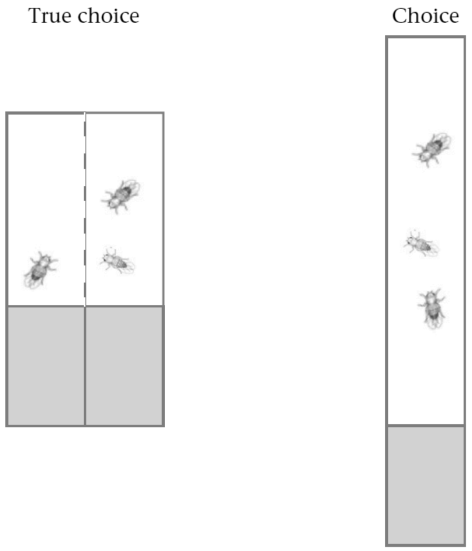

Figure 3.10

Diagram of the protocol for choice vs. true-choice treatments. Note that males have a dark posterior, but, due to the experimental manipulation, they are larger than females.

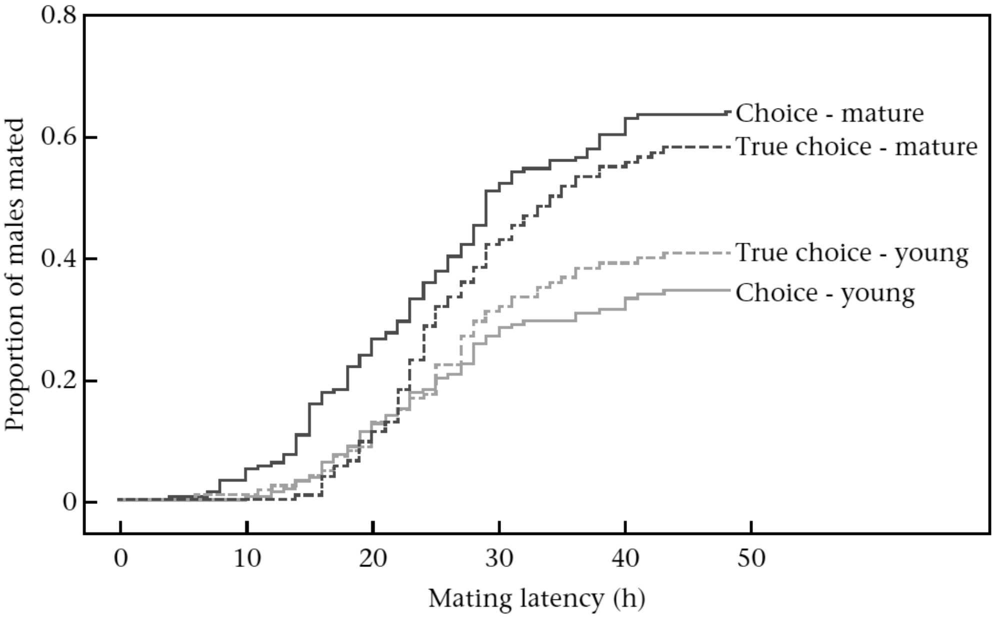

Figure 3.11

Proportion of matings between young and mature males over time in the choice (N=157) and true-choice (N=124) treatments.

3.7 Discussion

We documented robust but subtle aggressive interactions between males that affected their ability to court females: males persistently interfered with each other's courtship, attempting and often succeeding in taking over courtship from another male (Fig. 3.1). That is, we believe that the classical characterization of fruit fly mating system under high density as scramble competition (Spieth 1974; Partridge et al. 1987) should be revised to interference competition. Furthermore, we think that careful observations will necessitate reclassifying many other apparent scramble competition cases (Thornhill & Alcock 1983; Shuker & Simmons 2014) as interference competition. In fruit flies, we and others had failed to notice courtship takeover in many previous experiments using a choice protocol, which, in spite of its name, cannot distinguish between choice by females and subtle male-male competition. To detect the courtship takeovers, we required close-up video recordings. Once we established the protocol for measuring courtship takeovers, we could quantify highly replicable patterns of large males performing more takeovers than small males (Fig. 3.3), and of mature males performing more takeovers than young males (Fig. 3.7). Our results are consistent with data on fruit fly aggression in the context of resource defence polygyny, where large males outcompete small males (Hoffmann 1987; Asahina 2017) and mature males win over young males (Baxter & Dukas 2017).

Courtship interference is known in a variety of taxa (Wong & Candolin 2005). For example, although females appear to choose among males occupying a lek, courtship interruption is prevalent (Foster 1983). In the Guianan cock of the rock (*Rupicola rupicola*), males that disrupted other males' courtship or copulation had a higher mating success, and males that engaged in more aggressive and persistent interference were more likely to mate with the females they interrupted (Trail 1985). In the water boatman (*Sigara falleni*), males frequently engaged in overt aggression, interrupting courtship and mounting attempts. In choice trials dominated by male courtship interference, large males with smaller palae for their size had the highest mating success while in no-choice trials, large males with large relative palae had the highest mating rates (Candolin & Tregenza 2004). Finally, in the

Pacific blue-eye (*Pseudomugil signifier*), courtship bouts under no-choice were much longer than under choice trials. While dominant and subordinate males were equally likely to disrupt each other's courtship, dominant males monopolized courtship in the choice trials (Wong 2004). An important difference between our findings and previous reports on courtship interference is that we have documented subtle courtship interference, which could be revealed only through careful analyses of video recordings. Such covert male-male interactions may be prevalent, with their effects on sexual selection underappreciated.

Our three sets of experiments revealed a complex pattern, which most likely reflects the actual intricacy of the dynamics that determine mating success. In a choice protocol involving small and large males and an immature female, large males took over courtship (Fig. 3.1) four times more often than small males (Fig. 3.3). Remarkably, however, in spite of the persistent interference from large males, the small males were determined at resuming courting females. This is reflected in the fact that, in the no-choice trials, females received courtship during less than half of the trial duration (the average of the 2 left bars in Fig. 3.4) compared to about 0.85 of the trial duration in the choice trials (the sum of the 2 right bars in Fig. 3.4). Thus the persistence of the small males resulted in no relative loss in mating success in the choice compared to no-choice trials (Fig. 3.5). This indicates that there is no simple positive correlation between courtship interference and either the proportion of time spent courting, or mating success, at least not under our controlled experimental conditions.

The outcomes of matches between young and mature males were different from those of the small and large males (Figs. 3.6–3.9). Here mature males took over courtship twice as often as young males (Fig. 3.7) and this resulted in the mature males spending over twice as much time as young males courting females under the choice conditions compared to only a small courtship bias under no-choice (Fig. 3.8). Moreover, courtship interference translated into a significant mating advantage for mature over young males in choice than in no-choice settings (Fig. 3.9). We should note that, because we also recorded and reported in the text females' frequencies of terminating courtship, we know that females did not decamp more often when courted either by small than by large males, or by young than by mature males. There was even a marginally significant tendency of females to decamp more often when courted by mature than by young males.

The most likely explanation for the distinct effects of courtship interference on the relative mating success of small and young males (Fig. 3.5 vs. Fig. 3.9) is the age difference between the males. Our previous work on the life history of aggression indicated that, compared to young males, mature males that have been deprived of females show a higher motivation to gain matings, which translates into higher levels of aggression in male-male contexts over resources, and greater tendencies by males to persistently pursue females with low receptivity and to engage in forced copulations (Baxter & Dukas 2017). It is likely that the small, mature males responded to courtship interference by persistently resuming courtship when the large males discontinued their female pursuit, whereas the young males responded to the more aggressive mature males by retreating.

Finally, although our data revealed robust, subtle male contest, and suggested that it could influence male mating success in the context of the female choice protocol, the data did not show a direct effect of courtship interference on mating success. To test for such direct influence, we had to fully separate between male contest and female choice, a requirement that the traditional choice and no-choice protocols do not accomplish. To this end, we developed a novel protocol, true-choice, which met that requirement (Fig. 3.10). Using the true-choice protocol, we found that male-male interactions indeed affected mating success (Fig. 3.11). We think that true-choice protocols should replace choice protocols in future studies assessing traits determining mating success in species where physical contact is essential for courting and mate assessment.

We noted some difficulties with the true-choice protocol. Male harassment of females is prevalent in settings where females cannot escape pursuing males (Dukas & Jongsma 2012; Baxter & Dukas 2017). We thus think that the perfect true-choice protocol should provide females with the option of evading males. Our preferred prototype for the true-choice apparatus had a central female compartment and two side compartments for each male, as this design provides females with a male-free shelter. The weakness of that apparatus, however, was that we had to drop a high proportion of trials in which females mated prior to assessing both males. Another possible concern is the fact that the true-choice protocol required us to reverse the typical pattern of females being larger than males. Such size reversal, however, may occur in nature in cases where some females have a high-density larval environment while some males happen to develop at low density. We and others have successfully used small females previously. While males find small females less attractive than large females as indicated by lesser courtship and mating preference (Byrne & Rice 2006; Baxter et al. 2015a), both small-female behaviour and males' responses to them seem natural.

We think that our data indicating covert courtship interference should be considered in future mate choice studies. Distinguishing between female choice and male-male interactions is important only if there is a conflict between the sexes such that traits preferred by females are distinct from traits selected through male-male contests (Qvarnström & Forsgren 1998; Arnqvist & Rowe 2005). For example, if both male competition and female choice select for larger body size as is the case in many studies (Hunt et al. 2009), then separating the mechanisms may not be crucial. It is likely, however, that research focusing on sexually selected traits utilized by males and females under realistic settings will reveal distinct roles of traits affecting male competition and females choice. While such work has been encouraged in the past (Lande & Arnold 1983; Hunt et al. 2009), we still lack sufficient data. We suggest that future research on the topic employ true female choice protocols, which allow for female assessment of males and choice while fully eliminating male competition.

3.8 Acknowledgments

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CHAPTER 4 – AGGRESSION, MATE GUARDING, AND FITNESS IN MALE FRUIT FLIES

Baxter, C. M., Barnett, R. & Dukas, R. (2015) Aggression, mate guarding, and fitness in male fruit flies. Animal Behaviour. 109: 235–241.

4.1 Abstract

Aggression is a central trait affecting fitness, which has been well studied in many animals. As a part of a research program integrating mechanisms and fitness consequences of aggression, we examined the adaptive functions of antagonistic interactions in fruit flies (*Drosophila melanogaster*), a species in which aggression has been studied primarily in the context of territorial behaviour. In our experiments, males at an attractive food patch were more aggressive towards other males when in the presence of their recent mates than with females mated with other males. Furthermore, while recently mated males accompanied by their mates were more aggressive than virgin males, recently mated males and virgin males showed similar levels of aggression in the presence of females mated with other males. When we allowed focal males to mate inside experimental arenas and then added intruder males, the intruder males spent less time on the food patch, remated with the resident females at lower frequencies, and fathered a smaller proportion of offspring when the focals males remained in the arenas than when we removed the focal males. Our results reveal a novel adaptive function of aggression in fruit flies: in addition to fighting to defend attractive food sources that attract prospective mates, males rely on aggression to guard their mates, and such mate guarding enhances their fitness.

Key words: aggression; *Drosophila melanogaster*; fighting; fruit flies; mate guarding; resource-defence polygyny; territorial behaviour

4.2 Introduction

Aggression has long been recognized as a primary trait influencing fitness, especially in males, who often fight for territories and prospective mates (Darwin 1871; Howard 1920). Fruit flies (*Drosophila melanogaster*) have recently been adopted for a close examination of the genetics and neurobiology of aggression (Chen et al. 2002; Dierick & Greenspan 2006; Zwarts et al. 2011; Alekseyenko et al. 2014). Because fruit flies are also highly amenable for behavioural, ecological and evolutionary research, we have a unique opportunity for integrating the rapidly accumulating knowledge about the mechanisms that control the varieties of aggressive behaviours with their functional aspects.

Since the first experimental analysis of fruit fly aggression (Dow & Schilcher 1975), the primary focus in laboratory protocols has been on aggression in the context of territorial behaviour (Hoffmann 1987a; Chen et al. 2002; Dierick & Greenspan 2006; Certel & Kravitz 2012). The limited field work is consistent with the notion that male aggression serves for defending fruits frequented by prospective mates (Markow 1988). Male aggression, however, can also contribute to other activities such as mate guarding. Male fruit flies defend fruits that are highly suitable for feeding and oviposition. This means that females will most likely remain at their location of mating because after mating, they increase feeding and then initiate egg laying (Gioti et al. 2012). Remating, however, may be common (Harshman & Clark 1998) even though recently mated females have lower receptivity than virgin females (Chapman et al. 2003). Because there is a strong last male sperm precedence in fruit flies (Gromko et al. 1984; Price et al. 1999), the earlier male to mate will gain little paternity if his recent mate is quick to remate with another male. Thus males can benefit from guarding their mates that remain at the fruit they defend.

Mate guarding has been well studied in many species (Alcock 1994; Simmons 2001) and can be expressed in different ways. The most overt way involves cases such as in the dragonfly, *Pachydiplax longipennis*, in which the male remains close to the female after mating and during her oviposition and chases away approaching males (Sherman 1983). Similarly, in Idaho ground squirrels (*Spermophilus brunneus*), the males stay close to their mates and attack approaching males. Field observations corroborated with genetic tests indeed indicated that males sired the pups born to females that they guarded (Sherman 1989). An alternative form of mate guarding involves the males simply remaining mounted to females after copulation. This probably reduces the guarding males' need for using aggression. For example, male dung flies (*Scatophaga stercoraria*) remain mounted on the females after copulation for the duration of oviposition and thus physically block mounting by other males. Calculations indicate that such mate guarding is advantageous over the alternative strategy of searching for other females (Parker 1970). In soapberry bugs (*Jadera haematoloma*), copulations can last up to a few days even though sperm transfer takes only a few minutes. The males also remain close to the females during oviposition and remate if other males approach (Carroll 1991). Another form of mate guarding occurs in a variety of territorial birds. In addition to aggressively defending territories, the males closely follow their mates during their fertile period in order to reduce extra pair copulations (Birkhead 1979; Beasley 1996; Dickinson & Leonard 1996; Dickinson 1997; Chuang-Dobbs et al. 2001). For example, in house wrens (*Troglodytes aedon*), short-term experimental detention of males resulted in higher rates of extra pair copulations and paternity (Brylawski & Whittingham 2004).

A recent study focusing on the mechanisms of aggression (Yuan et al. 2014) hinted at the possibility of mate guarding in fruit flies. Because the natural history of fruit flies described above implies that mate guarding may be beneficial under some realistic field settings, we conducted a set of experiments to critically test the role of aggression in mate guarding. Overall, our goal was to expand the scope of research on aggression in fruit flies in order to place it in a broader ecological perspective. This can help us understand both the mechanisms and fitness consequences of aggression in many animals. Specifically, we predicted (i) that males with their recent mates will be more aggressive than control males; (ii) that aggression in the context of mate guarding will decrease female remating frequency with other males; and (iii) that aggression in the context of mate guarding will increase the paternity of mate guards.

4.3 General methods

We used descendants of wild-caught *Drosophila melanogaster* collected in several Southern Ontario localities in August, 2014. We housed the flies in population cages containing several hundred flies per cage. We kept the cages in an environmental chamber at 25°C and 60% relative humidity with a 12:12 hour light:dark cycle, with the lights turning on at 10:00 a.m. We reared the experimental flies at a low density of about 300 eggs per 240-ml bottle containing 50 ml of standard fly medium made of water, sucrose, cornmeal, yeast, agar and methyl paraben.

We sexed flies within 4 hours of eclosion to ensure virginity. We used gentle aspiration to sex and transfer the males into individual food vials, and CO_2 to sex and place females in groups of 20 per food vial. Each 40-ml vial contained 5 ml of the standard fly medium, and the females' vials also contained a dash of live yeast. At the time of testing all flies were 4 days old. We used small amounts of pink fluorescent powder to mark males to allow us to distinguish between males when two males shared an arena. Male colouring was counterbalanced with male treatment.

We conducted all tests in cylindrical arenas made of Plexiglas 3 cm in diameter and 2.5 cm high. To deter flies from climbing on the arenas' walls and ceilings, we coated the walls with Insect-a-Slip (Fluon; BioQuip) and the ceilings with Surfasil (Sigma). The floor of each arena had a piece of moist filter paper, and each arena contained a circular food patch 1.3 cm in diameter and 1.5 mm high covered with a live-yeast suspension. We recorded all trials using webcams (Logitech HD Pro C920 and iPad Air). Then observers blind to fly treatment scored the videos using Noldus software. We used generalized linear models (GLMs) when there were independent measures, and generalized estimating equations (GEEs) when there were repeated measures (IBM-Corp. 2011). Unless noted otherwise, the models assumed gamma distributions with log linked functions.

4.4 Aggression in the context of mate guarding

4.4.1 Methods

We began by examining whether males were more aggressive towards other males in the presence of their recent mates than were control males. We used a protocol modified from Yuan et al (2014). In the mate guarding treatment (n=30 arenas), we placed two focal males in an arena with two virgin females and allowed them to mate (Fig. 4.1). After both males had finished mating, we waited 10 min and then began video recording the arenas for 30 min. In the non-mates treatment (n=30 arenas), we placed one female and one male in each of two vials and allowed them to mate. Following mating, we discarded the males, placed the two mated females and two virgin focal males in an arena, let them acclimate for 10 min and then began video recording for 30 min (Fig. 4.1). From these videos, observers who were blind to the male treatment recorded the total duration of aggression, which included all occurrences of lunging, wing threat, high level fencing, holding, boxing, tussling and charging (Chen et al. 2002; Dierick & Greenspan 2006). To assess changes in aggression over time, we separated the 30-min trials into 6 blocks of 5 min. We predicted higher aggression levels in the mate-guarding than in the non-mates condition.

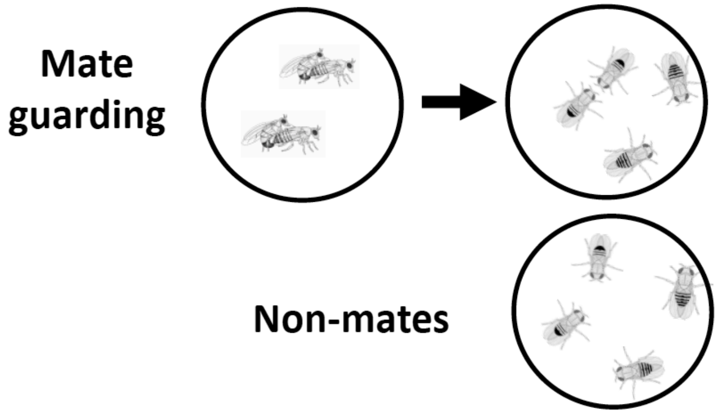

Figure 4.1

The mate-guarding treatment involved two males in the presence of their recent mates whereas the non-mates treatment had two males together with females recently mated to other males (note that males are distinguished from females by their smaller size and the black tip of their abdomen).

4.4.2 Results

Males were significantly more aggressive towards other males in the mate guarding than in the non-mates treatment (GEE; Wald χ^2_1=20.7, n=60, P<0.001, Fig. 4.2). Aggression varied significantly throughout the trials but the interactions between male type and time throughout the trials were not significant (GEE; Wald χ^2_5=18.3, n=60, P<0.005 and Wald χ^2_5=8.5, n=60, P=0.13 respectively, Fig. 4.2).

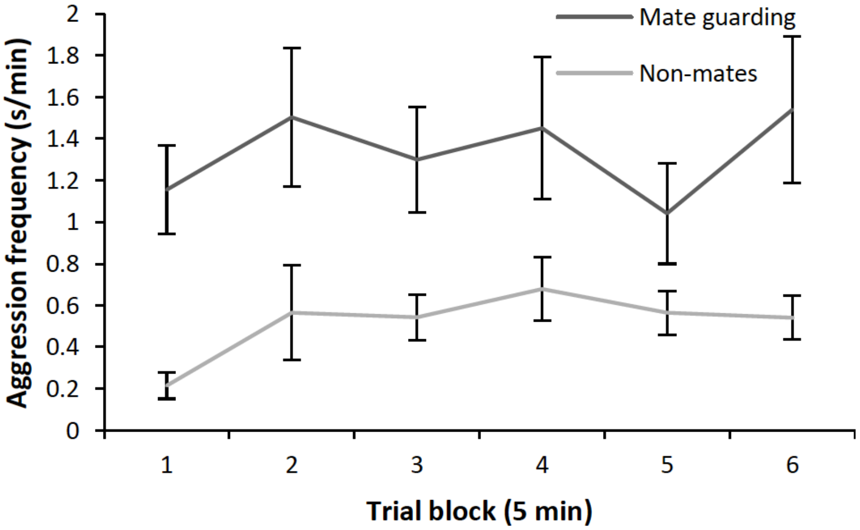

Figure 4.2

Mean ± SE aggression frequency (s/min) per 5 min block per arena in the mate guarding and non-mates treatments (n = 60 arenas).

