The Function and Evolutionary Ecology of Intra-Sexual Aggression in *Drosophila melanogaster*

Danielle Edmunds
Lincoln College
University of Oxford
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Abstract

Across the animal kingdom, competition for reproductive resources often results in intra-sexual aggressive contests. The extent to which individuals engage in aggression varies in response to ecological conditions. However, as individuals live and compete in highly complex environments, understanding how aspects of an individual's ecological surroundings shape aggression is a constantly advancing field with many unresolved questions. Furthermore, while much previous research has focused on male aggression, it is now acknowledged that females fight too, but whether female aggression reflects male aggression in its response to the environment is largely unknown. In this thesis, I use the fruit fly, *Drosophila melanogaster*, to investigate how the prevailing environment drives short term responses and long term evolutionary change in aggression in both sexes.

Sexual selection plays a key role in shaping traits involved in reproductive competition, such as aggression, and I begin by investigating how the sex ratio influences the operation of sexual selection in males, and whether these patterns are reflected in females. Traditional sexual selection theory predicts that as the sex ratio becomes more biased, the abundant sex should experience stronger sexual selection. I find support for this prediction in males, but, in females, I find sexual selection arising from mating competition to be weak and insensitive to the sex ratio, illustrating differences in intra-sexual competition in the two sexes. I then investigate how aggression evolves in response to the population sex ratio, and find that a male-biased sex ratio increases the propensity for males to focus aggression around food patches but does not

influence male aggressive intensity, while a female-biased environment increases the magnitude by which mating elevates female aggression.

Furthermore, kin selection has the potential to shape aggressive interactions, and high population relatedness can reduce harmful aggressive behaviours. I find some evidence that high relatedness can reduce harmful behaviours in *D. melanogaster*, but find this response to be highly sensitive, with no evidence that it is driven solely by olfactory-based discrimination. Finally, I show that nutrition can determine aggressive ability and motivation, with aggression decreasing in response to food deprivation during development but increasing in response to adult food deprivation.

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Contents

Declaration and author contributions

I declare that I composed this thesis, and the work included is my own, with the following acknowledgements and contributions:

Chapter 2: Sexual selection is sensitive to the sex ratio

Authors: Danielle Edmunds, Juliano Morimoto, Grant McDonald, Tommaso Pizzari, Stuart Wigby, Jennifer C. Perry

I conceived the ideas and designed the methodology with Stuart Wigby and Jennifer Perry based on previous work by all authors; I collected the data and lead the data analysis, with advice from all authors; I drafted the original version of the manuscript and all authors contributed to later versions of the manuscript

Chapter 3: Sex ratio and the evolution of aggression in fruit flies

Authors: Danielle Edmunds, Eleanor Bath, Jessica Norman, Charlotte Atkins, Lucy Harper, Wayne G. Rostant, Tracey Chapman, Stuart Wigby, Jennifer C. Perry

I conceived the ideas and designed the methodology with Eleanor Bath, Stuart Wigby and Jennifer Perry; Jessica Norman, Charlotte Atkins and Lucy Harper, assisted in designing the methodology; Tracey Chapman and Wayne Rostant designed and performed the experimental evolution; I collected data along with Eleanor Bath, Jessica Norman, Charlotte Atkins and Lucy Harper; I analysed the data with advice from Eleanor Bath and Jennifer Perry; I drafted the original version of the manuscript and all authors contributed to later versions of the manuscript. Thanks to two anonymous reviewers whose insightful feedback helped to improve this chapter.

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Chapter 4: Are flies kind to kin because of their smell?

Authors: Danielle Edmunds, Stuart Wigby, Jennifer C. Perry, Pau Carazo

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Chapter 5: A poor larval diet reduced adult aggression in male *Drosophila melanogaster*

Authors: Danielle Edmunds, Stuart Wigby, Jennifer C. Perry

I conceived the ideas and designed the methodology with Stuart Wigby and Jennifer Perry; I collected the data; I analysed the data with input from Jennifer Perry and Stuart Wigby; I drafted the initial manuscript and all authors contributed to the final manuscript. Many thanks to Rachel Loos-Bennett who assisted in collecting fly weight data. Many thanks to anonymous reviewers whose feedback helped improve this chapter.

Chapter 6: '*Hangry Drosophila*': food deprivation increases male aggression

Authors: Danielle Edmunds, Stuart Wigby, Jennifer C. Perry

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Note on References

For consistency, all references are in the style used by *Evolution*. For brevity and to save duplication, all references are in a single reference list at the end of this thesis.

Chapter 1: General Introduction

Aggression is a widespread and conspicuous behaviour in the animal kingdom, playing an integral role in determining access to resources required for survival and reproduction (Briffa & Sneddon, 2007; Fernández & Kravitz, 2013; Georgiev et al., 2013; Huntingford et al., 2012). Aggression occurs in many contexts, both between and within species, between parents and offspring, between siblings, and between males and females (Briffa & Sneddon, 2007). One important form of aggression is that involved in intra-sexual competition over mates and other resources that can increase reproductive success (Qvarnström et al., 2012). Indeed, the dramatic spectacle of intra-sexual contests in species such as elephant seals (*Mirounga spp.*) and red deer (*Cervus elaphus*) has long attracted the attention of biologists (Boeuf & Peterson, 1969; Clutton-Brock & Albon, 1979; Haley, 1994; McCann, 1981). As behaviours carrying substantial fitness consequences, aggressive contests are of much interest in the field of behaviour, evolution and ecology (Huntingford et al., 2012; Marler & Moore, 1988; Neat et al., 1998). Furthermore, the study of animal aggression can help us understand aggression and antisocial behaviour in humans (Blanchard & Blanchard, 2003; Georgiev et al., 2013; Sluyter et al., 2003), because there is overlap in genes involved in social responsiveness and aggressive behaviour between animals and humans (Crespi, 2017; O'Kane, 2011; Rohde et al., 2017).

Notably, there is a vast degree of variation in aggression displayed across animals in response to intra-sexual competition (Andersson, 1994; Kilgour et al., 2018; Weir et al., 2011). Some species exhibit high risk fighting behaviours, which can result in large costs in the terms of energy expenditure and injury, such as the brutal contests between male musk oxen (*Ovibos moschatus,* (Wilkinson & Shank, 1976), and the face-to-face combat of male baboons (*Papio cynocephalus*, (Drews, 1996), or ultimately in death, such as the lethal combat in fig wasps (Reinhold, 2003; Knell, 2009). In contrast, some species rarely fight, such as some mobile pelagic fish (Huntingford et al., 2012), or highly monogamous vertebrates (Georgiev et al., 2013). Even between closely related species, there is considerable variation the size and development of fighting armaments such as enlarged chelae or horns (e.g. chernetid pseudoscopion and the South African dung beetle; Knell, 2009).

Importantly, variation in aggression goes beyond the species level, with variability in aggressive strategies between populations within species, between individuals in populations, and within individuals over successive competitive encounters (Briffa et al., 2015; Dall et al., 2012; Huntingford et al., 2012). For example, between populations of the fruit fly, *Drosophila melanogaster*, from two different microclimates at Israel's Evolution Canyon, there are distinct differences in aggression and courtship (Palavicino-Maggio et al., 2019). Within populations, such as those of the mite, *Sancassiana berlesei*, competitive strategies vary between individuals with some developing into aggressive fighter morphs, and others into benign scrambler morphs (Lukasik et al., 2006). Within individuals, behavioural plasticity allows temporary and reversible flexibility in aggression (Briffa et al., 2015; Huntingford et al., 2012; Kilgour et al., 2018).

When viewed from the perspective of evolutionary ecology, such behavioural variation is inevitable. It has long been acknowledged that ecology is critical in understanding behavioural mechanisms, with a close interdependence between behaviour and ecology (Bradshaw, 1984; Knell, 2009). Animals live, compete, and reproduce in complex ecological environments, and factors such as resource availability, the population structure, and the social context are integral in determining the relative benefits and costs of different behavioural strategies in reproductive competition (Cornwallis & Uller, 2010; Evans & Garcia-Gonzalez, 2016; Planka, 2011; Miller & Svensson, 2014; Palmer & Kristan, 2011).

Recently, the importance of the relationship between ecology, competition, and selection has grown in prevalence in the attempt to understand aggressive behaviours, producing exciting hypotheses (Cornwallis & Uller, 2010; Kilgour et al., 2018; Knell, 2009). The dynamic interplay between the many aspects of an individual's ecology, selective forces, and aggressive behaviours makes the evolutionary ecology of aggression a broad and constantly advancing field (Cornwallis & Uller, 2010). Despite considerable interest, there are many unanswered questions and conflicting hypotheses to be resolved. An integrated study of aggression, ecology, development, and evolution is necessary for a complete understanding of the aggressive behaviours we observe in reproductive competition (Cornwallis & Uller, 2010; Fricke et al., 2009).

In my thesis, I use the fruit fly, *Drosophila melanogaster*, to explore how the environment acts to shape aggressive behaviour, using the framework of evolutionary ecology to investigate what determines evolutionary, developmental and plastic variation in aggression. Variation in aggressive strategies reflects how the nature of contested resources, individual phenotypes, and the phenotypes of conspecifics interact to determine the overall benefits and costs of aggressive fighting (Georgiev et al., 2013). Different populations can vary with respect to the physical environment and the population structure, which can cause divergence in aggressive traits (Qvarnström et al., 2012). For example, differences in the population structure and relatedness can shape social behaviours including aggression (Billeter et al., 2012; Carazo et al., 2014, 2015; Eldakar et al., 2009; Krupp et al., 2008; Saltz, 2013; Simmons, 1989). Likewise, differences in spatial distribution, migration and recruitment can alter the operational sex ratio, influencing the intensity of intra-sexual competition and subsequent aggression (Emlen & Oring, 1977; Forsgren et al., 2004; Kvarnemo & Ahnesjo, 1996). Furthermore, populations themselves are made up of many individuals that differ in sex, condition, age, and genotype, all of which can influence aggression (Briffa et al., 2015; Camerlink et al., 2016; Dall et al., 2012; Huntingford et al., 2012; Scott et al., 2018; Wolf & Weissing, 2012). Individual competitive abilities, motivations and contexts differ throughout their lives due to seasonal fluctuations in resource availability and quality (Cain & Ketterson, 2012; Forsgren et al., 2004; Kilgour et al., 2018), previous experiences and stresses (Penn et al., 2010; Stevenson et al., 2005; Tudor et al., 2018; Yuan et al., 2014; Yurkovic et al., 2006), and ontogenic shifts in internal state and needs (Asahina, 2017; Huntingford et al., 2012; Kilgour et al., 2018), meaning that the scope for intra-specific variation in aggression is vast.

I investigate how individual aggressive behaviours respond to environmental variables on a range of timescales, from short-term plastic responses, to medium-term developmental effects, to long-term evolutionary change. I compare the behavior of males and females, testing for the potential for sexual conflict, and explore the underlying mechanisms which enable individuals to alter aggressive behaviours in response to the environment. I aim to understand when, why and how different aggressive strategies evolve using *D. melanogaster* to explore fundamental properties of the evolutionary ecology of aggression that might be generalized over a broader taxonomic scale, potentially improving our understanding of aggression in humans.

My research falls into three main aspects of an individual's ecology:

- **Population sex ratio**

The population sex ratio plays an important role in determining the intensity of competition for mates, and in determining the extent to which success in this competition can contribute to reproductive success. Thus the population sex ratio can influence the dynamic interplay between the strength of sexual and social selection and the intensity of aggressive intra-sexual competition (chapters 2 & 3).

- **Kin structure**

The identity of conspecific mates and rivals in the population influences the behavioural optima that best promote reproductive success. The degree of genetic relatedness between conspecifics can alter the balance of the relative contributions of direct and indirect fitness, determining whether it's best to cooperate or fight (chapter 4).

- **Resource availability and nutrition**

Resource availability and nutrition can determine both individual ability and motivation to compete, influencing the relative costs and benefits of engaging in aggressive contests (chapter 5 & 6).

Population sex ratio, sexual selection, and aggression

In order to understand how population ecology influences the evolution of intra-sexual aggression involved in competition over resources relating to reproductive success, it is essential to understand how sexual selection operates in the population. The concept of sexual selection has been central to biology since Darwin first coined the term in the 19th century. Darwin noted the presence of traits that did not appear to confer an advantage in the struggle for existence, and proposed that these traits were selected due to the advantage they conferred to individuals over rivals of the same sex and species 'solely in respect of reproduction' (Carranza, 2009; Clutton-Brock, 2004; Darwin, 1871; Evans & Garcia-Gonzalez, 2016). He referred to the process by which such traits were selected as 'sexual selection', operating through male-male competition (intra-sexual selection) and female choice (inter-sexual selection; Evans & Garcia-Gonzalez, 2016). Originally, sexual selection was considered to operate mainly in males, selecting for traits involved in mating success. Indeed, Bateman, (1948) used *Drosophila* to demonstrate that there is a stronger correlation between mating success and reproductive success in males, a relationship now known as the Bateman gradient, and suggested variance in mating success drives the higher variance in reproductive success in males.

Over the past century, our understanding of sexual selection has progressed, acknowledging that it is a much broader process than simply male-male competition over mates (Shuker, 2010; Stockley & Bro-Jørgensen, 2011; Westneat et al., 2010). A vast body of research has highlighted the importance of post-copulatory components of sexual selection in the form of sperm competition and cryptic female choice arising due to polyandrous mating systems (e.g., McDonald & Pizzari, 2016; Miller & Svensson, 2014; Morimoto et al., 2019). Furthermore, there has been a recent acknowledgement that sexual selection and analogous processes occur in females (Clutton-Brock, 2009; Stockley & Bro-Jørgensen, 2011). In some species, females, like males, compete for mates, and this leads to sexual selection in the traditional sense (Kvarnemo & Ahnesjo, 1996; Aronsen et al., 2013; Jones et al., 2000). Furthermore, although in most species reproductive competition differs in males and females, with females largely competing for resources that allow successful reproduction rather than directly competing for mates, the variation in reproductive success created by such resource competition can be large, leading some biologists to propose an alternative conceptual framework acknowledging the importance of sexual selection in both sexes (Clutton-Brock, 2009; Stockley & Bro-Jørgensen, 2011; Tobias et al., 2012). There is movement towards considering a broader definition of sexual selection, potentially as a component of a more general form of social selection resulting from all social interactions between members of the same sex (Tobias et al., 2012). Indeed, there is some argument as to whether the distinction between sexual and natural selection should be abandoned in favour of exploring and comparing the ways in which selection operates in the two sexes (Clutton-Brock, 2009; Clutton-Brock &

Huchard, 2013). This field is contentious, but as it is clear that there is competition between females which results in variance in female reproductive success, a broad definition of ‘sexual selection’, such as that of Stockley & Bro-Jørgensen (2011) which refers to selection arising from competition between same sex individuals in the context of reproduction, is useful to allow a full exploration of the selective forces that shape traits such as intra-sexual aggression.

The strength, direction and patterns of sexual selection can be influenced by environmental factors, with aspects of the social and physical environment determining the relative forces of sexual selection (Emlen & Oring, 1977; Evans & Garcia-Gonzalez, 2016; Miller & Svensson, 2014). Recent research has demonstrated that the operation of sexual selection displays sensitivity to many ecological variables. For example, in the fruit fly, *Drosophila melanogaster*, the developmental environment and the degree of polyandry (females mating with multiple males; Taylor et al., 2014) influence the relative strength of pre- and post-copulatory components of sexual selection (Morimoto et al., 2016, 2019). In the guppy, *Poecilia reticulata*, dietary stress increases the opportunity for sexual selection and modifies selection on condition-dependent traits (Cattelan et al., 2020). In the small seed beetle, *Stator limbatus,* temperature can influence the strength of sexual selection on male body size (Moya-Laraño et al., 2007). In the field cricket, *Gryllus pennsylvanicus*, experience of females affects the operation of sexual selection on male weaponry (Judge, 2010).

However, one of the variables that has attracted the most interest amongst biologists in determining sexual selection forces is the operational sex ratio, defined as the ratio of males and females available for reproduction (Emlen & Oring, 1977). The sex ratio influences interaction networks and the frequency of encounters with potential mates and rivals, and its role in shaping the operation of sexual selection has long sparked interest (Emlen & Oring, 1977; Kvarnemo & Ahnesjo, 1996). However, the relationship between the operational sex ratio and sexual selection is highly debated. Some bodies of theoretical work suggest that the operational sex ratio is a key determinant of sexual selection, with a greater imbalance in the sex ratio increasing the intensity of mating competition and the variance in reproductive success in the abundant sex, leading to an increased level of sexual selection in that sex (Emlen & Oring, 1977; Janicke & Morrow, 2018; Kvarnemo & Ahnesjo, 1996). In contrast, other bodies of theory suggest that this proposed relationship between the sex ratio and sexual selection is over-simplified, providing only an estimate of the opportunity for sexual selection to occur rather than a direct measure of selection intensity (Krakauer et al., 2011) and does not take into account factors such as the degree of mate monopolization (Klug et al., 2010) or parental investment (Fitze & Le Galliard, 2008). Experimental and observational studies into the relationship between sexual selection and the operational sex ratio reflect this debate, with some offering support for the influence of the operational sex ratio on sexual selection (Aronsen et al., 2013; Evans & Garcia-Gonzalez, 2016; Janicke & Morrow, 2018; Punzalan et al., 2010), and others reporting a more tenuous role, or no role at all (Debuse et al., 1999; Fitze & Le Galliard, 2008; Mills & Reynolds, 2003). Thus, the nature of this potentially important relationship between the sex ratio and sexual selection remains highly contentious, and in chapter 2, I explore this relationship in depth, using groups of *Drosophila melanogaster* held at different sex ratios and investigating the effects on the opportunity for and intensity of sexual selection in both sexes.

Furthermore, because the population sex ratio influences the operation of sexual selection, this should shape the evolution of sexually selected traits such as the aggressive behaviours involved in intra-sexual competition for reproductive resources (Tobias et al., 2012; Weir et al., 2011). For example, in the Japanese medaka, *Oryzias latipes*, a biased sex ratio leads to higher intra-sexual aggressive competition in the dominant sex (Grant & Foam, 2002). Likewise, aggressive male-male interactions are more frequent in the sand goby, *Pomatoschistus minutus*, under a male-biased sex ratio (Kvarnemo et al., 1995). However, the variability in aggression can take the form of both temporary reversible changes, and evolutionary fixed changes (Huntingford et al., 2012). Whether the intensity of competition results in plastic modulation of aggressive behaviour, or in evolved differences in aggressive propensity in *D. melanogaster* remains unresolved, and this is a question I address in my thesis. This distinction has important implications for understanding how differences in population structure can shape evolution, potentially driving diversification and speciation. Therefore, in chapter 3, I explore aggression from an evolutionary perspective, comparing the levels of aggression in both male and female fruit flies from populations evolved under different intensities of intra-sexual competition.

Kin structure

In attempting to understand the evolution of aggressive competition, it is important to acknowledge that such behavioural traits are not solely controlled by sexual selection, but could also be influenced by kin selection. Since the pioneering work of Hamilton (1964), it has been acknowledged among biologists that genetic relatedness plays a key role in the evolution of social interactions, such as aggression, as these social interactions can significantly affect the fitness of conspecifics. Individuals gain fitness through their own reproductive success (direct selection), but also gain fitness by promoting the reproductive success of genetic relatives (indirect selection; Pizzari & Gardner, 2012). Therefore, the degree of genetic relatedness in populations can determine the indirect fitness costs and benefits of aggressive intra-sexual competition (Hamilton, 1964; Pizzari & Gardner, 2012; Rankin, 2011; Wild et al., 2011).

Selection may favour aggression as a competitive strategy where it enhances the actor's reproductive success relative to that of rival conspecifics, meaning that individual fitness gains can come with a cost to the fitness of competitors. In some cases, the harm to rivals can be extreme, such as that seen in the lethal fights of male entomopathogenic nematodes (*Steinernema longicaudum*) over mating opportunities and nutritional resources (Kapranas et al., 2016). However, not only does harm occur directly to rivals (intra-sexual conflict), but also to mates (Parker, 1979; Pizzari et al., 2015). Parker introduced the idea of sexual conflict in 1979, describing the notion that males and females have different evolutionary optima which cannot be achieved simultaneously, resulting in conflict. The evolutionary implications of this conflict received a resurgence of interest at the turn of the century (Arnqvist & Rowe, 2005; Tregenza et al., 2006), highlighting the notion that traits that allow one sex to maximise success in reproductive competition can result in costs to the opposite sex. In some cases, these costs can occur through direct physical harm. For example, male bed bugs (*Cimex lecturlarius*) pierce the female abdomen during mating (Morrow & Arnqvist, 2003; Stutt & Siva-Jothy, 2001), and male seed beetles (*Callosobruchus maculatus*) harm females via damaging genital spikes, (Crudgington & Siva-Jothy, 2000; Edvardsson & Tregenza, 2005), reducing female survival. Similarly, male-male contests can result in collateral damage to females, such as male fights in dung flies (*Scatophagus stercorcaria*), which can severely harm females (Parker, 1979; Wild et al., 2011). The harm males inflict to females in sexual conflict can have further detrimental effects on male conspecifics, as the pool of mates represent a shared resource amongst males, with attempts of individuals to increase their own share of such a resource resulting in reduction of this resource as a whole, analogous to the tragedy of the commons (Eldakar et al., 2009; Rankin & Kokko, 2006).

Because aggression and harassment can result in such significant costs to conspecifics, it is clear that the genetic relatedness of the population can determine the degree to which these aggressive behaviours can be adaptive in a population (Pizzari et al., 2015). In populations with a degree of genetic structure where competition might occur between kin or non-kin, high local genetic relatedness can reduce behaviours that harm conspecifics by increasing the indirect reproductive benefits an individual stands to gain from the reproduction of conspecifics (Rankin, 2011). Therefore, the genetic structure of populations has the potential to influence the evolution of aggression (Pizzari et al., 2015), and in chapter 4, I investigate how the degree of male relatedness in groups of *D. melanogaster* influences male-male aggression, male-female harassment, and the resultant reproductive outcomes.

The interaction between sexual conflict and genetic relatedness depends on the ability of individuals to recognise relatives, and the proximate mechanisms of kin discrimination are currently poorly understood (Pizzari et al., 2015). Individuals will receive the greatest gains to inclusive fitness if they can reduce aggressive competition only when competing with genetic relatives (Hamilton, 1964; Rousset & Roze, 2007), which requires the ability to discriminate kin from non-kin. When dispersal is low and relatives remain near their birth site, kin discrimination based on spatial distribution in possible is possible (Pizzari et al, 2015). In cases where these is a larger degree of dispersal, three main potential mechanisms have been proposed for kin recognition, including recognition based on familiarity gained from previous encounters or shared previous environments, phenotypic matching by distinguishing those similar to themselves as kin, and via recognition alleles (Blaustein, 1983; Pizzari et al., 2015; Rousset & Roze, 2007). However, while there has been some experimental work to empirically test these theories, much remains inconclusive (Blaustein, 1983; Holmes & Sherman, 1982; Wu et al., 1980), and the conditions (e.g. population structure and genetic structure) required for these mechanisms to successfully operate are highly specific and complex (Pizzari et al., 2015; Rousset & Roze, 2007). Furthermore, the sensory pathways and specific cues for such kin recognition remain elusive. Excitingly, there has been recent work suggesting the odours produced by gut microbiota could play a key role in kin recognition in insects (Lizé et al., 2013, 2014), but a clear consensus of the mechanisms that can allow kin discrimination in order to reduce sexual conflict and increase cooperation in response to relatedness has not yet been reached. Therefore, I address this in chapter 4, where I investigate the influence of male relatedness on male-male aggression and male-female harm, comparing the response to relatedness in wild-type and olfactory-deficient groups of *D. melanogaster*.

Resource availability, nutrition and aggression

The quantity and quality of food available to an individual determine the pool of resources available for allocation to all potential traits, life-history processes and behaviours (Han & Dingemanse, 2015; Kolluru & Grether, 2005; Rol & Houle, 1996; Zikovitz & Agrawal, 2013). Thus, nutrition has the potential to have large influences on aggressive ability. An individual's ability to aggressively compete for resources, referred to as resource holding potential, depends on traits that are directly influenced by nutrition including body size, energy reserves, physiology, and the development of appendages (Arnott & Elwood, 2008; Briffa & Sneddon, 2007; Kemp & Alcock, 2003; Stockermans & Hardy, 2013). For example, larger body mass can improve aggressive initiation, escalation and success in a range of species including fruit flies, *D. melanogaster* (Bath et al., 2018; Hoffmann, 1987b; Hoyer et al., 2008), elephant seals, *Mirounga leonina* (McCann, 1981), squids, *Loligo plei* (DiMarco & Hanlon, 1997), and copperheads, *Agkistrodon contortrix* (Schuett, 1997). Likewise, individuals with higher energy reserves have a greater ability to win fights in damselflies, *Calopteryx spp.,* (Marden & Waage, 1990; Plaistow & Siva-Jothy, 1996).

Furthermore, as food provides energy and micronutrients essential for life, individuals should be highly motivated to attain further nutrition when current reserves are low and the potential to gain high quality nutrients is high (Arnott & Elwood, 2008). The amount an individual has to gain or lose from winning a contested resource, referred to as resource valuation (Maynard & Parker, 1976), is determined by the nature of contested resources and the current state of the individual (Scharf, 2016). For example, hunger increases fighting success in the house cricket, *Acheta domesticus* (Nosil, 2002), and can cause subordinates to outcompete dominants in the American goldfinch, *Carduelis tristis* (Arnott & Elwood, 2008; Popp, 1987). Therefore, because past and present levels of nutrition play key roles in determining both resource-holding potential and resource valuation of individuals and potential rivals, it is important to interpret aggressive behaviours in the context of the nutritional history of all individuals involved.

Throughout an individual’s lifetime, the potential impacts of nutrition on aggression can change, as nutritional requirements vary as organisms progress through their life history (Poças et al., 2020). For example, holometabolous and semelparous insects have a distinct food acquiring larval or nymphal stage, in which nutrient acquisition is paramount in determining adult body size (Boggs, 1981; Kemp & Alcock, 2003; Nestel et al., 2016; Poças et al., 2020). Female metabolic requirements can increase during pregnancy and gestation, altering nutritional needs (Barnes et al., 2008; Mainardi et al., 1996; Seebacher et al., 2013; Timmerman & Chapman, 2003). Similarly, long distance migratory species require increased calorific intake surrounding the migratory period (Blem, 1980).

Therefore, the magnitude and nature by which nutrition influences resource holding potential and resource valuation should be highly sensitive to an individual's life-history stage. Distinguishing how the relationship between nutrition and aggression varies at distinct life stages is important for understanding how ecology shapes the evolution of aggression, and carries potential implications for human behaviour and health (Gesch, 2017; Iribarren et al., 2004; Oddy et al., 2009; Schroeder & Higgins, 2017). In chapter 5 and 6, I investigate how nutritional deprivation at either the developmental or adult stage influences male-male aggression in *D. melanogaster*.

Study Organism – The fruit fly, Drosophila melanogaster

The fruit fly, *Drosophila melanogaster*, provides an excellent system in which to study aggression, with individuals engaging in aggressive contests over food, territories and mates (Chen et al., 2002; Dow & von Schilcher, 1975; Hoffmann, 1987a, 1987b; Hoffmann & Cacoyianni, 1990; Yurkovic et al., 2006). Over the past 50 years, male aggression has been studied in *D. melanogaster* (Dow & von Schilcher, 1975; Hoffmann, 1987b; Hoffmann & Cacoyianni, 1990), and more recently, female aggression has received growing attention (Bath et al., 2017, 2018; Nilsen et al., 2004). This wealth of research has allowed the development of ethograms describing competitive behaviours in both males and females (Andrews, 2016; Certel & Kravitz, 2012; Nilsen et al., 2004; Yurkovic et al., 2006), providing a well-established framework for studying aggression. *Drosophila melanogaster* are amenable to controlled laboratory systems, meaning the social and physical environmental can be manipulated to explore how such ecological components determine behavioural strategies (Carazo et al., 2014, 2015; Hoffmann, 1987b; Hoffmann & Cacoyianni, 1990). This facilitates both short-term studies and long-term studies tracking evolutionary changes in response to different selection pressures (experimental evolution; Dierick & Greenspan, 2006; Holland & Rice, 1999; Wensing et al., 2017). Furthermore, advancements in the study of *D. melanogaster* genetics and neurobiology offer exciting potential for studying the proximate mechanisms underlying modulation of aggressive behavioural strategies in response to environmental conditions (Alekseyenko et al., 2010; Asahina, 2017; Asahina et al., 2014; Hoopfer et al., 2015). As aggression displays a degree of conservation among species (Edwards et al., 2009), and genes involved in *D. melanogaster* aggression have orthologues associated with neurological human disorders (Rohde et al., 2017), the study of aggression in *D. melanogaster* might reveal fundamental processes parallel to those in humans (Dierick, 2007; O'Kane, 2011).

Thus, the application of the techniques and processes available in *D. melanogaster*, combined with our current wealth of knowledge of this species, create exciting new possibilities for answering previously unexplored questions as to how ecological factors shape the evolution of aggression in the fruit fly and on a broader taxonomic scale.

Thesis Overview

In my thesis, I explore how the prevailing social and nutritional environment act to determine the selective forces that shape aggression in male and female *Drosophila melanogaster*.

In chapter 2, I investigate how the sex ratio relates to the operation of sexual selection in both males and females. I find that, in males, the opportunity for and maximum potential strength of sexual selection increases as the sex ratio becomes male biased, and this is largely driven by greater variance in mating success. I find no evidence for sexual selection arising from mating competition in females in my experimental set up, and no influence of the sex ratio on the variance in female mating success, reproductive success, and the relationship between the two.

In chapter 3, I investigate how male and female aggression evolves in response to the population sex ratio. I find that males performed more aggression on a central food patch after evolution in more male-biased environments, demonstrating differences in how aggression relates to food, but find no evidence for evolution in the intensity or frequency of male aggression. In females, I find that a female-biased sex ratio supports the evolution of a greater increase in aggression post-mating, but only after mating to co-evolved males. Furthermore, I find evidence consistent with underlying genetic correlation in aggression levels between the sexes.

In chapter 4, I investigate the ability of relatedness to reduce sexual conflict, and the role of olfaction in this response. I find evidence inconsistent with olfactory-based responses to kin, as I find lower male-male aggression and increased mate longevity in related compared to unrelated groups of olfactory-deficient males, suggesting olfaction is not the sole mechanism for reduced conflict in response to relatedness. I find no evidence for a response to relatedness in wild-type males, despite previous evidence of this, suggesting a sensitivity and context-dependent nature of this response.

In chapters 5 and 6, I investigate the relationship between aggression and nutritional deprivation at different life stages. In chapter 5, I find that nutritional deprivation in the developmental stage decreases adult aggression, but in chapter 6, I find that nutritional deprivation in the adult stage increases aggression. I discuss how the life stage at which nutritional stresses are experienced might determine how they influence resource holding potential and resource valuation, and how this relates to subsequent aggression.

Chapter 2: Sexual selection is sensitive to the sex ratio

ABSTRACT

Theory predicts that biases in the Operational Sex Ratio (OSR) increase the strength of sexual selection in the abundant sex, such that many empirical studies have used OSR as a proxy for the strength of sexual selection. However, it remains unclear whether this assumption is appropriate. To test this principle, we manipulated the sex ratio in *Drosophila melanogaster* populations to directly investigate the relationship between OSR and the operation of sexual selection in both sexes. We found that, as the OSR becomes male-biased, the opportunity for and maximum potential strength of pre-copulatory sexual selection increased for males, as expected by theory. We also found a trend for a greater maximum potential strength of post-copulatory sexual selection acting on males that obtained a mate in male-biased populations. However, the OSR did not influence male or female Bateman gradients. Our findings in females contrasted with those in males: we found no evidence of sexual selection arising from mating competition at any sex ratio, consistent with previous studies demonstrating that sexual selection arising from mate competition is weak or absent in females. This likely reflects differences in intra-sexual competition in the two sexes: while males predominantly compete for mates and fertilisation opportunities, females predominantly compete for resources that increase reproductive success. Our results provide empirical support for the long-held assumption that the OSR influences the force of sexual selection operating on males, but, in our study system, this pattern is not reflected in females.

INTRODUCTION

Sexual selection, described as competition between individuals of the same sex in the context of reproduction (Darwin, 1871; Evans & Garcia-Gonzalez, 2016; Stockley & Bro-Jørgensen, 2011), plays an integral role driving the evolution of reproductive traits, such as ornaments, weapons and behaviours (Jennions & Kokko, 2010; Miller & Svensson, 2014; Morimoto et al., 2019). Sexual selection is sensitive to variation in the local environment (Emlen & Oring, 1977; Evans & Garcia-Gonzalez, 2016; Morimoto et al, 2016; Procter et al., 2012), and understanding how the strength, direction and patterns of sexual selection change with environmental variation is essential for a full evolutionary understanding of reproductive strategies.

One feature of the social environment widely considered to influence sexual selection is the operational sex ratio (OSR), defined as the ratio of reproductively available males and females in a population (Emlen & Oring, 1977; Klug et al., 2010; Weir et al., 2011). The influential work of Emlen and Oring (1977) suggested that variation in the OSR changes the potential for intra-sexual competition for reproductive opportunities, and the degree to which certain individuals can monopolise such opportunities by limiting the access of other members of their sex to reproductive opportunities (Emlen & Oring, 1977; Krakauer et al., 2011; Kvarnemo & Ahnesjo, 1996). This traditional view of sexual selection suggests that as the OSR becomes skewed, the abundant sex will experience more intense reproductive competition, resulting in greater variance in reproductive success, and therefore stronger sexual selection (Aronsen et al., 2013; Emlen & Oring, 1977; Kvarnemo & Ahnesjo, 1996; Weir et al., 2011). Some empirical evidence is consistent with these views (Carroll & Salamon, 1995; Kvarnemo & Ahnesjo, 1996), and, accordingly, many empirical studies use sex ratio as a proxy for the strength of sexual selection, adjusting the sex ratio to investigate the evolutionary impacts of varying intensities of sexual selection (Linklater et al., 2007; McNamara et al., 2016; Nandy, Chakraborty, et al., 2013).

Despite traditional views, more recent advances in the field of sexual selection have challenged the role of the OSR (Klug et al., 2010; Kokko et al., 2012; Krakauer et al., 2011). Recent studies have found more limited, context-dependent patterns, and even patterns opposite to those of traditional views, with stronger sexual selection in the less abundant sex (Fitze & Le Galliard, 2008; Klug et al., 2010; Krakauer et al., 2011; Mills et al., 2007). Therefore, there is a general lack of consistency on the effects of the sex ratio on the operation of sexual selection, and few direct empirical tests, especially in species without sex-role reversal. Because the OSR can be highly variable in natural populations due to inter-sexual differences in migration, distribution, longevity, and sexual receptivity timings (Kasumovic et al., 2008; Kvarnemo & Ahnesjo, 1996), it is important that we resolve the implications for the operation of sexual selection.

Furthermore, while traditional sexual selection theory predicts that the OSR determines sexual selection on males through its influence on mating success, post-copulatory processes and the fecundity of female mates are integral in translating male mating success to reproductive success (Collet et al., 2012; Collet et al., 2014; Kokko et al., 2012; Morimoto et al., 2016, 2019; Webster et al., 1995). Because the OSR describes the ratio of available mates, which predominantly influences pre-copulatory mate competition, its usefulness in predicting post-copulatory competition and variation in mate fecundity might be limited (Kvarnemo & Ahnesjo, 1996). That said, if intense male-bias increase female polyandry through male harassment and convenience polyandry (Wigby & Chapman, 2004), then sperm competition intensity might be elevated, increasing post-copulatory sexual selection (Morimoto et al., 2019). In contrast, because a male's ability to obtain a highly fecund mate depends partly on the degree of variation in female fecundity within a population (Webster et al., 1995), the role of mate fecundity in sexual selection on males might increase as the population becomes more female-biased and has increased scope for female variation. Thus, the sex ratio might simultaneously influence multiple pre- and post-copulatory aspects of sexual selection on males.

Importantly, although much previous research has focused on sexual selection on males, there has recently been an increased appreciation that an analogous form of selection also operates in females (Clutton-Brock, 2009; Stockley & Bro-Jørgensen, 2011). The nature of selection arising from intra-sexual competition differs between the sexes, with males typically competing for mates and gametes, and females competing for access to resources necessary for reproduction. However, in both sexes, this competition strongly impacts reproduction, and such social selection, describing selection resulting from intra-sexual competition for all forms of reproductive resources, is an important component of evolution in both sexes (Clutton-Brock, 2009; Stockley & Bro-Jørgensen, 2011). Additionally, in polyandrous species, females can gain fitness benefits from mating multiply (Hosken & Stockley, 2003), meaning sexual selection arising from mating competition can act on females as well as males. Thus, a high degree of reproductive skew can exist among females (Clutton-Brock et al., 2006), and high female-female competition in female-biased sex ratios might strengthen sexual or social selection on female traits that confer competitive advantages (Clutton-Brock, 2009). Much empirical research on the relationship between OSR and female sexual selection focuses on sex-role-reversed species, where extreme female-bias result in females competing for mating opportunities (e.g., pipefish *Syngnathus typhle*, Aronsen et al., 2013; Jones et al., 2000, Wilson's phalarope, Kvarnemo & Ahnesjo, 1996). However, we have limited knowledge of how female social selection responds to the sex ratio in species with traditional sex roles where females predominantly compete over resources (but see Milner-Gulland et al., 2003; Rusu & Krackow, 2004; Stockley & Bro-Jørgensen, 2011). The sex ratio predominantly influences the ratio of available mates relative to competitors, but might also influence intra-sexual competition for other resources such as food and breeding sites if resources are limited. In environments without limitation of such resources, it is likely that the sex ratio will have a stronger influence on mating competition than intra-sexual competition for other resources. Thus, the social selection arising from female-female resource competition might be less sensitive to the sex ratio than sexual selection in the traditional sense in males. Studying sexual and social selection in both sexes simultaneously can reveal the potential for sexual conflict over mating rates and demonstrate how the targets of selection arising from intra-sexual competition differ between the sexes (Morimoto et al., 2016).

Currently, we lack a consensus based on experimental evidence of the influence of the sex ratio on the operation of sexual selection in males, and of whether trends in male sexual selection are reflected in females. To address this gap, we measured the opportunity for and strength of male pre- and post-copulatory sexual selection, and applied these measures to females where applicable, in fruit flies, *Drosophila melanogaster,* held at different experimentally-manipulated sex ratios. *D. melanogaster* are amenable to laboratory experimentation, allowing effective manipulation of the sex ratio and full observations of mating behaviours (Cramer et al., 2020). Males are polygynous and court females to obtain mating success (Manning, 1960; Markow & O'Grady, 2008), with greater mating success increasing reproductive success (Bateman, 1948; Morimoto et al., 2016, 2019; Pischedda & Rice, 2012). The transfer of the seminal fluid protein 'sex peptide' at mating renders females unreceptive to further mating for up to 5 days post-mating (Kubli & Bopp, 2012; Manning, 1962; Wigby & Chapman, 2005) meaning that over a short timescale, the adult sex ratio approximates the OSR, as the number of adult females is equal to the number of females ready to mate at that time. Male harassment can overcome female resistance during the refractory period (Wigby & Chapman, 2004), and females regain receptivity after the effect of sex peptide subsides, such that females exhibit polyandry (Imhof et al., 1998). Therefore, male competition continues post-mating in the form of sperm competition (Bretman et al., 2009; Gilchrist & Partridge, 2000; Morimoto et al., 2019). Previous studies suggest that male sexual selection is stronger than analogous selection arising from intra-sexual competition in female *D. melanogaster* (Bateman, 1948; Morimoto et al., 2016). Likewise, previous studies show that the sex ratio is an important factor in shaping the evolution of components of *D. melanogaster* reproduction, such as sperm competitive abilities (Chechi et al., 2017; Linklater et al., 2007; Nandy, Chakraborty, et al., 2013), courtship and mating rates (Rostant et al., 2020) and female resistance (Wigby & Chapman, 2004). Furthermore, genetic markers allow precise estimation of paternity share and post-copulatory selection. Therefore, the wealth of knowledge of *D. melanogaster* mating and reproductive behaviour, along with the experimental tools available for this organism, mean that it represents an ideal system for the experimental study the operation of sexual and social selection.

We studied 82 experimentally-constructed groups of 8 flies at three sex ratios – male-biased (6 male:2 female), equal (4 male: 4 female), and female-biased (3 male:5 female) – each containing one focal individual of each sex. All flies were painted for identification and focal males carried genetic eye markers, allowing us to record mating behaviour of all individuals, and reproductive success of all females and focal males. If the sex ratio is central to determining the operation of sexual and social selection in males and females, we predicted that as the sex ratio becomes increasingly biased towards one sex, the opportunity for and strength of sexual and social selection should increase in that sex. Specifically, in males, we predicted that this should be driven by an increase in pre-copulatory sexual selection as the intensity of mating competition increases, which might be enhanced by greater post-copulatory sexual selection if male harassment increased polyandry. However, a reduced potential for variance in mate fecundity as the pool of available mates decreases might lessen the expected increase in male sexual selection in male-biased conditions. In females, we predicted that the increased female-female resource competition as female abundance increases might result in greater female sexual or social selection in female-biased conditions, but we expected the intensity of selection and the degree to which it responds to OSR to be weaker than in males, with a weaker relationship between competition for mates and reproductive success.

RESULTS AND DISCUSSION

We assembled groups of 8 flies at either male-biased, equal or female-biased sex ratios by placing 6, 4, or 3 males with 2, 4 or 5 females respectively (male-biased, n=28, equal, n=27, female-biased, n=27). Each vial contained a focal individual of each sex, identified by paint-marking, and, in males, by a genetic marker to allow focal paternity to be tracked (see methods and fig. S1). All males and females were paint-marked for identification, and we observed all groups for 4h per day for 3 consecutive days, recording all matings and the identity of the flies involved. Each 4h observation period was followed by an egg laying period for each individually-housed female, allowing us to measure the reproductive success of individual females, and estimate the reproductive success and relative paternity share of each focal male from his number of daughters (genetic eye markers allowed us to assess paternity of daughters but not sons; see methods). The number of daughters reflected the total progeny: we found an even ratio of daughters to sons (binomial test, p=0.420), and this was consistent across sex ratios (χ^2_1=3.5, p=0.177). Although eye-colour markers were essential for paternity assessment, eye mutations impair visual acuity and might affect behaviour. Therefore, we assessed whether focal and rival male genotypes influenced their mating frequencies. This revealed that although focal males had a lower mating frequency than rivals (binomial tests; table S1), the mating advantage of rival males over focal males was consistent across sex ratios (χ^2_2=0.5, p=0.769). Furthermore, ≥50% focal males mated at least once in each sex ratio, showing that rival competition was not so strong as to eliminate variation in focal mating success across vials, an important component of sexual selection.

Mating increased for males as the sex ratio became female-biased

For most species, the number of fertile females is the limiting factor for male reproduction (Darwin, 1871; Kokko et al., 2006), and our findings largely reflect this, with mating rates and reproductive success per vial and per male increasing as the sex ratio becomes female-biased. We first tested the influence of sex ratio on total vial mating frequency (total number of matings per vial), and found that this increased as the sex ratio shifted from male-biased to female-biased ($\chi^2_{2,77}$=23.7, p<0.0001; fig.1a). This largely reflected increased male polygyny, as across all males (focal and rival), both average male mating success, M, and mating frequency per male increased in line with the greater abundance of potential mates (M: $F_{2,77}$=116.7, p<0.0001; Frequency: $F_{2,77}$=129.3, p<0.0001; fig.1b-c), a pattern also apparent in focal males (M:$\chi^2_{2,77}$=20.5, p<0.0001; Frequency: $\chi^2_{2,77}$=22.2, p<0.0001). This supports the assumption that, in this system, the adult sex ratio reflects the operational sex ratio.

Mating success and frequency capture both the number of mates and matings, and whether or not mating is achieved at all. This latter component is integral to reproductive success, and we found that the likelihood of males obtaining at least one mate increased as the sex ratio became more female-biased (focal males:$\chi^2_{2,77}$=10.9, p=0.0042; all males:χ^2_{2}=43.6, p<0.001; table S2). Reproductive success largely reflected mating patterns, as both total vial productivity and focal male reproductive success increased as the sex ratio became increasingly female-biased (total vial productivity: $\chi^2_{2,77}$=210.0, p<0.0001; focal male siring success: $\chi^2_{2,77}$=18.0, p=0.0001; fig1.d-e). Increased focal siring success likely reflected that of all males, as opposed to an increase in focal male paternity share relative to rivals, because focal paternity share was consistent over the three sex ratios (for focal males that obtained a mate; $\chi^2_{2,}$=2.1, p=0.354; fig. 1f; excluding mates of sterile females:$\chi^2_{2,}$=2.1, p=0.356).

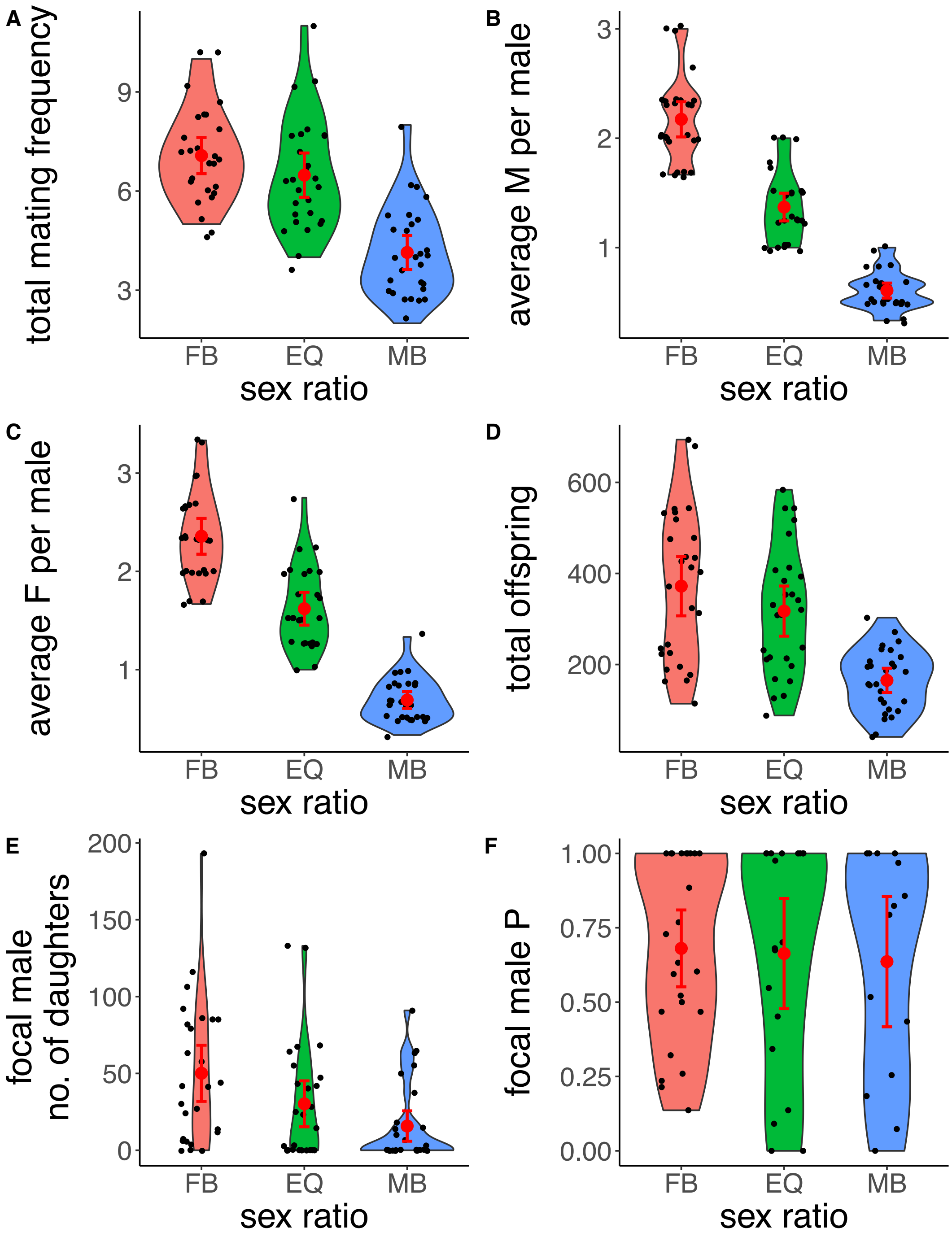


Figure 1

The influence of sex ratio on total vial and male mating behaviour and reproduction. (A) shows total vial mating frequency, (B-C) show the per male average (mean) mating success and mating frequency respectively, (D) shows total vial productivity, (E) shows focal male reproductive success (measured as the number of daughters), (F) shows the paternity share of focal males that *obtained at least 1 mate. Red points show means; error bars show confidence intervals.*

Conversely, in females, there was an increase in mean mating success and frequency per female as the sex ratio became more male-biased, with higher polyandry in male-biased sex ratios (M:$\chi^2_{2,77}$=30.2 p<0.0001; Frequency: $\chi^2_{2,77}$=28.5 p<0.0001; fig.2a-b). In female *D. melanogaster*, mating renders females temporarily unreceptive to further mating (Avila et al., 2011; Kubli & Bopp, 2012; Morimoto et al., 2019; Wigby & Chapman, 2005). Thus, increased female mating rates in male-biased populations are likely driven by greater male harassment in male-dense environments, suggesting reduced female resistance when intense harassment renders resistance uneconomic or ineffective (i.e., convenience polyandry; Lauer et al., 1996; Rowe, 1992). This increased female mating rate did not translate into reproductive success: mean female reproductive success remained constant across all sex ratios ($\chi^2_{2,77}$=2.4, p=0.303; fig.2c), consistent with previous studies (Bateman, 1948; Mills et al., 2007). Furthermore, female reproductive success per mating was significantly lower in male-biased conditions where mating rates where highest ($F_{2,77}$=4.2, p=0.019; fig.2d). This suggests that, after the point of the first mating, repeated mating doesn't further increase female reproductive success (Arnqvist & Rowe, 2005; Bateman, 1948; Chapman & Partridge, 1996; Sirot et al, 2011).

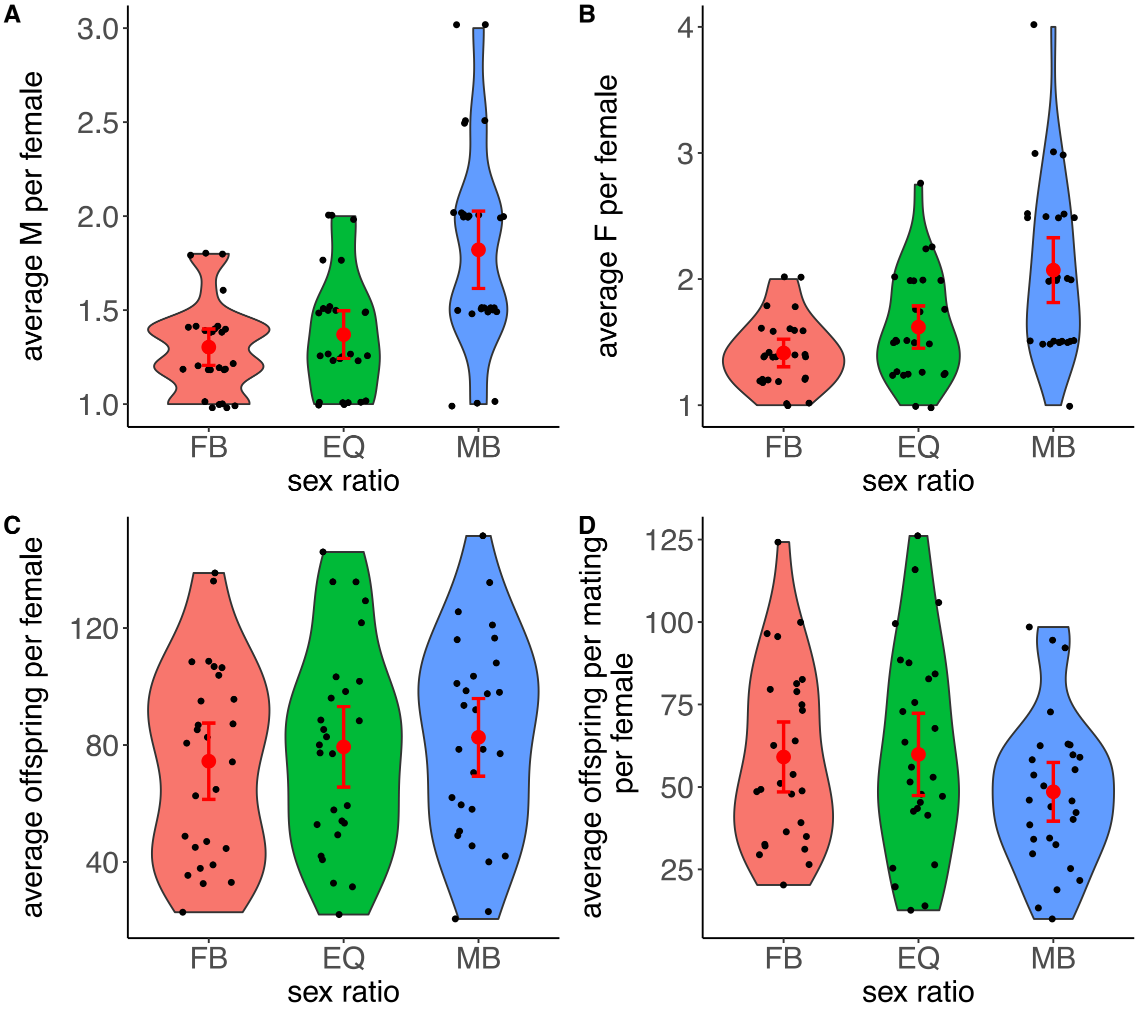


Figure 2

The influence of sex ratio on female mating behaviour and reproduction. A-B show the per female average (mean) mating success and mating frequency respectively, C shows the per female average (mean) reproductive success, and D shows the per female average (mean) reproductive success per mating. Red points show means, error bars show confidence intervals.