4.5 Aggression by mate guards vs. mated males

4.5.1 Methods

The previous experiment indicated that recently mated males in the presence of their mates are more aggressive than focal males in the presence of females recently mated to other males (Fig. 4.2). To assess the relative effects of mate guarding and recent mating on male aggression, we compared male aggression under two conditions. In the mate guarding treatment (n=30), we placed a focal male (guard) and a virgin female in an arena and allowed them to mate. Following mating, we added a virgin intruder male, allowed the flies to acclimate for 10 min and then began video recording for 30 min (Fig. 4.3). In the non-mate treatment (n=30), we placed one virgin female and one virgin male in each of two vials and allowed them to mate. Following mating, we discarded the female from one vial and the male from the other vial, and placed the focal male and non-mate female along with a virgin male in the arena, allowed them to acclimate for 10 min and then began video recording for 30 min (Fig. 4.4). Observers blind to treatment and male role recorded the duration of aggression performed by each male. We predicted more aggression by the guard males than by the intruder males in the mate guarding treatment and no difference in aggression levels between the focal males and other males in the non-mate condition. We had to exclude three replicates from the analysis because the male colours were not distinct in two replicates and the video file was corrupted in another replicate.

Intruder males can respond to aggressive male guards by reducing further contact with these males (Fig. 1 in Yurkovic et al. 2006). This means that the duration of aggression may not be the best measure of male behaviour. We thus also quantified the relative dominance of the two males, measured by the time they spent on the food patch. Because our preliminary data indicated that the females spent much of their time on the food patch, we predicted a longer food-patch residency by the guard males than by the intruder males in the mate guarding treatment and no difference in food-patch residency between the focal males and other males in the control condition.

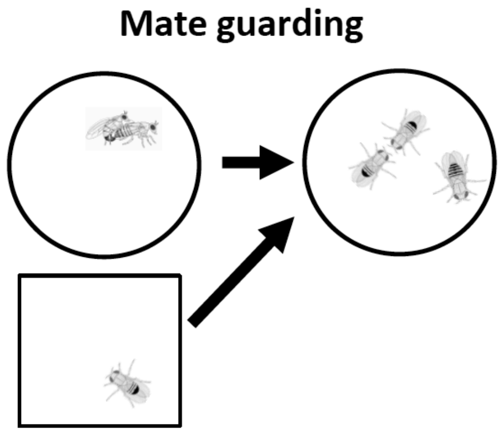

Figure 4.3

In the mate-guarding treatment, we introduced an intruder male into an arena containing a guard male and his recent mate.

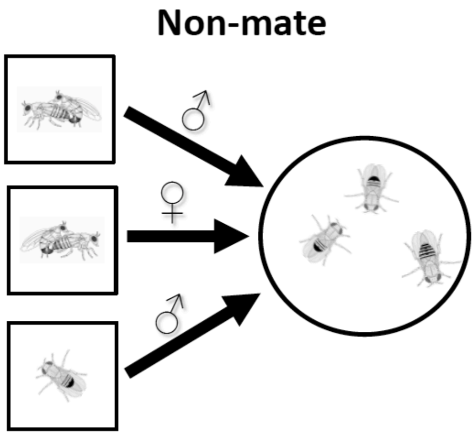

Figure 4.4

In the non-mate treatment, we placed in an arena a focal recently mated male, a female recently mated to another male, and another male.

4.5.2 Results

The male guards were more aggressive than the intruder males in the mate guarding treatment (GEE; Wald χ^2_1=5.3, n=58, P<0.05, Fig. 4.5). Aggression did not vary significantly throughout the trials (GEE; Wald χ^2_5=5.5, n=58, P=0.4) but the interaction between male type and time throughout the trials was significant (GEE; Wald χ^2_1=20.5, n=58, P<0.001). In contrast, there was no difference in aggression levels between the focal males and the other males in the non-mate treatment (GEE; Wald χ^2_1=0.8, n=56, P=0.4, Fig. 4.6). Both the levels of aggression throughout the trials (GEE; Wald χ^2_5=19.9, n=56, P<0.01) and the interaction between male type and time throughout the trials were significant (GEE; Wald χ^2_5=14.1, n=56, P<0.02).

The food-patch residency data paralleled those of the male aggression. The male guards spent significantly more time on the food patch than the intruder males (GEE; Wald χ^2_1=5.8, n=58, P<0.02, Fig. 4.7). Patch residency varied significantly throughout the trials (GEE; Wald χ^2_5=34, n=58, P<0.001) and the interaction between male type and time throughout the trials was significant as well (GEE; Wald χ^2_5=19.5, n=58, P<0.002). In contrast, there was no difference in the food-patch residency between the focal males and the other males in the non-mate treatment (GEE; Wald χ^2_1=0.6, n=56, P=0.4, Fig. 4.8). Patch residency varied significantly throughout the trials (GEE; Wald χ^2_5=18.3, n=56, P<0.005) but the interaction between male type and time throughout the trials was not significant (GEE; Wald χ^2_5=2.3, n=56, P=0.8). The food-patch residency of females was nearly identical in the two treatments (33±1.6 s/min with the mates present, 32.6±1.7 s/min with non-mates present; GEE; Wald χ^2_1=0.01, n=57, P=0.9) and did not vary throughout the 30 min trials (GEE; Wald χ^2_5=2.6, n=57, P=0.8 and Wald χ^2_5=3.8, n=57, P=0.6 for time throughout the trials and treatment by time interaction respectively).

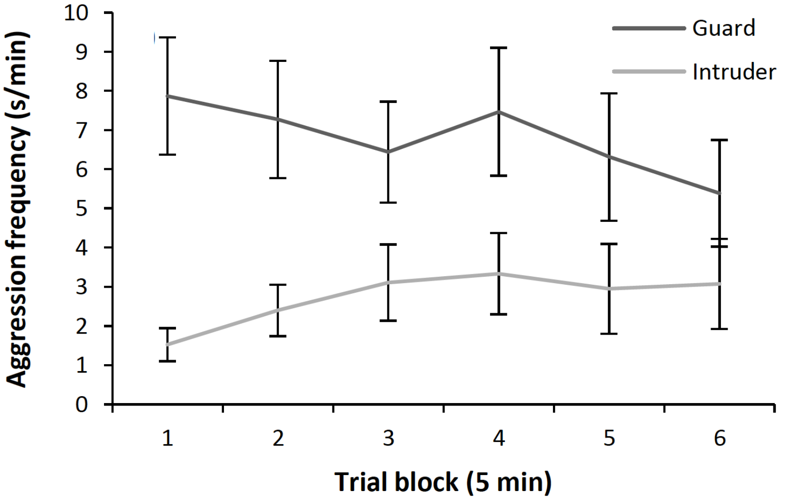

Figure 4.5

The mean ± SE aggression frequency (s/min) per 5 min block by each male in the mate guarding treatment (n = 58).

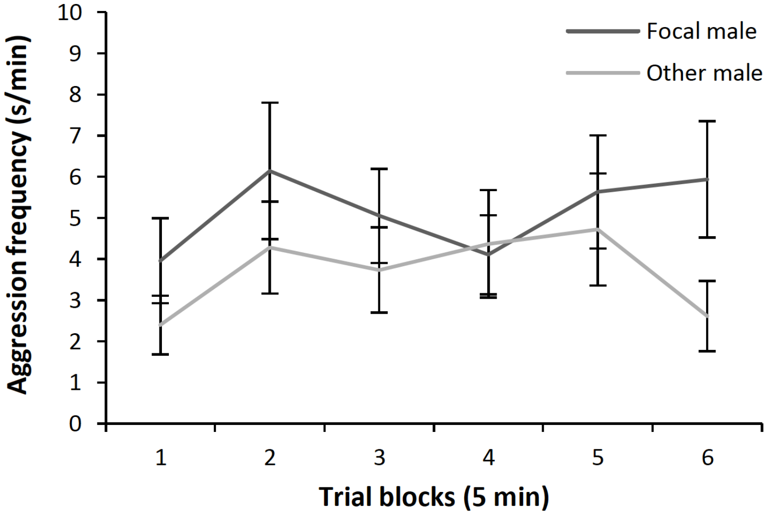

Figure 4.6

The mean ± SE aggression frequency (s/min) per 5 min block by each male in the non-mate treatment (n = 56).

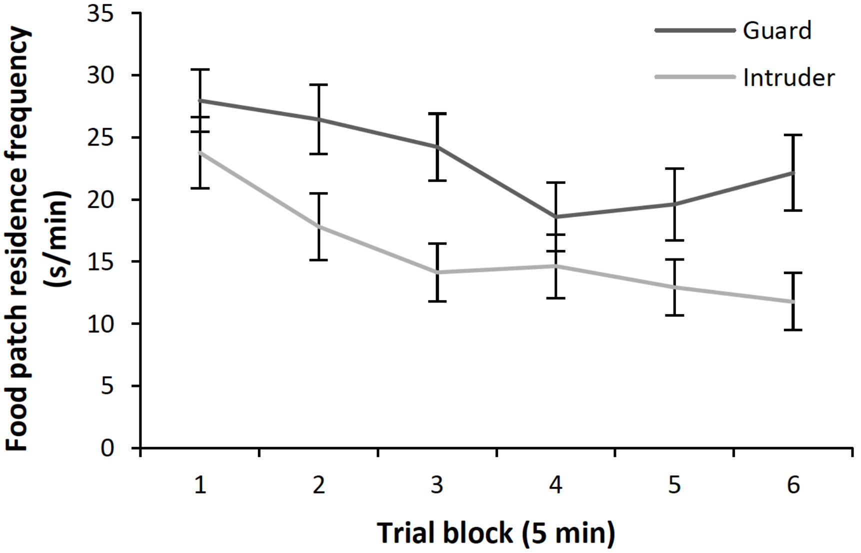

Figure 4.7

Food-patch residency (s/min) per 5 minute block in the mate guarding treatment (n = 58).

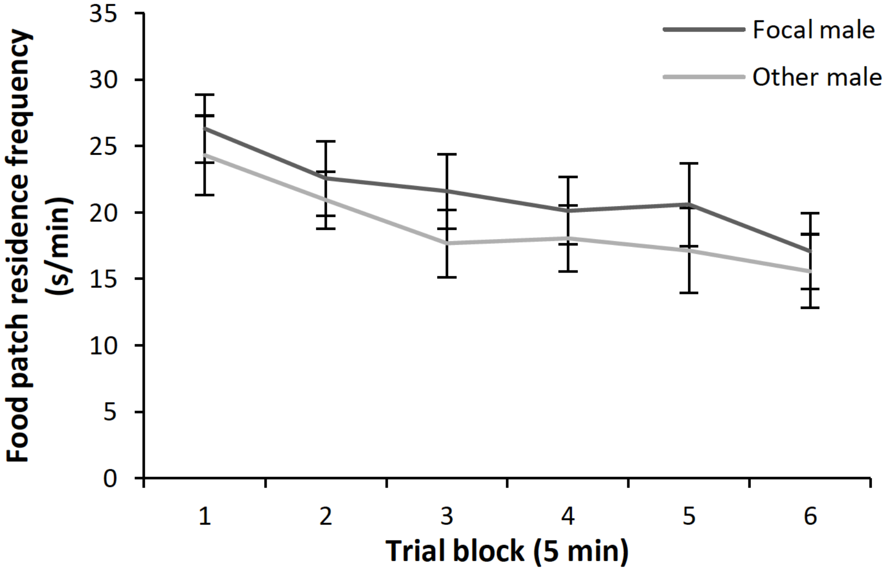

Figure 4.8

Food-patch residency (s/min) per 5 minute block in the non-mate treatment (n = 56).

4.6 Fitness benefit of mate guarding

4.6.1 Methods

The above experiments indicated that mated males show elevated levels of aggression in the presence of their recent mates and intruder males. Such increased aggression can deter intruder males and thus ensure the mated male's paternity. Here we wished to critically test this possibility. Specifically, we predicted, first, that a female will be less likely to remate with intruder males when guarded by her recent mate than when her recent mate is removed, and, second, that a male remaining to guard his recent mate at a patch will father a higher proportion of her offspring than a male removed from the patch after mating.

We conducted two experiments, the first monitoring only rematings and the second measuring both rematings and paternity. In the first experiment, we used the same wild-derived flies used in all previous experiments. We introduced one male and one female into each arena and allowed them to mate. Following mating, we randomly divided the arenas into two treatments. In the guard-present treatment, we kept the male (guard) and his recent mate and added an intruder into each arena (n=30). In the guard-absent treatment, we removed the mated male and introduced an intruder male into each arena (n=30). Observers blind to arena treatment scanned each arena for matings every 5 min for 4 hours. Because matings in *D. melanogaster* last about 15 min (Ashburner 1989), we could record all matings. As a supplementary information, the observers also recorded during each 5 min scan the locations of each fly (on or off the food patch), and the occurrence of courtship in each arena.

In the second experiment, we used a combination of our regular wild-derived flies and wild-derived eye mutants with partially white eyes. We had identified the eye mutation in a few flies descended from our field-collected population and isolated them in a separate population cage maintained under the same conditions described above for the wild-type flies. Our analyses indicated that the eye mutation is autosomal recessive with simple Mendelian inheritance. The protocol was similar to that described above except for the following. We used eye mutant females, wild-type males as the guards and eye mutant males as the intruders. After the observation phase had finished, we removed the females from each arena and placed them individually in labelled food vials with a dash of live yeast. We transferred these mated females to fresh vials every other day until they no longer laid fertilized eggs. Observers blind to female treatment counted all wild-type and eye mutant adult offspring. We analyzed the remating data with generalized linear models with multinomial distributions and probit link functions and analyzed the progeny data and courtship and location data using generalized linear models with gamma distributions and log link functions.

4.6.2 Results

In the first experiment, females' remating rates with intruders were lower in the presence than in the absence of guard males (GLM; Wald χ^2_1=3.3, n = 60, P=0.07; Fig. 4.9). Intruder males spent significantly less time on the food patch in the presence than in the absence of the guards (GLM; Wald χ^2_1= 46.3, n = 60, P < 0.001; Fig. 4.10) but courted for similar durations in both treatments (GLM; Wald χ^2_1=0.026, n = 60, P=0.9; Fig. 4.10).

In the second experiment, females' remating rates with intruder males were also lower in the presence than in the absence of guard males (GLM; Wald χ^2_1=11.5, n = 60, P < 0.001; Fig. 4.11). Intruder males spent less time both on the food patch and courting when the guard was present than when he was absent (GLM; Wald χ^2_1= 35.8, n = 60, P <0.001, and Wald χ^2_1=8.5, n = 60, P < 0.01 respectively; Fig. 4.12). Finally, the guard males fathered a greater proportion of the females' offspring when they were present than when they were absent (GLM; Wald χ^2_1=4.3, n=59, P<0.05; Fig. 4.13).

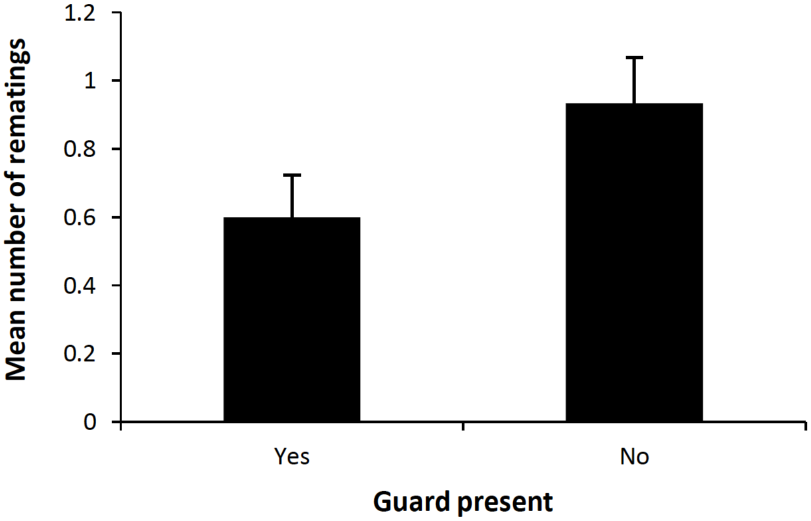

Figure 4.9

Mean ± SE number of rematings by intruder males when the guard male was either present or absent (n = 60 males).

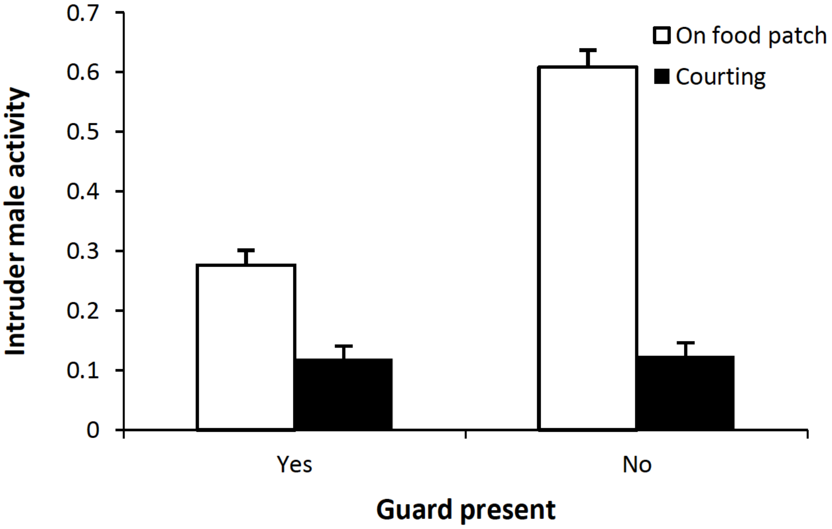

Figure 4.10

Mean ± SE proportion of observations that intruder males were seen on the food patch and courting when the guard male was either present or absent (n = 60 males).

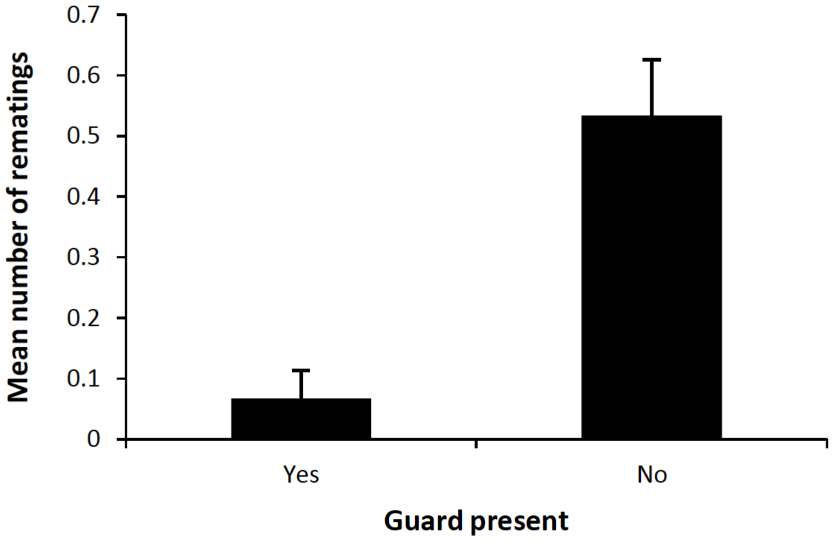

Figure 4.11

Mean ± SE number of rematings by intruder males when the guard male was either present or absent (n = 60 males).