Sex ratio influences variance in male, but not female, reproductive success

To test our prediction that a more biased sex ratio should increase the total opportunity for sexual and social selection in the abundant sex, we initially calculated the standardised variance in reproductive success, *I*, for both sexes. In males, our results supported the predictions, with an increased *I* as the sex ratio became more male-biased, and non-overlapping confidence intervals revealing a significantly greater *I* in male-biased relative to female-biased sex ratios (fig.3a). This pattern was similar when males with sterile mates were excluded (some males mated only with unproductive females, see methods; fig. S2a). This is consistent with predictions from the field of sexual selection (Emlen & Oring, 1977; Kvarnemo & Ahnesjo, 1996), resulting from the increased intensity of male-male competition as the relative number of males to females increases (Clutton-Brock & Parker, 1992). In contrast, in females, we found no influence of the sex ratio on focal female *I* (fig.3b), and this was reflected by the results for all females in the vial (fig. S3a). This suggests that variance in female reproductive success does not reflect male reproductive success in its response to the sex ratio, with *I* showing no sensitivity to the sex ratio in this system. As predicted, across all sex ratios, values of *I* were lower in females relative to males, consistent with previous evidence of weaker selection arising from intra-sexual competition in females of this species (Bateman, 1948; Morimoto et al., 2016).

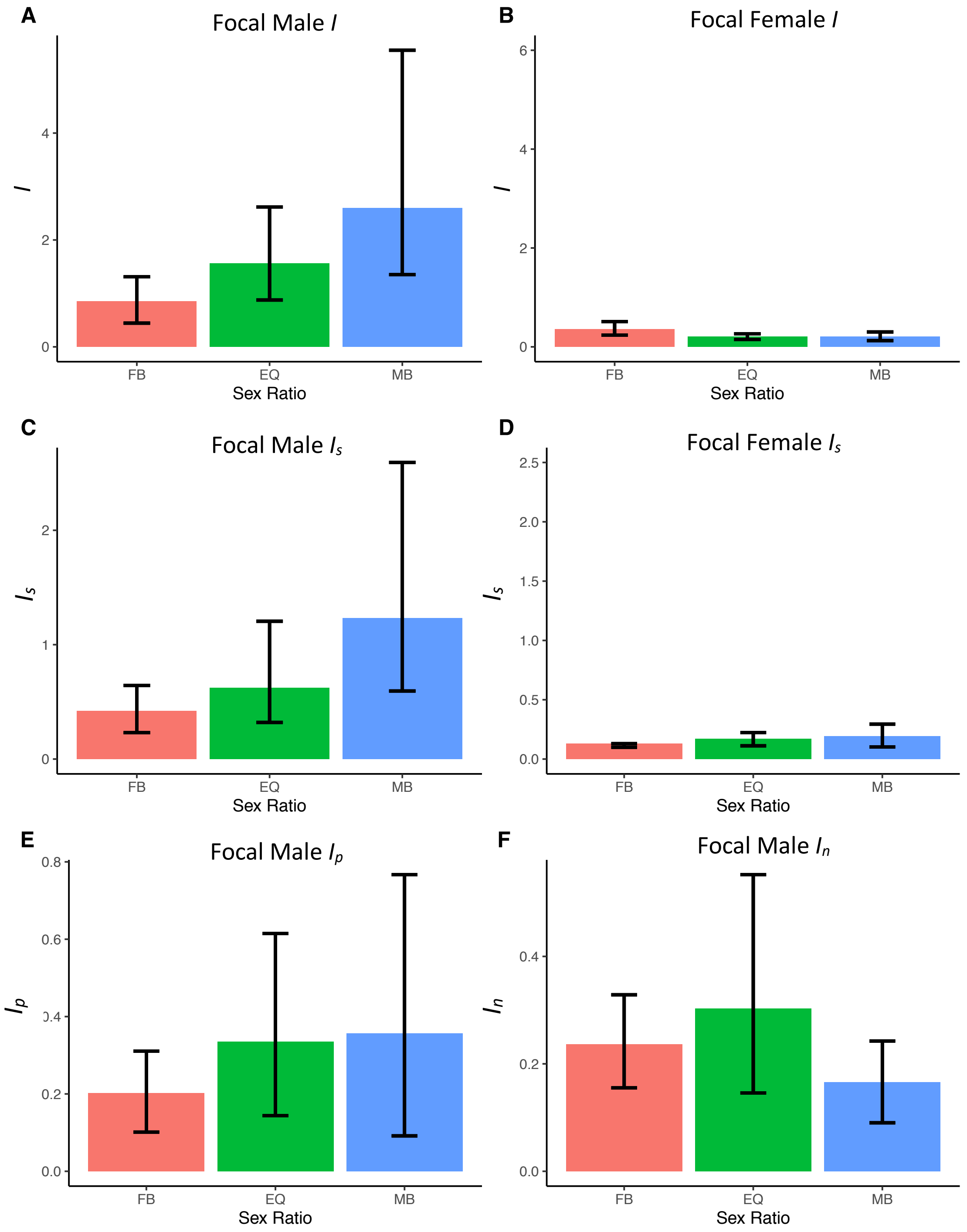


Figure 3

The influence of sex ratio on the opportunity for total sexual selection, I, in males (A) and females (B), the opportunity for pre-copulatory sexual selection, I_s*, in males (C) and females (D), the opportunity for male post-copulatory sexual* *selection, I_p (E), and the opportunity for selection on male mate choice, I_n (F). Error bars show bootstrapped 95% confidence intervals.*

Variance in mating success largely explains reproductive variance in males

Mating success represents an important component of reproductive success, and we predicted that, in males, the increase in the total opportunity for sexual selection in the abundant sex might be largely driven by an increased opportunity for pre-copulatory sexual selection (i.e., selection on traits contributing to mating success). We assessed this by calculating the standardised variance in mating success, I_s, for both sexes. Consistent with the prediction, we found a trend for greater male I_s as the sex ratio became increasingly male-biased, but between-group differences did not meet the level of significance in focal males (overlapping bootstrapped 95% confidence intervals; fig.3c). This trend was consistent across all males (both focals and rivals; fig.S3b), suggesting that the ability to obtain a mate contributes to variability in male reproductive success (Bateman, 1948). In contrast, we found no influence of the sex ratio on I_s in focal females or across all females (fig.3d & fig.S3c), consistent with our results for female I. Values for female I_s were nearly an order of magnitude lower than males, demonstrating lower opportunity for pre-copulatory sexual selection arising from mating competition in females. This is consistent with the notion that females predominantly compete over reproductive resources as opposed to mates (Clutton-Brock, 2009; Stockley & Bro-Jørgensen, 2011).

Next, because paternity share and mate fecundity for males (N, the average fecundity of a male's mates) also represent an important component of male reproductive success and might contribute to the total variance in reproductive success (Collet et al., 2012; Collet et al., 2014; Morimoto et al., 2019; Parker, 1970; Pélissié et al., 2014; Webster et al., 1995; Wigby & Chapman, 2004), we calculated the standardised variance in paternity share (I_p, opportunity for post-copulatory sexual selection) and mate fecundity (I_n) for all focal males that obtained a mate. In moderately polyandrous species like *D. melanogaster*, sexual selection can continue post-copulation, and because we detected increased polyandry in the male-biased sex ratio, we predicted that the opportunity for post-copulatory sexual selection, I_p, would increase as the sex ratio became increasingly male-biased (Morimoto et al., 2019). Our results offered no support for this prediction, with no significant influence of the sex ratio on I_p (overlapping bootstrapped confidence intervals; fig.3e). We found the same lack of relationship after excluding males with sterile mates (fig.S2b). This suggests that I_p plays at most a minor role in driving the increased opportunity for total sexual selection as the sex ratio becomes more male-biased. Furthermore, we predicted that the greater number of potential mates in female-biased conditions might increase the opportunity for selection to occur on a male's ability to choose more fecund mates, I_n. However, our results revealed no influence of the sex ratio on I_n (overlapping 95% confidence intervals; fig.3f). This was also the case after excluding males with sterile mates (fig.S2c). These results are consistent with the hypotheses of Kvarnemo & Ahnesjo, (1996): while sex ratio can predict competition for mates, it will not generally predict other components of sexual selection.

To qualitatively assess how the relative variance in M, P and N, and their covariances, contribute to variance in male reproductive success, T, we decomposed the variance into these relative components (e.g. Bohrnstedt & Goldberger, 1969; Collet et al., 2012; Morimoto et al., 2016; Webster et al., 1995; see methods). From our results for I_s, I_p, and I_n we predicted that variance in M would explain a larger percentage of the variance in reproductive success than variance in P and N. Our decomposition of variance was consistent with this prediction: variance in M explains just under half the variance in T across all sex ratios, with lower contributions of P and N (table 1). Furthermore, the differences in the contribution of variance in M relative to variance in P and N is largest in male-biased sex ratios. Variance in P plays a smaller role in male-biased sex ratios relative to equal and female-biased sex ratios, likely because fewer males obtained a mate and entered into post-copulatory competition in the male-biased sex ratio. We predicted that the relative contribution of variance in N to variance in reproductive success would be greatest in the female-biased sex ratio and lowest in the male-biased sex ratio, and decomposition of variance analysis was consistent with this prediction. This might reflect that in a female-biased sex ratio, male choice for the most fecund female mates is a more important component of reproductive success, whereas in a male-biased sex ratio, high polyandry and potential mating group saturation might result in a greater proportion of males sharing the same mates, minimising the variance in N. Covariances between M, P and N explained only a small percentage of the total variance in reproductive success (table 1). Excluding males with sterile mates from this decomposition of variance increased the contribution of variance in M, and decreased the contribution of variance in P and N to variance in total reproductive success, with a small negative covariance between M and P (table 1).

Table 1

Decomposition of variance illustrating the percentage of variance in reproductive success (var(T)) that can be accounted for by variance in mating success, paternity share, mate fecundity (var(M), var(P), var(N)) and their covariances, and an error term ε *for each sex ratio treatment*

	Male-biased		**Equal**		**Equal *(excluding sterile)***		**Female-biased**	
	value	%	value	%	value	%	value	%
Var T	2.600		1.562		1.374		0.849	
Var M	1.231	47.3	0.621	39.8	0.681	49.6	0.421	49.6
Var P	0.356	13.7	0.335	21.4	0.190	13.8	0.202	23.8
Var N	0.166	6.38	0.303	19.4	0.163	11.9	0.2368	27.9
Cov(M,P)	0.0549	2.11	0.0182	1.17	-0.0113	-0.82	0.0200	2.36
Cov(M,N)	-0.0419	-1.61	0.0315	2.02	0.0019	0.14	0.00435	0.51
Cov(N,P)	-0.0034	-0.13	0.2196	14.1	0.0867	6.31	0.0256	3.02
error	0.8374		0.0337		0.2637		-0.0608	

Sex ratio influences the maximum potential strength of sexual selection in males, but not females

The above analyses show the maximum opportunity for sexual selection to occur. However, this opportunity will only be realised if success in pre- or post-copulatory processes translates into reproductive success. Therefore, we calculated selection gradients to assess how mating success and, where applicable, paternity share and mate fecundity influence reproductive success in both sexes in each sex ratio treatment. For males, paternity share and mate fecundity only contribute to reproductive success if a male is successful in mating. As not all males obtained a mate, and the likelihood of obtaining a mate is arguably the most important component of reproductive success, we initially analysed the univariate mean-standardised linear regression between reproductive success and mating success (i.e., the Bateman gradient). Results from this analysis did not support our prediction of stronger male pre-copulatory sexual selection as the sex ratio becomes male-biased: male univariate mean-standardised M gradients were significantly positive, showing that mating success was associated with increased reproductive success, but this relationship was not influenced by sex ratio ($\chi^2_{2,73}$=0.2, p=0.887; fig.4a; excluding males with sterile mates: $\chi^2_{2,71}$=0.3, p=0.857; fig.S4a). Although our findings of steep and positive male Bateman gradients is consistent with Bateman's principle of a high correlation between male mating success and reproductive success (Bateman, 1948), our results suggest that males gained the same increase in reproductive success per unit increase in mating success across all sex ratios.

However, mean-standardised gradients do not fully account for differences in variance across sex ratios. For example, a Bateman gradient of 1 would show that a 1% increase in M results in a 1% increase in T. But, while a 1% increase in M might result in a similar increase in T across all sex ratios, the degree to which these reproductive gains reflects a relative reproductive advantage compared to mating competitors also depends on the variance in M in the population. Because a more male-biased sex ratio increased variance in male mating success, mean-standardised selection gradients might not fully detect differences in selection arising from changes to the sex ratio. Therefore, we repeated our analysis of selection gradients using variance-standardised data, also known as s'max or the Jones index (Henshaw et al., 2016; Jones, 2009; Morimoto et al., 2016, 2019). This takes into account both the variance in pre- and post-copulatory indices and how they relate to T, estimating the maximum potential strength of sexual selection (Arnold, 1994; Collet et al., 2012; Henshaw et al., 2016; Jones, 2009; Morimoto et al., 2016, 2019).

In contrast to the mean-standardised analysis, our results from male variance-standardised univariate M gradients provide evidence for the increased maximum potential strength for sexual selection with increasing male-bias, consistent with results for *I* and I_s. Variance-standardised M gradients were positive across all sex ratios, and were significantly steeper as the sex ratio became more male-biased (χ^2_2=7.9, p=0.019; fig.4b; excluding sterile: χ^2_2=8.0, p=0.018; fig.S4b), showing that an increase per unit standard-deviation in mean male mating success resulted in a larger relative increase in reproductive success in male-biased sex ratios. Thus gaining more mates results in a similar increase in reproductive success across all sex ratios, but gaining relatively more mates than other males in the population results in a greater relative increase in reproductive success in male-biased conditions.

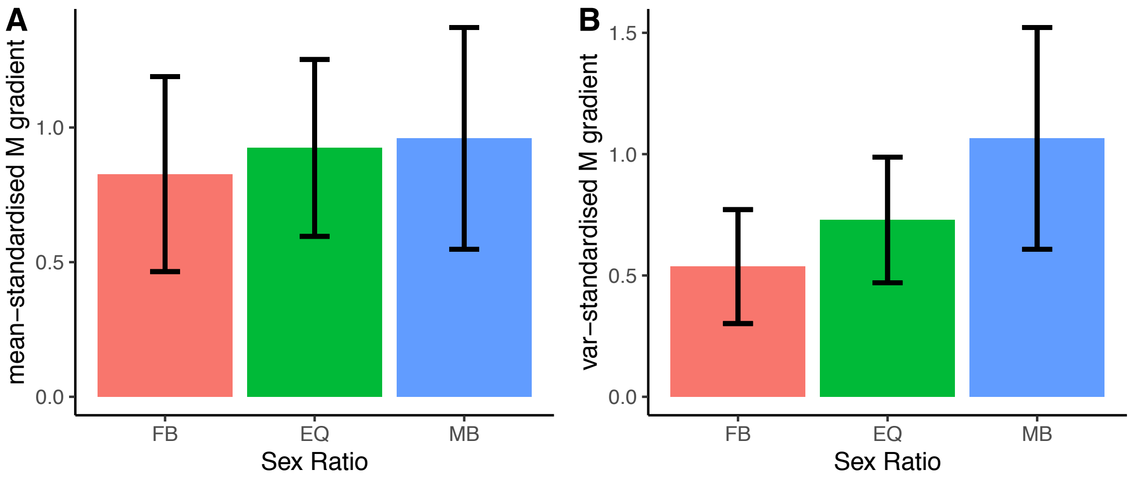


Figure 4

The influence of sex ratio on male mean-standardized (A) and variance-standardised (B) univariate gradient between mating success and reproductive success (the Bateman gradient and pre-copulatory s'max respectively). Error bars show confidence intervals.

Next, because paternity share and mate fecundity are important components of reproductive success in those males successful in mating, and because M, P and N can all interact and do not occur in isolation, we calculated multivariate mean-standardised linear regressions to measure the effect of each of M, P and N on T, while controlling for the effect of the other indices and their covariances, for the focal males that obtained at least one mate. Similar to univariate analyses, multivariate mean-standardised M gradients were not influenced by the sex ratio ($F_{2,42}$=0.3, p=0.780; fig.5a; excluding males with sterile mates: $F_{2,40}$=0.1, p=0.872; fig.S5a). The multivariate M gradient did not significantly differ from 0 in the male-biased sex ratio. However, this may result from restricting multivariate analysis to focal males that mated, such that the full variance in M is not captured, especially in male-biased sex ratios where only

50% of focal males mated. Likewise, we found no influence of sex ratio on multivariate mean-standardised P gradients ($F_{2,42}$=2.4, p=0.105; fig.5b; excluding sterile females: $F_{2,40}$=1.4, p=0.257; fig.S5b). This analysis revealed a significantly positive relationship between paternity share and reproductive success in male-biased and female-biased sex ratios, but this gradient was only significantly positive in the equal sex ratio after exclusion of males with sterile mates. Similarly, we found no influence of sex ratio on multivariate mean-standardised N gradients ($F_{2,42}$=0.6, p=0.537; fig.5c; excluding sterile females: $F_{2,40}$=0.9, p=0.431; fig.S5c). N gradients were positive in equal and female-biased sex ratios, but did not significantly differ from 0 in the male-biased sex ratio, suggesting a slight trend towards greater variability in the reproductive advantage of mating with more fecund females in male-biased sex ratios.

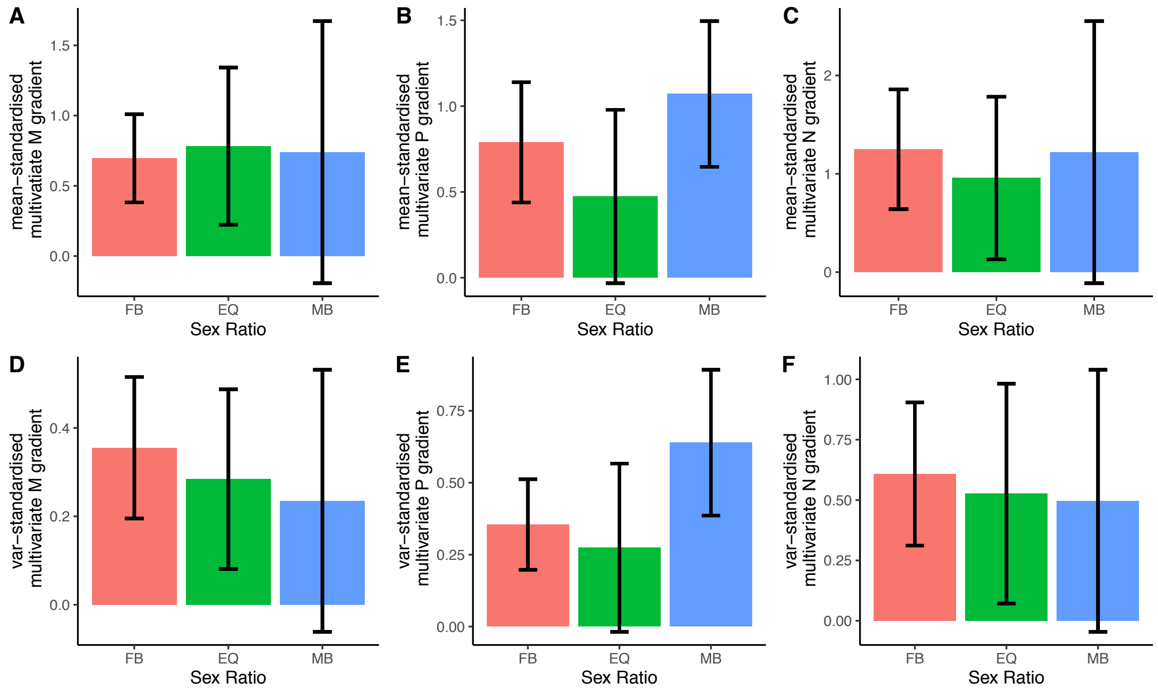


Figure 5

The influence of sex ratio on male mean-standardized (A-C) and variance-standardized (D-F) multivariate gradients between mating success (A&D),

paternity share (B&E), and mate fecundity (C&F) and reproductive success, while controlling for all other indices, their covariances and replica block. Gradient apply only to males that obtained at least 1 mate. Error bars show confidence intervals.

Variance-standardised multivariate M and N gradients were similar to mean-standardised gradients, with no significant influence of sex ratio (M: $F_{2,42}$=0.2, p=0.819; fig.5d, excluding sterile: $F_{2,40}$=0.3, p=0.756; fig.S5d; N: $F_{2,42}$=0.1, p=0.940; fig.5f, excluding sterile: $F_{2,40}$=0.0, p=0.968, fig.S5f). However, while mean-standardised P gradients were not influenced by sex ratio, we detected a steeper variance-standardised P gradient in the male-biased sex ratio, which reached the level of significance after excluding males that mated with sterile females ($F_{2,42}$=3.0, p=0.058; fig.5e, excluding sterile: $F_{2,40}$=3.7, p=0.033, fig.S5e). This suggests that post-mating, males in male-biased conditions might experience a relatively greater increase in reproductive success accompanying an increase per unit standard-deviation in mean paternity share. This provides some evidence consistent with the prediction that as the sex ratio becomes increasingly male-biased, the maximum strength of both pre- and post-copulatory sexual selection increase in males.

For females, post-copulatory components and mate quality are not easily determinable. Therefore, we performed only univariate analysis on females, calculating the mean- and variance-standardised linear regression between reproductive success and mating success (i.e. the Bateman gradient and pre-copulatory s'max). Mean-standardised analysis showed no influence of sex ratio on the relationship between mating success and reproductive success ($F_{2,73}$=0.0, p=0.953, fig.6a). In contrast to males and in line with predictions, we found no evidence that mating success translated into increased reproductive success in any sex ratio ($F_{1,73}$=0.1, p=0.819), with the female mean-standardised M gradients not significantly differing from 0. As female I_s was low and similar across all sex ratios, variance-standardised univariate M gradients reflected mean-standardised analysis, with no influence of sex ratio on the relationship between variance-standardised mating success and reproductive success ($F_{2,73}$=0.0, p=0.958; fig.6b), and no evidence that increased mating success translated into increased reproductive success in any sex ratio ($F_{1,73}$=0.1, p=0.800), with variance-standardised univariate M gradients not significantly differing from 0, suggesting no sexual selection on female mating rates (Bateman, 1948; Jones et al., 2000). This reflects the differences in intra-sexual competition between the sexes, with females seldom competing for mates (Clutton-Brock, 2009; Stockley & Bro-Jørgensen, 2011).

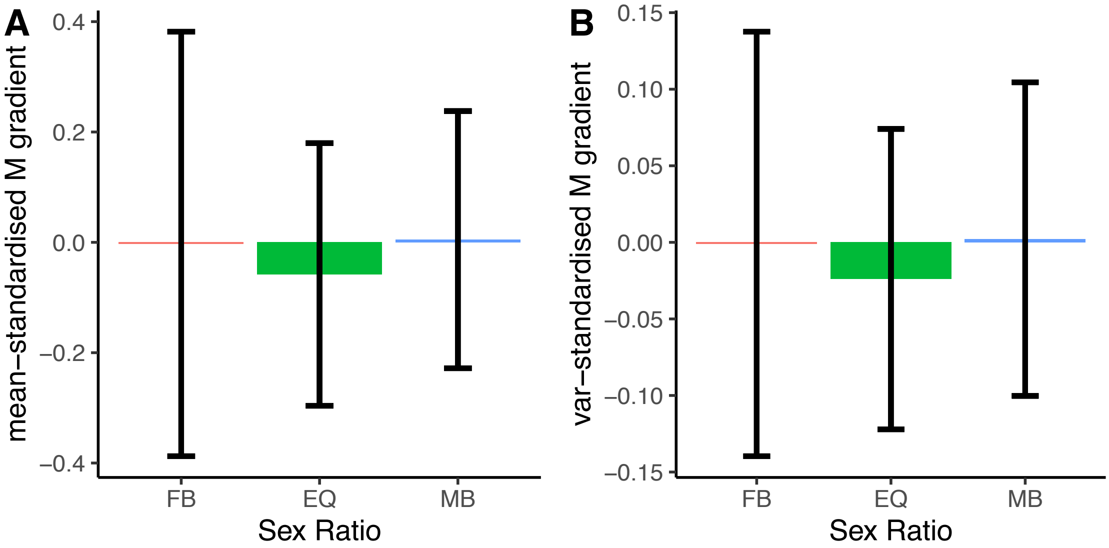


Figure 6

The influence of sex ratio on male mean-standardized (A) and variance-standardized (B) univariant gradient between mating success and reproductive success (the Bateman gradient and pre-copulatory s'max respectively). Error bars show confidence intervals.