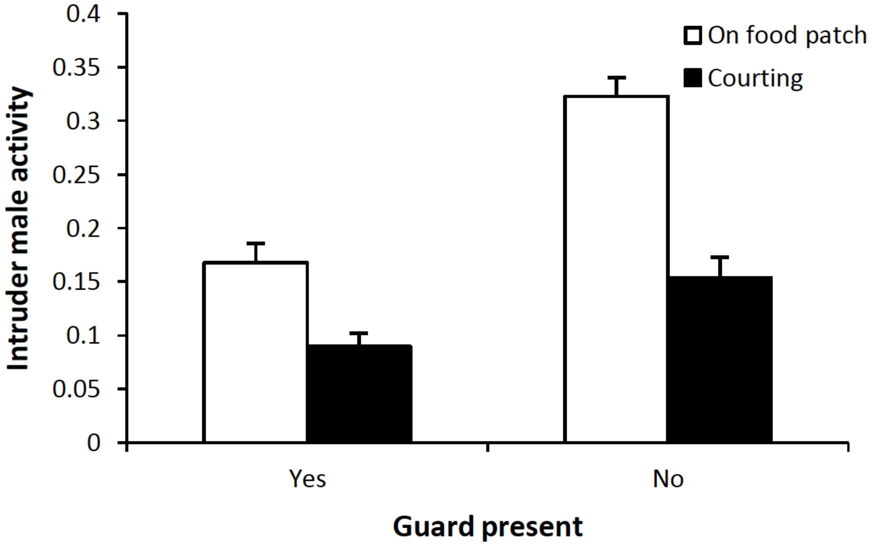

Figure 4.12

Mean ± SE proportion of observations that intruder males were seen on the food patch and courting when the guard male was either present or absent (n = 60 males).

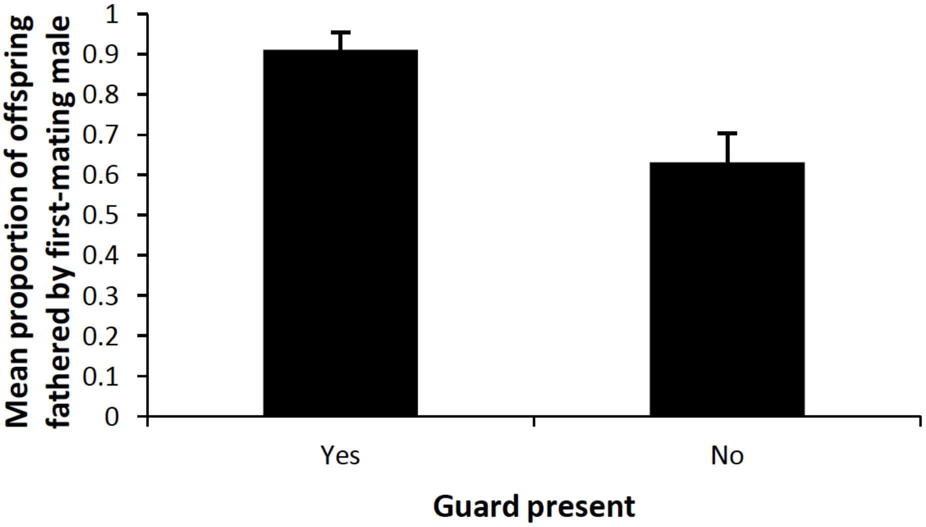

Figure 4.13

Mean ± SE proportion of offspring fathered by the guard male when he was either present or absent.

4.7 Discussion

Our major findings were that male fruit flies occupying a food patch with their recent mates were more aggressive towards other males than were males with non-mate females (Figs 4.1–4.8). The removal experiments indicated that such elevated aggression served for mate guarding because removing the male guards increased the food-patch occupancy, remating rates and paternity of intruder males (Figs 4.9–4.13). We should note that our experiments do not fully resolve the effects of either mating or experimental transfer into a new resource on male aggression. While mate guarding has been studied in a large variety of species (Alcock 1994; Simmons 2001), very few studies have experimentally tested its fitness consequences. Exceptions include a few bird studies in which short term detentions of males resulted in increased extra pair paternity (Chuang-Dobbs et al. 2001; Brylawski & Whittingham 2004).

Our experimental results are consistent with the limited information about the natural history of fruit flies (*D. melanogaster*). In settings with dispersed, small, decaying fruits, large males defend the fruits most attractive for feeding and egg laying, which are frequented by females. These males are more likely to mate than are smaller males (Markow 1988; Hoffmann & Cacoyianni 1990). The focus of previous behavioural (e.g. Hoffmann 1987a; Hoffmann 1987b; Hoffmann & Cacoyianni 1990) and neurogenetic (Chen et al. 2002; Dierick & Greenspan 2006; Zwarts et al. 2011; Yuan et al. 2014) studies was on male territoriality. From an evolutionary ecological perspective, however, the fruit fly (*D. melanogaster*) mating system is a classical resource-defence polygyny (Emlen & Oring 1977), in which some males monopolize decaying fruit that females require for feeding and egg laying. While the previous research implied that males that monopolize attractive food sources are more likely to acquire mates, our work suggests that an equally or even more important function of male aggression is in reducing the frequency at which his recent mates remate with other males.

One can argue that, just by maintaining his control of a fruit, the occupying male ensures his paternity. That is, males merely defend their fruit and there is no true mate guarding. Our data, however, indicate that the males show context-dependent aggression, with males at an attractive resource in the presence of their recent mates being more aggressive than males with non-mate females. The best explanation for this context-dependent aggression is that males elevate their levels of aggression in order to deter other males attracted to their recent mate. It is indeed likely that, in nature, a fruit occupied by a male and recently mated females attracts more intruder males than a fruit with only a male because recently mated females emit *cis*-vaccenyl acetate (cVA), which serves as an aggregation pheromone in fruit flies (Bartelt et al. 1985; Wertheim et al. 2006). Hence an evolved mechanism that generates increased aggression in a resource-defending male fruit fly after mating seems adaptive. In general, mate guarding in male fruit flies is somewhat similar to mate guarding in territorial birds (e.g. Birkhead 1979; Sundberg 1994; Dickinson 1997; Brylawski & Whittingham 2004). In both systems, males engage in a conspicuous defense of either a resource (fruit flies) or territory (birds) and somewhat less conspicuous protection of their mate.

To further assess the importance of aggression for mate guarding, we will require field data on two key behaviours of recently mated females, which are their tendency to stay at the fruit where they have just mated and their frequency of remating. Because males fight for the possession of the most desirable fallen fruits, which provide both adult and larval nourishment, we would expect females to frequent these fruits after mating in order to feed and lay eggs. There are currently no field data pertaining to this issue. The other key female behaviour for which we desire field data is the frequency of remating as a function of mating recency. Laboratory data provide a somewhat conflicting picture. On one hand, mating reduces female receptivity, and males find recently mated females much less attractive than virgin females (Manning 1962; Chapman et al. 2003). Indeed, in fly populations recently established from the wild, short-term tests lasting 15-60 min reveal no rematings in females mated up to 24 h beforehand (Dukas 2005). On the other hand, at least in small arenas, recently mated females that are incessantly courted by males for a few hours often remate (Billeter et al. 2012). This was true also in our experiments, in which trials lasting only 30 min had close to zero rematings (data not shown) whereas longer trials lasting 4 h had a high proportion of rematings (Figs 4.9, 4.11). The most critical field data, which ensured that there were no rematings during trapping, did indicate that females remate (Ochando et al. 1996; Harshman & Clark 1998), but they did not provide the desired information about the frequency of remating as a function of mating recency.

While our results suggest a novel function of aggression in male fruit flies, we expect that its relative importance will vary depending on the ecological settings, which, in turn, will determine the typically plastic mating system. For example, aggression in the context of resource-defence polygyny will be most common in settings with distinct resources and relatively low male density (Emlen & Oring 1977). Environments with other conditions including those most common in fruit fly laboratories, which have a single food source and high male density, might select for either other types of aggression or a baseline of low aggression. That is, to achieve our goal of producing a comprehensive synthesis of the types of aggression and their biological bases, we must attend to the subtleties of fruit fly natural history in an evolutionary ecological context in addition to the mechanistic foundations of aggression.

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CHAPTER 5 – LIFE HISTORY OF AGGRESSION: EFFECTS OF AGE AND SEXUAL EXPERIENCE ON MALE AGGRESSION TOWARDS MALES AND FEMALES

Baxter, C. M. & Dukas, R. (2017) Life history of aggression: effects of age and sexual experience on male aggression towards males and females. *Animal Behaviour*, *123*:11–20.

5.1 Abstract

Aggression is a prominent behaviour well studied in a large variety of animals, but it has not been well integrated within life history research. To address this shortcoming, we conducted a series of experiments simultaneously quantifying the effects of age and sexual experience on the use of aggression in male fruit flies. We studied three types of aggression relevant to male fruit flies' natural history: fighting in the context of resource defence, forced copulation with newly eclosed females, and coercion of recently mated females. Young, sexually mature flies were initially reluctant to use aggression towards either males or females. Within a few days, however, their use of aggression increased and then plateaued. While sexual experience caused males to decrease aggression towards females, it did not affect their aggression towards males. It is likely that aggression involves some cost, which leads males to favour peaceful over aggressive pursuit of females. However, sexually experienced males are probably highly motivated to deter other males from their apparently attractive resource. Our results highlight the importance of studying multiple types of aggression and the need for careful assessments of the relative fitness benefits and costs of aggression versus peaceful alternatives in shaping the life history of aggression.

Key words: aggression, coercion, *Drosophila melanogaster*, fighting, forced copulation, fruit fly, life history, remating, resource-defence polygyny

5.2 Introduction

Life history research has been instrumental in identifying key features characterizing animals as they go through the major life transitions of growth, reproduction and aging (Stearns 1992; Roff 2002). The major focus of life history studies has appropriately been on topics such as age and size at sexual maturity, the number and size of offspring, trade-offs between current and future reproduction, and senescence (Stearns 1992). Although aggression is a prominent and well-studied animal behaviour (Nelson 2005; Hardy & Briffa 2013), it has received scant attention in life history research. Notable research on aggression includes extensive work on territorial fights in a variety of spider species (Riechert 1986; Elwood & Prenter 2013) and butterflies (Davies 1978; Kemp 2013), shell fights in hermit crabs, *Pagurus bernhardus* (Elwood & Neil 1991; Briffa & Elwood 2001), contests in red deer, *Cervus elaphus*, and other ungulates (Clutton-Brock, Guinness & Albon 1982; Jennings & Gammel 2013), and violence in humans (Quetelet 1833; Daly & Wilson 1988; Baron & Richardson 2004; Daly 2016).

The two relevant life history models of aggression predict that younger males should be less willing to fight than older males. This is because younger males have a higher residual reproductive value than older males and can thus lose more from injuries (Parker 1974; Kemp 2006). Kemp (2006) noted, however, that young individuals should be more willing to fight under a narrow set of conditions where there is a steep decline in resource-holding potential with age and the benefit from holding a resource is relatively small. While the existing models are insightful, it is not clear how relevant they are to species that lack weapons. In such species, males cannot readily inflict injuries on their opponents, so the costs of fighting are not clear. Another limitation of the current models is that they do not consider a broad range of realistic population dynamics parameters, such as growth rate and density dependence, which strongly affect predictions of life history models (Abrams 1993; Williams, Day, Fletcher & Rowe 2006). Indeed, although both Kemp (2006) and others (Huntingford & Turner 1987; Hardy & Briffa 2013) highlighted a general pattern of fighting tending to increase with male age in a variety of species, they also noted many exceptions. Two well-studied species in which peak aggression occurs in young rather than older males are the lizard *Anolis aeneus* (Stamps 1978) and humans (Farrington 1986; Reiss & Roth 1993).

While many studies reported on variation in aggression with age (reviewed in Huntingford & Turner 1987; Kemp 2006; Hardy & Briffa 2013), most did not carefully account for correlates of age that can affect aggression. Such possible confounds include selective mortality (Huntingford & Turner 1987), mating experience, as well as subtle effects of social housing conditions prior to tests. For example, if subjects are housed in groups, social interactions within the group might determine subsequent aggression. Another limitation of most studies of aggression is that they focus on a single context, most commonly male fighting associated with access to females. It is widely agreed, however, that aggression is not a unitary phenomenon (Moyer 1968; Huber & Kravitz 2010). Hence it is highly appropriate that we incorporate multiple, ecologically relevant types of aggression into routine research protocols.

To broaden our knowledge on the life history of aggression, we have adopted fruit flies (*Drosophila melanogaster*) as a model system and focused on three types of aggression. Several factors make fruit flies an ideal species for examining the life history of aggression. The flies are short-lived and cheap to maintain. Ethical difficulties are limited because the flies do not inflict injuries during fights. There are numerous tools for functional and mechanistic research in this species. Finally, fruit flies have recently been adopted for genetic and neurobiological research on aggression (Chen, Lee, Bowens, Huber & Kravitz 2002; Dierick & Greenspan 2006; Zhou, Rao & Rao 2008; Edwards et al. 2009; Anholt & Mackay 2012), which, combined with our evolutionary ecological approach, can help illuminate general features of aggression and its trajectory throughout life.

We focused on three realistic types of male aggression, fighting in the context of resource defence, forced copulation with recently eclosed (teneral) females, and coercion of recently mated females. In settings with small, dispersed fruit and low fly density, capable male fruit flies use aggression to monopolize the attractive patches that females seek for feeding and egg laying (Dow & Schilcher 1975; Hoffmann 1987; Markow 1988).

Resource-holding males also rely on aggression to guard females they have recently mated with (Baxter, Barnett & Dukas 2015a). By far, fighting in the context of resource defence has dominated the mechanistic literature on fruit fly aggression, although most papers refer to it as territorial aggression (Hoffmann 1987; Chen et al. 2002; Edwards, Rollmann, Morgan & Mackay 2006).

In addition to the male–male aggression protocol, we also examined two types of male aggression towards females. Forced copulation with teneral females has been well characterized in the field and laboratory. Such forced copulation occurs primarily within 2 h post eclosion. During this short period, the vaginal plates of teneral females are still soft. Hence the females cannot physically resist intercourse by males that succeed in mounting them in spite of the females' persistent efforts to dislodge the males. We chose this type of aggression because it occurs at a sufficiently high frequency and thus can have significant effects on both male and female fitness (Markow 2000; Seeley & Dukas 2011; Dukas & Jongsma 2012b, a). While the role of coercion in the remating of recently mated females has not been well addressed, there is ample evidence that such rematings are more likely to occur at high male-to-female ratios, after long periods of persistent male mating attempts, and when females have no refuge from males (Gromko, Gilbert & Richmond 1984; Wigby & Chapman 2004; Byrne, Rice & Rice 2008). These features are typically associated with convenience polyandry (Thornhill & Alcock 1983; Arnqvist & Rowe 2005), where females give in to males' persistent harassment and remate. We should note that aggression and forced copulation are typically discussed in distinct bodies of literature, so one might question whether it is appropriate to call forced copulation aggression. We feel, however, that when physical force is used by one actor (the aggressor), which the other actor (the victim) attempts to evade, and when the aggressor inflicts injuries that reduce the expected life span of the victim (Dukas & Jongsma 2012b), the term aggression is appropriate.

Overall then, we tested the effects of male age and mating experience on three types of aggression. The theoretical models suggest that, generally, male aggression should increase with age (Parker 1974; Kemp 2006). Furthermore, we have previously documented that sexually mature, 1-day-old males spent less time than did 4-day-old males courting recently mated females that were unlikely to remate. Males' mate choosiness decreased with age and reached asymptote by age 7 days (Dukas & Baxter 2014; Baxter, Barnett & Dukas 2015b). This suggests that young males are less willing to incur costs associated with access to females than are mature males. We thus predicted that, as with our mate choosiness data (Baxter et al. 2015b), both male–male and male–female aggression would initially increase and then asymptote with male age. Our predictions for the effects of sexual experience were more complex. Previous data indicated a higher mate choosiness in previously mated than in virgin males (Byrne & Rice 2006; Baxter et al. 2015b). Hence we predicted that, compared to virgin males, sexually experienced males would show less coercion of teneral and recently mated females because such females clearly reject pursuing males. As for male–male aggression in the context of resource defence, we predicted no effect of sexual experience owing to the operation of two opposing factors. On the one hand, we expected sexual deprivation to increase males' motivation to use aggression in order to secure access to females. On the other hand, because encountering and mating with females informs males that they occupy an attractive resource, we expected previously mated males to show a higher motivation to use aggression for resource defence than males that had not encountered females previously.

5.3 Methods

5.3.1 General

We used descendants of wild-caught *D. melanogaster* collected in several southern Ontario localities in August 2014. We housed the flies in population cages containing several hundred flies per cage. We kept the cages in an environmental chamber at 25 °C and 60% relative humidity with a 12:12 h light:dark cycle, with the lights turning on at 1000 hours. We reared the experimental flies at a low density of about 300 eggs per 240 ml bottle containing 50 ml of standard fly medium made of water, sucrose, cornmeal, yeast, agar and methyl paraben. We sexed flies within 4 h of eclosion to ensure virginity and minimal experience with other flies. We used gentle aspiration to sex and transfer males into individual 40 ml vials each containing 5 ml of medium, and CO_2 to sex and place females in groups of 20 per vial, which also contained 5 ml of medium and a dash of live yeast.

We conducted all tests in cylindrical arenas made of Plexiglas (3 cm in diameter and 2.5 cm high). To deter flies from climbing on the arenas' walls and ceilings, we coated the walls with Insect-a-Slip (Fluon, BioQuip, Gardena, CA, U.S.A.) and the ceilings with Surfasil (Sigma Aldrich, Oakville, ON, Canada). We covered the floor of each arena with filter paper, and placed at its centre a circular food patch (1.3 cm in diameter, 1.5 mm high) coated with a suspension made of 3 g of live yeast in 100 ml of grapefruit juice. Throughout the study, we provided flies with ad libitum food optimized for fruit flies' preferences and needs, kept flies at low densities and kept to a minimum the duration of aggressive encounters.

Our experiments comprised two dimensions. First, we examined independently the effects of male age and sexual experience. Second, we assessed the effects of age and experience on three types of aggression: male–male aggression in the context of resource defence, and male–female aggression in the contexts of forced copulation of teneral females and coercive matings with recently mated females. Below we first detail our protocols for manipulating male age and experience, and then present specific methods for each of the three types of aggression.

5.3.2 Effects of age on aggression

As individuals age, they gain further experience. To separate the effects of age and experience on aggression, we conducted two sets of experiments. In the first set, we varied male age while holding mating experience constant whereas in the second set, we manipulated male mating experience while holding age constant. In the experiments on male age, we used males that were 1, 4 and 7 days old (see Results, Fig. 5.1). We housed these males individually in regular food vials until the time of testing. Our previous work indicated that males are sexually mature and have a high mating success and fertility when they are 1 day old (Dukas & Baxter 2014; Baxter et al. 2015b). We used males that were 1–7 days old because this represents a realistic age range for wild fruit fly populations. The limited field data suggest a median life span of 3–6 days in *D. melanogaster* (Rosewell & Shorrocks, 1987). In the similarly sized antler flies (*Protopiophila litigate*), median life span in the field was 6 days. Finally, in a few honeybee field studies, median forager life span was 5–7 days (Dukas & Visscher 1994; Dukas 2008a; Dukas 2008b). We had to limit the number of male age classes used because it was crucial that we conduct tests of all age groups simultaneously due to day and time of day effects.

5.3.3 Effects of mating experience on aggression

In the experiments on male age and aggression, we equalized males' experience by keeping the males away from females prior to testing. Age and experience, however, were positively correlated, meaning that older males had been deprived of females longer than younger males. We thus conducted another set of experiments in which we manipulated males' experience with females while keeping male age constant. On day 1, we randomly assigned newly eclosed males into either an experienced treatment or a deprived treatment. In the experienced male treatment, we added one 3-day-old virgin female to each male vial on days 2, 3 and 4, without removing the females added on the previous days (see Results, Fig. 5.9). This means that each male of the experienced treatment had ample opportunities to court and mate with both virgin and recently mated females. After the experience phase, we checked the food vials for the presence of larvae, which indicated that each male did in fact mate with females in his vial. In the deprived male treatment, we simply left the males alone in their vials, and thus they never had the opportunity to interact with females prior to the test (see Results, Fig. 5.9). We conducted the tests on day 5, when the males were 4 days old.

5.3.4 Resource defence

Our basic protocol involved aspirating two males and a 4-day-old, recently mated female into each arena. The female had mated with a nonfocal male 1–3 h prior to the test. After a 10 min habituation, we videorecorded each arena for 15 min using webcams (Logitech HD Pro C920). Later, observers blind to fly treatment scored the videos using

Noldus software (Noldus Information Technology, Wageningen, The Netherlands). The observers recorded the total duration of aggression by the two males, which included all occurrences of lunging, wing threat, high-level fencing, charging, holding, boxing and tussling (Chen et al. 2002; Dierick & Greenspan 2006). We analysed the data with a generalized linear model (GLM) with gamma distribution and log link function (IBM 2013), and applied sequential Bonferroni corrections to the post hoc comparisons.

In the test for the effect of male age, our three treatments included pairs of males that were 1, 4 or 7 days old (*N* = 36 pairs of males per treatment). We conducted two experiments assessing the effect of sexual experience. The first experiment consisted of our preferred protocol, in which we added to each arena one experienced and one deprived male, and a 4-day-old, recently mated female (*N* = 40 arenas). The female had mated with a nonfocal male 1–3 h prior to the test. After a 10 min habituation, we videorecorded each arena for 15 min. Later, observers blind to male treatment recorded from the videos the total duration of aggression displayed by each male. We analysed the data with a generalized linear model (GLM) with gamma distribution and log link function (IBM 2013), with male treatment as a repeated measure within each arena.

In our second test for the effect of sexual experience, we replicated the protocol of Yuan et al. (2014), because their results, unlike ours, suggested that males housed with females are less aggressive towards other males than are males deprived of females. To each arena, we added two males and two 4-day-old virgin females. We had three treatments in which the two contesting males within an arena were both deprived of females, both experienced with females, or one was deprived and one was experienced with females. One male per arena was coloured with pink fluorescent powder, and this colouring was counterbalanced across male experience. After adding the males to the arenas, we observed the arenas for mating. After the matings ended, we videorecorded each arena for 30 min. Later, observers blind to male treatment recorded from the videos the total duration of aggression displayed by each male.

We followed the protocol of Yuan et al. (2014) and discarded all trials in which one of the males did not mate. The sexually experienced males failed to mate in 23 trials, and the sexually deprived males failed to mate in 12 trials. We also excluded eight trials in which the female mated more than once. After these exclusions, we ended up with *N* = 16 experienced versus experienced arenas, *N* = 18 deprived versus deprived arenas and *N* = 17 experienced versus deprived arenas. The need to discard trials in which one of the males did not mate is an obvious weakness of the Yuan et al. (2014) protocol. Another complication is that matings with virgin females creates a conflicting, unnatural dynamic in which both males mate-guard their recently mated female (see Baxter et al. 2015a). We analysed the data with a generalized linear model (GLM) with gamma distribution and log link function (IBM 2013), with male treatment as a repeated measure within each arena.

5.3.5 Forced copulation with teneral females

We collected teneral females within a few minutes post eclosion and aspirated one teneral female and one male into each arena. Observers blind to male age continuously scanned the arenas for 2 h and recorded the mating latencies and durations. In the test for the effect of male age, our three treatments included males that were 1, 4 or 7 days old (*N* = 96 arenas per treatment). In the test for the effect of sexual experience, our two treatments were experienced and deprived males (*N* = 128 arenas per treatment). We analysed the data with Cox regressions, and used simple contrasts when comparing three treatments.

5.3.6 Coercive mating with recently mated females

5.3.6a *Evidence for male coercion*

To critically assess whether mating with recently mated females is coercive, we compared courtship and matings with recently mated and virgin females. We had three treatments (Fig. 5.5): mated females; virgin females matched to the mated female treatment for trial start time (virgin 1 treatment); and virgin females matched to the mated female treatment for approximate mating start time (virgin 2 treatment). This protocol allowed us to simultaneously compare the initial courtship of mated and virgin females, courtship just prior to mating, and behaviour during mating while controlling for time of day given that the mating latency of previously mated females is much longer than that of virgin females (see below). The initial matings of the recently mated females occurred 1 day before the test with nonfocal, 4-day-old males in regular food vials containing a dash of live yeast. After the matings, we discarded the males and kept the females in their individual food vials overnight. We later examined the vials for the presence of larvae to ensure that the initial matings were fertile. Only one female was not fertile, and she was excluded from the analysis.

For the test phase, we placed in each arena a 4-day-old virgin male and a 4-day-old female belonging either to the mated or virgin 1 treatment. We then began videorecording, which continued until mating ended or until 4 h passed. Based on preliminary data, we set up the virgin 2 treatment about 1 h later. This treatment was similar to the virgin 1 treatment except that it started later such that matings occurred at about the same time as matings in the mated treatment (Fig. 5.5). Later, we generated three sets of similar-length video clips for analyses (squares in Fig. 5.5). The initial courtship consisted of the courtship during the first few minutes of trials with virgin 1 and mated females (purple squares in Fig. 5.5). The length of these clips was determined by the short mating latencies of the virgin females. The final courtship consisted of the courtship during the last few minutes prior to mating with virgin 2 and mated females (blue squares in Fig. 5.5). Again, the length of these clips was determined by the short mating latencies of the virgin females. Finally, the mating clips (green squares in Fig. 5.5) included the full mating by each female as we did not expect a major difference in mating duration between the treatments (Bretman, Fricke & Chapman 2009). Separating the courtship and mating portions and equalizing the lengths within each set allowed us to remain blind to female treatment given the obvious differences in mating latencies.

Observers blind to female treatment recorded from each of the courtship clips the duration of male courtship (Dukas & Dukas 2012). While we also examined a variety of other male and female behaviours during courtship as we have done in the past (Seeley & Dukas 2011; Dukas & Scott 2015), we found no substantial differences between the treatments. For the mating clips, we recorded the duration of time that females kicked the males and had their wings closed. We considered closed wings as evidence for coercive mating because, prior to consensual mating, females spread their wings and this allows males to firmly hold the females during copulation (Spieth 1974). We also recorded from the mating clips the duration of unstable male mounting, which included males struggling to stay mounted on the females or being noticeably at an angle to the females rather than being securely mounted on her dorsal side between her wings. Because these data violated normality assumptions even after transformations, and showed no good fit to other distributions, we used nonparametric statistics in the analysis of coercive mating behaviours.

Our sample sizes for the virgin 1, virgin 2 and mated female treatments were 22, 22 and 28, respectively. One of the females from the mated female treatment did not produce offspring as a result of her first mating, and therefore was excluded from the analyses. There were also one, two and three trials in the virgin 1, virgin 2 and mated female treatments, respectively, that did not result in mating during the test phase. Therefore, these trials are not included in the analysis of coercive mating behaviours, giving us sample sizes of 21, 20 and 24 for virgin 1, virgin 2 and mated female treatments. However, these trials were still included in mating latency comparisons, as we performed Cox regressions to compare mating latencies between treatments, which take the absence of mating into consideration.

5.3.6b Effects of age

This experiment had two phases, the first involving the initial mating of virgin females and the second being the actual remating test. Theoretically, rematings could be affected by the age of the males that the females had initially mated with. Hence we had three types of mated females that initially mated with virgin, nonfocal males that were either 1, 4 or 7 days old. The initial matings occurred in regular food vials containing a dash of live yeast, and each containing one male and one female that was 3 days old. After the matings, we discarded the males and kept the females in their individual food vials overnight. We later examined the vials for the presence of larvae to ensure that the first matings were fertile and excluded from the analysis the 30 females (15% of the total sample) who were infertile (11 mated to 1-day-old males, 9 mated to 4-day-old males and

10 mated to 7-day-old males).

As before, our three main treatments included males that were 1, 4 or 7 days old (*N* = 56 1-day-old males, *N* = 57 4-day-old males and *N* = 52 7-day-old males, after infertile females were excluded). In addition, we also had three categories within each male age, of females initially mated with a male that was 1, 4 or 7 days old. This generated a total of nine treatment combinations. We aspirated one recently mated female and one focal male into each arena. Observers blind to fly treatment scanned the arenas continuously for 4 h, recording mating latencies and durations. We analysed the data using a Cox regression with simple contrasts. Note that we recorded forced copulations with teneral females over 2 h and coercive matings with recently mated females for 4 h owing to relevant natural history factors. By definition, the teneral state is transitional, and most forced copulations with teneral females occur within 2 h (Markow 2000; Seeley & Dukas 2011). In contrast, the frequency of coercive matings with recently mated females is positively correlated with the duration of persistent male coercive attempts (Gromko et al. 1984).

5.3.6c Effects of experience

This experiment, like the previous one, had two phases. The initial matings occurred during the first phase in regular food vials containing a dash of live yeast, with each vial containing one virgin 4-day-old male and one female that was 3 days old. We discarded 22 females (15% of the total sample) who did not produce larvae after the initial mating. In the second phase, we aspirated one male from either the experienced or deprived treatment and a recently mated female into each arena (*N* = 62 experienced and *N* = 58 deprived males after infertile females were excluded). Observers blind to male treatment scanned the arenas continuously for 4 h, recording the latency and duration of any matings that occurred. We analysed the data with a Cox regression.

5.4 Results

5.4.1 Effects of age on aggression

5.4.1a Resource defence

Aggression varied significantly with age (GLM: Wald $\chi^2_2 = 47.7$, $N = 108$, $P<0.001$; Fig. 5.2). Four-day-old males were more aggressive than 1-day-old males ($P<0.001$), but the 4- and 7-day-old males showed similar levels of aggression ($P = 0.33$).

5.4.1b Forced copulation with teneral females

The frequency of forced copulations varied significantly with male age (Cox regression: Wald $\chi^2_2 = 9.7$, $N = 288$, $P<0.01$; Fig. 5.3). Four- and 7-day-old males force-copulated significantly more frequently than 1-day-old males (Wald $\chi^2_1 = 7.7$, $N = 192$, $P<0.005$ and Wald $\chi^2_1 = 9.5$, $N = 192$, $P<0.005$, respectively), but 7-day-old males did not force-copulate significantly more frequently than 4-day-old males (Wald $\chi^2_1 = 0.76$, $N = 192$, $P = 0.38$).

5.4.1c Coercive mating with recently mated females

We first tested whether remating involved male coercion. While males spent similar proportions of time courting virgin and previously mated females during the initial few minutes of trials (purple squares in Fig. 5.5; Mann–Whitney *U* test: $U = 227$, $N_1 = 21$, $N_2 = 24$, $P = 0.57$; Fig. 5.6), they showed significantly more intense courtship of the previously mated than of the virgin females in the few minutes just prior to mating (blue squares in Fig. 5.5; $U = 45$, $N_1 = 20$, $N_2 = 24$, $P<0.001$; Fig. 5.6).

Mating latency varied significantly with female treatment (Cox regression: Wald $\chi^2_2 = 20.6$, $N = 71$, $P<0.001$; Fig. 5.7). The mating latencies in the virgin 1 and virgin 2 treatments (means ± SEs of 3.5 ± 1.3 and 3.0 ± 0.8 min, respectively) were much shorter than those in the mated female treatment (48.1 ± 8.3 min; Cox regression: Wald $\chi^2_1 = 17.4$, $N = 49$, $P<0.001$ and Wald $\chi^2_1 = 13.4$, $N = 49$, $P<0.001$, respectively; Fig. 5.7). During mating, the previously mated females spent a larger proportion of time kicking and with their wings closed (Mann–Whitney *U* test: $U = 177$, $N_1 = 20$, $N_2 = 24$, $P = 0.095$ and $U = 144$, $N_1 = 20$, $N_2 = 24$, $P<0.05$, respectively; Fig. 5.8). Consequently, the males were more likely to have an unstable hold of the previously mated than of the virgin females ($U = 168.5$, $N_1 = 20$, $N_2 = 24$, $P = 0.063$; Fig. 5.8). The mating durations in the mated female treatment (14.8 ± 0.58 min) were significantly longer than in the virgin 2 female treatment (12.7 ± 0.46 min; $U = 129$, $N_1 = 20$, $N_2 = 24$, $P<0.01$).

Next, we assessed the effects of male age on the frequency of matings with recently mated females. Mating frequency varied significantly with male age (Cox regression: Wald $\chi^2_2 = 20.2$, $N = 165$, $P<0.001$; Fig. 5.4). Four- and 7-day-old males mated with recently mated females more frequently than did 1-day-old males (Cox regression: Wald $\chi^2_1 = 14.4$, $N = 113$, $P<0.001$ and Wald $\chi^2_1 = 18.7$, $N = 108$, $P<0.001$, respectively). There was no significant difference in the mating frequency between 4- and 7-day-old males (Cox regression: Wald $\chi^2_1 = 0.50$, $N = 109$, $P = 0.48$). The age of the first-mating male in each treatment did not affect the likelihood of remating (Wald $\chi^2_2 = 2.3$, $N = 165$, $P = 0.32$).

The trials for coercive remating with recently mated females were 240 min in duration; however we also analysed the frequency of remating after 120 min to allow for direct comparisons between these results and the results for forced copulation of teneral females. We found that after 120 min, mating frequency varied significantly with male age (Cox regression: Wald $\chi^2_2 = 8.5$, $N = 165$, $P<0.05$; Fig. 5.4). Four- and 7-day-old males mated more frequently than 1-day-old males (Cox regression: Wald $\chi^2_1 = 3.2$, $N = 113$, $P<0.073$ and Wald $\chi^2_1 = 8.1$, $N = 108$, $P<0.005$, respectively). The mating frequencies of 4- and 7-day-old males did not vary significantly (Cox regression: Wald $\chi^2_1 = 2.3$, $N = 109$, $P = 0.13$). The age of the first-mating male in each treatment did not affect the likelihood of remating (Wald $\chi^2_2 = 0.78$, $N = 165$, $P = 0.68$).

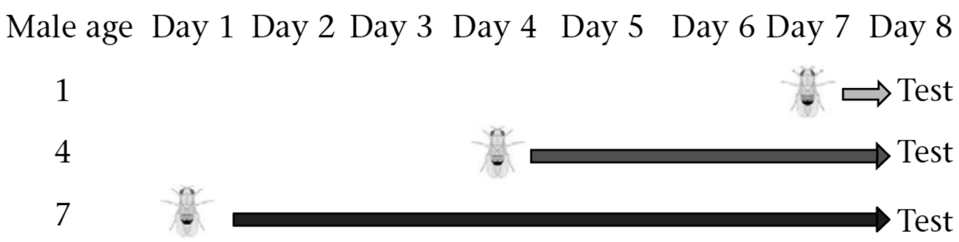

Figure 5.1

Three treatments for the effects of age on aggression: males were 1, 4 and 7 days old when tested on day 8.

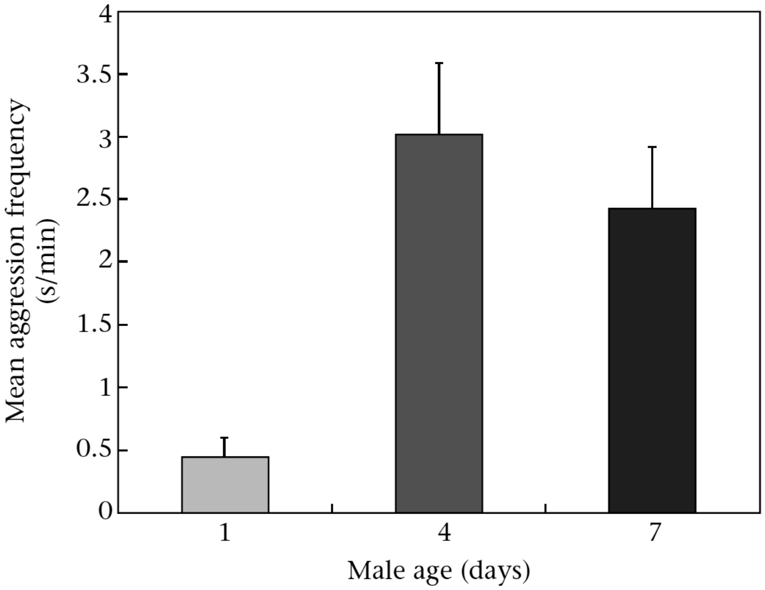

Figure 5.2

Mean + SE aggression frequency per arena in the three male age treatments (*N* = 108 arenas, 36 per treatment).

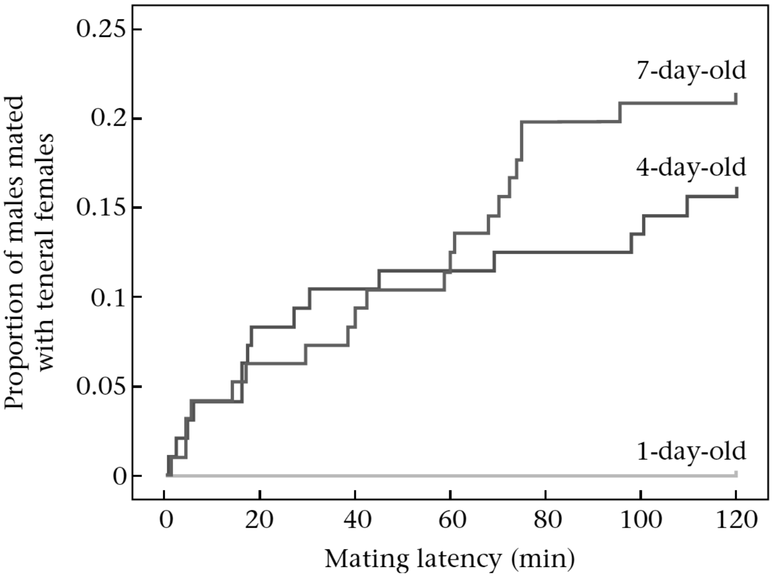

Figure 5.3

The cumulative proportion of 1-, 4- and 7-day-old males that force-copulated with teneral females across a 120 min trial duration (*N* = 288, 96 per treatment).

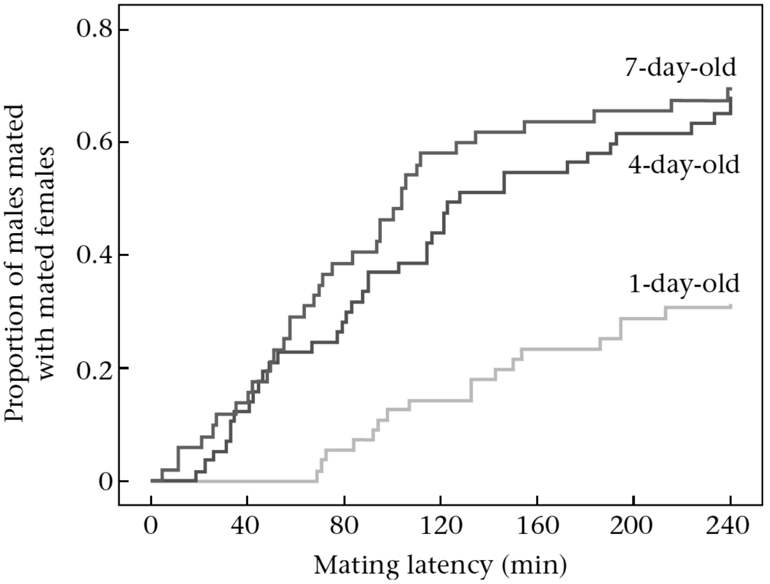

Figure 5.4

The cumulative proportion of 1-, 4- and 7-day-old males that mated with recently mated females across a 240 min trial duration (*N* = 165, with 56 1-day-old males, 57 4-day-old males and 52 7-day-old males).

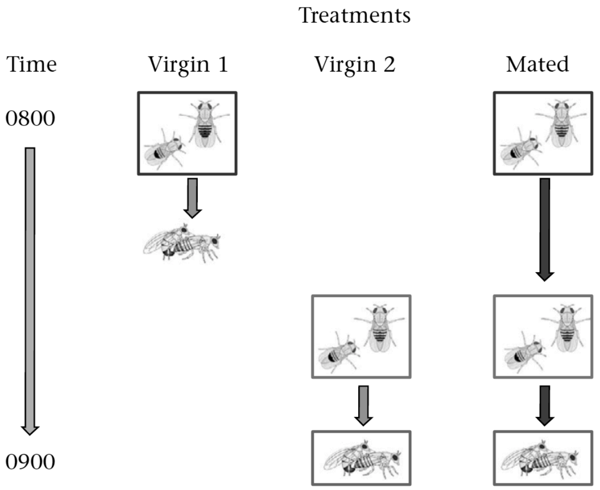

Figure 5.5

Three treatments for testing whether mating with recently mated females is coercive. The virgin 1 female treatment was matched to the mated female treatment for trial start time (purple squares) whereas the virgin 2 female treatment was matched to the mated female treatment for the approximate times of courtship just prior to mating (blue squares) and mating (green squares).