Sex ratio influences sperm competition intensity, but not patterns of assortative mating

It is possible that the influence of sex ratio on sexual selection could be partly explained by changes in the patterns of mate sharing. For example, if mating occurs non-randomly, a male with high mating success might mate with the most polyandrous females, and therefore face the highest level of sperm-competition post-mating, or vice versa, and assortative mating patterns might alter with the sex ratio. To address this possibility, we first investigated how the polyandry level of a male's mates, which reflects the sperm competition intensity (SCI) he faces, influences his reproductive success and paternity share. As female polyandry was higher in male-biased conditions, we expected SCI to increase with the degree of male-bias, and our results were consistent with this. Across all males that obtained a mate, SCI was greatest in male-biased sex ratios (χ^2_2=32.9, p<0.001; fig.S6a), and a similar but non-significant trend was seen in focal males (F_2=2.0, p=0.145; fig.S6b). In all sex ratios, focal males facing a higher SCI experienced decreased reproductive success while controlling for mating success ($F_{1,47=}$17.91 p<0.001; fig.S6c; excluding sterile mates: $F_{1,45=}$17.0, p<0.001), but this relationship was not influenced by sex ratio (interaction: $F_{2,47=}$2.4, p=0.101; excluding sterile mates: $F_{2,45=}$ 2.348, p=0.107). Likewise, in all sex ratios, males facing a higher SCI experienced decreased paternity share

(χ^2_1=40.7 p<0.001; fig.S6d; excluding sterile mates: χ^2_1=39.4, p<0.001), but this relationship was not influenced by sex ratio (interaction: χ^2_2=3.0, p=0.220; excluding sterile mates: χ^2_2= 3.2, p=0.207). These results demonstrate that increased sperm competition reduces a male's paternity share and resultant reproductive success across all sex ratios. Then, to test whether more polygynous males are more or less likely to mate with more polyandrous females, and whether this relationship is influenced by the sex ratio, we assessed the correlation between a male's mating success and SCI, which reflects the polyandry of his mates (SCI correlation, SCIC; see methods (McDonald & Pizzari, 2016, 2018; Morimoto et al., 2019)). Across all males (focal and rival), we found evidence of a negative SCIC in male- and female-biased conditions, with similar trends in the equal sex ratio (MB: Estimate=-0.284±0.123, p=0.020; EQ: Estimate=-0.071±0.075, p=0.341; FB: Estimate=-0.188±0.062, p=0.003; fig.S6e), but we found no interaction between sex ratio and M (χ^2_2=1.1, p=0.586). This suggests that in all sex ratios, more polygynous males are more likely to mate with less polyandrous females (i.e. a negative SCIC), which should strengthen the correlation between a male's mating success and reproductive success because those with high mating success are more likely to enjoy lower sperm competition. However, we found no evidence for an influence of the sex ratio on this relationship, providing no support that the sex ratio shapes sexual selection by determining assortative mating patterns.

OVERALL DISCUSSION

Sexual selection theory predicts that as the sex ratio becomes increasingly biased, sexual selection should increase in the abundant sex, and we investigated this prediction using *Drosophila melanogaster*. In males, our results offered support to this hypothesis: we found that as the sex ratio becomes increasingly male-biased, the opportunity for sexual selection in males increases, largely due to greater variance in mating success, resulting in a greater maximum potential strength for pre-copulatory sexual selection. This did not translate into a greater reproductive advantage per unit increase in mating success in male-biased sex ratios (i.e. the Bateman gradient), suggesting that sexual selection didn’t reach its full potential strength in the males in this experimental system, and highlighting the importance of accounting for systematic differences in variance when measuring sexual selection intensity. Furthermore, among males that obtained a mate, we found evidence consistent with a greater maximum potential strength of post-copulatory sexual selection in the male-biased sex ratio, suggesting an increased importance of post-copulatory processes as increased rival density intensifies sperm competition. Thus, our results offer support to the hypothesis that male sexual selection increases as the sex ratio becomes more male-biased. Conversely, in females, we found no influence of the sex ratio on the variance in reproductive success, mating success, or the relationship between the two. We found no evidence of positive Bateman gradients, suggesting that increased female mating success did not increase reproductive success. This is consistent with our prediction that the intensity of selection arising from intra-sexual competition, and the degree to which it responds to OSR should be weaker in females, with mating competition playing less of a role in reproductive success. This might reflect differences in intra-sexual competition between the two sexes, with males largely competing for fertilisation opportunities and females for resources that increase reproductive success, meaning factors that influence selection arising from female-female competition might be less sensitive to the sex ratio.

A male-biased sex ratio increases sexual selection in males

A common assumption in the field of sexual selection is that the opportunity for and strength of sexual selection in males should increase as the sex ratio becomes more male-biased (Aronsen et al., 2013; Emlen & Oring, 1977; Kvarnemo & Ahnesjo, 1996; Weir et al., 2011). This has led to the sex ratio being used as a proxy for sexual selection when studying the evolution of sexual traits (Linklater et al., 2007; Nandy, Chakraborty, et al., 2013; Nandy, Gupta, et al., 2013; Reuter et al., 2008). Here, we provide empirical support for the relationship between the sex ratio and the operation of sexual selection in males.

In this system, the influence of the sex ratio on sexual selection was predominantly the result of increased variance in mating success, as reflected by I_s, decomposition of variance analysis, and variance-standardised M gradients (pre-copulatory s'max). The increased mating competition in the male-biased conditions resulted in 50% of focal males failing to obtain a mate, whereas this was reduced to ~10% in female-biased conditions. Because obtaining a mate determines whether a male can reproduce or not, this likely causes the greater relative variance in reproductive success between 'winners' and 'losers' in mating competition in male-biased conditions. An underlying assumption in the predicted relationship between sex ratio and pre-copulatory sexual selection is that mate monopolisation is high, allowing some individuals to obtain a high share of fertilisation opportunities at the expense of others (Emlen & Oring, 1977; Klug et al., 2010). In male *D. melanogaster*, high mate monopolisation is plausible as males form dominance hierarchies (Nilsen et al., 2004) with strong winner-loser effects (Penn et al., 2010; Yurkovic et al., 2006), and, in nature, losers of aggressive contests are excluded from mating sites, potentially allowing winners to monopolise mates (Markow, 1988).

Although variance-standardised selection gradients suggested that the increased variance in male mating success in male-biased conditions translated into a greater maximum potential for pre-copulatory sexual selection, this was not reflected in Bateman gradients (i.e. the univariate relationship between mating success and reproductive success), which did not differ between sex ratios. This could suggest that sexual selection did not reach its full potential strength in the males in male-biased conditions. Possibly, this might be a consequence of this closed system, meaning that increased mating success often resulted in mating with females that had frequently mated. This was increasingly likely in the male-biased sex ratio in which >70% females mated multiply compared to <40% in the female-biased sex ratio. Because females might suffer harm from frequent mating and male harassment (Arnqvist & Rowe, 2005; Carazo et al., 2014; Chapman et al., 1995; Wigby et al., 2009; Wigby & Chapman, 2005), this could prevent males from reaping the full potential reproductive advantage of increased mating. This is consistent with our findings of lower female productivity per mating in the male-biased sex ratio. Possibly, a male-biased sex ratio in an open system where there is a flux of individuals entering and leaving the population, such as asynchronous mate arrival (Emlen & Oring, 1977), might results in Bateman gradients better reflecting the maximum opportunity for pre-copulatory sexual selection, as each additional mating could be with a fully receptive, fertile female. However, in our closed study system, Bateman gradients fail to capture the increased potential for pre-copulatory sexual selection reflected in variance-standardised gradients. This highlights the importance of using a range of measures of sexual selection to obtain a full picture of this process (Aronsen et al., 2013; Janicke & Morrow, 2018; Klug et al., 2010).

In species where polyandry occurs, such as *D. melanogaster* (Imhof et al., 1998), sexual selection continues post-copulation (Collet et al., 2012; Collet et al., 2014; Kokko et al., 2012; Morimoto et al., 2016, 2019; Webster et al., 1995). Our results suggest that post-copulatory sexual selection plays only a small role in total sexual selection relative to pre-copulatory processes. This might be explained by the low polyandry in this system: across all sex ratios, females mated with ~1.5 males on average, and although polyandry was relatively higher in male-biased conditions, the absolute size of this increase was small and likely had only a small influence on sperm competition intensity. As mated females are likely to remain unreceptive to further mating for the duration of the observation period, as is shown by the low observed female re-mating rates, consistent with other studies (e.g., Chapman et al., 2003; Manning, 1962), we captured a small degree of female polyandry relative to studies over longer timescales or in which female polyandry is elevated via genetic manipulation (Morimoto et al., 2019). However, that said, we found evidence that among the males that obtained at least one mate, after accounting for differences in mating success and mate fecundity, the maximum opportunity for post-copulatory selection was greatest in male-biased conditions. This is consistent with our finding of increased sperm competition intensity in male-biased conditions, reflecting the ability for greater polyandry to increase the potential for post-copulatory sexual selection (Gay et al., 2009; Hosken & Ward, 2001; Morimoto et al., 2019).

The relationship between sex ratio and sexual selection in males is not reflected in females

Across all sex ratios, we detected no relationship between female mating success and reproductive success (i.e., neutral Bateman gradients), suggesting no evidence of selective forces on female mating rates. This is consistent with the expectation that sexual selection arising from mate competition is weak or absent on females and consistent with previous studies (Bateman, 1948; Jones et al., 2000). Possibly, over longer timescales, increased female mating might increase reproductive success in a moderately polyandrous species such as *D. melanogaster* (Chapman et al., 1995; Harshman & Clark, 1998; Imhof et al., 1998) if additional matings replace depleted sperm supplies (Manning, 1962). Alternatively, a longer timescale might result in a negative relationship between mating and reproductive success if cumulative costs of mating and harassment reduce female fitness (Carazo et al., 2014, 2015; Partridge & Fowler, 1990; Wigby & Chapman, 2005).

The lack of influence of the sex ratio on selection arising from intra-sexual competition in females might reflect the nature of female-female competition compared to that seen between males (Clutton-Brock, 2009; Stockley & Bro-Jørgensen, 2011). The higher energy demands of reproduction for females results in food resources, rather than mates, limiting female reproduction (Stockley & Bro-Jørgensen, 2011). While it is plausible that the sex ratio could influence the intensity of female-female resource competition (and, thus, sexual selection), the sex ratio directly changes the relative numbers of mates and same-sex competitors, with changes in resource availability only occurring as a by-product if resources are sex-specific and limited. Sex ratio manipulations in our study system are unlikely to impact female resource availability because food sources were ample, likely resulting in low female-female competition for resources required for reproduction across all sex ratios. Future work manipulating the sex ratio under nutritional limitation might capture other potential consequences of changes in the sex ratio on female-female resource competition.

It is possible that sexual selection arising from mating competition might operate on females in even more female-biased sex ratios The life history of some species includes periods in which the sex ratio is dramatically skewed towards females, resulting in sex role reversal with females becoming the dominant competitor for mating opportunities (Kvarnemo & Ahnesjo, 1996). Under such conditions, female competitive behaviours and variance in mating and reproductive success increase with the degree of female-bias (e.g. in pipefish, *Syngnathus typhle*, Aronsen et al., 2013; Vincent et al., 1994; Ahnesjö & Berglund, 1994). Because male *D. melanogaster* have a higher mating rate than females, females are likely to remain the limiting factor for male reproductive success even in our female-biased sex ratio, such that this sex ratio did not cause role reversal and female mate limitation: across all sex ratios, no females went unmated, and the average reproductive success per female remained constant. Although our sex ratios reflect those of natural popuations, in which populations rarely become more female-biased than 80% female (Markow, 1988), it is possible that if we skewed the sex ratio towards females to the extent that high quality sperm are limited, females might compete intensely over mates, increasing the operation of sexual selection in females (Stockley & Bro-Jørgensen, 2011). Future work using an extreme female-biased sex ratio might allow the exploration of fundamental properties of the influence of sex ratio on sexual selection that are generalisable across a wider range of populations and species (Morimoto et al., 2019).

Importantly, although we found no evidence of female pre-copulatory sexual selection arising from mating competition, processes analogous to sexual selection could still occur in females. Because female reproductive success is typically limited by ecological resources as opposed to mating opportunities, regressing mating success against reproductive success might fail to capture important aspects of female competition, and a broader framework of social selection might be more appropriate (Cain & Rosvall, 2014; Clutton-Brock, 2009; Tobias et al., 2012). Variation in female reproductive success might better correlate with traits related to a female's access to ecological resources, such as body size. In female *D. melanogaster*, a large body size signals a high quality developmental diet (Bath et al., 2018; Morimoto et al., 2016), can improve success in resource competition in adulthood (Bath et al., 2018), and increases fecundity (Lefranc & Bundgaard, 2000). A regression between female size and reproductive success might be a better metric of selection resulting from intra-sexual competition in females, analogous to traditional sexual selection in males.

CONCLUSIONS

Our results provide support for the predictions of traditional sexual selection theory in males, with increased sexual selection as the sex ratio becomes male-biased. However, there is a limit to the appropriateness of using the sex ratio as a proxy for the strength of sexual selection because, in our study, the increased potential for sexual selection did not translate into a steeper Bateman gradient. Thus, to get a complete understanding of how sexual selection operates in different ecological settings, it is important to measure a range of selection indices and take into account the reproductive biology of the species. In females, we found no evidence of a relationship between sex ratio and sexual selection, with sexual selection related to mate acquisition absent in the females in this system. This reflects the differences in female-female and male-male competition, illustrating the sex-specific nature by which the social environment can shape selective forces arising from intra-sexual competition.

METHODS

Fly populations

Flies were maintained at 25°C, uncontrolled humidity, on a 12:12h light:dark cycle. We used a Dahomey wild-type population and two mutant lines: one homozygous for the recessive w^{1118} allele, which is a loss-of-function allele for the *white* gene in the X chromosome that confers white eyes in homozygotes (hereon '*white'*), and another homozygous for the recessive *sparkling*^{poliert} allele (hereon '*spa'*), which produces a rough-looking eye phenotype (Fu et al., 1998). Stock populations for both mutant lines were created by serially back-crossing *w*^{1118} or *sparkling*^{poliert} alleles into Dahomey for 5 generations to standardise genetic backgrounds. Stocks were maintained on standard food media in large population cages with over-lapping generations.

Experimental flies

To generate experimental flies, we collected eggs from *white* and *spa* stock populations using yeasted grape-agar plates, and transferred them at a standard density to separate vials or bottles (50/vial or 200/bottle depending on experimental replica block) containing standard food media. We collected *white* males and females and *spa* males on ice as virgins, then housed them in same-sex vials of 10-20 individuals. To differentiate individuals in behavioural trials, we marked all flies with a small dot of acrylic paint on the dorsal thorax at 1-4 days post-eclosion under CO_2 anaesthesia. *spa* males were marked with a black dot and designated as 'focal' males. *white* males (designated as 'rival' males) and *white* females were randomly assigned a red, yellow, white, green or orange dot. Chi squared analysis showed no influence of paint colour on mating frequency (rival males:χ^2_4= 2.7, p = 0.618; females:χ^2_4= 1.3, p=0.858), consistent with previous work (Morimoto et al., 2016; Nilsen et al., 2004). After painting, we assigned flies to a sex ratio: male-biased (6M:2F), equal (4M:4F), or female-biased (3M:5F), and housed male and female counterparts of each group in same-sex vials until behavioural observations. Each male group contained one *spa* focal male with 5, 3 or 2 *white* rival males respectively. Group assembly design was based on (Bjork & Pitnick, 2006) and (Morimoto et al., 2016, 2019). As *spa* males sired red-eyed daughters, while *white* males sired white-eyed daughters, this enabled differentiation of focal male daughters, facilitating estimates of paternity (fig.S1).

Behavioural observations

We observed groups of flies for 4h per day for 3 consecutive days. We set up groups beginning at 0h Zeitgeber time on day 1 by aspirating 5-8 day old female and male counterparts of each sex ratio into new food vials containing yeast, forming groups of 8 flies of either male-biased, equal or female-biased sex ratios. During the observation periods, a single observer scanned vials for copulations, recording the colour of the flies involved. Each day, we separated males and females after observation using light CO_2 anaesthesia, housing the male counterparts together and female counterparts individually in yeasted egg-laying vials. We incubated egg-laying vials at 25°C for 12-14 days to allow offspring to eclose, before freezing them. We counted offspring, using the eye-colour of daughters to assess the paternity of focal and rival males. We performed the experiment over 3 blocks, with 27-28 groups per sex ratio after excluding vials in which individuals died or escaped.

Data analysis

Assessing the effect of male genotype on mating

Eye-colour markers were essential for paternity assessment, but impair visual acuity and might affect behaviour. Our experimental design should tolerate such behavioural differences as long as they are not too extreme. We assessed whether focal and rival male genotypes (*spa* vs *white*) influenced their mating frequencies using separate binomial tests for each sex ratio, and then used a quasibinomial general linear model (GLM) to test whether the magnitude of any differences between focal and rival male mating frequencies is influenced by the sex ratio.

Assessing the effect of sex ratio on mating and reproduction

We tested the influence of sex ratio on mating success (number of unique mates, M), mating frequency (total number of matings, including re-mating) and total reproductive success (total number of offspring (females) or daughters (males) in the 3 day period, T) for both males and females, and male paternity share (proportion of a focal male's mate's daughters sired by the focal male, P). We performed these analyses using poisson or, for P, binomial (or Quasi equivalents when data were over/under-dispersed) GLMs with sex ratio and block as fixed effects. For M, frequency and T, we performed these analyses on three response variables: for total values per vial (summed across all individuals of both sexes) to assess responses at a group level; for focal individual values per vial to assess behaviour at an individual level (assigning the female marked with yellow paint as the focal female); and for mean values per males or females per vial to test whether focal individual behaviours were representative of all individuals of that sex in the group. For P, we performed this analysis for focal males and included only vials in which the focal male mated because paternity share only applies post-mating (male-biased n=14, equal n=19, female-biased n=24). As T can vary with both mating frequency and the number of offspring produced per mating, and both might be influenced by the sex ratio, we also analysed the effect of sex ratio on total, average and focal reproductive success per mating, dividing T by mating frequency. This provides an estimate of the additional reproductive success gained per additional mating, a measure distinct from the Bateman gradient which estimates additional reproductive success per mate (Morimoto et al., 2016).

Mating success captures both the number of mates an individual obtains and whether or not an individual managed to obtain a mate at all. As this latter component is integral to reproductive success, we assessed the influence of sex ratio on the probability of mating in a quasibinomial GLM, including block as a fixed factor, and used post-hoc Tukey tests for pairwise comparisons between sex ratios. No females failed to mate so this analysis was only performed for males.

Assessing the effect of sex ratio on the opportunity for sexual selection

We assessed the influence of sex ratio on the total opportunity for sexual selection (I, the standardised variance in reproductive success ($var(T)/(mean(T))^2$) for both sexes. Because variance in reproductive success can depend on variance in mating success for both sexes, and variance in paternity share and mate fecundity for males (N, the average fecundity of a male's mates), we also assessed the influence of sex ratio on the opportunity for pre-copulatory sexual selection (I_s, the standardised variance in mating success, $var(M)/(mean(M))^2$) for all males and females, and the opportunity for post-copulatory sexual selection (I_p, the standardised variance in paternity share, $var(P)/(mean(P))^2$) and the opportunity for sexual selection on mate fecundity (I_n, the standardised variance in mate fecundity, $var(N)/(mean(N))^2$) for focal males that mated. All opportunity for selection indices were calculated using data from the focal individual of each sex per vial. However, for I_s and female I, we could also assess whether patterns detected in focal individuals were representative of all individuals of that sex by calculating I_s and female I for each individual in the vial. To test whether these indices significantly differed between sex ratios, we generate 95% confidence intervals from 1000 bootstrapped iterations of the data (bootstrap package, Canty & Ripley, 2020; Davison & Hinkley, 1997). To control for block, we generated these replications for each block individually and then combined them, mirroring the way data were collected. We considered groups significantly different when there was no overlap of 95% confidence intervals.

To qualitatively assess how the relative variance in M, P and N, and their covariances, contribute to variance in reproductive success, T, we decomposed the variance in reproductive success into these relative components, by calculating the percentage of var(T) that can be accounted for by var(M), var(P), var(N) and their covariances and an error term ε for each sex ratio treatment (Bohrnstedt & Goldberger, 1969; Collet et al., 2012; Morimoto et al., 2016; Webster et al., 1995). We used mean-standardised values for T, M, P and N to allow comparison between these indices. We included all vials in calculations of the variance in T and M, and only vials in which the focal males mated in calculations of the variance in P, N, and all covariances. Variation among replicate blocks would have contributed to the error term (ε).

We found two vials, both in the equal sex ratio, in which focal males mated only with females that produced one or no offspring, giving these males an N of 0 or 1. These values fell below 2 standard deviations of mean N. These data points might result from random variation in female fecundity that is not an effect of sex ratio. Both instances occurred in the same sex ratio and thus could cause systematic bias in the results. As a result we repeated all opportunity for selection analysis and further analysis which involve focal male reproduction while excluding these vials.

Assessing the effect of sex ratio on the strength of selection

To assess how pre- or post-copulatory processes translate into reproductive success for focal individuals, we calculated standardised univariate M gradients (Bateman gradients and pre-copulatory s’max) and standardised multivariate selection gradients using linear models. Multivariate gradients allow us to measure the effect of each of M, P and N on T while controlling for the effect of the other indices and their covariances and thereby accounting for interactions between them. Multivariate analysis is not applicable to males who did not obtain a mate so were conducted only for males that mated. As the ability to obtain a mate is a key component of mating success, it was necessary to use univariate male M gradients in addition to multivariate analysis in order to capture the full variation in M. We calculated selection gradients using both mean-standardised and variance-standardised indices. Variance-standardised analysis takes into account both the variance in M/P/N and how they relate to T, and estimates the maximum potential strength of sexual selection (Arnold, 1994; Collet et al., 2012;

Jones, 2009; Morimoto et al., 2016, 2019). Variance-standardisation was performed for each of M, P and N by subtracting the mean value from the i^{th} value, and dividing by the standard deviation. In all univariate and multivariate regressions, we included block as a fixed factor and mean-standardised total vial productivity (TVP) as a covariate to account for variation in female fecundity between vials. However, as TVP and N showed a strong positive correlation, which could mask the effects of N on T, we replaced TVP with 'residual TVP' (the residuals from a linear model of mean- or variance-standardised N (depending on whether we were fitting mean- or variance-standardised selection gradients) on mean-standardised TVP, representing the remaining variance in TVP after N had been accounted for) in all multivariate regressions. Although mating data could be standardised within vials, paternity share, reproductive success and mate productivity could only be standardised across vials. Therefore, to ensure all metrics were comparable, for all sexual selection gradients, all indices (M,N,P,T and TVP) were standardised across vials within each sex ratio (Morimoto et al., 2019). To test whether sex ratio influenced the strength of these selection gradients, we added sex ratio as an interaction term with M, N and P in our regression model.

Because sexual selection gradients are normally measured as linear regressions, all multivariate and univariate gradients were calculated from gaussian linear models, but when these regressions included interaction terms between the selection indices and sex ratio, we assessed model fit using diagnostic plots, and where data were over- or under-dispersed, we used GLMs with quasipoisson (for count data) and quasibinomial (for proportion data) distributions.

The relationship between the sex ratio and sexual selection might partly result from sex ratio influence on assortative mating. For example, if more polygynous males preferentially mate with more polyandrous females, they will face higher sperm competition, which might reduce the reproductive benefits accompanying increased mating success. Alternatively, if more polygynous males preferentially mate with less polyandrous females, they will face reduced sperm competition, increasing the reproductive benefits accompanying increased mating success. Therefore, we first calculated the sperm competition index (SCI), an estimate of the polyandry of a male's mates, as the reciprocal of 1/M*(the sum of $1/M_{mate}$ for all a male's mates), where M is a male's mating success, and M_{mate} is mating success of each of a male's mates. If a male has multiple mates, 1/Mmate of each mate is summed together (McDonald & Pizzari, 2016; Morimoto et al., 2019; Shuster & Wade, 2003). We calculated SCI for both focal males that mated and all males that mated (focal and rivals). We first investigated the influence of sex ratio on SCI while controlling for block, using a linear model for focal male data, and a linear mixed effect model for all male data (square-root transformed to improve model fit), controlling for vial as a random effect to account for using data from multiple males per vial. To assess how SCI relates to reproductive success, we used linear models to calculate the influence of SCI on T while controlling for M, TVP and block. To assess how SCI relates to post-copulatory competition, we used quasibinomial GLMs to calculate the influence of SCI on P while controlling for TVP and block. We calculated both of these regressions for each sex ratio individually, and then for focal males of all sex ratios, including sex ratio as an interaction term with SCI to test for the influence of sex ratio on these relationships. For both regressions, all indices were mean-standardised across vials within sex ratio treatments to facilitate comparison among treatments. Next, to assess the relationship between a male’s polygyny and the polyandry of his mate(s), we calculated the SCI correlation (SCIC) in linear mixed effect models of mean-standardised SCI on mean-standardised M, while controlling for block as a fixed effect and vial ID as a random effect, for all mated males (focal and rival) in each sex ratio individually, and then for all mated males from all sex ratios, including sex ratio as an interaction term with standard M to test for an influence of sex ratio on this correlation.

SUPPLEMENTARY MATERIAL

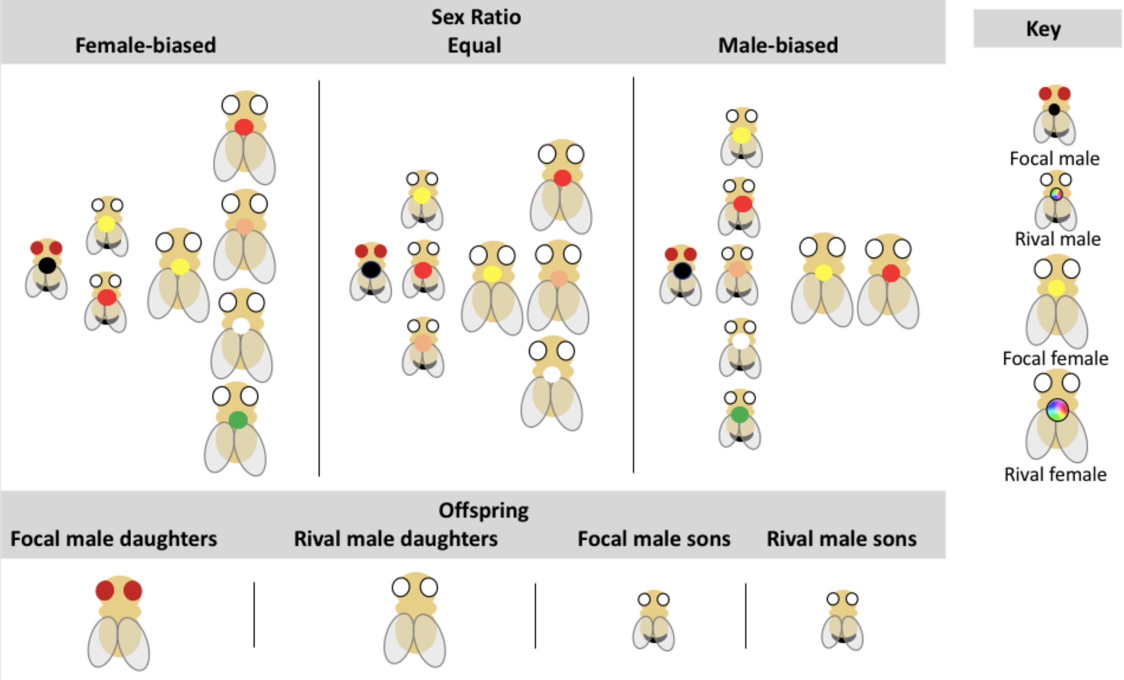


Figure S1

A schematic representation of the experimental set up. The top panel illustrates the sex ratio treatments. Coloured dots represent paint marking. Focal males are homozygous for the recessive sparkling^{poliert} allele, producing rough-looking red eyes (spa), rival males and all females are homozygous for the recessive w^{1118} allele, producing white eyes (white). The bottom panel illustrates offspring eye colour. All sons have white eyes, while daughters have red or white eyes depending on their paternity, allowing us to track the paternity of the focal male.

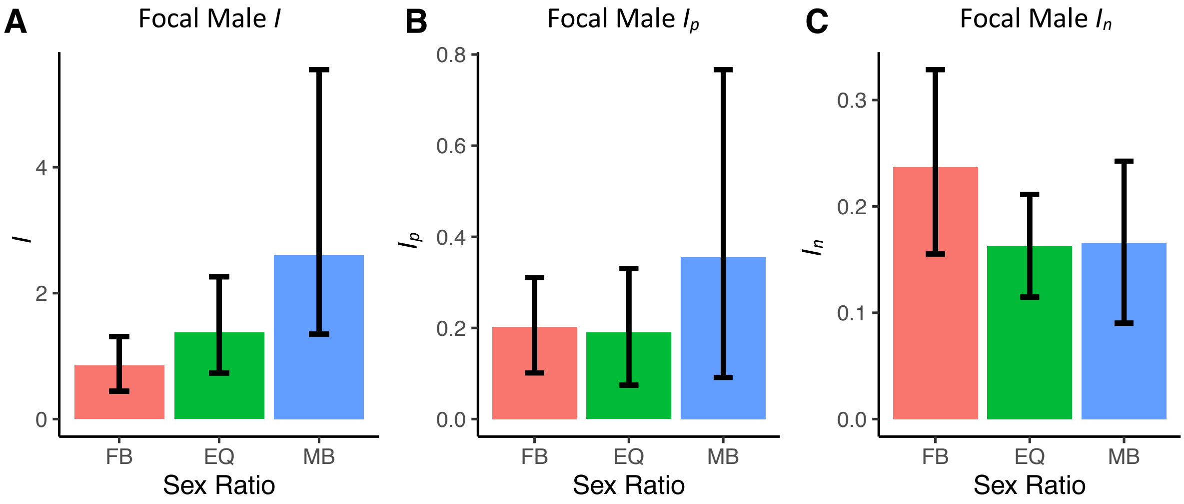


Figure S2

The influence of sex ratio on the opportunity for male total sexual selection, I (A), post-copulatory sexual selection, I_p *(B), and the opportunity for selection on male mate choice,* I_n *(C) excluding males with sterile mates. Error bars show bootstrapped 95% confidence intervals.*

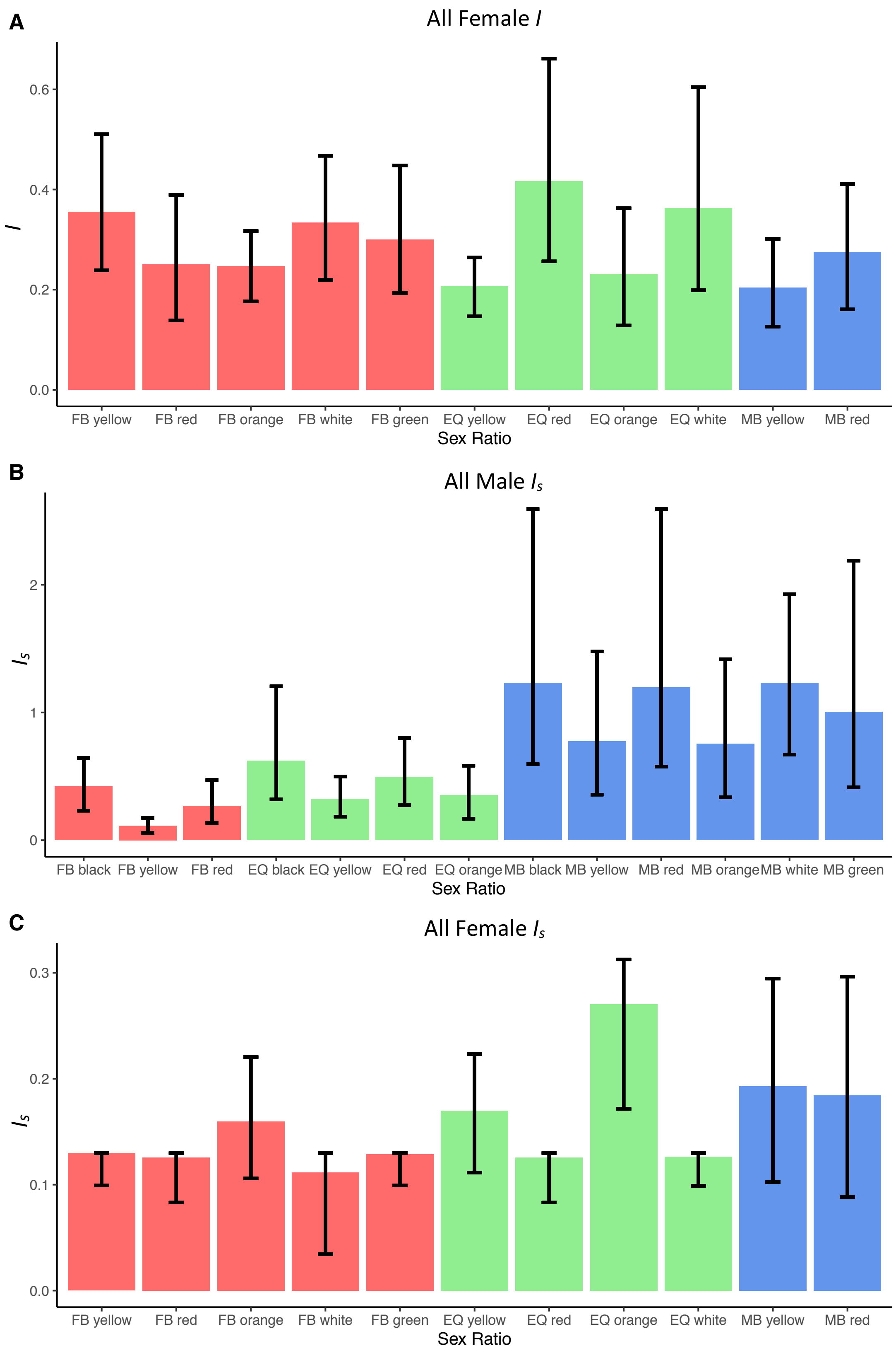

A
All Female I
I
0.0
0.2
0.4
0.6
FB yellow
FB red
FB orange
FB white
FB green
EQ yellow
EQ red
EQ orange
EQ white
MB yellow
MB red
Sex Ratio
B
All Male Is
Is
0
1
2
FB black
FB yellow
FB red
EQ black
EQ yellow
EQ red
EQ orange
MB black
MB yellow
MB red
MB orange
MB white
MB green
Sex Ratio
C
All Female Is
Is
0.0
0.1
0.2
0.3
FB yellow
FB red
FB orange
FB white
FB green
EQ yellow
EQ red
EQ orange
EQ white
MB yellow
MB red
Sex Ratio

Figure S3

The total opportunity for sexual selection, I, in females (A), and the opportunity for pre-copulatory sexual selection, I_s, in males (B) and females (C) for all individuals (identified by paint colour). Red bars represent male biased vials, green bars represent equal vials, blue bars represent female-biased vials. Error bars show bootstrapped 95% confidence intervals

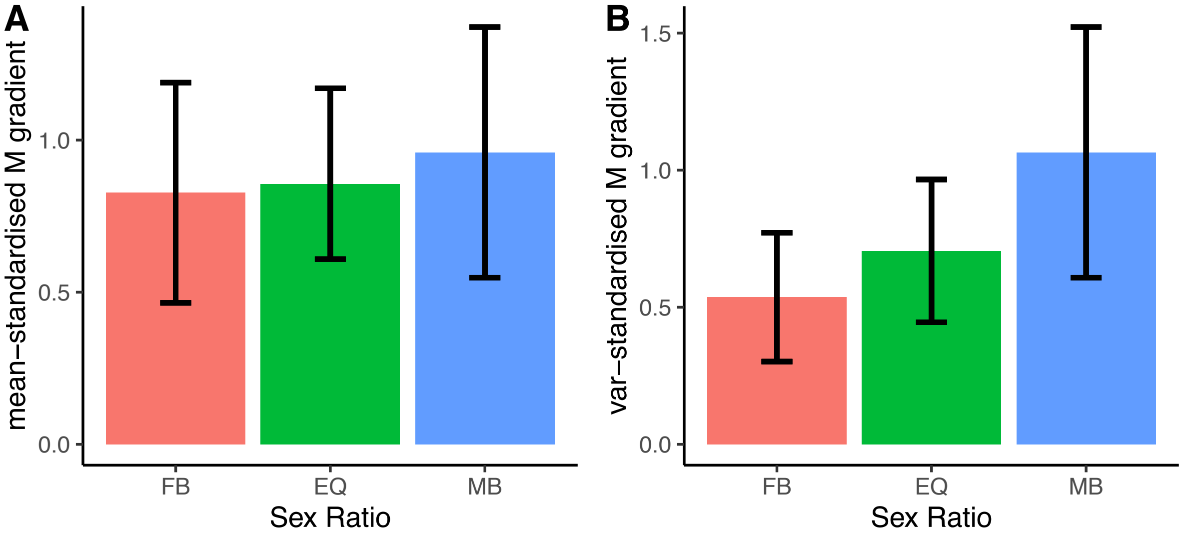


Figure S4

The influence of sex ratio on male mean-standardized (A) and variance-standardized (B) univariate gradient between mating success and reproductive success (the Bateman gradient and pre-copulatory s'max respectively) excluding males with sterile mates. Error bars show confidence intervals.

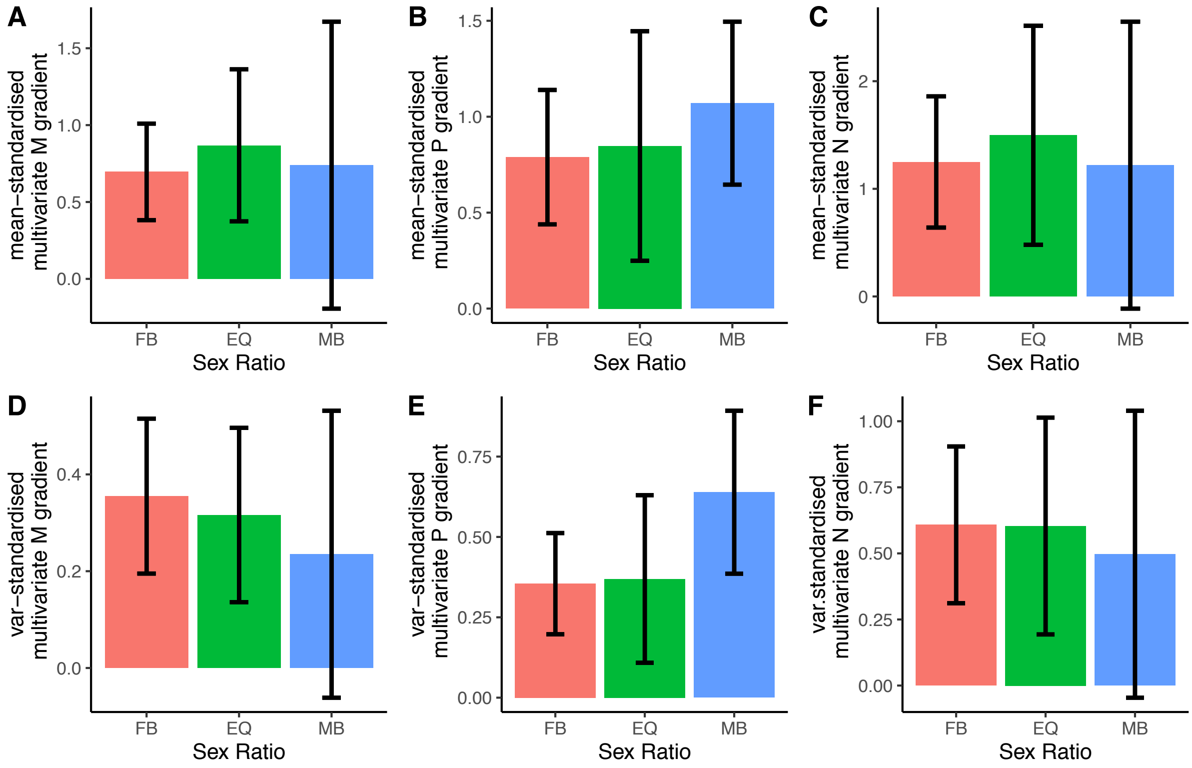


Figure S5

The influence of sex ratio on male mean-standardized (A-C) and variance-standardized (D-F) multivariate gradients between mating success (A&D), paternity share (B&E), and mate fecundity (C&F) and reproductive success, while controlling for all other indices, their covariances and replica block. Gradient apply only to males that obtained at least 1 mate, and males with sterile mates are excluded. Error bars show confidence intervals.

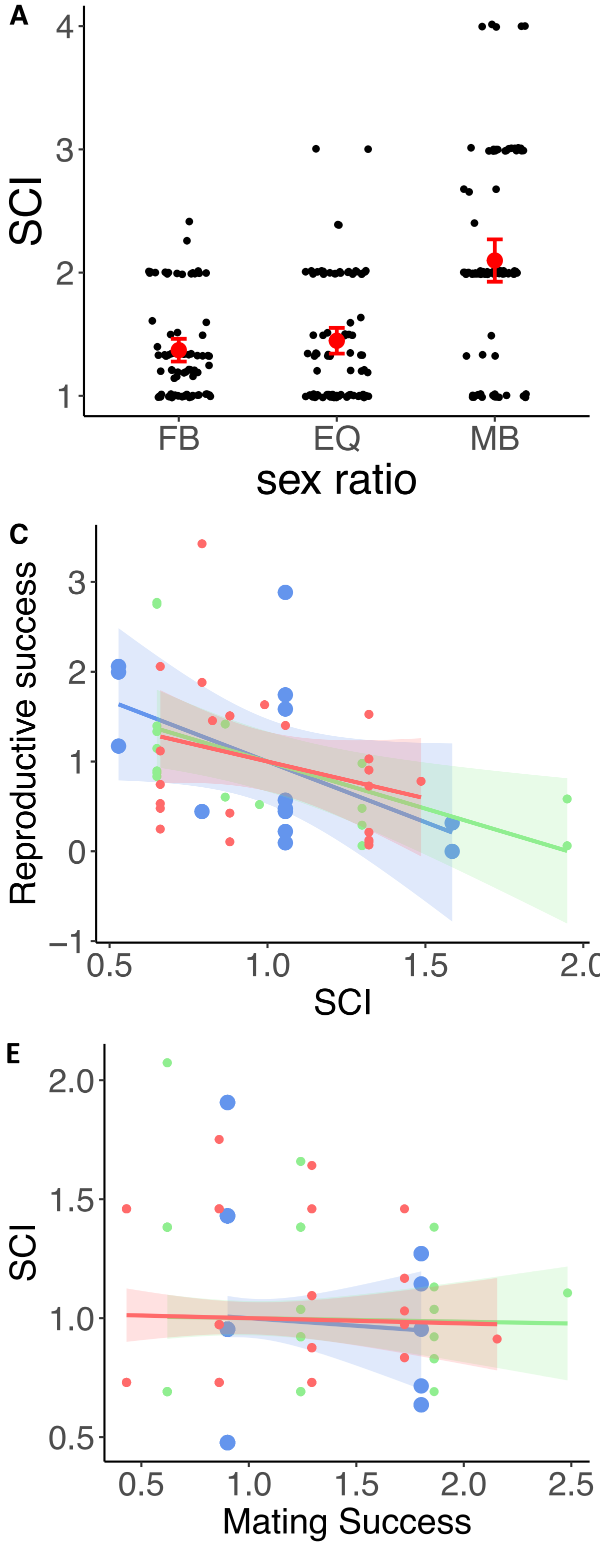


Figure S6

The influence of sex ratio on (A-B) sperm competition intensity (SCI) of all males (A) and focal males (B), (C) the relationship between focal male SCI and *reproductive success, T, (D) the relationship between focal male SCI and paternity, and (E) the relationship between mating success, M, and SCI for all males (i.e. the sperm competition intensity correlation, SCIC, showing the relationship between male polygyny and the polyandry of his mates). In (c-f), red lines and points show FB, green show EQ, blue shows MB. Shaded areas show confidence intervals.*

Supplementary table 1

The mating frequencies (F) of spa focal males and white rivals in each sex ratio relative to expected mating frequencies if matings were distributed evenly among all rival and focal males in accordance to their ratio.

Sex ratio	Focal: rival ratio	Focal male F	Rival male F	% focal male F	Expected % focal male F	Binomial P values
MB	1:5	16	100	13.8	16.7	0.457
EQ	1:3	30	145	17.1	25.0	0.018
FB	1:2	52	139	27.2	33.3	0.078

Supplementary table 2

The % of focal and total males that obtained at least 1 mate in each sex ratio

Sex ratio	% focal males successful in mating	% total males successful in mating
MB	50.0	54.2
EQ	70.4	83.3
FB	88.9	93.8

Chapter 3: Sex ratio and the evolution of aggression in fruit flies

ABSTRACT

Aggressive behaviours are among the most striking displayed by animals, and aggression strongly impacts fitness in many species. Aggression varies plastically in response to the social environment, but we lack direct tests of how aggression evolves in response to intra-sexual competition. We investigated how aggression in both sexes evolves in response to the competitive environment, using populations of *Drosophila melanogaster* that were experimentally evolved under female-biased, equal, and male-biased sex ratios. We found that after evolution in a female-biased environment – with less male competition for mates – males fought less often on food patches, although the total frequency and duration of aggressive behaviour did not change. In females, evolution in a female-biased environment – where female competition for resources is higher – resulted in more frequent aggressive interactions among mated females, along with a greater increase in post-mating aggression and food patch occupation. These changes in female aggression could not be attributed solely to evolution either in females or in male stimulation of female aggression, suggesting that co-evolved interactions between the sexes determine female post-mating aggression. We found evidence consistent with a positive genetic correlation for aggression between males and females, suggesting a shared genetic basis. This study demonstrates the experimental evolution of a behaviour strongly linked to fitness, and the potential for the social environment to shape the evolution of contest behaviours.

INTRODUCTION

Aggressive contests occur in males and females across diverse animal taxa (reviewed by Clutton-Brock, 2007). The nature of aggressive contests differs between the sexes: males largely compete for reproductive opportunities and females largely for reproductive resources (Tobias et al., 2012). Because aggression significantly impacts fitness in both sexes (Cain & Ketterson, 2012; Clutton-Brock & Huchard, 2013; Haley, 1994), aggressive contests form an important part of reproductive competition (Hoffmann, 1987a, 1987b; Stockley & Campbell, 2013). Hence, the intensity of reproductive competition in a population should determine the strength of sexual and social selection on aggressive behaviours (Fitze & Le Galliard, 2008; Tobias et al., 2012; Weir et al., 2011).

More intense reproductive competition is predicted to lead to heightened aggression (Kvarnemo & Ahnesjo, 1996). This prediction has received empirical support. For example, comparative studies of chernetid false scorpions and dung beetles have found that the presence and size of male weapons is positively correlated with population density and degree of male bias in the sex ratio across species (Pomfret & Knell, 2008; Zeh, 1987). Behavioural studies have reported increased aggression in the sex in excess within populations in fish (Grant & Foam, 2002; Kvarnemo et al., 1995). However, comparative studies cannot eliminate the possibility that variation in aggression is due to other factors that covary with the intensity of competition. Likewise, behavioural studies do not show how the competitive environment shapes diversity in aggression across groups. Hence, direct tests of how aggression evolves in response to the intensity of competition are lacking.

An additional challenge to studying adaptive variation in aggression is that male and female aggression might be constrained by their shared genome, preventing either or both sexes from reaching their optimum (Bonduriansky & Chenoweth, 2009). Indeed, intra-sexual aggression has sometimes been considered a predominantly male trait, with female aggression assumed to arise as a by-product of an intersex genetic correlation (Cain & Ketterson, 2012, and references therein). Recently, female-female aggression has gained attention as an adaptive strategy for maximising access to resources required for reproduction (Rosvall, 2011; Stockley & Campbell, 2013), leading to improved reproductive success or offspring survival (Ketterson et al., 2005; Sinn et al., 2008; Stockley & Bro-Jørgensen, 2011). However, we currently lack data on the independence of the evolution of aggression in each sex.

Beyond constraints through the shared genome, female aggression might also depart from the female optimum if female behaviour is subject to manipulation by males (Perry & Rowe, 2015). In polygynous mating systems, the optimal level of female-female aggression will be higher for males than for females whenever female aggression confers immediate reproductive benefits that both mating partners experience, but incurs longer-term costs to females in lifetime reproduction. Mating offers males an opportunity to influence female behaviour through ejaculate transfer, and ejaculate-stimulated changes in female behaviour are well-documented (Gillott, 2003). In several species, shifts in female aggression are associated with mating (Mainardi et al., 1996; Seebacher et al., 2013; Sinn et al., 2008). Overall, because female aggression has been under-researched relative to male aggression, key facets of the evolution of female aggression, including sexual conflict, the intersex genetic correlation, and responses to intra-sexual competition, are not yet fully understood.

Here, we used experimental evolution to ask how male and female aggression evolve in response to the intensity of intra-sexual competition. We used replicate populations of fruit flies, *Drosophila melanogaster*, exposed to different competitive environments for >75 generations via manipulation of the population sex ratio, a common proxy for the intensity of competition (Kvarnemo & Ahnesjo, 1996; Kvarnemo & Simmons, 1999; Linklater et al., 2007). Aggression is heritable in *D. melanogaster* and can evolve rapidly under laboratory conditions (Dierick & Greenspan, 2006). Both sexes engage in contests over food patches. For females, food patches provide nutrition required for egg production (Jensen et al., 2015). For males, which display limited adult feeding (Carvalho et al., 2006), food patches predominantly provide access to mates (Chen et al., 2002; Dow & von Schilcher, 1975; Hoffmann, 1987a, 1987b). Both sexes display aggressive behaviours including fencing, male lunging, and female headbutting ((Hoffmann, 1987b; Nilsen et al., 2004); Table S1). Mating increases female aggression (Bath, Biscocho, et al., 2020; Nilsen et al., 2004) due to the effects of sperm and seminal fluid proteins received at mating (Bath et al., 2017). Therefore, changes in female aggression could represent a response to evolved differences in male stimulation of aggression – mediated by sexual conflict – as well as the direct evolution of female behaviour.

We addressed the following questions: Does the evolutionary sex ratio drive the evolution of male and female aggression? Does the evolutionary sex ratio affect the post-mating increase in female aggression? Is there evidence for a genetic correlation between male and female aggression? We predicted, first, that males and females evolving in a population biased towards their sex would display heightened aggression. Second, if increased aggression after mating is adaptive for females, then we expected a greater increase in aggression after mating in females from female-biased populations. Third, if female aggression responds to the sex ratio through female adaptation, then we expected that sex ratio effects would occur when experimentally-evolved females mated with males from stock populations, whereas if female aggression responds to the sex ratio through male adaptation to the sex ratio, then we expected that experimentally-evolved males would induce altered aggression in female mates from stock populations. Finally, if the sexes share a genetic basis for aggression, then we expected congruent changes in aggression across populations.

METHODS

Overview

We conducted two experiments. First, we measured intra-sexual aggression in virgin females, mated females, and mated males that had evolved under male-biased, equal and female-biased evolutionary sex ratios (the ‘coevolved experiment’). In this experiment, all mated individuals were mated with partners from the same replicate population. We tested both virgin and mated females because females show a distinct increase in aggression post-mating (Bath et al., 2017; Nilsen et al., 2004), but tested only mated males because, to our knowledge, male aggression does not change with mating. We then conducted a second, two-stage experiment to test whether differences in female aggression among sex ratio treatments arise from the evolution of female aggression itself or of male stimulation of female aggression. To do this, we mated experimentally-evolved females with stock males (the ‘evolved female experiment’), and stock females to experimentally-evolved males (the ‘evolved male experiment’), and measured female aggression before and after mating. The stock population was derived from the same wild-type Dahomey background from which the experimentally-evolved populations were generated and was maintained in a large population cage with overlapping generations. Experimentally-evolved flies were maintained under male-biased, equal and female-biased sex ratios, with 3 independent replicate populations for each sex ratio. The experimental evolution protocol is detailed in the supplementary methods (see also Rostant et al., 2020). We assayed behaviour after 78 generations for the coevolved experiment and 92 generations for the evolved female and evolved male experiments. All fly husbandry and experiments were conducted at 25^{o}C on a 12:12h light:dark cycle with uncontrolled humidity.

Generation of experimental flies

We collected eggs from each of the 9 replicate populations and the stock population using grape-agar plates. To ensure a standard larval density, we transferred 12.5µl of eggs suspended in phosphate-buffered saline solution into separate bottles containing 45ml standard laboratory medium (Clancy & Kennington, 2001).

At eclosion (day 1), we collected virgin flies under ice anaesthesia and placed them in vials containing standard laboratory medium. All flies used in aggression trials were housed singly. Males that were used as mates only (in the evolved female and evolved male experiments) were housed in pairs. We randomly assigned females to either the virgin or the mated treatment. Females assigned to the virgin treatment were housed singly in vials and transferred to new vials on day 3 after eclosion (to mirror how mated females were handled). On day 3, we transferred pairs of males and females (those assigned to the mating treatment) from the same replicate population into fresh vials by gentle aspiration and observed them until mating, recording mating latency and duration. We discarded pairs that did not mate within 3h. To allow only a single mating, we separated pairs into individual vials when copulation ended.

Aggression Trials

On day 4, we placed flies of all treatments singly into food deprivation vials containing only damp cotton wool for 2h to increase aggressive motivation. We randomly assigned flies to a same-sex dyad, with both flies in the dyad coming from the same replicate population and mating status (N=10-29 per replicate population per treatment for the co-evolved experiment (Table S2), 15 per replicate population per treatment for the evolved female experiment, and 25 per replicate population per treatment for the evolved male experiment) to standardize the difference between competitors within a contest and to expose individuals to the type of competitor encountered in their recent evolutionary history. We transferred dyads into observation chambers (20mm diameter, 5mm depth) containing a central food cup (5mm diameter, standard laboratory medium and live yeast paste). We allowed dyads 5 minutes to acclimatise before aggression trials of 15 minutes began, in which we video-recorded behaviours (Toshiba Camileo X400 cameras). We observed each dyad once, randomly assigning each dyad a trial time between 2-6h Zeitgeber time.