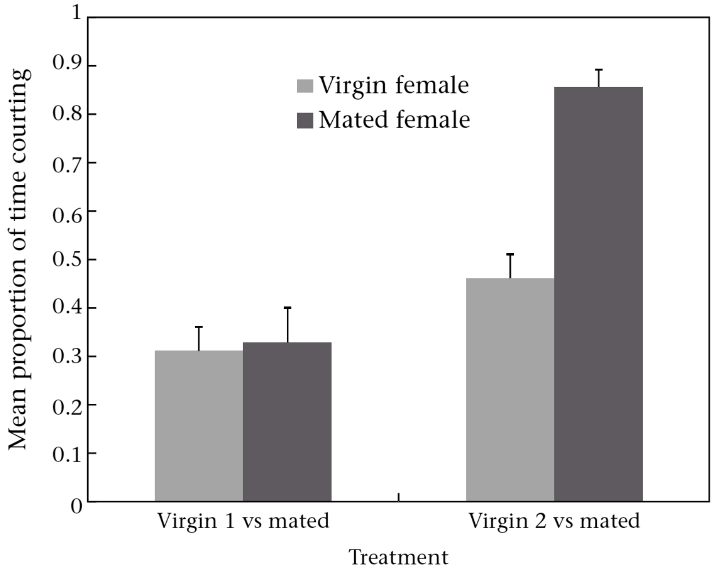

Figure 5.6

Mean + SE proportion of time males spent courting virgin and mated females at the commencement of trials (virgin 1 versus mated) and just prior to mating (virgin 2 versus mated) (*N* = 65, with 21 virgin 1, 20 virgin 2 and 24 mated female trials).

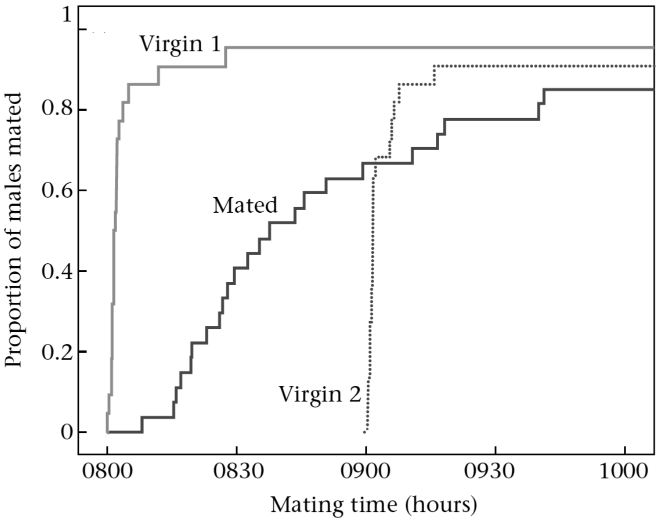

Figure 5.7

The cumulative proportion of virgin 1, virgin 2 and mated females matings. Note that trials of the virgin 1 and mated females started at 0800 hours while trials of the virgin 2 treatment started at 0900 hours.

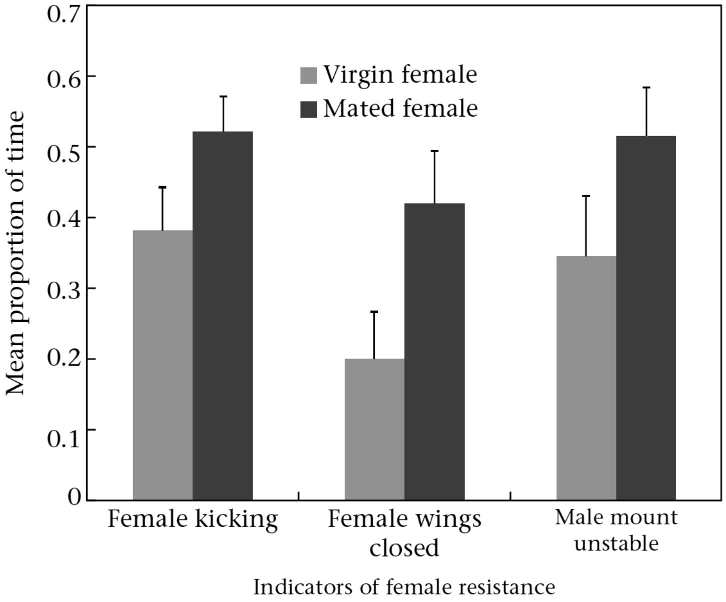

Figure 5.8

Mean + SE proportion of time females resisted during mating (through kicking and having their wings closed) and proportion of time males struggled to maintain mounting of females. Matings occurred at approximately the same time of day (green squares in Fig. 5.5).

5.4.2 Effects of mating experience on aggression

5.4.2a Resource defence

In the first experiment with a single mated female in each arena, the experienced and deprived males showed similar levels of aggression (GLM: Wald $\chi^2_1 = 0.34$, $N = 40$ arenas, $P = 0.56$; Fig. 5.10). In the second experiment with two virgin females in each arena, the experienced and deprived males showed similar levels of aggression (GLM main effect of experience: Wald $\chi^2_1 = 1.4$, $N = 49$ deprived and 47 experienced males, $P = 0.24$; Fig. 5.13).

5.4.2b Forced copulation with teneral females

Males of the deprived treatment force-copulated with teneral females significantly more frequently than males from the experienced treatment (Cox regression: Wald $\chi^2_1 = 14.3$, $N = 256$, $P<0.001$; Fig. 5.11).

5.4.2c Coercive mating with recently mated females

Deprived males were significantly more likely to mate with recently mated females than were experienced males after the 240 min trial duration (Cox regression: Wald $\chi^2_1 = 4.2$, $N = 120$, $P<0.05$; Fig. 5.12). We also analysed the frequency of remating after 120 min and found that the difference between deprived and experienced males approached, but did not reach, significance (Cox regression: Wald $\chi^2_1 = 2.2$, $N = 120$, $P = 0.14$).

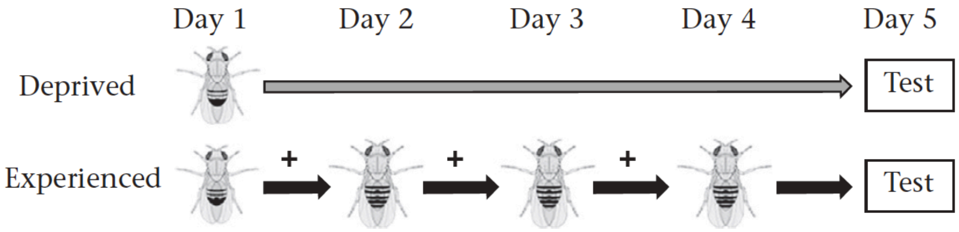

Figure 5.9

Two treatments for the effects of mating experience on aggression. Males were either housed alone (deprived treatment) or housed with females (experienced treatment) until the test on day 5. One female was added to each experienced male's vial on days 2, 3 and 4. Males of both treatments were 4 days old when tested on day 5. Note that males are smaller than females and have a dark posterior.

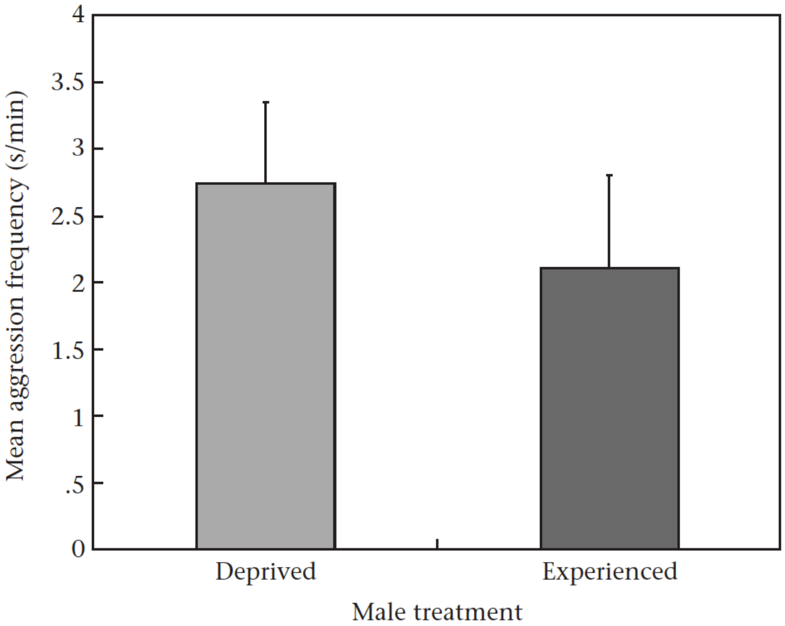

Figure 5.10

Mean + SE aggression frequency per male for mating-deprived and mating-experienced males, which were paired together in arenas ($N = 40$ arenas).

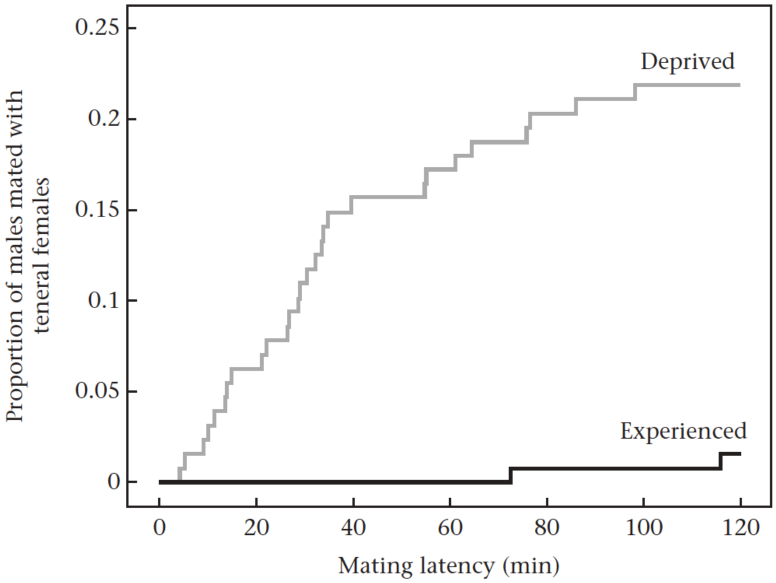

Figure 5.11

The cumulative proportion of previously mating-deprived and mating-experienced males that force-copulated with teneral females across a 120 min trial duration (*N* = 256, 128 per treatment).

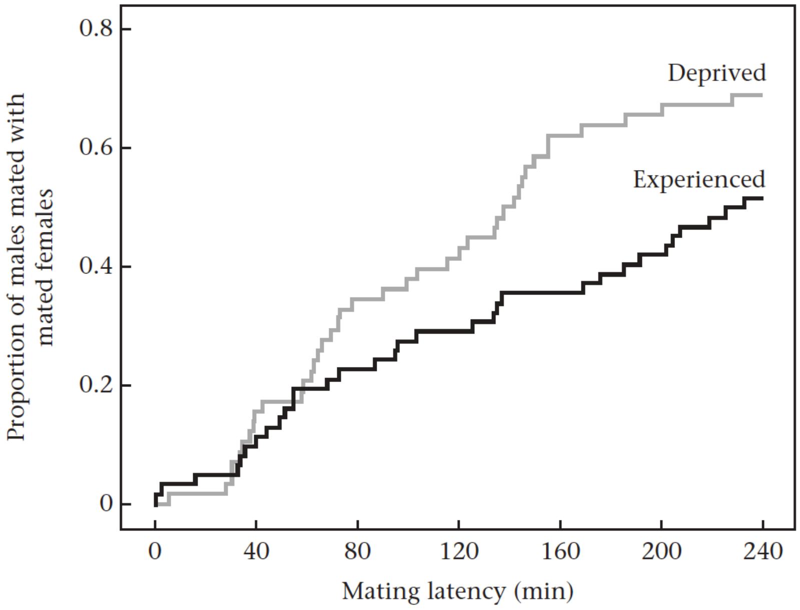

Figure 5.12

The cumulative proportion of previously mating-deprived and mating-experienced males that mated with recently mated females across a 240 min trial duration (*N* = 120, with 58 deprived males and 62 experienced males)

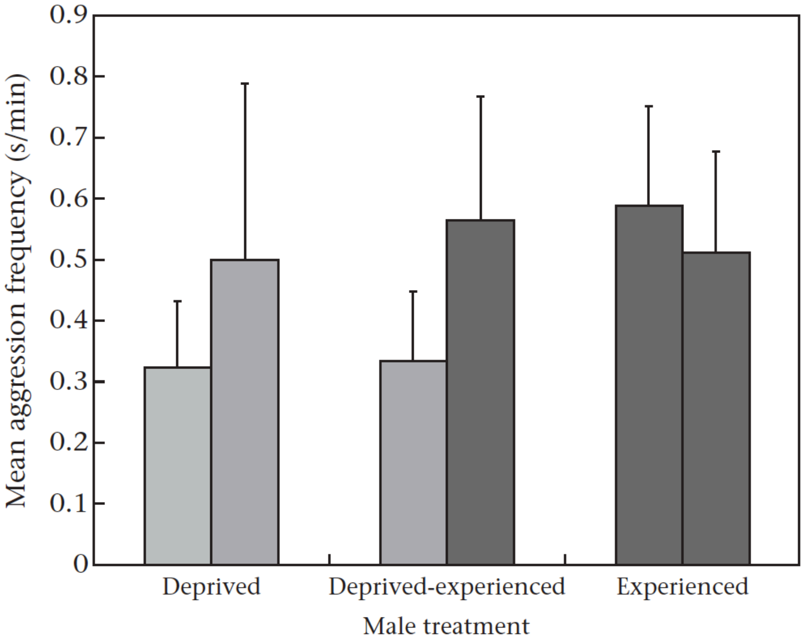

Figure 5.13

Mean + SE aggression frequency per male for mating-deprived and mating-experienced males. There were three arena combinations that males could be placed in: two deprived males (N = 17 arenas), one deprived and one experienced male (N = 16 arenas), or two experienced males (N = 16 arenas).

5.5 Discussion

Our major results were, first, that for all three aggression types, aggression initially increased with age and then plateaued (Figs 5.1–5.4). Second, sexually experienced males were less aggressive towards females but not towards males than were virgin males (Figs 5.9–5.13). The distinct effects of sexual experience on aggression towards females and males underline the importance of studying multiple types of aggression. That is, given the complexity of the proximate and ultimate mechanisms underlying aggression (Moyer 1968; Huber & Kravitz 2010), we should not expect different types of aggression to covary. We should note that, because we did not subdivide aggression into distinct categories (e.g. wing threat and lunging), we cannot judge whether there was age-specific variation in qualitative aspects of aggression.

We studied aggression using three protocols representing distinct features of fruit fly natural history. As noted in the Introduction, the role of aggression in resource defence and forced copulation had been well established. Furthermore, Hoffmann (1990) documented that 3- and 4-day-old males are more likely to be resource holders when competing with 1- and 2-day old males, respectively. This is consistent with our direct data on aggression, which indicate increased aggression between age 1 and 4 days. The role of aggression in female remating, however, had not been studied. We thus compared male–female interactions during courtship and mating in pairs consisting of a male and a virgin female versus pairs consisting of a male and a mated female. The most obvious difference between the female categories was an average mating latency of about 3 min with the virgin females and 45 min with the mated females. Intriguingly, males courted the previously mated females much more intensely than they courted virgin females in the few minutes just prior to mating (right bars in Fig. 5.6). This novel observation will require future elaboration because it might help explain males' mating success with reluctant females. The longer mating latencies and higher frequencies of kicking and lack of wing spreading with recently mated than with virgin females (Fig. 5.8) are consistent with the hypothesis that rematings by recently mated females are caused by male coercion (Thornhill & Alcock

1983; Gromko et al. 1984; Arnqvist & Rowe 2005). Nevertheless, the issue of rematings in fruit flies as well as in other species requires further examination because it is not fully clear why females that can control rematings succumb to male coercion (Boulton & Shuker 2016). We discuss the effects of age, sexual experience, and their interactions on the three types of male aggression below.

5.5.1 Effects of age on aggression

The life history of age-specific aggression within and among species is not well understood. The limited theory focuses on male–male aggression and assumes significant injury risks. Under these conditions, models generally predict peak fighting later in life (Parker 1974; Kemp 2006). While there are excellent data about the cost of aggression in animals with the capacity to inflict injury (Table 3.1 in Huntingford & Turner 1987), the cost of aggression in species in which participants do not get wounded is unknown. In a variety of species, fighting is associated with physiological costs that increase mortality rate (Huntingford & Turner 1987). In male fruit flies, pursuit of females is associated with increased mortality rate (Cordts & Partridge 1996). It is thus likely that the major cost of male–male and male–female aggression in fruit flies is increased mortality rates associated with heightened activity. Another possible cost is the increased tendency to lose a fight after a previous loss (Hsu, Earley & Wolf 2006; Trannoy, Penn, Lucey, Popovic & Kravitz 2016). Such loser effects may vary in magnitude with age and experience (Fawcett & Johnstone 2010). In nature, another cost of aggression may be elevated predation rates caused by a combination of increased detection rates by visual predators, reduced attention devoted to approaching predators (Dukas & Kamil 2000; Dukas 2002) and reduced flight initiation distance (Ydenberg & Dill 1986; Jakobsson, Brick & Kullberg 1995; Brick 1998; Cooper 1999).

Assuming that aggression, even with no injury risk, increases mortality rate, then the reluctance of young male fruit flies to engage in aggression towards either males or females can be explained by their higher residual reproductive value compared to that of older males (Parker 1974; Kemp 2006). It is likely that experience plays a role as well.

Under this scenario, the default strategy of young males is to initially seek reproductive opportunities that do not involve aggression. After failing to secure matings with the peaceful tactic, the males gradually increase their motivation to fight with males over high-quality resources and to coerce females. Our experiments on the effect of sexual experience indeed indicate that it plays a key role in shaping the trajectory of age-specific aggression.

5.5.2 Effects of sexual experience on aggression

Assuming that aggression is more costly than peaceful alternatives, males should avoid fighting and coercion if they can gain copulations using peaceful options. The obvious peaceful tactics are direct pursuit of females without engaging in aggression with males (i.e. scramble competition; Spieth 1974; Thornhill & Alcock 1983; Andersson 1994) and quick departure from females that provide clear rejection signals (Spieth 1952; Connolly & Cook 1973; Dukas & Scott 2015). The relative fitness costs to males of the aggressive and peaceful strategies, however, are unknown. Nevertheless, our results are consistent with the notion that aggression in fruit flies is more costly than peaceful options because males that had had access to virgin females were much less likely to engage in forced copulation with teneral females or to engage in coercion of recently mated females (Figs 5.11 and 5.12). A nonmutually exclusive alternative is that the fitness benefit from coercive matings is lower than that from consensual matings with virgin females. This is indeed the case for forced copulations with teneral females, which result in lower paternity than do consensual matings with virgin females (Dukas & Jongsma 2012b). Owing to last-male precedence, however, paternity from coercive matings with previously mated females may be comparable to that from consensual matings with virgin females because the latter females may end up remating as well (Gromko et al. 1984).

Two studies have documented that previously mated male fruit flies exercise more stringent mating criteria than do virgin males as indicated by their stronger preferences for large over small females and virgin over mated females (Byrne & Rice 2006; Baxter et al. 2015b). These data agree with our interpretation that males consider aggressive pursuit of females as a lesser option than seeking sexually receptive females. Consequently, males that have encountered and mated with virgin females are less likely to engage in coercive matings than are virgin males of the same age.

The effect of sexual experience on male–male aggression is more complicated than its effect on male–female aggression. As with male–female aggression, we expected previously mated males to be less willing than virgin males to engage in presumably costly fighting with other males. The major function of male–male aggression in fruit flies, however, is securing an attractive resource frequented by females seeking nutritious food and egg-laying sites. Hence males successful at mating probably perceive a food resource as more valuable than do virgin males. Such higher estimates of a resource value by mated males can lead to a greater motivation to defend it via aggression. At least one other study documented that experience with females increased males' motivation to fight. In the speckled wood butterfly, *Pararge aegeria*, males exposed to females in the territory persisted in fighting against intruders longer and were more likely to win the contest than were males that did not encounter a female (Bergman, Olofsson & Wiklund 2010). Data from house crickets, *Acheta domesticus*, however, have been inconsistent, with one study suggesting decreased aggression (Brown, Smith, Moskalik & Gabriel 2006) and another indicating increased aggression by sexually experienced males (Killian & Allen 2008).