Behavioural data extraction

All videos were scored by observers blind to treatment group using event-logging software JWatcher v.1.0 (Macquarie University & UCLA) and BORIS v.7.7.3 (Friard & Gamba, 2016). We recorded aggressive behaviours as described in Table S1. To avoid pseudoreplication, the dyad was taken as the unit of replication, with behaviour measures summed for the two individuals. Lunging, chasing and tussling (in males) and headbutts (in females) are classed as high-intensity aggression, and fencing in both sexes represents low-intensity aggression (Chen et al., 2002). We calculated a total male high-intensity aggression score by summing the amount of time each dyad spent lunging, chasing and tussling. Because a food patch can represent a breeding territory for males (Hoffmann & Cacoyianni, 1990; Markow, 1988), and an attractive nutritional resource for females (Bath et al., 2017; Nilsen et al., 2004), we calculated food patch occupancy as the average duration the two flies in a dyad spent on the food patch so that we could assess the relationship between aggression and resource defence. We recorded the sum of the duration the two flies in a dyad spent walking, allowing us to test for locomotor differences that might influence aggression. For females, all videos were scored for headbutts as the main high-intensity aggressive behaviour (Bath et al., 2017; Nilsen et al., 2004). A subset was also scored for female fencing so that we could assess whether differences extended to low-intensity aggression (the time-intensive nature of extracting low-intensity aggression data prevented us from assaying it in the full dataset).

Statistical analyses

Statistical analyses were carried out in R (version 3.6.2 (2019-12-12)), using the packages ‘MASS’ (Venables & Ripley, 2002) ‘emmeans’ (Lenth, 2020), ‘lme4’

(Bates et al., 2014), ‘survminer’ (Kassambara et al., 2020) and ‘coxme’ (Therneau, 2020).

For all experiments, we ran linear mixed effects models (LMMs; lme4 *lmer()* function) to test the influence of the evolutionary sex ratio on the number of lunges (in males) or headbutts (in females), fencing duration, intense male aggression duration, locomotion duration and food patch occupancy. We ran binomial general linear mixed effect models (GLMMs) to test the influence of evolutionary sex ratio on the proportion of male total aggression (fencing, chasing, lunging and tussling) or female headbutting performed on the food patch. For models of female behaviour in the coevolved and evolved female experiments, we included evolutionary sex ratio, mating status, their interaction, and observer as fixed factors. For models of male behaviour in the coevolved experiment, we included evolutionary sex ratio as a fixed factor (a single observer extracted data for males). All models included replicate population and day as random factors and Zeitgeber time as a covariate. For the evolved male experiment, we had a single virgin female treatment and three mated female treatments (i.e., stock females mated to males from each evolutionary sex ratio). We first assessed the effect of mating on aggressive and food occupancy behaviours in an LMM that included mating status as a fixed factor. For mated females, we then ran a model including evolutionary sex ratio as a fixed factor. Both models included replicate population and day as random factors and Zeitgeber time as a covariate. We found no influence of evolutionary sex ratio on mating latency or duration (Table S3), and hence no evidence that differences in aggression resulted from differences in mating behaviour among treatments, so we did not include mating behaviour as a covariate in any models.

When we found an effect of evolutionary sex ratio on food patch occupancy, we investigated the relationship between aggression and food patch occupancy. We used binomial general linear mixed models as described above to test whether the individual that performs the greatest proportion of total aggression (in males) or headbutts (in females) within a dyad also spends the highest proportion of time on the food patch, and whether this relationship was influenced by evolutionary sex ratio. Individuals that performed an equal amount of aggression (16 male dyads, 24 female dyads) were excluded from this analysis.

For all analysis, we checked for outliers by visual inspection of boxplots or, where data were non-normally distributed, adjusted boxplots (Hubert & Vandervieren, 2008). We assessed the influence of outliers by re-running models on a winsorised dataset (replacing points outside 1.5* the interquartile range with the value of the lower or upper 1.5*interquartile range, respectively (Khan et al., 2007; Wilcox, 2005)), which revealed no qualitative impacts of outliers on analyses. Therefore, all reported results are from analyses of the non-winsorised data. For all models, we examined model fit by inspection of diagnostic plots, and where necessary, applied transformations. We analysed LMMs with Wald F tests with Kenward-Roger degrees of freedom (Arnqvist, 2020) (type III for models with significant interactions, type II for models without significant interactions), and analysed GLMMs with Wald χ^2 tests. In female models, when we found a significant interaction between the evolutionary sex ratio and mating status, we re-ran models separately for virgin and mated females to better understand effects of the evolutionary sex ratio within each group. When evolutionary sex ratio was significant, we explored the effect using post-hoc Tukey tests. For females, we compared the magnitude of the post-mating changes in behaviours among evolutionary sex ratios using post-hoc effect size tests.

To explore whether the evolution of sex-specific aggression might be constrained by a shared genetic basis between the sexes, we assessed the correlation between the aggressive behaviour of males and females that evolved in the same replicate population, using data from the coevolved experiment. A positive correlation might arise from a shared genetic basis, from similar effects of the time and day of behavioural observations in both sexes, or from congruent evolution in response to the evolutionary sex ratio. To control for the influence of time and day (and observer, for female data for which multiple observers were involved) on variation in aggression among vials, we ran linear models of lunging, headbutting and fencing against time and day (and observer, for female data), and used model residuals to calculate a mean behaviour score for males, virgin females, and mated females for each replicate population (N=9). We controlled for effects of the evolutionary sex ratio on variation in aggression among replicate populations by extracting the residuals from linear models of these 9 data points against evolutionary sex ratio. We used residual values from this model to test for correlations in aggression (female headbutts and male lunges, and fencing in both sexes) between males and virgin females and males and mated females. We tested for a correlation between virgin and mated female aggression to assess evidence for a shared genetic basis to female aggression pre- and post-mating.

RESULTS

Male aggression and food patch occupancy

We detected no significant influence of the evolutionary sex ratio on the frequency of lunges ($F_{2,6.0}=1.3$, $p=0.339$, square root transformed data; Fig. 1A), the duration of high-intensity aggression (chasing, lunging and tussling; $F_{2,6.0}=1.4$, $p=0.317$, log-transformed data), or the duration of low-intensity fencing ($F_{2,6.0}=3.8$, $p=0.085$, log-transformed data).

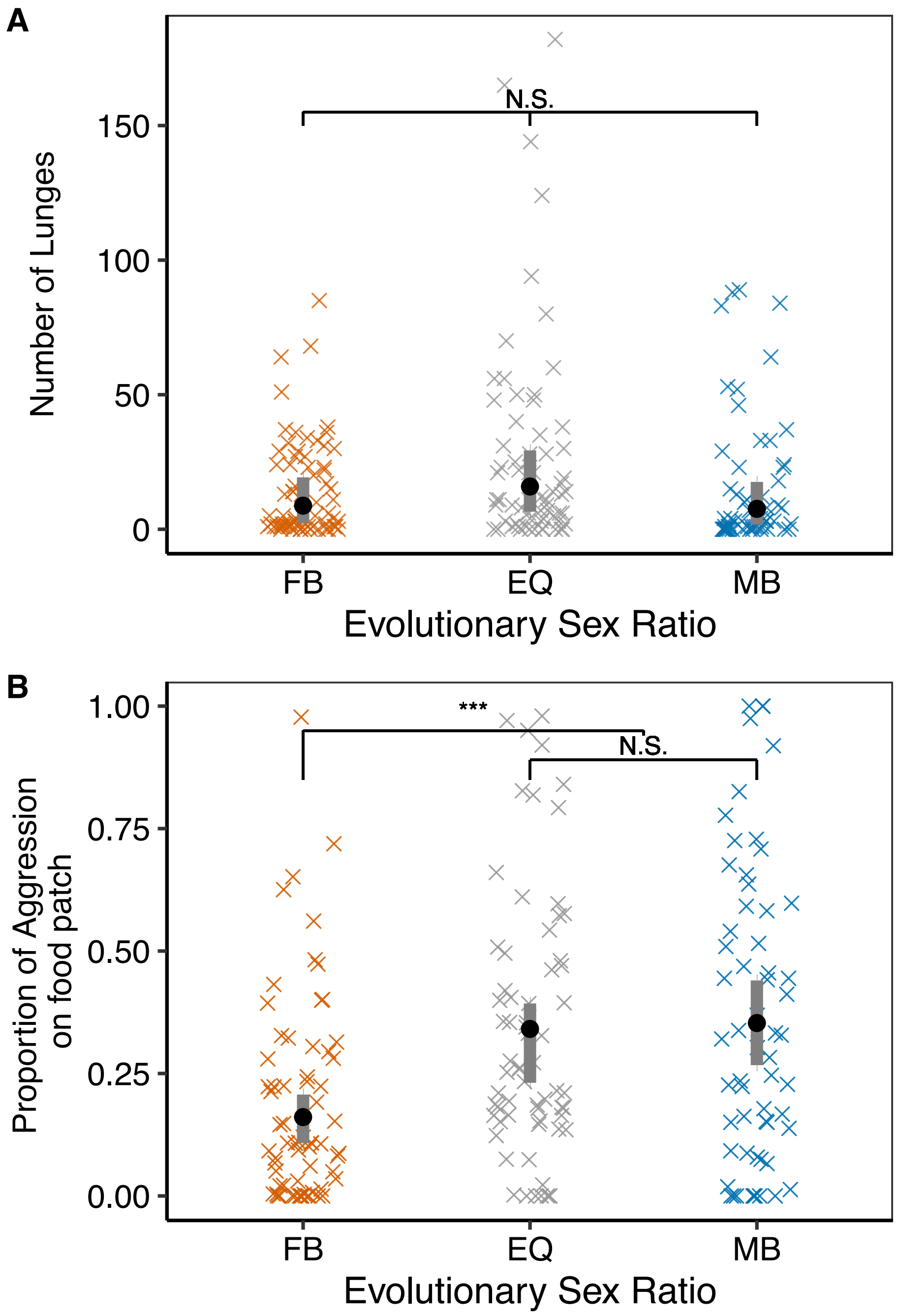


Figure 1: Male aggressive behaviour in the coevolved experiment

*Male aggressive behavior after experimental evolution at female-biased (FB), equal (EQ), or male-biased (MB) sex ratios: lunging (A, back-transformed data), and the proportion of aggression performed on the food patch (B). Circles indicate means and grey bars indicate 95% confidence intervals. *** indicates p<0.001, * indicates p between 0.01-0.05, N.S. (not significant) indicates p>0.05.*

We found that males from female-biased populations spent less time on the food patch compared with male-biased and equal sex ratio populations ($F_{2,5.9}$=13.2, p=0.007, square root transformed data, Fig. S1B). Males from female-biased populations also performed a lower proportion of total aggression on the food patch relative to males from the other treatments (χ^2_2=44.4, p<0.001; Fig. 1B), suggesting differences in resource defense. Aggressive behaviour was related to food patch occupancy. Across all sex ratios, the individual that performed relatively more aggression within a dyad spent relatively more time on the food patch (χ^2_1=56.5, p<0.001), and this relationship was weaker as the evolutionary sex ratio became more female-biased (χ^2_2=113.8, p<0.001, Fig. 2A). The reduction in food patch use by males from female-biased populations was accompanied by a weak trend towards increased locomotion in these males, relative to those from equal and male-biased evolutionary sex ratios ($F_{2,6.0}$=4.8, p=0.056, Fig. S1A).

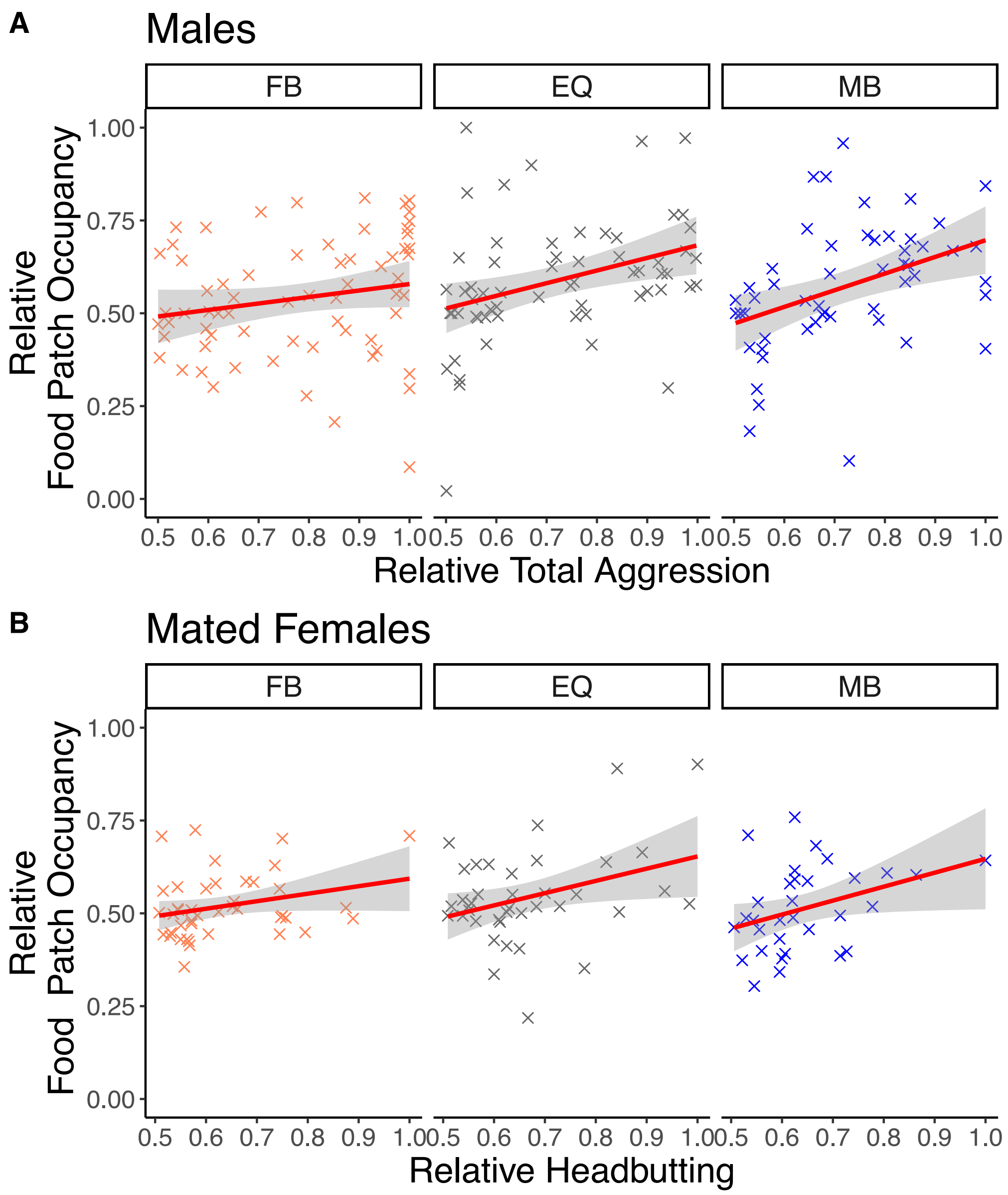


Figure 2: The relationship between aggression and food patch occupancy within dyads

The relationship between the proportion of aggression (male total aggression and female headbutts) performed by the most aggressive individual in a pair and the proportion of food patch occupancy for that individual, for males (A), and mated females (B) at female-biased (FB), equal (EQ) or male-biased (MB) sex ratios. Grey shading indicates the 95% confidence interval.

Female aggression and food patch occupancy in the coevolved experiment

We found that mating status and evolutionary sex ratio interacted to influence female headbutt frequency (mating status: $F_{1,335.1}$=47.8, p<0.001; sex ratio: $F_{2,10.8}$=1.8, p=0.213; interaction:$F_{2,335.6}$=5.3, p=0.005, Fig. 3A). Headbutting increased after mating in all evolutionary sex ratios, but females from female-biased populations increased headbutting twice as much females from male-biased or equal sex ratio populations (Fig. 3A; Table S4). In virgin females, we found no significant effect of the evolutionary sex ratio on headbutt frequency ($F_{2,6.1}$=2.7, p=0.149), but after mating, females from female-biased populations performed more headbutts than females from male-biased populations ($F_{2,6.0}$=5.1, p=0.050; post-hoc male-biased vs. female-biased comparison: t=3.2, df=6.1, adjusted p=0.043).

There was only weak evidence of an interaction between mating status and evolutionary sex ratio for female fencing duration (mating status: $F_{1,238.6}$=46.8, p<0.001; sex ratio: $F_{2,5.8}$=2.8, p=0.140; interaction: $F_{2,239.8}$=3.0, p=0.053, square root-transformed data; Fig. 3B). Fencing duration increased after mating within all evolutionary sex ratios, and as with headbutting, this increase was greatest in dyads from female-biased populations (Fig. 3B; Table S4). The evolutionary sex ratio had no significant influence on fencing within females of either mating status (virgin females: $F_{2,5.9}$=3.2, p=0.117; mated females: $F_{2,5.9}$=0.8, p=0.507).

Our results revealed an interaction between mating status and evolutionary sex ratio for food patch occupancy, suggesting evolved changes in feeding behaviour ($F_{2,240.0}$=3.6, p=0.028; Fig. S2B). Food patch occupancy increased post-mating in all evolutionary sex ratios, with a larger increase in females from female-biased populations, relative to females from male-biased or equal sex ratio populations (Fig. S2B). The evolutionary sex ratio had no significant influence on food patch occupancy within females of either mating status (virgin females: $F_{2,6.0}=1.1$, $p=0.380$; mated females: $F_{2,6.0}=1.8$, $p=0.249$). As in males, the more aggressive mated female within a dyad spent relatively more time occupying the food patch ($\chi^2_1=197.5$, $p<0.001$), with the strongest positive correlation in mated females from male-biased sex ratios (sex ratio: $\chi^2_2=27.3$, $p<0.001$; interaction: $\chi^2_2=28.4$, $p<0.001$; Fig. 2B). However, virgin females showed the opposite pattern: more aggressive virgin females within a dyad spent relatively less time occupying the food patch ($\chi^2_1=7.1$, $p=0.008$), with the strongest negative correlation in male-biased sex ratios (sex ratio: $\chi^2_2=15.5$, $p<0.001$; interaction: $\chi^2_2=35.6$, $p<0.001$; Fig. S3).

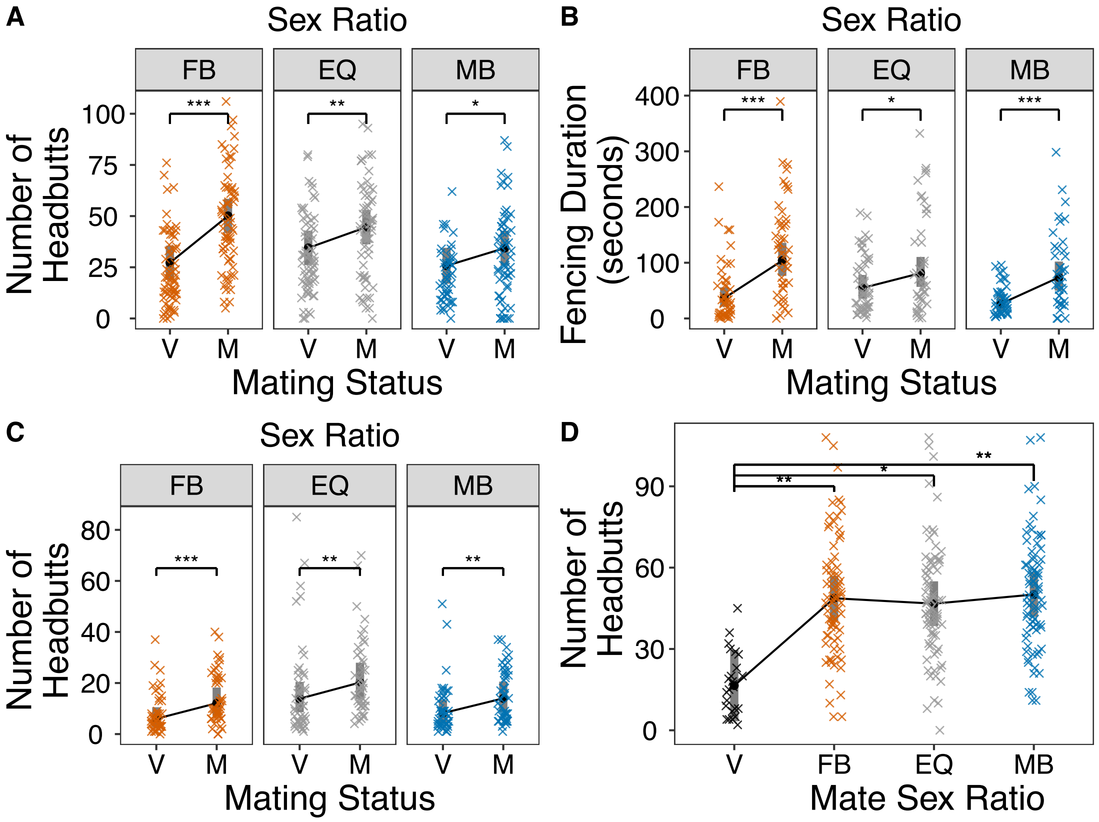


Figure 3: Female aggressive behaviour in the coevolved experiment

*Female aggression after experimental evolution at female-biased (FB), equal (EQ), or male-biased (MB) sex ratios, assayed when females were virgins (V) or mated (M). Female headbutting (A, C, D) and fencing behaviour (B, back-transformed data) was measured when experimentally-evolved females mated with experimentally-evolved males (A, B; the coevolved experiment), when experimentally-evolved females mated with stock males (C; the evolved female experiment; back-transformed data), and when stock females mated with experimentally-evolved males (D). Circles indicate means and grey bars indicate 95% confidence intervals. *** indicates $p<0.001$, ** indicates p between 0.001-0.01, * indicates p between 0.01-0.05, N.S. (not significant) indicates $p>0.05$.*

Mating reduced female locomotion ($F_{1,237.9}=40.0$, $p<0.001$, square root-transformed data; Fig.S2A), but we detected no influence of evolutionary sex ratio on locomotion, and no interaction between mating and evolutionary sex ratio (evolutionary sex ratio: $F_{2,5.9}=3.9$, $p=0.083$; interaction:$F_{2,239.0}=2.1$, $p=0.129$).

Female aggression and food patch occupancy in the evolved female experiment

In the coevolved experiment, the effect of sex ratio on female headbutting might have arisen from evolutionary change in females, from changes in male stimulation of female aggression, or from changes in both sexes. To test whether differences arose from females alone, we mated experimentally-evolved females to stock males. As expected, mating caused a general increase in headbutting

($F_{1,248.3}$=32.7, p<0.001). However, the evolutionary sex ratio did not influence the magnitude of this post-mating increase (evolutionary sex ratio x mating interaction: $F_{2,248.3}$=0.2, p=0.789, square root-transformed data; Fig. 3C, Table S4). Females from equal sex ratio populations tended to headbutt more, relative to female-biased and male-biased females ($F_{2,6.0}$=4.9, p=0.056), regardless of mating status.

We observed no significant increase in fencing post-mating ($F_{1,250.9}$=0.2, p=0.699, log(constant-x)-transformed data; Fig. S4A), in contrast to results from the previous experiment. We found no overall effect of evolutionary sex ratio on female fencing ($F_{2,6.0}$=0.4, p=0.708), nor an interaction between evolutionary sex ratio and mating ($F_{2,250.8}$=0.8, p=0.471).

In the coevolved experiment, we found an interaction between evolutionary sex ratio and mating status for female food patch occupancy, but we found no significant interaction when evolved females mated with stock males ($F_{2,247.5}$=1.0, p=0.360, Fig. S4C). Although mating caused a general increase in food patch occupancy ($F_{1,247.5}$=10.6, p=0.001), we found no effect of evolutionary sex ratio ($F_{2,6.0}$=2.0, p=0.218).

Female aggression and food patch occupancy in the evolved male experiment

To test whether the differences in female headbutting observed in the coevolved experiment were due to evolved differences in male stimulation of female aggression, we mated experimentally-evolved males to stock females. All females showed a similar increase in headbutting post-mating ($F_{1,7.9}$=40.1,

$p<0.001$). There was no effect of male evolutionary sex ratio on headbutt number post-mating ($F_{2,6.1}=0.3$, $p=0.751$, Fig. 3D).

Males did not stimulate a significant increase in fencing in stock females post-mating ($F_{1,7.9}=0.4$, $p=0.553$), and we found no effect of male evolutionary sex ratio on female post-mating fencing duration ($F_{2,6.1}=1.1$, $p=0.401$; Fig. S4B).

The interaction between evolutionary sex ratio and mating status on food patch occupancy detected in the coevolved experiment was not detectable when stock females were mated with experimentally-evolved males. Regardless of evolutionary sex ratio, all males stimulated increases in food patch occupancy in stock females post-mating ($F_{1,7.8}=8.7$, $p=0.019$), but there was no significant effect of male evolutionary sex ratio on female post-mating food-patch occupancy ($F_{2,6.1}=0.3$, $p=0.719$; Fig. S4D).

The correlation between male and female aggression

We found a positive correlation between the number of male lunges and female headbutts across replicate populations (Spearman's rank correlation, males and virgin females, $\varrho=0.72$, $S=34$, $p=0.037$; males and mated females, $\varrho=0.63$, $S=44$, $p=0.076$; Fig. 4A,B), but found no correlation in fencing duration between the sexes (males and virgin females, $\varrho=-0.10$, $S=132$, $p=0.810$; males and mated females, $\varrho= -0.25$, $S=150$, $p=0.521$).

The correlation between virgin and mated female aggression

We found a positive correlation between pre- and post-mating female headbutting frequency across replicate populations (Spearman's rank correlation, ϱ=0.70, S=36, p=0.043, Fig. 4C), but found no correlation in fencing behaviour (Spearman's rank correlation, ϱ= 0.07, S=112, p=0.880).

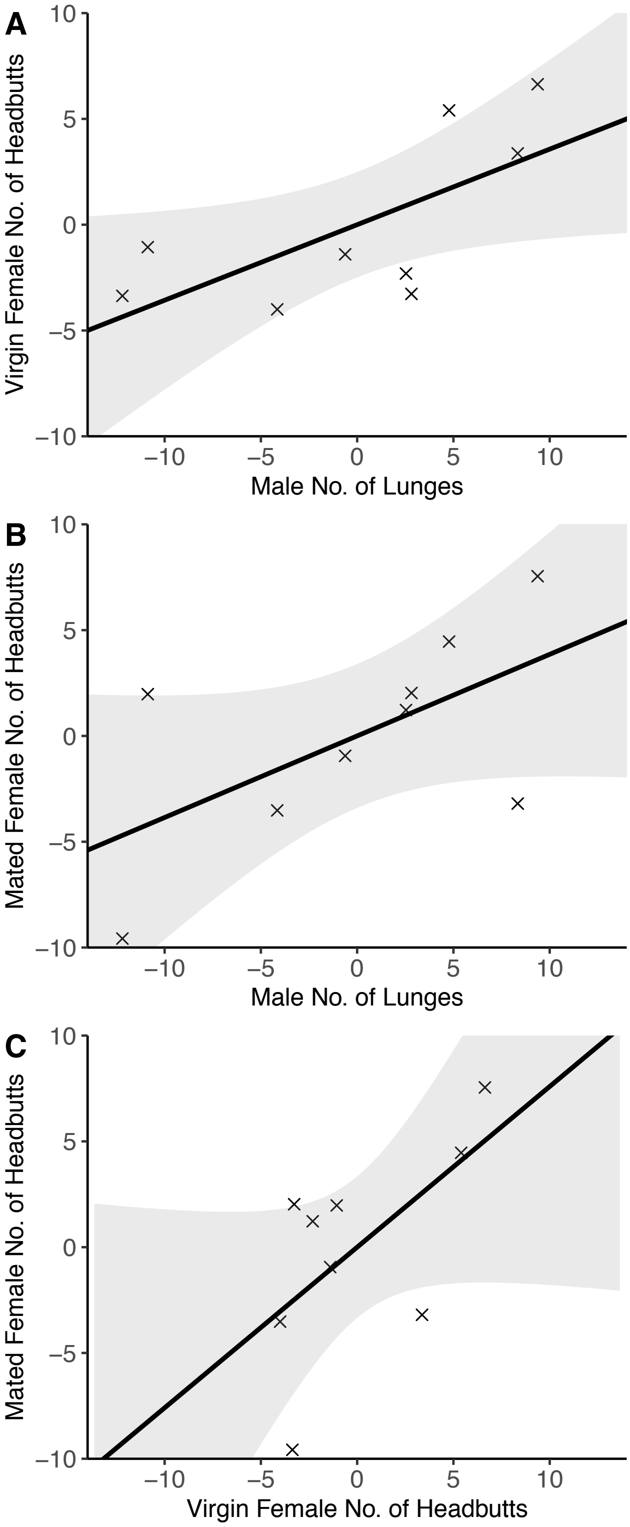


Figure 4: Correlations between male and female aggressive behaviours

The relationship between male and female aggressive behaviour (male lunges and headbutts by virgin (A) or mated females (B); and between virgin and mated female headbutts (C). All points are residual values from models controlling for day, time and sex ratio (see text). Lines indicate the monotonic fit from Spearman's correlation; grey shading indicates the 95% confidence interval.

DISCUSSION

We investigated how aggression evolves in response to the intensity of intra-sexual competition by assaying aggression after experimentally manipulating the population sex ratio for >75 generations. We predicted that males and females would evolve increased aggression after evolution in populations biased towards their sex, and our results support this prediction strongly in females and weakly in males. Although male aggressive behaviour showed little change in frequency or duration, male food patch occupancy and the aggression associated with the food patch was reduced in males from female-biased populations, suggesting that the evolutionary sex ratio influences the relationship between aggression and access to resources. We observed a greater increase in aggression after mating in females from female-biased populations, as predicted if higher post-mating aggression is adaptive for females. Surprisingly, differences in the magnitude of this increase among sex ratios occurred only after matings between experimentally-evolved males and females, and not when experimentally-evolved individuals mated with stock flies. These results suggest that differences in the post-mating increase in aggression do not arise through evolved changes in either sex independently, but might depend on co-evolved interactions between the sexes. We found positive correlations in aggression between the sexes, consistent with a shared genetic basis for aggression. Our results suggest that the intensity of competition can determine the strength of sexual and social selection on aspects of aggression and food patch occupancy in both male and female *D. melanogaster*, shaping the evolution of these behaviours.

The evolution of male aggression in response to biased sex ratios

We predicted that evolution under stronger sexual selection, through more intense competition for mates in more male-biased populations, should lead to increased male aggression, mirroring plastic changes in response the sex ratio in a wide range of species (e.g., sand gobies (Kvarnemo et al., 1995), medflies (Gaskin et al., 2002), Japanese medaka (Grant & Foam, 2002), water striders (Wey et al., 2015)). The results offer only weak support for this prediction. On the one hand, the absence of evolved differences in the frequency and duration of male aggression in response to sex ratio does not support the prediction. Two possible explanations for the absence of response are that selection favours the plasticity in aggression rather than fixed increases or decreases (Nandy et al, 2016); or that there is little change in net selection on aggression with sex ratio because changes in the strength of competition for mates are balanced by changes in rival density and costs of fighting (Grant, 1993; Kilgour et al., 2018; Knell, 2009; Weir et al., 2011). However, neither hypothesis accounts for our observations of sex ratio effects on the evolution of female aggression and male aggression in relation to food patches.

On the other hand, we observed the evolution of reduced food patch occupancy, a reduced proportion of aggression performed on food, and a weaker relationship between aggression and food occupancy, in males from female-biased populations relative to other males. The function of male aggression in gaining access to food resources is supported both by our finding that more aggressive males spend relatively more time occupying the food patch, and by previous reports that aggressive male *D. melanogaster* win access to food-patches (Hoffmann, 1988; Lim et al., 2014), which increases their access to mates (Hoffmann & Cacoyianni, 1990; Lim et al., 2014; Markow, 1988). Our results are consistent with weaker selection for the use of aggression to attain access to food patches under female-biased conditions, in which weaker competition for mates is expected to reduce the benefits of dominating breeding sites (Aronsen et al., 2013; Grant & Foam, 2002). An alternative hypothesis is that reduced male food patch occupancy after evolution in female-biased populations might reflect reduced female aggregation on food patches. However, females aggregate more, not less, on food patches in our female-biased populations (Rostant et al., 2020).

The evolution of female aggression in response to biased sex ratios

Females increase aggression after mating in many species (Bath et al., 2017; Mainardi et al., 1996; Nilsen et al., 2004; Seebacher et al., 2013; Sinn et al., 2008). Our results are consistent with this pattern. Increased aggression post-mating might represent an adaptive response that relates to the acquisition or defence of nutritional resources required for reproduction, as the switch to a post-mating reproductive state increases female feeding and protein requirements (Barnes et al., 2008; Jensen et al., 2015; Lee et al., 2008). Our findings that females from all sex ratio treatments display increased food patch occupation post-mating, and that aggression is positively related to food occupancy in mated females, support this idea.

We found that the evolutionary sex ratio influences both the level of aggression in mated females and the magnitude of the post-mating increase in aggression, with more headbutts and a greater increase in headbutt frequency post-mating in females from female-biased populations. The greater intensity of female competition in female-biased populations might impose stronger selection favouring aggression in the nutritionally-demanding mated state. Consistent with these hypotheses, we found that females evolved under female-biased conditions also showed the greatest increase in food patch occupancy post-mating. Our results suggest that the intensity of intra-sexual competition can shape the evolution of female aggression, and that this might relate to nutritional defence, although causality in this relationship is unclear. Future work testing the relationship between female aggression, defence of food resources, and reproductive success would improve understanding of the function of aggression in this species.

The influence of sex ratio on the post-mating increase in aggression led us to ask whether this pattern arose through evolved changes in females, or evolved changes in male stimulation of female aggression, or coevolved interactions between the sexes. Our findings are inconsistent with the hypotheses that evolution in either sex alone explains the observed changes. We detected no influence of the evolutionary sex ratio on the post-mating increase in aggression when experimentally-evolved females mated with stock males, or when experimentally-evolved males mated with stock females. Previous work has demonstrated that the receipt of male sperm and the seminal fluid protein ‘sex peptide’ directly influence female aggression in *D. melanogaster* (Bath et al., 2017). Moreover, properties of the male ejaculate such as sperm competitiveness and ejaculate expenditure show evolvability in response to the sex ratio (Chechi et al., 2017; Linklater et al., 2007; Nandy, Chakraborty, et al., 2013; Nandy, Gupta, et al., 2013). However, a male’s ability to stimulate female aggression did not appear to evolve in the conditions of our experiment.

We are left with the hypothesis that the female post-mating behaviours observed when both sexes had been subjected to experimental evolution reflect coevolved interactions between the sexes, such that evolved changes occur only after matings between individuals from the same social environment. Similar complex interactions between male and female genotypes are known in *Drosophila*. For example, the effect of some male sex peptide alleles on sperm competitiveness depends on the female sex peptide receptor allele (Chow et al., 2010). Likewise, sperm success can depend on interactions between male and female genotypes (Clark et al., 1999). Although we know that female post-mating aggression is linked to the receipt of male ejaculates (Bath et al., 2017), the downstream mechanism within females remains elusive. Further research into the post-mating regulation of female aggression would help to evaluate the co-evolution hypothesis.

A positive correlation in aggression between the sexes

Studying the evolution of male and female aggression simultaneously allowed us to evaluate the hypothesis that aggression is genetically correlated between the sexes. This is especially relevant because female aggression has sometimes been considered a non-adaptive by-product of selection for male aggression (Bateman, 1948; Cain & Ketterson, 2012) and has only recently been studied as an adaptive female trait (Stockley & Bro-Jørgensen, 2011).

We observed a positive correlation between male lunging and female headbutting across replicate populations, consistent with a shared genetic basis for aggression. There is evidence that selection for aggressive behaviour in male *D. melanogaster* results in correlated responses in female aggression (Edwards et al., 2006), supporting this idea. This suggests the possibility that genetic constraints might impede the evolution of sex-specific optimal aggression. Alternatively, a positive correlation could arise if aggression forms a behavioural syndrome with other coevolving inter-sexual behaviours, such as male harassment of females and female resistance. However, this seems unlikely because there is little evidence for aggression covarying across contexts in *D. melanogaster* (Baxter & Dukas, 2017) and intra-sexual aggressive behaviours are rarely directed at the opposite sex (de la Paz Fernández et al., 2010). Furthermore, the positive correlation between headbutting by virgin and mated females suggests a consistent genetic basis for female aggression pre- and post-mating, such that females have a baseline level of aggression that is enhanced by mating.

In contrast, the absence of correlations in fencing behaviour between males and females, and between virgin and mated females, across replicate populations might reflect differences in the function of this low-intensity aggressive behaviour between the sexes, and within females depending on their mating status. Fencing is performed by both sexes, but there are distinct differences in the aggressive strategies of males and females (Nilsen et al., 2004) and in females pre- and post-mating (Bath et al., 2017). If there are distinct genetic pathways underlying low- and high-intensity aggression, then the extent to which sex-specific aggression is constrained by a shared genetic basis may vary for different aggressive behaviours.

Our study provides evidence that the strength of sexual and social selection, mediated by competition for mates and resources, can shape the evolution of aggressive behaviours in both male and female *D. melanogaster*. These effects differ between the sexes, which might reflect the different routes by which aggression influences reproductive success in the two sexes (Tobias et al., 2012). The higher energy demands of reproduction in females might result in greater reproductive costs from energetically expensive aggression in females than in males, causing reduced female aggression with greater sensitivity to the ecological setting.

Furthermore, although we found evidence consistent with a shared genetic basis for aggression, our observation of divergent responses to sex ratio for males and females suggests that a genetic correlation for aggression does not completely restrict its independent evolution. Our study also highlights that increased female aggression in response to mating might be sensitive to adaptations in both sexes. This underscores the value of future study into the mechanisms behind the female post-mating increase in aggression, and reveals insights gained from studying behaviour in both sexes simultaneously.

SUPPLEMENTARY METHODS

Experimental evolution

Colleagues established replicate populations in 1L cages of 100 individuals drawn from the stock population. The sex ratio treatments were female-biased

(FB, 25:75 male:female), equal sex ratio (EQ, 50:50 male:female), and male-biased (MB, 70:30 male:female). For each sex ratio, there were 3 replicate populations that evolved independently. Populations were maintained on sugar-yeast-agar medium, provided in 3 70 ml bottles per cage, which were renewed on the 4th and 7th day for each generation, generating spatial and temporal variation in food availability and quality. On the 10th day of each generation, eggs were collected from each cage using grape agar plates and transferred first-instar larvae to food vials (100 larvae per vial). At eclosion, virgin adults were collected and placed them in the population cages in the assigned sex ratio. This procedure was repeated each generation for 78 generations for the coevolved experiment, and 92 generations for the evolved female and evolved male experiments.

SUPPLEMENTARY TABLES

Supplementary Table 1

Ethogram of aggressive behaviours observed in this study (Andrews, 2016; Chen et al., 2002; Dow & von Schilcher, 1975; Nilsen et al., 2004)

Aggressive behaviours	**Sex**	**Description**	**Metrics**
Lunging	Male	*The male raises up on hind legs and rapidly thrusts his upper body at his opponent*	Number of lunges per dyad, converted to lunge duration for summed behavioural metrics by multiplying by average lunge duration (0.3s)
Chasing	Male	*One male rapidly pursues his opponent, remaining in close proximity. Contact and even lunging may occur during chasing*	Total time (s) that each fly in the dyad spent chasing, summed together
Tussling	Male	*Both opponents raise up on hind legs and become interlocked in a prolonged struggle*	Total time (s) that each fly in the dyad spent tussling, summed together
Fencing	Both	*A male or female uses their forelimbs to bat their opponent, including front-on and side-on action with any of the two front or middle legs. Fencing can be performed by one or both opponents, alone or in combination with other behaviours.*	Total time (s) that each fly in the dyad spent fencing, summed together
Headbutting	Female	*Females thrust their bodies towards each other, physically contacting each other with their heads*	Number of headbutts per dyad

Supplementary Table 2

Sample sizes for aggression trials for the coevolved experiment. For females, the first number illustrates the total number of trials all of which were scored for headbutting, and a subset (259 trials, represented by the number in brackets) were also scored for low-intensity behaviours.

Sex Ratio	**Replicate Population**	**Males**	**Virgin Females**	**Mated females**
FB	FB1	24	18 (14)	22 (14)
	FB2	22	23 (15)	19 (13)
	FB3	23	23 (18)	23 (18)
EQ	EQ1	22	20 (12)	20 (15)
	EQ2	29	20 (16)	19 (10)
	EQ3	26	23 (20)	23 (16)
MB	MB1	20	16 (14)	20 (13)
	MB2	19	16 (13)	18 (13)
	MB3	21	16 (14)	19 (11)

Supplementary Table 3

The influence of evolutionary sex ratio on mating duration and mating latency for each experiment. Mating latency results are from Cox mixed survival models, and mating duration results are from linear mixed effect models

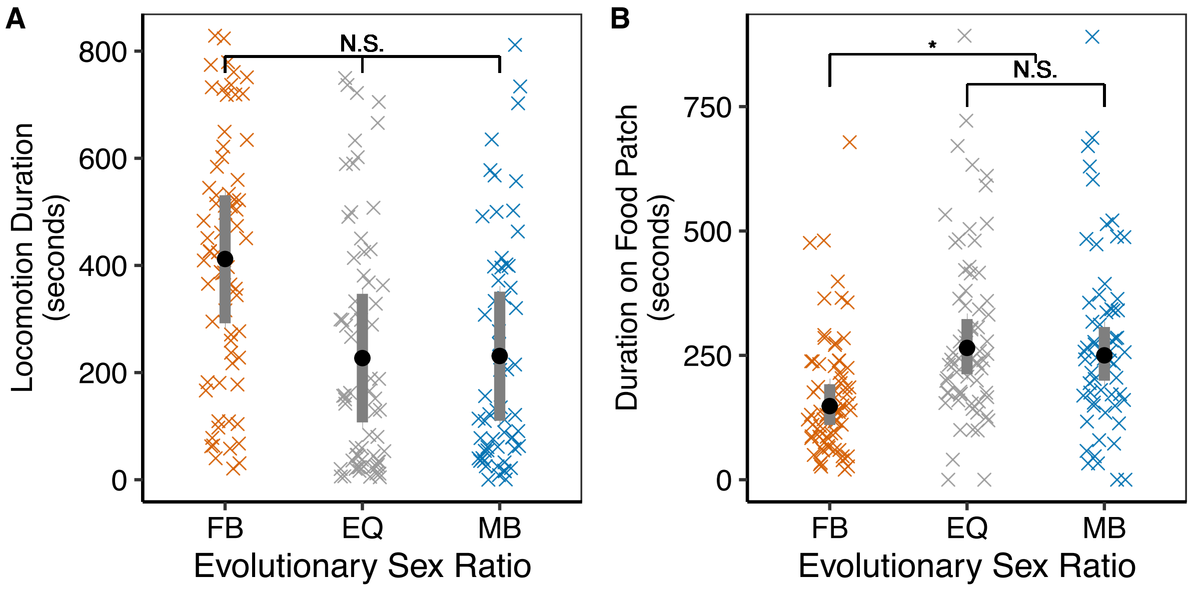
	Co-evolved experiment	**Evolved female experiment**	**Evolved male experiment**
Mating latency	χ^2_2=1.8, p=0.40 χ^2_2=0.9, p=0.63	χ^2_2=0.2, p=0.90	χ^2_2=0.2, p=0.90 χ^2_2=0.2, p=0.88
Mating duration	$F_{2,6.0}$=1.67, p=0.27	$F_{2,5.9}$=0.72, p=0.53	$F_{2,5.9}$=0.72, p=0.53 $F_{2,6}$=0.7, p=0.53

Supplementary Table 4

Effect sizes for magnitude of post-mating changes in female behaviour.

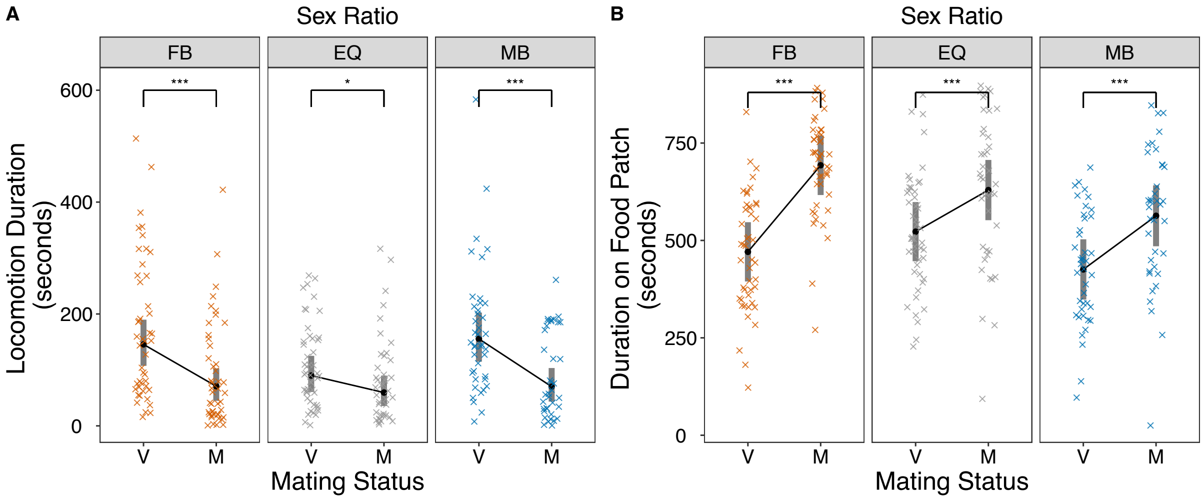
Experiment	Behaviour	Sex Ratio	Effect Size	Std. Error
Coevolved experiment	Post-mating increase in headbutts	FB	-1.23	0.18
		EQ	-0.55	0.18
		MB	-0.47	0.20
Coevolved experiment	Post-mating increase in fencing	FB	-1.17	0.22
		EQ	-0.46	0.22
		MB	-0.98	0.23
Coevolved experiment	Post-mating increase in feeding	FB	-1.51	0.23
		EQ	-0.73	0.22
		MB	-0.94	0.23
Evolved female experiment	Post-mating increase in headbutts	FB	-0.82	0.22
		EQ	-0.61	0.22
		MB	-0.70	0.22

SUPPLEMENTARY FIGURES



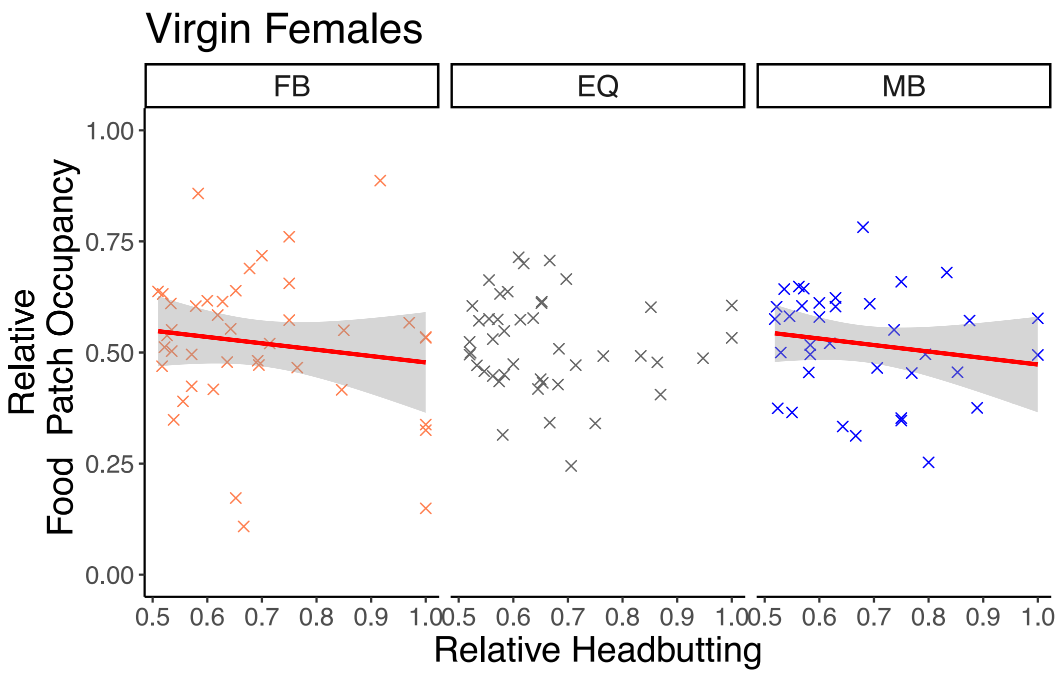
Supplementary Figure 1

*Locomotion duration (A) and food patch occupancy duration (B) of males from each evolutionary sex ratio (coevolved experiment). Black circles indicate means. Grey bars represent 95% confidence intervals. * indicates p between 0.01-0.05, N.S. (not significant) indicates p>0.05*



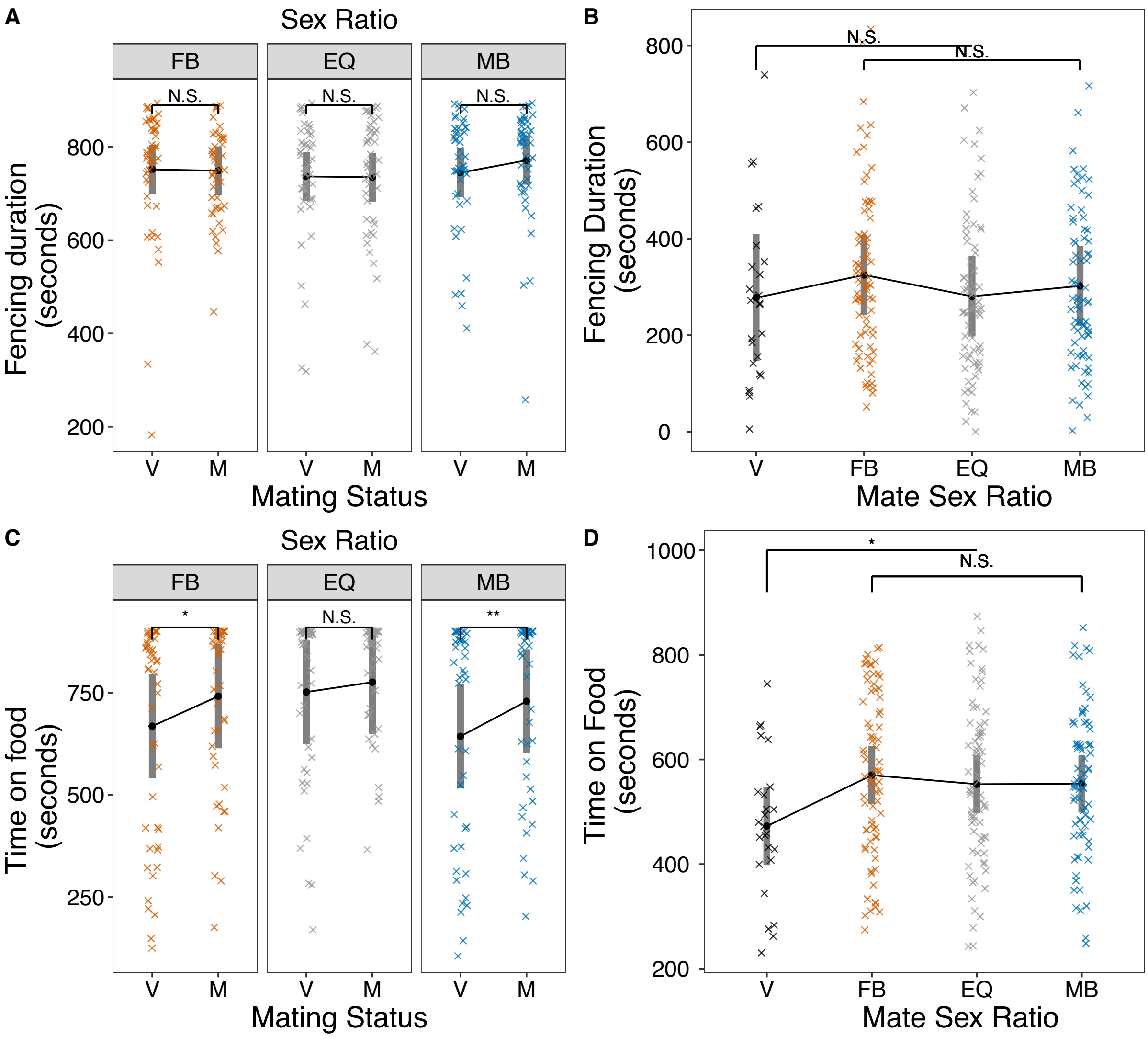
Supplementary Figure 2

*The locomotion duration (back-transformed data, A) and food patch occupancy duration (B) of females from each evolutionary sex ratio (coevolved experiment). Black circles indicate means. Grey bars represent 95% confidence intervals. *** indicates p<0.001, ** indicates p between 0.001-0.01, * indicates p between 0.01-0.05.*



Supplementary Figure 3: The relationship between aggression and food patch occupancy within dyads

The relationship between the proportion of aggression (female headbutts) performed by the most aggressive individual in a pair and the proportion of food patch occupancy for that individual for virgin females. Grey shading indicates the 95% confidence interval.