Overall then, sexual experience may not affect male–male aggression in the context of resource defence because both female-deprived and mated males should have a high motivation to fight. Indeed, we observed no effects of sexual experience on male-male aggression in two experiments (Figs 5.10 and 5.13). We conducted another experiment assessing the effect of experience on male aggression in the context of resource defence in order to resolve the disagreement between our results (Fig. 5.10) and those of Yuan et al. (2014), who reported decreased aggression in males previously housed with females. Yuan et al. (2014), however, employed a novel protocol in which each male had to mate with a virgin female just prior to the recording of contest. A weakness of this protocol is that, if one of the two males did not mate, the trial was discarded. If a male's reduced probability of mating is associated with his aggressiveness, this can lead to biased results. Furthermore, we have shown that males in the presence of their recent mates show heightened aggression associated with adaptive mate guarding (Baxter et al. 2015a). Nevertheless, even when we employed the protocol of Yuan et al. (2014), we unequivocally failed to find decreased aggression in males previously housed with females (Fig. 5.13). We cannot resolve this discrepancy at this point.

5.5.3 Conclusions

Our simultaneous assessment of the effects of age and sexual experience on three types of aggression allows us to draw a general picture of the life history of aggression in fruit flies. This depiction may be relevant for many other weaponless species with a plastic mating system of resource defence polygyny under low density and dispersed, defendable resources, or scramble competition otherwise. Young males shun aggression towards both males and females most likely because it inflicts costs that decrease expected life span. Males that succeed in matings may maintain their low aggression levels. Males that fail at acquiring mates gradually increase their motivation to persist in pursuing both teneral females for forced copulations and previously mated females, which may be coerced into remating. It is likely that, in settings where most matings occur at resources defended by capable males, males' motivation to fight increases with age so that they can either acquire or maintain an attractive resource. In that setting, we do not expect males to ever decrease their level of aggression because matings inform them of the high attractiveness of the resource they currently defend. While the divergent effects of sexual experience on aggression towards females versus other males is clearly adaptive, it suggests distinct underlying mechanisms that can be examined in future work.

5.6 Acknowledgments

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CHAPTER 6 – GENETIC VARIATION IN SEXUAL AGGRESSION AND THE FACTORS THAT DETERMINE FORCED COPULATION SUCCESS

Baxter, C. M., Yan, J. L. & Dukas, R. (in press) Genetic variation in sexual aggression and the factors that determine forced-copulation success. *Animal Behaviour*.

6.1 Abstract

Sexual conflict is common in nature and sometimes results in sexual aggression. An extreme case is forced copulation, where one individual forcibly mates with another individual who resists the mating. To understand what makes some males sexually aggressive, we established an experimental system that allowed us to quantify the characteristics that contribute to males' forced copulation success. In fruit flies (*Drosophila melanogaster*), sexually mature females can choose to accept or reject courting males; however, males can forcibly copulate with newly eclosed, sexually immature, teneral females. We tested males from 59 genotypes and found significant genetic variation in forced copulation success, with a broad sense heritability of 0.16. We then chose three genotypes with the lowest and three with the highest forced copulation success rates and compared the behaviour of males from these two groups. Males from genotypes with high forced copulation success were more persistent in their pursuit of teneral females and mounted them more frequently than did males from the low-success genotypes. Males of the two categories, however, were similar in their attractiveness to both teneral and sexually mature females. Our results suggest that males vary in their pursuit strategies. Some males respond to female rejection signals by giving up and searching for receptive females, while other males persist in pursuit and coercion in spite of female objection. Our work highlights the practicality of using forced copulation in fruit flies as a model for further research on the mechanisms affecting variation in sexual coercion and forced copulation success and their evolutionary consequences.

Keywords: aggression, coercion, *Drosophila melanogaster*, forced copulation, fruit flies, genetic variation, heritability, sexual conflict

6.2 Introduction

Sexual reproduction was once thought to involve cooperation between males and females in their mutually beneficial endeavor to create offspring. However, it has long been understood that the optimal reproductive strategies for males and females differ, creating an opportunity for conflict between the sexes (Parker, 1979). Studies of sexual conflict have since greatly influenced our understanding of evolution via sexual selection (Arnqvist & Rowe, 2005).

Sexual conflict can be quite subtle, such as males exploiting pre-existing sensory biases in females for their own reproductive advantage. An example of this is seen in guppies (*Poecilia reticula)*, where females are attracted to males with orange spots due to a general, innate attraction to orange objects, which is common to both sexes (Rodd, Hughes, Grether, & Baril, 2002). However, conflict can also be overt, such as when males aggressively coerce, force or intimidate females into mating. Males can use harassment and aggression to coerce unreceptive females into accepting a mating, as seen, for example, in water striders (Rowe, Arnqvist, Sih, & Krupa, 1994). *Gerris gracilicornis* females are under greater predation risk than males. Males exploit this higher vulnerability by creating ripples that attract predators while mounting females, and only stop this activity once mating begins (Han & Jablonski, 2009, 2010). Aggression can be used to directly forcibly copulate with females, such as in Lake Eyre dragons (*Ctenophorus maculosus)* (McLean, Chan, Dickerson, Moussalli, & Stuart-Fox, 2016; Olsson, 1995), waterfowl (Mckinney, Derrickson, & Mineau, 1983; McKinney & Evarts, 1997) and wolf spiders (*Schizocosa ocreata*) (Johns, Roberts, Clark, & Uetz, 2009). In such cases, males can attack and pin down a female, causing injury to the female and forcibly mate with her while she resists the mating. Finally, males can also use aggression as a long-term sexual intimidation tactic, where their aggression towards females is temporally-decoupled from their mating, and leads to increased mating success in Chacma baboons (*Papio ursinus*) (Baniel, Cowlishaw,

& Huchard, 2017), and increased paternity in Kasekela chimpanzees (*Pan troglodytes schweinfurthii)* (Feldblum et al., 2014).

Fruit flies (*Drosophila melanogaster)* are an important model system for studying sexual conflict, with a strong focus on post-mating conflict (Chapman, Liddle, Kalb, Wolfner, & Partridge, 1995; Filice & Long, 2016; Laturney & Billeter, 2016; Rice et al., 2006). As for examples of sexual coercion in fruit flies, it has been shown that females are more likely to remate after long periods of persistent male pursuit, when females have no refuge from males, and particularly when the ratio of males to females is high, which suggests that males are harassing females into remating (Byrne, Rice, & Rice, 2008; Wigby & Chapman, 2004). Recently mated females are also more likely than virgin females to resist during mating by kicking the males and keeping their wings closed, hence reducing the ability of the males to mount securely (Baxter & Dukas, 2017). However, it can be difficult to distinguish between female resistance to male coercion and female mate screening, where females reject all males initially and then only accept males who demonstrate they are of high quality by persisting in their mating attempts. Since mate screening relies on the ability of a female to exercise control over the occurrence of mating, situations in which the female cannot prevent copulation can provide us with unambiguous examples of male coercion. Female fruit flies are typically able to reject males and prevent intromission. Therefore, although they can still be coerced into mating, using them as a model to study male coercion presents the difficulty of ruling out possible mate screening. In the first two hours post eclosion, however, females are in a teneral stage where their wings are still curled, their cuticle is soft, and their vaginal plates have not yet hardened. Since teneral females cannot either fly or physically prevent intromission by males who have mounted them, they can be forcibly copulated by sexually mature males, and these forced copulations occur both in nature and under laboratory conditions (Markow, 2000). Teneral females attempt to flee from males who persistently pursue them, and females who are forcibly copulated suffer significant costs, including wing damage, early mortality and reduced reproductive success, while males benefit from these matings because they father some offspring (Dukas & Jongsma, 2012a; Seeley & Dukas, 2011). Because matings with sexually immature, teneral females are unambiguously forced, they provide us with a clear model system for experimental research on aggressive manifestations of sexual conflict.

In laboratory studies with fruit flies, approximately 20-40% of males forcibly copulate when presented with a teneral female (Dukas & Jongsma, 2012b; Markow, 2000; Seeley & Dukas, 2011). So, why is it that some males aggressively force copulate while others do not? One potential explanation is that males who cannot obtain consensual matings resort to aggressively coercing females to mate, such as in scorpion flies (*Panorpa latipennis*), where males who have not obtained the nuptial gifts necessary for consensual mating will attempt to aggressively force copulate with females (Thornhill, 1980). However, this is certainly not true in all cases; for example, in waterfowl, it is generally the males who are already pair-bonded who engage in forced copulations (Mckinney et al., 1983; McKinney & Evarts, 1997). And in wolf spiders, only approximately 30% of males who have been rejected by a female end up forcibly copulating with her (Johns, 2007; Johns et al., 2009), so not all males faced with rejection perform forced copulations. What contributes to the variation in forced copulation rates across males who are faced with the same circumstances?

Our goal was to examine the behavioural determinants of forced copulation success. We first assessed the genetic variation in forced copulation success across 59 male genotypes (using a standard genotype for all teneral females), allowing us to estimate the broad-sense heritability of forced copulation success. Based on these results, we chose three genotypes with the highest and three genotypes with the lowest forced copulation rates to perform follow-up experiments with detailed behavioural observations of forced copulation trials. We predicted that males from the ‘high’ success genotypes would be more persistent and perform more coercive actions when pursuing teneral females than males of the ‘low’ success genotypes. Additionally, we quantified teneral females’ behaviour while being pursued by males and predicted that females would be less resistant to males with ‘high’ success genotypes, thus facilitating these males’ higher forced copulation rates. Finally, we tested males’ mating success with mature virgin females, who have full control over mating. If higher quality males are more likely to overcome teneral female resistance and thus succeed in forced copulations, then we would expect that these males would also have higher mating success with mature virgin females. However, if lower quality males are more motivated to perform forced copulations, then we would expect such males to have lower mating success with mature virgin females.

6.3 General methods

We used a subset of 60 Wolbachia-free lines from the *Drosophila* Genetic Reference Panel (DGRP). Mackay et al., (2012) generated these lines by collecting wild mated females in Raleigh, North Carolina, USA and then inbreeding their offspring for 20 generations of full-sibling mating. We housed all lines in standard fly vials with 5mL of our standard food medium (1L = 90 g sucrose, 75 g cornmeal, 10 g carrageenan, 32 g yeast, and 2 g methyl paraben dissolved in 20 mL ethanol). We maintained all flies in an environmental chamber at 25°C and 50% relative humidity with a 12h light:dark cycle with the lights turning on at 10:00am.

Zwarts et al., (2015) found that the majority of DGRP lines suffer from neurological defects, which they attributed to the fixation of recessive mutations affecting the architecture of the mushroom bodies. Their analyses indeed identified 24 genes directly linked to mushroom body defects. In order to lessen such deleterious effects, we crossed males from 59 distinct DGRP lines to females from a single line (DGRP-83) to create F1 hybrid flies. While such crossing generally reduces inbreeding depression (Charlesworth & Willis, 2009), its specific effect on the mushroom body has not been quantified. To generate the hybrids, we collected virgin females of DGRP-83 within eight hours of eclosion using light CO_2 anaesthesia and housed them in groups of 15 per food vial sprinkled with live yeast to stimulate egg laying. Once females were three to five days old, we transferred eight DGRP-83 females and five young males (one to four days old) from each one of 59 DGRP lines into food vials with live yeast. We transferred these parental flies of the 59 hybrid crosses to new food vials with live yeast daily and scraped excess eggs from the vials to ensure a consistent rearing density across lines. Eleven days after egg laying, we collected by gentle aspiration the F1 hybrid flies (hereafter hybrid genotypes) within 8h of eclosion to ensure virginity and aspirated them into individual food vials. We tested these males when they were four days old.

We conducted all trials within +/- 2h of the flies' subjective dawn. This period is associated with the morning peak of fly activity in most studies (Green et al. 2015) and has been verified in our laboratory (RD unpublished). We tested all flies in polystyrene Petri dishes that were 35mm in diameter and 8mm high. We covered the floor of each arena with a piece of filter paper and coated the walls and ceilings of the arenas with Surfasil (Sigma Aldrich, Oakville, ON, Canada). Surfasil is a siliconizing agent that, when dry, forms an odourless slippery film that the flies cannot walk on, thus restricting the flies to the base of the arena.

Since we wished to test for genetic variation in male propensity to forcibly copulate, males from the above-mentioned 59 hybrid genotypes were our focal individuals. In order to reduce variation in the response of the teneral females, we used females of a single reference line. We chose to use females of DGRP-83 as our teneral females, as they would be equally related by descent to males of each hybrid genotype. It is likely that females also possess genetic variation in susceptibility to forced copulation and that there is male by female interactions, but these topics are beyond the scope of our research.

Ethics Statement

Our research complied with all applicable laws and did not require approval from an ethics committee.

6.4 Genetic variation in forced copulation success

6.4.1 Methods

We collected teneral females of DGRP-83 within a few minutes of eclosion and aspirated one teneral female and one 4-day-old hybrid male into each arena. Observers blind to the focal males' hybrid genotypes continuously scanned the arenas for the presence of mating for a maximum of two hours. Trials occurred on 13 test days within a 15-day period. We tested two to four males per hybrid genotype per day, resulting in a final sample size of 43-46 males per hybrid genotype, with the exception of hybrid genotype 894 for which we were only able to collect and test 34 males.

6.4.2 Statistical Analyses

We used a generalized linear mixed-effects models (GLMM) in R version 3.3.2 (R-Core-Team, 2016) with the package lme4 version 1.1-12 (Bates et al., 2015). We used a binomial distribution to model the occurrence of forced copulation during the 2h trials, with hybrid genotype and test day as random factors. To assess the significance of the random effect of hybrid genotype, we report p-values calculated as the fraction of parametric bootstrapped likelihood ratio test (LRT) statistics (with 10 000 iterations) that were larger than the observed LRT values, using the package pbkrtest version 0.4-7 (Halekoh and Hojsgaard 2014).

We also calculated broad sense heritability (H^2) as $V_G / (V_G + V_E)$, where V_G is the genetic variance (i.e. the among-hybrid genotype variance) and V_E is the environmental variance (i.e. residual variance from the model). We multiplied the among-hybrid genotype variance by two because all hybrid genotypes had genetically identical mothers from DGRP-83.

6.4.3 Results

We found significant variation in forced copulation success across hybrid genotypes (LRT $p<0.01$, Fig. 6.1). The broad-sense heritability of forced copulation success was 0.16.

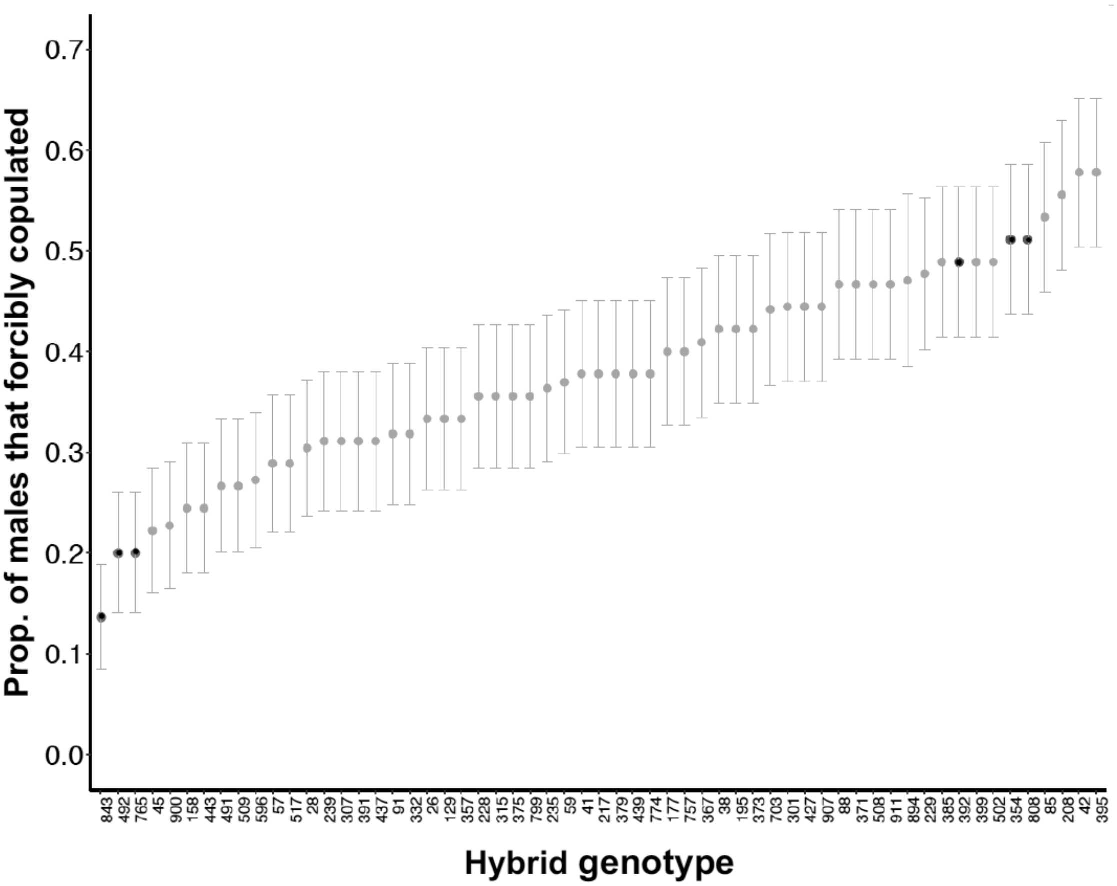

Figure 6.1

Genetic variation in male forced copulation success shown as the proportion ±1 SE of trials that resulted in forced copulation for 59 hybrid genotypes. The points are ordered along the x axis by increasing proportion and are labeled according to the paternal DGRP line of each hybrid genotype. The three 'low' and three 'high' forced copulation success genotypes used in the determinants of forced copulation success experiment are shown in black.

6.5 Determinants of forced copulation success of ‘low’ and ‘high’ hybrid genotypes

6.5.1 Methods

In order to perform detailed video observations of the males’ behaviours leading up to forced copulation, we chose to test a subset of six genotypes and shorten the trial duration to one hour. Based on the data from the previous experiment, we chose three hybrid genotypes that had the lowest and three that had the highest forced copulation rates within one hour (marked in Fig. 6.1 in white and dark grey, respectively, but note that Fig. 6.1 depict mating rates over 2 h). Our previous work on sociability (Scott et al., 2018) and aggression (Baxter et al., in prep) with the same DGRP hybrid genotypes indicated moderate repeatability of behavioral scores over time. We thus decided a priori that we would only score the videos from the hybrid genotypes that showed forced copulation rates consistent with their assignment into the low and high forced copulation rate categories. We considered the forced copulation rate of a genotype to be consistent if it was among the top three forced copulation rates for the ‘high’ genotypes, or among the bottom three for the ‘low’ genotypes.

We reared flies of the three ‘low’ and three ‘high’ success hybrid genotypes and collected and housed males as described above. We again aspirated one 4-day-old focal male and one DGRP-83 newly eclosed, teneral female into each arena. We then immediately began video recording for one hour using Logitech HD Pro c920 webcams and iPods. Trials occurred over six test days, where we tested four males per hybrid genotype per day, resulting in a sample size of 24 males per hybrid genotype.

Later, observers blind to the focal males’ hybrid genotypes observed the videos using BORIS observation software (Friard & Gamba, 2016). For each arena, an observer recorded the duration that each male pursued the female and the number of times he mounted the female as indicators of persistence and coercive actions, respectively. Observers also recorded the duration that the female spent running away from the male while he pursued her as an indication of resistance. We excluded two trials with hybrid genotype 354. In one trial, mating occurred at trial commencement, so we did not have behavioral observations. In the other trial, the male spent 43% of the trial duration on the ceiling of the arena and never interacted with the female. No other trial had a fly on the ceiling for more than 20% of the trial duration.

6.5.2 Statistical Analyses

We again used the lme4 package to perform linear mixed-effects models (LMM) and generalized linear mixed-effects models (GLMM). All models included hybrid genotype and test day as random factors and forced copulation success group ('low' vs. 'high') as a fixed factor. We report Wald χ^2 values generated with the Anova function from the car package version 2.1-4 (Fox and Weisberg 2011) for tests of the forced copulation success grouping (i.e. 'low' vs. 'high').

We used a binomial distribution to model the occurrence of forced copulation during the 1h trials with the 'low' and 'high' success hybrid genotypes. To assess males' persistence, we log transformed the duration that each male pursued the female to increase the normality of the distributions and added 1 second of pursuit to all males to eliminate 3 (out of 114) 0s. We used a LMM on the log transformed durations and included the log of trial duration as an offset (since males experience different trial durations depending on if/when they force copulated). For coercive actions, we modeled the number of mounts and mounting attempts males performed with a negative binomial distribution and included the log of the pursuit duration as an offset. Finally, to assess female resistance, we analyzed the duration that females spent running from males while they were being pursued. We log transformed both female running duration and male pursuit duration to increase the normality of the distributions and added 1 second of running to all females to eliminate 9 (out of 114) 0s. We used a LMM to assess female running duration and included male pursuit duration as an offset. We verified model fits by visually inspecting plots of model residuals.

As a secondary analysis, we grouped males based on whether or not they force copulated during the trial. The logic being that, if the behavioural differences between our 'low' and 'high' genotypes are representative of what varies between individual males who fail or succeed to forcibly copulate, then we would see similar trends when comparing between ‘unsuccessful’ and ‘successful’ males as we see when comparing between males from ‘low’ and ‘high’ success genotypes. We used binomial logistic regressions to assess whether forced copulation success varied with the proportion of pursuit, mounting frequency, or female evasion attempts. In each model, we also included hybrid genotype and test day as random factors.

6.5.3 Results

Only five of the six hybrid genotypes showed consistent forced copulation rates in the screening (Fig. 6.1) and follow-up experiments (Fig. 6.2). Following our protocol, we thus excluded hybrid genotype 765 from the video scoring. The two remaining hybrid genotypes with low forced copulation rates had half the forced mating success as the three hybrid genotypes with high forced copulation rates (GLMM, χ^2_1=6.58, p<0.05, N=119, Fig 6.2).

Males from the ‘high’ forced copulation hybrid genotypes spent significantly more time pursuing teneral females than males from the ‘low’ hybrid genotypes (LMM, χ^2_1 = 5.35, p<0.05, N=118; Fig 6.3). Males from the ‘high’ hybrid genotypes also performed significantly more mounting attempts while accounting for pursuit duration (GLMM, χ^2_1= 12.9, p<0.001, N=118; Fig. 6.4). Females showed a non-significant tendency to spend more time running from ‘high’ than ‘low’ hybrid genotype males when accounting for pursuit duration (LMM, χ^2_1=1.68, p=0.20, N=118; Fig 6.5).

When comparing males grouped by forced copulation success, we found that both male pursuit and mounting frequency significantly predicted forced copulation success (GLMM, z=4.4, p<0.001, N=118 and z=3.9, p<0.001, N=118, respectively). However, the proportion of time a female spent attempting to evade a male did not significantly predict whether or not she would be forcibly copulated (GLMM, z=0.50, p=0.62, N=118).

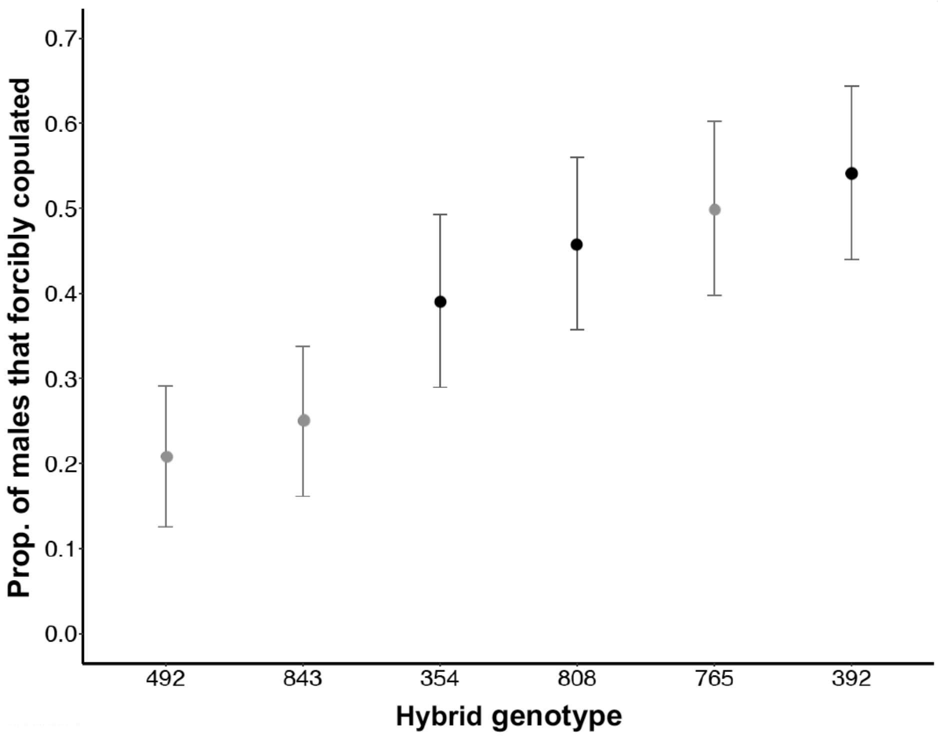

Figure 6.2

Proportion ±1 SE of trials that resulted in forced copulation for the chosen three ‘low’ and three ‘high’ genotypes (shown in grey and black, respectively).

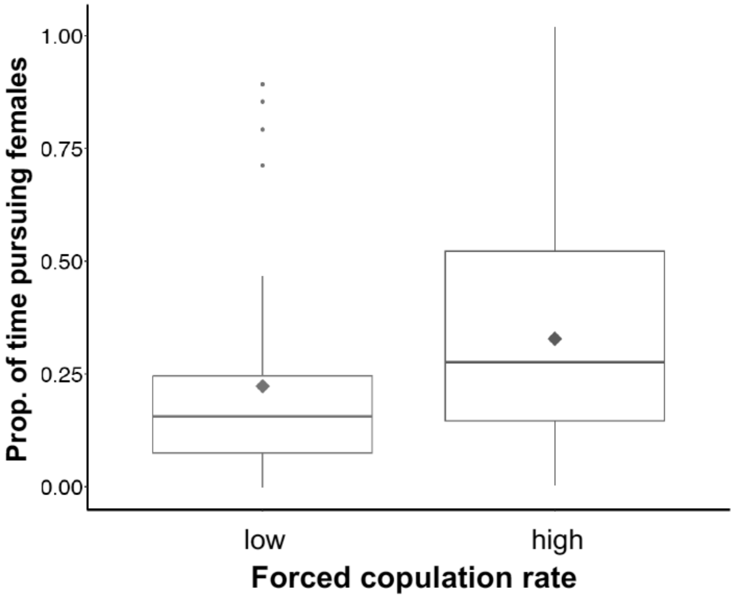

Figure 6.3

Comparing males from ‘low’ and ‘high’ forced copulation success genotypes (shown in blue and red, respectively). (a) Male persistence, shown as the proportion of time that males spent pursuing teneral females during the trials. The bold horizontal lines indicate the medians, the boxes represent the interquartile range (IQR) between the first and third quartiles, and the vertical lines extend to the minimum and maximum values. Outliers are indicated by small dots, and the means are indicated by the larger diamond shapes.

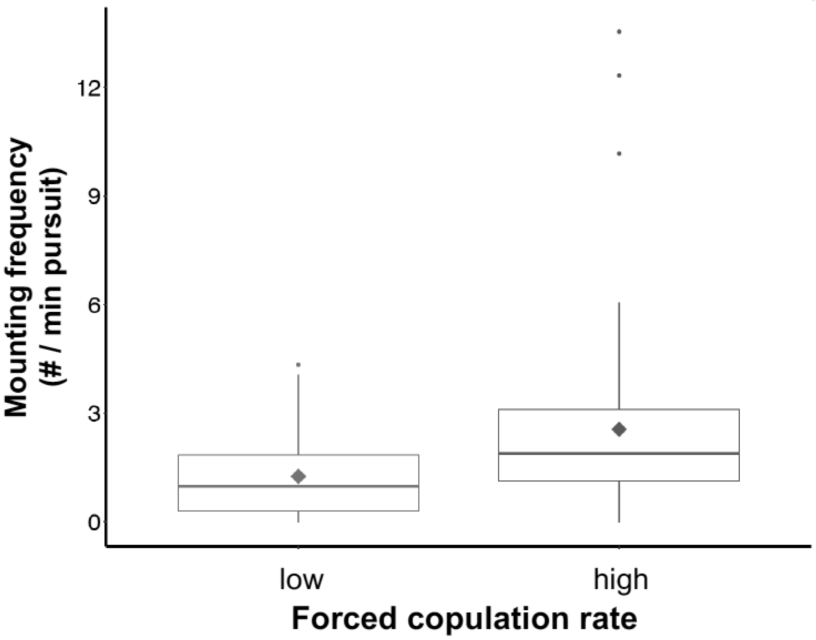

Figure 6.4

Male coerciveness, shown as the number of times males mounted teneral females per minute of pursuit.

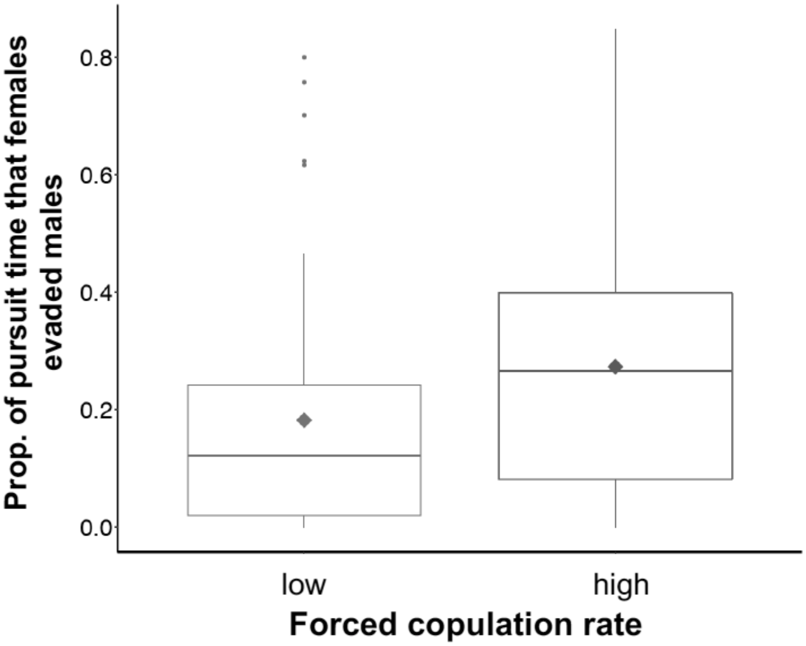

Figure 6.5

Teneral female resistance, shown as the proportion of male pursuit that females ran away from the males.

6.6 Mating success of 'low' and 'high' hybrid genotypes with mature virgin females

6.6.1 Methods

Simultaneous with the forced copulation trials described in the previous section (Figs. 6.2–6.5) we also tested males of the same hybrid genotypes with mature virgin females. We followed the same procedure as above, except that we placed each male in an arena with a 2-day-old virgin female of DGRP-83 instead of a teneral female. We used 2-day-old virgin females because they are sexually mature but choosier than older virgin females (unpublished data). We tested 48 males per hybrid genotype.

Observers who were blind to hybrid line identity scanned the mature female arenas and recorded whether or not the males mated within a one-hour trial.

6.6.2 Statistical Analyses

We used a binomial distribution to model the occurrence of mating during the 1h trials with the 'low' and 'high' success hybrid genotypes (fixed factor). The model also included test day and hybrid genotype as random factors. We only analyzed the five hybrid genotypes included in the analyses of the previous experiment.

6.6.3 Results

Males from the 'low' and 'high' forced copulation success genotypes mated at similar rates with mature females (GLMM, χ^2_1=2.55, p=0.11, N=240; Fig. 6.6).

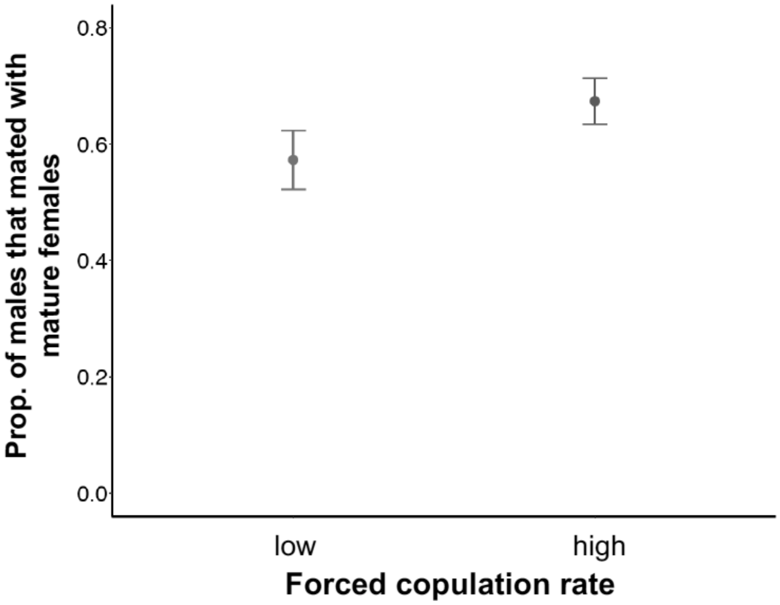

Figure 6.6

Male mating success with mature females, shown as the proportion ±1 SE of trials that resulted in matings with mature virgin females.

6.7 Discussion

In our initial screen of males from 59 hybrid genotypes we found significant variation in forced copulation success across genotypes (Fig 6.1). Such genetic variation is expected, and our lab has already documented genetic variation in sociability and activity levels (Scott, Dworkin, & Dukas, 2018), mating success, latency and duration (Filice & Dukas, 2019), and both male-male and female-female aggression (Baxter et al., in prep) in these same hybrid genotypes derived from DGRP lines. Many other studies using flies from the DGRP lines have also documented genetic variation in a variety of behaviours such as courtship (Gaertner et al., 2015), same-sex sexual behaviour (Hoskins, Ritchie, & Bailey, 2015), male-male aggression (Edwards et al., 2009; Shorter et al., 2015), odour aversion (Swarup, Huang, Mackay, & Anholt, 2013), behavioural plasticity (Saltz, Lymer, Gabrielian, & Nuzhdin, 2017), punishment and relief memory (Appel et al., 2016) and startle response (Mackay et al., 2012). Since we used teneral females from a single genotype in our screen, we can confidently attribute the variation in forced copulation success to genetic variation across the male genotypes, implying that males with different genetic backgrounds are predisposed to distinct mating strategies.

To examine the behavioural determinants of forced copulation success we chose three male genotypes with the lowest and three with the highest forced copulation rates within one hour (note that Fig 6.1 shows forced copulation rates within two-hour trials). When we re-tested these six genotypes, we found that their forced copulation rates were generally consistent with what we found in the 59 genotype assay, with the exception of one of the ‘low’ genotypes (765) which, in this replication, had the second highest forced copulation success rate (Fig 6.2). This inconsistency across experiments is likely due to gene by environment interactions that may have specifically affected hybrid genotype 765 in this case. We have observed such variation in behaviour over time in our previous work with the DGRP hybrids (Scott et al., 2018; Baxter et al., in prep). Since we wanted to compare males from ‘high’ and ‘low’ success genotypes, we chose to focus on the five genotypes that were consistent over time. As we predicted, males from the ‘high’ genotypes were more persistent (Fig 6.3) and mounted more frequently (Fig 6.4) than males from the ‘low’ genotypes. When we compared ‘unsuccessful’ to ‘successful’ males, we found that male pursuit duration and mounting frequency positively predicting forced copulation success. The fact that these results are in the same direction indicates that the behavioural differences we found between the ‘high’ and ‘low’ success genotypes are representative of what varies between individual males who either succeed or fail to forcibly copulate.

The results from comparing the ‘high’ and ‘low’ success genotypes support the idea that heritable behavioural differences across males contribute to their variation in forced copulation success. At the same time, we found no significant difference in how females responded to males from ‘low’ and ‘high’ genotypes with respect to running away from them during pursuit (Fig 6.5). This indicates that the variation in forced copulation success between male genotypes was not driven by variation in female resistance. This could be because males from ‘high’ success genotypes persist in their pursuit despite rejection signals from teneral females, which parallels what we have found in potentially coercive matings with recently mated females (Fig. 2 in Baxter & Dukas, 2017). Recently mated females typically reject males, and do so by extruding their ovipositor, which is a rejection signal specific to mated females (Bastock & Manning, 1955; Connolly & Cook, 1973). Due to their low receptivity, mating latencies with recently mated females are significantly longer than those with virgin females. Males who succeed in mating with recently mated females not only spend a greater cumulative amount of time pursuing them despite their clear rejection behaviour, but also spend a significantly greater proportion of time in active pursuit in the few minutes prior to mating than males who mate with virgin females (Baxter & Dukas, 2017). This suggests that male persistence, in spite of clear rejection signals, plays a role in the potentially coercive mating of recently mated females, and may also play a similar role in the forced copulation of teneral females.

Finally, when we paired males with mature virgin females (who can prevent unwanted matings) we found no significant difference in mating success between males from the ‘low’ and ‘high’ genotypes (Fig 6.6). We did find that males from the ‘high’ genotypes had non-significantly greater mating success with mature females, and, if this trend is indicative of a true difference, it would imply that males with high forced copulation success also have high mating success with mature virgins. However, further experiments are needed to clarify whether this is a true biological difference, and if so, what male behaviours and/or female preferences are contributing to it.

For this study, we chose to focus on behavioural differences that contribute to variation in forced copulation success. Morphological variation, however, may also be a contributing factor. For example, larger males may be able to more easily forcibly copulate with smaller females. Larger male to female body size ratios increase the likelihood of mating after a pre-mating struggle in the seaweed fly (*Coelopa ursina*) (Crean & Gilburn, 1998), and correlate with increased durations of forced copulations in Lake Eyre dragons (Olsson, 1995). Variation in the morphology of specific body parts could also aid male fruit flies in overcoming female resistance, like in water striders (*Gerris odontogaster*), where males with longer abdominal processes can more firmly grasp females who attempt to dislodge them, and as a result have greater mating success than males with shorter abdominal processes (Arnqvist, 1989). A potential candidate structure in fruit flies (*Drosophila melanogaster)* are the sex combs, an array of bristles on the forelegs of males, which can vary in the number and length of bristles across individuals (Ahuja, de Vito, & Singh, 2011; Snook, Gidaszewski, Chapman, & Simmons, 2013). Males that have had their sex combs removed have drastically reduced mating success compared to intact males, despite displaying persistent courtship and mounting attempts (Cook, 1977; Hurtado-Gonzales, Gallaher, Warner, & Polak, 2015; Ng & Kopp, 2008), demonstrating that that sex combs are important for mating success. While no studies have examined how variation in sex comb structure relates to forced copulation success, it is possible that the structures of either the sex combs or other physical traits affect a male's ability to overcome teneral female resistance. We will examine this possibility in our ongoing research on the mechanisms underlying forced copulation success.

We consider the forced copulation of teneral female fruit flies to be an aggressive act since males inflict harm, such as wing damage, upon them and females who are forcibly copulated while teneral are more likely to experience early mortality than females who consensually mate once mature (Dukas & Jongsma, 2012a). So, given that forced copulation is an aggressive act, are males who perform forced copulations simply more aggressive in general? It is important to note that aggression is not a unitary trait, but a broad categorization of behaviours that can occur under different contexts with distinct goals (such as gaining access to mates, defending territory, protecting offspring, etc.), and that different forms of aggression may have distinct genetic, neurobiological and physiological underpinnings (Moyer, 1968). Despite this, aggressive behaviours under different contexts are sometimes positively correlated. For example, in western bluebirds (*Sialia mexicana*) males who aggressively defend their nests against heterospecifics are also more aggressive in conspecific male-male competition (Duckworth, 2006). In mice, transgenic males who lack the monoamine oxidase A (MAOA) enzyme are more aggressive towards males they are housed with, towards intruder males, and more sexually aggressive towards unreceptive females (Cases et al., 1995). Additionally, St. John and Corning (1973) found a positive correlation between male and female aggression levels across inbred mouse lines. However, Hyde and Ebert (1976) found that lines selected for increased female aggression did not show increased male aggression, and Gammie et al. (2003) did not find support for an association between male-male and maternal aggression. In fruit flies, we have found that older males are more aggressive towards other males and also have higher forced copulation rates than younger males (Baxter & Dukas, 2017), which could be taken as support for a positive correlation between male-male aggression and male sexual aggression towards females. However, in the same series of experiments, we also found that males with previous mating experience showed reduced forced copulation rates compared to mating-deprived males, while showing similar levels of aggression towards other males, indicating that prior experience can have distinct effects on sexual aggression and male-male aggression. Therefore, it is not obvious whether males who are genetically predisposed to engage in high levels of sexual aggression would also display more aggression under different contexts, making this a valuable avenue for future studies.

Given the prevalence of sexual coercion and aggression in nature, we feel it is vital to gain a better understanding of the genetics, neurobiology and evolutionary biology of sexual aggression and forced copulations. However, studying aggressive sexual coercion can be complicated by many factors. First, it may not always be possible to reliably generate sexual coercion under controlled laboratory settings for certain species. Additionally, unless it is clear that the female cannot prevent an unwanted mating, it may not be possible to fully disentangle female mate screening from male sexual coercion. Finally, for many species, it is not feasible to subject females to males who may sexually coerce them. Our goal for these experiments was not only to assess genetic variation and behavioural differences contributing to forced copulation success, but to demonstrate that the forced copulation of teneral females is an excellent model for studying aggressive sexual coercion. In particular, given that fruit flies are a well-established model organism, this lesser-studied model of forced copulation with teneral females provides many opportunities for future studies to take advantage of the numerous resources and tools that exist for examining sexual conflict and its evolutionary consequences in fruit flies.

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CHAPTER 7 – DISCUSSION

7.1 Overview

This thesis has examined the roles of male persistence and aggression in a variety of conspecific interactions in fruit flies. We began by examining how males of distinct ages varied in their persistence in courting unreceptive, recently mated females, finding that older males were more persistent than younger males. (Chapter 2). Then we moved on to test for male persistence in courting a female in the presence of a competitor male, and quantified direct male-male courtship interference, which is a novel finding for *Drosophila melanogaster* where it is often assumed that males engage in scramble competition for mates (Spieth, 1974) (Chapter 3). We then placed males in 'mate guarding' circumstances by adding an intruder male to an arena with a recently mated male with either his mate or a foreign mated female. We observed increased aggression by males in the presence of their recent mate and quantified how being present to guard one's mate can increase paternity success (Chapter 4). Next we examined how male aggression towards other males as well as towards females would be affected by male age and previous mating experience (Chapter 5). We found that male age was positively correlated with male aggression towards other males and sexual aggression towards females, and that previous mating experience was associated with reduced male sexual aggression but did not appear to affect male-male aggression. Finally, we found that males who were more persistent and aggressive in their pursuit of teneral females were more likely to succeed in forcibly copulating with them (Chapter 6).

7.2 The role of persistence

In the introduction I broadly defined persistence as the continuation in a course of action in spite of difficulty or resistance. From this definition, we can divide persistence into two broad categories: (i) persistence in performing a task in spite of external difficulties, and

(ii) persistence in performing a task in spite of difficulty or resistance from the 'object' of the task. For the first category of persistence, the external difficulties could be interruptions or interference by conspecifics while trying to complete a task. For example, foraging bats (*Tadarida brasiliensis*) will emit ultrasonic signals to interfere with the echolocation of conspecifics, making it more difficult for them to catch prey (Corcoran & Conner, 2014), while male satin bowerbirds (*Ptilonorhynchus violaceus*) will steal decorative feathers from one another's bowers during bower-building (Borgia & Gore, 1986). For the second category of persistence, difficulty from the 'object' of the task could be, for example, difficulty in consuming certain seeds because of a hard shell, as seen in bullfinches (Greig-Smith, 1987) or grey squirrels (*Sciurus carolinensis*, Jacobs, 1992), or difficulty in achieving copulation because the potential mate is resisting (Arnqvist, 1992; Kumano, Kuriwada, Shiromoto, Haraguchi, & Kohama, 2011).

In our earliest experiments, our primary goal was to compare male mate choosiness between younger and older males (Chapter 2). However, in one of our experiments, we compared the courtship durations of younger and older males when they were placed together with a female in a vial. We found that older males spent a significantly longer duration courting than did younger males under this competitive condition, while both younger and older males spent the same amount of time courting when placed individually with females (Fig. 2.6). This result implied that older males were more persistent in courtship in the face of difficulty from a competitor than were younger males (i.e. category (i) persistence). At that point in time, we were not yet aware of what the males were doing that was affecting the courtship of their competitor. When we followed-up on this experiment in Chapter 3, we replicated the result of older males being more persistent in courting a female in spite of the presence of a competitor male and found that the males were directly interfering with one another's courtship via courtship takeovers (Fig. 3.7). In addition to comparing younger versus older males, we compared smaller versus larger males competing for access to a single female. Paralleling our results with older versus younger males, we found that larger males interfered more with the courtship of smaller males (Fig. 3.3). Interestingly, however, we found that small males persisted in spite of this interference by larger males and spent just as much time courting the female when competing against a larger male as they did when they were alone with the female (Fig 3.4). This highlights the interesting and potentially complex interactions that can occur between males' subtly aggressive courtship interference and their persistence in courting in spite of interference by their competitor. Older and larger males interfere more than younger and smaller males. However, this interference does not deter persistence by small males, while it does appear to reduce courtship by younger males. Therefore, it is not obvious how a male's courtship will be affected by the presence of a competitor male. This is particularly important when studying female mate choice in fruit flies, which are often used as models for sexual selection research (e.g. Filice & Long, 2017; Friberg & Arnqvist, 2003; Qvarnström & Forsgren, 1998). To infer a female's preference between males that vary in some way, researchers will sometimes place a female with males from two distinct categories and observe which male she mates with (e.g. male age (Long, Markow, & Yaeger, 1980; Somashekar & Krishna, 2011), and previous male mating experience (Markow, Quaid, & Kerr, 1978)). However, our findings demonstrate the results of such tests may not be determined exclusively by the female's preferences but may also be affected by the ability of males to interrupt each other's courtship, and by how persistent each male is in spite of difficulty caused by the interference from his competitor. Therefore, due to the complex interplay between female preference, male competitive ability and male persistence, the results from such female choice assays must be interpreted cautiously.

With respect to category (ii) persistence, we documented a number of instances in which males were persistent in courting and pursuing females in spite of resistance by those females. Again, in our earliest experiments designed to explore male mate choosiness, we found that older males were more persistent in courting recently mated females than were younger males, while both male ages courted virgin females vigorously (Fig. 2.1). Note that recently mated females are relatively unreceptive and perform a variety of well-documented rejection behaviours when being pursued by males (Bastock & Manning, 1955; Connolly & Cook, 1973; Dukas & Scott, 2015). At the time, we interpreted these results as indicating that older males (4-day-olds) were less choosy in their courtship than were younger males (1-day-olds). However, we can also interpret the same results as indicating that older males are more persistent in pursuing unreceptive females than are younger males. In Chapter 5, we specifically tested the courtship persistence of 4-day-old males by placing them with mature virgin or mature recently mated females and observing for courtship and mating. We found that matings with recently mated females occurred after significantly longer trial durations than matings with virgin females (Fig. 5.7), and that males in the mated female treatment spent significantly more time courting, especially in the few minutes prior to mating, than did males in the virgin female treatment (Fig. 5.7). This demonstrates that mating with recently mated females typically requires higher courtship persistence from males than does mating with virgin females. When comparing mating success with recently mated females across male ages, we found that older males had higher mating success than younger males, in spite of long mating latencies (Fig. 5.4). We also found that males that had been previously deprived of female contact and mating also had higher mating success with recently mated females compared to males who had prior mating experience (Fig. 5.12). Again, these matings had long latencies, implying that the mating-deprived males may have been more persistent in their pursuit of recently mated females than were males with previous mating experience.

Another example of males pursuing resistant females is when males pursue recently eclosed, teneral females. Teneral female flies resist mating by running from males and kicking them during courtship, and by walking and kicking during mating if it occurs (Seeley & Dukas, 2011). In Chapter 5, paralleling our results with recently mated females, we found that older males, and mating-deprived males had higher mating success with teneral females than did younger and mating-experienced males. These matings also had long latencies, which, combined with our knowledge of the intense resistance by teneral females, implies that older and mating-deprived males are more persistent in their pursuit of teneral females despite resistance. We further supported this conclusion by comparing males of different genotypes that differed in their forced copulation success with teneral females (Chapter 6). When we compared males from genotypes that had relatively high forced copulation success to those with relatively low forced copulation success, we found that males from the 'high' genotypes were more persistent in their pursuit of teneral females than males from 'low' genotypes.

Overall, the results in this thesis have demonstrated that male fruit flies are able to utilize persistent behavioural patterns under a variety of circumstances. Males can persist in their courtship attempts in spite of interference by competitors, and they can also persist in pursuing females who attempt to resist or flee from their mating attempts. However, our results also demonstrate that this persistence is consistently variable across males, based on their age, size, prior experience, and genotype. Therefore, male persistence behaviours merit research under a variety of contexts in order to fully understand when, why, and how males will persist, and to uncover how these distinct persistent behavioural patterns can affect their fitness.

7.3 The role of aggression

Many studies of aggression tend to focus on interactions between males, likely because males are often the more aggressive sex in many species (e.g. humans (Frisell, Pawitan, Långström, & Lichtenstein, 2012; Niv, Tuvblad, Raine, & Baker, 2013; Tuvblad, Grann, & Lichtenstein, 2006), Lake Eyre dragons (Olsson, 1995), deer (family: Cervidae, Clutton-Brock, 1982) and dragonflies (family: Libellulidae, Alcock, 1987; Marden & Cobb, 2004)). This is certainly the case in fruit flies, where the majority of research focuses on male-male aggression (e.g. Chen, Lee, Bowens, Huber, & Kravitz, 2002; Davis, Thomas, Liu, Campbell, & Dierick, 2018; Hoffmann, 1987; Saltz, 2013; Shorter et al., 2015; Wang & Sokolowski, 2017, but see Bath et al., 2017; Nilsen, Chan, Huber, & Kravitz, 2004; Ueda & Kidokoro, 2002 for exceptions). In my work, I have also mainly focused on aggression performed by males. However, rather than focusing solely on male-male aggression, I have expanded the scope of my studies to include male sexual aggression towards females, which is not often studied explicitly with fruit flies. I will discuss my findings on both male-male and male-female sexual aggression in turn below.

Our first foray into studying explicitly aggressive interactions was prompted by an interesting finding by Yuan et al., (2014) who were studying the effects of experience with females on male-male aggression from a neurological perspective. One of their secondary findings was that males who had just mated with virgin females became significantly more aggressive after mating, and they suggested that this could possibly be a guarding behaviour. Based on their finding we decided to explicitly test for male-male aggression under a mate-guarding context (Chapter 4). First, we compared the aggression of a pair of mated males in the presence of their recent mates to the aggression of a pair of males who had not mated but were in the presence of recently mated females. We found that the recently mated males were more aggressive than the virgin males (Fig. 4.2). Next we compared the aggression performed by a guard male in the presence of his recent mate to the aggression displayed by an intruder. We found that guard males were significantly more aggressive than intruders (Fig. 4.5) and that the presence of a guard male reduced the likelihood that his recent mate would remate with the intruder male (Figs. 4.9 and 4.10). This exciting finding about an undocumented potential function of male-male aggression in fruit flies inspired us to look for other instances of male aggression in flies that may have been previously overlooked.

Conflict between males and females over reproduction is highly prevalent across species (Arnqvist & Rowe, 2005). When pre-mating sexual conflict involves the potential of one sex physically coercing and potentially harming the other sex, it is also reasonable to describe such behaviours as aggressive. It was through this perspective that we quantified male sexual aggression towards both recently mated females (Chapter 5) and teneral females (Chapters 5 and 6)

The remating of recently mated female fruit flies has been well-described as a form of sexual conflict between males and females (e.g. Byrne, Rice, & Rice, 2008; Wigby & Chapman, 2004). However, to my knowledge, we were the first to quantify female fruit fly resistance behaviours during remating, demonstrating that such matings are sometimes coercive. We found that, when compared to mating with previously virgin females, females who were previously mated spent more time kicking males during mating and were also more likely to keep their wings closed (Fig. 5.8). These resistance behaviours performed by recently mated females during remating parallel those performed by teneral females during forced copulations, specifically with respect to females kicking during mating (Seeley & Dukas, 2011). In this series of experiments, we found that older and mating-deprived males had higher potentially coercive mating success with recently mated females, and higher forced copulation success with teneral females than did younger or mating-experienced males, respectively (Figs. 5.3, 5.4, 5.11 and 5.12). Therefore, a male's propensity to sexually coerce recently mated females or to forcibly mate with teneral females may be affected by similar factors.

In Chapter 6 we chose to focus exclusively on forced copulation, rather than remating, as it is a more definitive case of male sexual aggression towards females. When comparing males from 'high' and 'low' forced copulation success genotypes we found that males from the 'high' success genotypes performed more mounting attempts per minute of pursuit than males from the 'low' success genotypes (Fig. 6.4). Females who are force copulated while teneral sustain significant wing damage from copulation (Dukas & Jongsma, 2012). However, even females who are only pursued by males while teneral, and not force copulated, receive significantly more wing damage than those who are not. Therefore, even males from the 'high' forced copulation success genotypes who did not force copulate during the trial may have caused more harm to females through their higher mounting attempts and pursuit durations than males from the 'low' success genotypes.

Throughout my graduate studies, it has been my goal to study aggressive behaviour using a variety of different approaches. Through these different approaches we have demonstrated that male fruit flies use aggression towards other males, either subtly as in the case of courtship interference, or more overtly as seen in aggression under mate guarding contexts. We have also examined sexual conflict over mating rate in flies by categorizing such interactions as male sexual aggression towards both teneral and recently mated females. Our results demonstrate that there are many ways in which males can use different aggressive behavioural patterns to achieve goals, often related to their mating success. However, as with persistence, we have also shown that the ways in which males utilize aggressive behaviours are variable, and can be affected by their age, size, genetic background and both their long-term and immediate prior mating experiences.

7.4 Future directions

Overall, the work included in this thesis covers a wide range of topics and is exploratory in nature. Due to its breadth, it opens many avenues for future research on many of the topics it touches upon. In this section I will focus on a three key areas for future work.

One of the main focuses of this thesis has been on male aggressive behaviours towards both males and females. Although we have studied a wide breadth of instances in which males can be aggressive, by including both male-male and male-female aggression, we have still mostly overlooked the aggression performed by females.

Aggressive behaviours are important for securing resources for both males and females in many species. For example, in Texas cichlids (*Herichthys cyanoguttatum*) both males and females will fight with conspecifics over access to spawning sites (Draud, Macías-Ordóñez, Verga, & Itzkowitz, 2004) and in beaugregory damselfish (*Stegastes leucostictus*) both males and females chase intruders away from their territories (Horne & Itzkowitz, 1995). Female fruit flies also perform aggressive behaviours, though few studies have examined it directly. Studies that have focused on female-female aggression have shown that females utilize distinct behavioural patterns and aggressive actions compared to males (Nilsen et al., 2004), their aggression levels are influenced by prior social experience (Ueda & Kidokoro, 2002), and that male sperm and sex peptide transferred during mating increases female-female aggression levels (Bath et al., 2017). However, how is female aggression affected, for example, by female age? In Chapter 5 we demonstrated that older males are more aggressive than younger males in male-male aggression; do females show the same pattern with age? One could predict a positive association between age and aggression in females following the same logic for this prediction in males, namely that older individuals have lower reproductive value owing to their lower potential for future reproductive success, and therefore they may be more willing to sustain damage from fighting (Kemp, 2006; Parker, 1974). Additionally, female aggression can depend on previous experience. If, for example, a female encounters a high-quality food source for the first time at an older age it may indicate to her that such high-quality food is rare, and therefore she would be more motivated to fight over access to it than a younger female encountering the same high-quality food for the first time. Future work on female-female aggression in fruit flies could attempt to answer these questions.

Aside from understanding whether male and female aggression levels are similarly affected by age, environment or previous experiences, we can also ask whether they are genetically correlated. In other words, does variation in male-male and female-female aggression levels have shared or distinct genetic underpinnings? Fruit flies are an excellent model for examining such genetic correlations because of our ability to maintain a large number of distinct genetic lines, thereby allowing us to test multiple individuals per genotype. Shorter et al., (2015) performed aggression assays with males from the *Drosophila* Genetic Reference Panel (DGRP) (Mackay et al., 2012) and found significant genetic variation in male-male aggression. In Chapter 6 we demonstrated significant genetic variation in male-female sexual aggression using hybrid flies generated from the DGRP. However, I am aware of no previous research on genetic variation in female-female aggression in fruit flies or studies that test for a genetic correlation between males and females. Therefore, as part of my graduate studies (not included in this thesis), we tested for both male-male and female-female aggression in hybrid offspring generated from the DGRP. We tested males and females from 24 hybrid genotypes simultaneously, and video recorded trials to allow for detailed observations of their behaviours. We hope that our results will help us understand the relationship between genetic variation in male-male and female-female aggression.

A second area that calls for future research is courtship interference and persistence in the face of a competitor. The results from Chapter 3 demonstrate that males vary in their persistence in the face of a competitor and that they vary in the frequency with which they interfere with their competitor's courtship. However, perhaps more interestingly, the results demonstrate that this persistence and interference are not necessarily positively associated, meaning that a male who interferes less is not necessarily one who will be less persistent. For example, in Chapter 3 we saw that small males interfered less than large males, but courted females in the presence of a competitor just as much as if they were placed alone with the female, demonstrating high persistence (Figs. 3.3 and 3.4). Therefore, this single study has introduced us to the complexities of male-male interactions during competitive courtship, but it certainly has not answered all of the questions.

Protocols where one female is placed with two males, and the identity of her mating partner is recorded, have been used to assess female mate preferences in fruit flies, leading authors to conclude that females prefer older males over younger ones (Long et al., 1980; Somashekar & Krishna, 2011) and that they prefer virgin males over recently mated ones (Markow et al., 1978). However, in order to gain a better understanding of the competitive courtship dynamics between, for example, virgin versus recently mated males, we need to compare the males' persistence in the face of a competitor as well as their frequency of interference. And even then, the resulting 'choice' in the test will still rely on a combination of female preference, male persistence and male interference, and none of these contributions should be ignored when making conclusions based on the result of the test. Further, rather than simply comparing males of two distinct groups (e.g. small vs. large), it would be beneficial for future studies to also observe equally matched males in a competitive courtship situation. The sequential assessment model from contest theory predicts that the relative strength or resource holding potential (RHP) of each individual affects the duration and intensity of the contest, with more equally matched individuals having longer and more intense fights than when RHP is highly skewed across the opponents (Enquist & Leimar, 1983; Leimar, Austad, & Enquist, 1991). Similar patterns could arise in the case of courtship interference and persistence. For example, equally matched males may interrupt one another more frequently or persist for longer durations than pairs with a high skew in ability. Therefore, our understanding of male-male courtship interference in flies is at its beginning, with many possible avenues for future work.

The final, and perhaps most apparent, area for future research is in males' persistence and sexual aggression towards females. The final data chapter of this thesis

(Chapter 6) is primarily a starting point for work on male sexual aggression via the forced copulation of teneral females. Given that it was a starting point for examining behavioural differences between males with high and low forced copulation success rates, there are many other relationships we could look for. For example, in Chapter 5 we saw that older males, and mating-deprived males had both higher forced copulation rates with teneral females, and higher potentially coercive mating rates with recently mated females. Does this pattern (of higher forced copulation and higher remating) hold true in comparisons other than age and previous mating experience? For example, will males who are genetically predisposed towards higher forced copulation success also have higher rates of coercive remating? Are males who are highly sexually aggressive also better able to aggressively defend food resources from other males? Are these males more persistent in their pursuit of mature females, or persistent in courtship in the face of a competitor? Is a male's tendency or ability to perform forced copulations related to his attractiveness to mature females? Given all of these unanswered questions, following the experiments included in Chapter 6 we decided to artificially select for high and low forced copulation success lineages. We used wild-caught starting populations collected by Andrew Scott and modified a protocol he created for artificial selection on sociability. Using those starting populations we have selected on three lineages for high forced copulation success, three lineages for low forced copulation success, and also included three control lineages. These lineages are now significantly diverged and can be used to compare a variety of behavioural, physical and genetic determinants of forced copulation success.

7.5 Conclusion

Fruit flies are a very common model organism and have been used extensively for studies on aggressive behaviour. However, many studies focus on a single context where males use aggression, while we know that males can use aggressive behaviours in a variety of different contexts. For this thesis, I have examined male aggression towards other males and towards females and have also examined male persistence under the same contexts.

Particularly in the study of male sexual aggression towards females, we can see how persistence and aggression tie together to allow more sexually aggressive and persistent males to have higher forced copulation success. Overall, this research has broadened our understanding of the ways in which flies can use persistent and aggressive behavioural patterns to obtain and secure resources and mates, paving the way for future work to examine these distinct scenarios more in-depth.

7.6 References

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