Supplementary Figure 4

*The fencing duration (A-B) and food patch occupancy duration (C-D) of females from the evolved female experiment (A&C) and the evolved male experiment (B&D). Black circles indicate means. Grey bars represent 95% confidence intervals. ** indicates p between 0.001-0.01, * indicates p between 0.01-0.05, N.S. (not significant) indicates p>0.05.*

Chapter 4: Are flies kind to kin because of their smell?

ABSTRACT

Across many species, sexual reproduction results in a conflict between the sexes, with male-male competition selecting for traits that maximise their reproductive success but consequently harm their mates. This male-female harm can decrease the reproductive success of male conspecifics by diminishing the fitness of shared mating partners in a tragedy of the commons. Kin selection theory predicts that genetic relatedness should reduce sexual conflict due to the inclusive fitness costs associated with decreasing the reproductive success of relatives. Reduced male-female harm amongst related males has been demonstrated in the fruit fly, *Drosophila melanogaster*. However, while such a response relies on the ability of individuals to recognize kin, the mechanisms underlying kin discrimination remain unknown. A prime candidate mechanism is olfaction. Olfaction plays an important role in mediating social interactions in many species, and recent work has identified differential expression of olfactory genes linked with reduced sexual conflict in related groups of *D. melanogaster*. Here, we tested the role of olfaction in reducing male-male aggression and male-female harm in related groups of *D. melanogaster*, comparing the response of both wild-type flies and olfactory-deficient flies to relatedness. We predicted that if olfaction is required for kin discrimination, relatedness should decrease male aggression and harm to females in wild-type groups, but not in olfactory deficient groups. Surprisingly, despite previous evidence, our observations of wild-type flies revealed no effect of relatedness on male-male aggression or male-female harm, suggesting a high

variability and sensitivity of this response across different studies. However, in females housed with olfactory-deficient males, we found evidence of greater female longevity with related males compared to unrelated males, consistent with previous studies on flies with full olfactory ability. Olfactory deficient related males tended to show lower levels of male-male aggression, but this did not translate into differences in the reproductive lifespan or lifetime reproductive success of their female mates. This provides some evidence for relatedness reducing sexual conflict, in line with kin selection theory, through mechanisms not solely based on olfaction. Future work exploring the role of other sensory modalities in kin recognition would increase our understanding of the mechanisms underlying behavioural responses to relatedness.

INTRODUCTION

In many species, selection favours males that engage in intense competition for mating and fertilisation opportunities. Such competition drives the evolution of traits that increase success in pre-copulatory mating competition and post-copulatory sperm competition (Andersson & Iwasa, 1996; Le Page et al., 2017). This can lead to sexual conflict via two routes. Firstly, differences in the reproductive optima of the sexes might select for males to directly influence females to change their reproductive investment so it is closer to the male optima. Secondly, males might harm females as a by-product of competition between males (Rankin, 2011; Wild et al., 2011). For example, male sexual aggression and coercion can severely damage females (e.g. dung flies *Scatophagus stercorcaria*, Parker, 1979; Wild et al., 2011), drive them from prime breeding sites (e.g. seed-eating true bugs, McLain & Pratt, 1999), and reduce their survival and fecundity (e.g. the common lizard *Lacerta vivipara*, Galliard et al., 2005). Importantly, when population dispersal is low and individuals mate locally, sexual conflict not only results in male harm of females, but can also reduce the fitness of male conspecifics by decreasing the value of shared resources, analogous to tragedy of the commons (Rankin & Kokko, 2006).

Kin selection theory predicts that genetic relatedness should shape the evolution of interactions that impact the fitness of conspecifics, such as the aggression, harassment and coercion involved in male harm to females (Hamilton, 1964). Relatedness is an important aspect of an individual's social environment, determining the indirect fitness costs and benefits of behavioural interactions (Hamilton, 1964; Pizzari & Gardner, 2012; Rankin, 2011; Wild et al., 2011). Individuals can increase their inclusive fitness both by increasing their own reproductive success, and by increasing the reproductive success of genetic relatives (Pizzari & Gardner, 2012). Because male harm to females has detrimental effects on the fitness of male conspecifics and related female mates, high local genetic relatedness should select for reduced male harm to females (Rankin, 2011), increasing a male's inclusive fitness by increasing the reproductive success of a male's relatives.

Recently, empirical evidence of relatedness reducing male-female harm and male-male aggression was reported in the fruit fly, *Drosophila melanogaster* (Carazo et al., 2014, 2015). Sexual conflict is common in *D. melanogaster*, with male-male competition harming females (Yun et al., 2017). Carazo and colleagues (2014, 2015) demonstrated that groups of related brothers housed with a female showed reduced aggression and female harassment, resulting in higher female lifetime reproductive success and slower reproductive aging, relative to groups of unrelated males. Further investigation demonstrated that these differences extend across generations, with daughters of females exposed to related familiar males having lower mortality (Carazo et al., 2015). Although similar responses have recently been demonstrated in a range of other species (e.g. entomophathogenic nematodes, *Steinernema longicaudum*, Kapranas et al., 2016; domestic fowl, *Gallus gallus domesticus*, Rosher et al., 2017; bulb mites *Rhizoglyphus robini*, \Lukasiewicz et al., 2017; and seed beetles *Callosobruchus maculatus*, Lymbery & Simmons, 2017), there is inconsistency in the replication of these patterns in *D. melanogaster* by other research groups. While some studies offer support for aspects of this response, demonstrating reduced male-male aggression (Martin & Long, 2015) or increased female fitness (Le Page et al., 2017) in *D. melanogaster* groups with high male relatedness, male behavioural and female fitness effects were not reported simultaneously, and others found that the response to relatedness was highly context-dependent (Hollis et al., 2015), or absent altogether (Chippindale et al., 2015).

Furthermore, kin selection theory (Hamilton, 1964) suggests that the ability to recognise kin from non-kin, and thus to direct beneficial actions only to close relatives, will increase inclusive fitness advantages. However, our understanding of the proximate mechanisms and sensory cues that allow kin discrimination lags behind demonstrations of its existence, especially in the context of sexual conflict (Daniel & Rodd, 2020; Mehlis et al., 2008). Despite work on *D. melanogaster* representing significant progress in demonstrating reduced sexual conflict and aggression in response to relatedness, the mechanisms behind kin discrimination among rivals in this key model species remain elusive. Revealing the underlying proximate mechanisms has important implications for understanding the evolutionary significance of this behaviour (Real, 1994; Tang-Martinez, 2001).

A prime candidate mechanism for rival kin discrimination in *D. melanogaster* is olfaction. Olfactory and pheromonal cues from rival males are important in mediating social interactions, such as courtship and mating, across multiple species including male meadow voles, *Microtus pennsylvanicus*, newts, *Lissotriton boscai*, and beetles, *Tenebrio molitor*, (Aragón, 2009; Bretman, Westmancoat, et al., 2011; Carazo et al., 2007; delBarco-Trillo & Ferkin, 2004). In the context of kin discrimination specifically, olfactory mechanisms have been proposed in a number of vertebrate species, facilitating inbreeding avoidance and group formation (e.g., Bonadonna & Sanz-Aguilar, 2012; Boulet et al., 2009; Daniel & Rodd, 2020; Green et al., 2015; Leedale et al., 2020; Makowicz et al., 2016; Mann et al., 2003; Mehlis et al., 2008; Mitchell et al., 2018; Waldman, 1985). In insects, olfactory signals, such as those stemming from gut bacteria and cuticular hydrocarbons, facilitate species and sex-specific recognition in solitary species (e.g. house flies, *Musca domestica*, tsetse flies, *Glossina spp.*, and fruit flies, *Drosophila spp.*, Ferveur, 2005; Lizé et al., 2014; Singer, 1998), and provide a promising mechanism for nest mate recognition in social insects (Couto et al., 2017; Getz & Smith, 1987; Kather & Martin, 2015; Ruther et al., 2002; Smith & Breed, 1995; van Zweden & d'Ettorre, 2010). Indeed, *D. melanogaster* use olfactory cues in mate choice (Gailey et al., 1986; Laturney & Billeter, 2016), detection of rival presence (Garbaczewska et al., 2013) and responding to sexual familiarity (Tan et al., 2013). However, although olfaction may allow some degree of recognition in *D. melanogaster,* there has been no empirical demonstration, to our knowledge, of olfactory kin discrimination this solitary insect species.

Recent research (Perry et al, unpublished) has suggested the first evidence of a role for olfaction in the interaction between relatedness and social behaviour in *D. melanogaster*. By measuring gene expression in the heads of males, Perry and colleagues (unpublished) identified several classes of genes with different expression profiles in males depending on whether rival males are kin or non-kin. One such class of genes is those involved in olfaction, suggesting a role for olfaction in reducing male-female harm and male-male aggression in response to high relatedness.

Here, we tested the role of olfaction in modulating social behaviours and sexual conflict in response to relatedness and familiarity in *Drosophila melanogaster*. The wealth of existing knowledge of *D. melanogaster* behaviour, genetics and neurobiology provides an excellent opportunity to investigate the interplay between the social environment, behavioural interactions, and the underlying mechanisms. We assessed the degree of male-male aggression, male-female harassment, and female harm (measured as a reduction in longevity and reproductive success) in groups of related and unrelated males housed with a female, comparing the influence of relatedness on behaviour in groups of wild-type and olfactory-deficient males. Firstly, we predicted that male-male aggression, male-female harassment, and female fitness costs would be reduced in related groups relative to unrelated groups as demonstrated by

Carazo et al (2014,2015), consistent with the hypothesis of relatedness reducing sexual conflict. Secondly, we predicted that if olfaction is necessary for this response to relatedness, differences in behaviours and fitness costs between related and unrelated groups should not occur in groups deficient in olfaction.

METHODS

Fly stocks

Two genotypes of *D. melanogaster* were used in this experiment: olfactory deficient flies with a mutation in the *orco* gene in a white Dahomey background (back-crossed for 6+ generations) containing a miniwhite marker to rescue red eye-colour (*orco*); and wild-type flies derived from an outbred red Dahomey stock population with over-lapping generations (wild-type, wt; (Carazo et al., 2014). The red as opposed to white Dahomey background of wild-type flies was necessary to ensure all flies (*orco* and wild-type) had red eyes and were not visually impaired. Fly populations were maintained on standard sugar-yeast medium at 25^{o}C on a 12h:12h light dark cycle. The *orco* gene codes for an olfactory receptor that acts with other olfactory receptors to enable responses to all odours, meaning flies lacking a functional version of this gene are deficient in olfaction (Larsson et al., 2004). Although previous research of Perry and colleagues (unpublished) highlights a number of specific olfactory genes that may be differentially expressed in response to relatedness, we used flies with a mutation in the *orco* gene to broadly test for the involvement of olfaction in the response to relatedness. Preliminary tests confirmed that *orco* flies were olfaction deficient: individual *orco* males were placed in a clean petri dish with small section of pipe cleaner covered in female odour on one side, and an identical but odourless section on the other side. We found no difference in the amount of time olfactory-deficient males spent in contact with each pipe cleaner (binomial test; p=0.864) compared to control males, who biased contact towards the odour-covered pipe cleaner (binomial test; p<0.0001). While we hypothesise that *orco* flies might be deficient in kin recognition, these flies typically show similar courtship behaviours and social responses as wild-type flies, provided that other cues are available (Bretman, Westmancoat, et al., 2011; Krstic et al., 2009; Larsson et al., 2004; Laturney & Billeter, 2016; Tan et al., 2013) suggesting they are a suitable comparison to our wild-type populations.

Experimental flies

To generate parental flies, we placed ~50 mated females from both the wild-type and *orco* populations in separate egg laying cages containing grape-agar petri dishes smeared with live yeast paste. We collected parental flies as eggs and transferred them to bottles containing standard sugar-yeast media (~200 eggs per bottle; Clancy & Kennington, 2001). We incubated bottles at 25-28^{o}C until eclosion, adjusting the temperature as necessary to ensure developmental synchrony between the two genotypes. Within 8h of eclosion, we collected parental flies under ice anaesthesia as virgins and placed them in single sex 36ml vials containing standard sugar-yeast media.

To generate experimental females, we placed groups of 10-20 parental wild-type flies into yeasted bottles, allowing them to mate and oviposit. We incubated the resultant eggs at 25-28^{o}C until eclosion, at which point we collected virgin females and placed them in single sex vials.

To generate experimental males, we placed pairs of 1 week old male and female virgin parental flies in yeasted vials for 36h and allowed them to mate and oviposit. We discarded parental flies, then incubated vials at 25-28^{o}C until eclosion, meaning that all larvae developed in family groups (i.e. the vials in which they were laid). We collected experimental males as virgins and placed them in vials in one of 4 treatment groups: related familiar *orco* (n=57), unrelated unfamiliar *orco* (n=57), related familiar wild-type (n=60), and unrelated unfamiliar wild-type (n=57). Familiar related triplets consisted of 3 males from the same rearing vial, and unfamiliar unrelated triplets consisted of 3 males from 3 different vials. To control for any genetic differences between parents, we created related familiar and unrelated unfamiliar flies as paired sets, with parental vials giving 3 males to the related familiar vials, and one to the unrelated, unfamiliar vials. The other 2 males of the unfamiliar, unrelated treatment were from different parental vials that contributed 1 male each. To ensure adult social experience was standardized across treatments, we left males in these triplets for 24-72h before trials began.

Experimental trials

To initiate behavioural trials, we added one female to each vial by gentle aspiration. Females were 48-72h old wild-type virgins unrelated to any of the males. We conducted trials in 2 blocks, with block 1 starting a day before block 2. We performed behavioural observations of flies every day for the first 5 days, with a further behavioural observation on day 8, after which flies were maintained in their groups until the death of the female and observed only for mortality but not behaviour. Behavioural observation periods started at 0h Zeitgeber time and consisted of 18 3 second spot checks conducted by 2 observers blind to treatment scanning each vial in turn, carried out at 25°C. Observers recorded male-male aggression (number of high intensity aggressive events per spot check (lunging or tussling)), male-female courtship (number of males courting per spot check), and copulation (whether or not copulation occurred during the spot check).

From the start of behavioural trials, we transferred flies to fresh vials every 3-4 days until death of the female. We recorded the day that each experimental male and female died up until the death of the female. When the female died, we terminated the vial, and discarded remaining males. We kept all occupied vials at 25°C, and, when flies were transferred to fresh vials, we incubated previously occupied vials at 28 °C for 11 days to allow offspring to eclose. After eclosion, we froze all vials and counted the number of offspring to measure the total lifetime reproductive success and age-specific reproduction of each group.

Statistical analysis

We performed analyses in R, version 3.6.2 (2019-12-12). For each vial, we calculated aggression and courtship rates over the 6 observation days as bouts per minute: (behavioural score/(total number of scans*3))*60, and we expressed mating behaviour as the total number of copulations. To allow us to track how behaviours changed over duration of the observation period, we calculated behavioural rates both per day (daily behavioural rates) and as a total for the whole 6 day observation period (overall behavioural rates).

To investigate behavioural responses to relatedness, we analysed overall aggression, courtship and mating rates in separate linear models with genotype, relatedness and their interaction as main effects. To investigate effects on proxies of female fitness, we tested the interaction between genotype and relatedness on female lifetime reproductive success (LRS), longevity (L), and reproductive lifespan (RL, days from the start of experimental trials to the point at which offspring were no longer present in vials). For LRS, we used general linear models with a quasipoisson distribution to account for over-dispersion, and for L and RL, we used survival analysis. For survival analysis, we tested whether the data met the proportional hazards assumption of Cox proportional hazard models using the "cox.zph" function (survival package, Therneau et al., 2020), and where assumptions were not met, we used parametric survival regression models following with Weiball distribution ("survreg" function, survival package, Therneau, 2015; Therneau & Grambsch, 2000).

In some vials, one, two or all three of the males died before the females (Supplementary table 1). Chi-squared analysis revealed that male death occurred in significantly greater percentage of vials in the *orco* relative to wild-type treatment (Supplementary table 1). Increased exposure to males increases harm to female (Chapman & Partridge, 1996; Partridge et al., 1986, 1987; Partridge & Fowler, 1990), and we found evidence of reduced reproductive success, reproductive lifespan, and longevity in females that spent a greater proportion of their lifespan with males (supplementary table 2). Therefore, we calculated the percentage of a female's life spent with all three, at least two, and at least one male, and included these three percentages as fixed factors in all models of female fitness to statistically control for male death. To further ensure our results were not affected by differences in male death, we re-ran linear models testing for the effect of relatedness, genotype and their interaction on LRS using only the subset of vials in which no males died before the female (*orco* related n=17, *orco* unrelated n=26, wild-type related n=45, wild-type unrelated n=39). Likewise, we re-ran survival models, censoring vials in which males died before the female at the time point at which the first male died, and testing for the effect of genotype, relatedness and their interaction on female longevity and reproductive lifespan up to this point. To get a more in-depth assessment of the effect of relatedness within each genotype, we then ran censored survival models for each genotype individually, testing for the effect of relatedness on longevity and reproductive lifespan.

As we made behavioural observations over 5 consecutive day, with a further observation on day 8, we could track changes in behaviour over this period. Similarly, as we placed all groups into new vials each 3-4 days, we could track reproduction throughout a female's lifespan, using data from each consecutive vial from the start of experimental trials to the point at which no vials in any treatment produced offspring. We compared daily behavioural rates and age-specific offspring count for related and unrelated treatments within each genotype using Wilcoxon rank sum tests. Due to the wide range of the data, we used median values for aggression, courtship and offspring counts, but used mean values for mating data which ranged between 0-3. We then compared the rate of change in daily behaviours and age-specific reproductive success between related and unrelated groups within each genotype by regressing standardised median (or mean for mating data) daily behavioural rates and age-specific reproductive success against time, including the interaction between relatedness and time as a fixed effect. Standardisation made relative rates of change comparable between groups. To ensure results were not influenced by male death, we repeated all age-specific reproductive success analyses using only vials in which no males died before females.

For both behavioural and fitness analysis, we visually assessed model fit with diagnostic plots and, when necessary, used square root transformations to ensure data met parametric assumptions. When models exploring the interaction between genotype and relatedness revealed effects of relatedness nearing significance within either genotype, we re-ran models for each genotype individually to further explore these trends.

RESULTS

Relatedness, olfaction, and behaviour

We predicted that if relatedness reduces male-male aggression and male-female harassment, and this is mediated through olfaction, then aggression, courtship and mating efforts should decrease with relatedness in wild-type but not *orco* groups. Our results were largely inconsistent with these predictions. We found no interaction between relatedness and genotype, and no main effect of relatedness on overall aggression, courtship and mating rates (aggression: interaction: $F_{1,216}$=1.76, p=0.19; relatedness: $F_{1,216}$=1.23, p=0.27; courtship: interaction: $F_{1,216}$=0.28, p=0.60; relatedness: $F_{1,216}$=1.78, p=0.18; mating: interaction: χ^2_1=0, p=0.98; relatedness: χ^2_1=0.019, p=0.89; Fig. 1). Genotype significantly influenced behaviours: *orco* groups showed higher overall aggression rates, but lower overall courtship and mating rates relative to wild-type groups (aggression: $F_{1,216}$=16.75, p<0.0001; courtship: $F_{1,216}$=5.65, p=0.018; mating: χ^2_1=37.37, p<0.0001). To further explore the effects of relatedness within each genotype, we analysed each genotype individually. *orco* males showed a trend for reduced overall aggression in related groups ($F_{1,106}$=3.63, p=0.059), and tended towards reduced overall courtship rates in related groups ($F_{1,106}$=3.09, p=0.082), but relatedness did not influence mating (χ^2_1=0.011, p=0.92). We found no influence of relatedness on any behaviours in wild-type groups (aggression: $F_{1,110}$=0.017, p=0.90; courtship: $F_{1,110}$=0.24, p=0.63; mating: χ^2_1=0.0082, p=0.93).

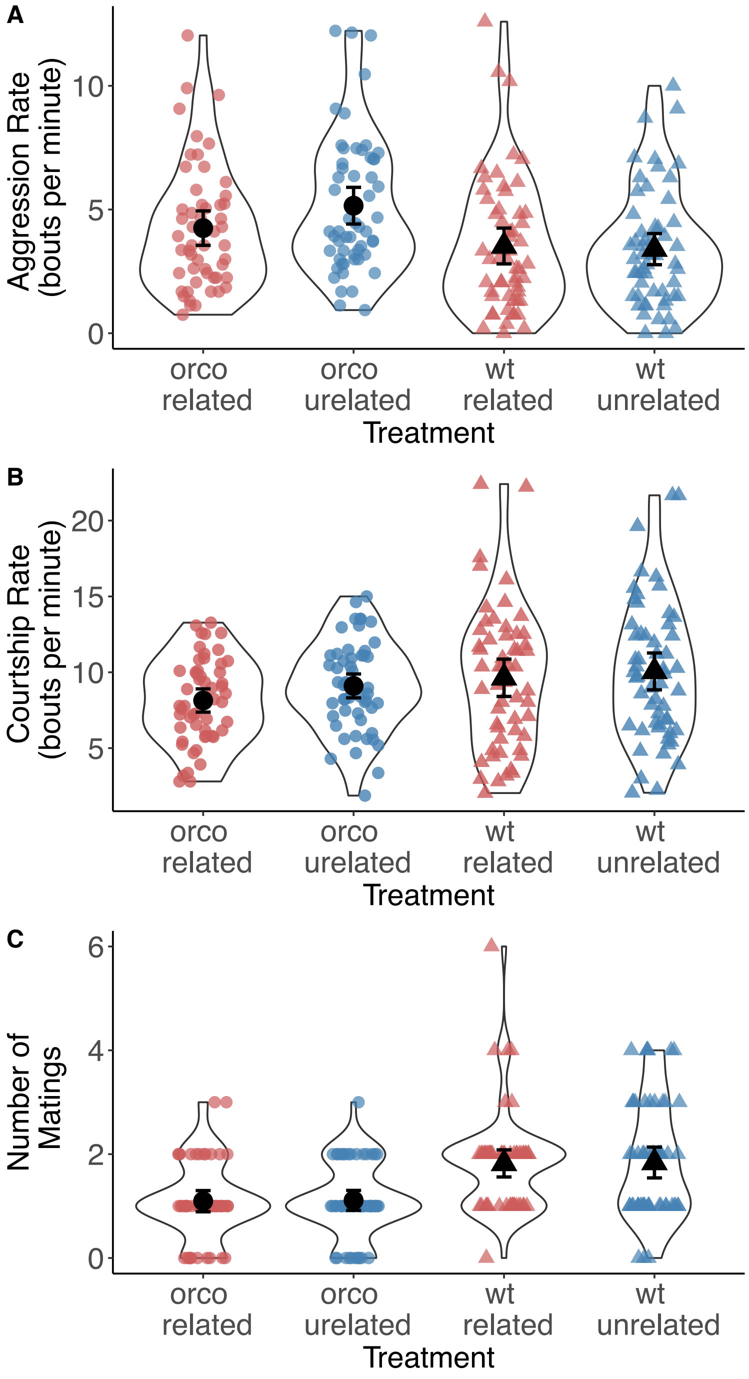


Figure 1

The relationship between genotype and relatedness, and overall aggression (A), courtship (B), and mating (C) rates. Red points illustrate related groups, blue points illustrate unrelated groups, circles illustrate orco groups, and triangles illustrate wild-type groups, 'violin' areas represent the shape of the distribution of data.

We found no influence of relatedness on daily aggression, courtship and mating rates in either genotype (*orco*: aggression W=15, p=0.69, courtship W=12.5, p=0.41, mating W=17, p=0.94; wild-type: aggression W=19.5, p=0.87, courtship W=19, p=0.93, mating W=16.5, p=0.87; fig. S1). In both genotypes, daily aggression rates remained consistent across the observation period (*orco*: $F_{1,8}$=3.67, p=0.09; wild-type: $F_{1,8}$=0.73, p=0.42; fig. S1a). Daily courtship rates remained consistent throughout the observation period in *orco* males ($F_{1,8}$=2.35, p=0.16) but showed a declining trend in wild-type males ($F_{1,8}$=4.46, p=0.07), but this rate of decline was not influenced by relatedness ($F_{1,8}$=0.04, p=0.85; fig. S1b). Daily mating rates showed a significant decline throughout the observation period in both genotypes (*orco*: $F_{1,8}$=6.95, p=0.03; wild-type: $F_{1,8}$=7.83, p=0.02), but the rate of decline was not influenced by relatedness (*orco*: $F_{1,8}$=0.001, p=0.98; wild-type $F_{1,8}$=0.0004, p=0.98; fig. S1c).

Relatedness influenced female longevity in orco but not wild-type treatments

We predicted that if olfaction is involved in the reduction in male-female harm in response to relatedness, relatedness should increase female longevity, lifetime reproductive success (LRS) and reproductive lifespan (RL) in wild-type but not *orco* groups. Again, our results were largely inconsistent with these predictions.

We initially censored groups at the point of female death, controlling for male death by including the percentage of a female's life in which males were present as a covariate. This revealed no interaction between relatedness and genotype, and no overall effect of relatedness (longevity: interaction: χ^2_1=0.033, p=0.86, relatedness: χ^2_1=1.56, p=0.21; LRS: interaction:$\chi^2_{1,224}$=1.22, p=0.27; relatedness: $\chi^2_{1,224}$=0.001, p=0.97; RL: interaction: χ^2_1=0.0008, p=0.98;

relatedness: χ^2_1=0.0007, p=0.98), but longevity, LRS and RL were all significantly greater in *orco* relative to wild-type groups (longevity: χ^2_1=9.79, p=0.0018; LRS: $\chi^2_{1,,224}$=108, p<0.0001, RL: χ^2_1=4.41, p=0.036; Fig, 2a,c&e).

Analysis of female longevity and RL censored at the point at which the first male died, and LRS analysis including only vials in which no males died, revealed similar patterns. However, when repeating this censored analysis for each genotype individually, we found that in orco *groups*, female longevity was significantly reduced when housed with unrelated males relative to related males (χ^2_1=4.83, p=0.028), but relatedness had no effect on longevity in wild-type groups (χ^2_1<0.001, p=0.98) or on LRS and RL in either genotype (*orco* LRS: $\chi^2_{1,41}$=2.9, p=0.090; *orco* RL: χ^2_1= 0.27, p=0.60; wild-type LRS: $\chi^2_{1,82}$=0.44, p=0.51; wild-type RL: χ^2_1= 0.141, p=0.71; Fig, 2b,d&f).

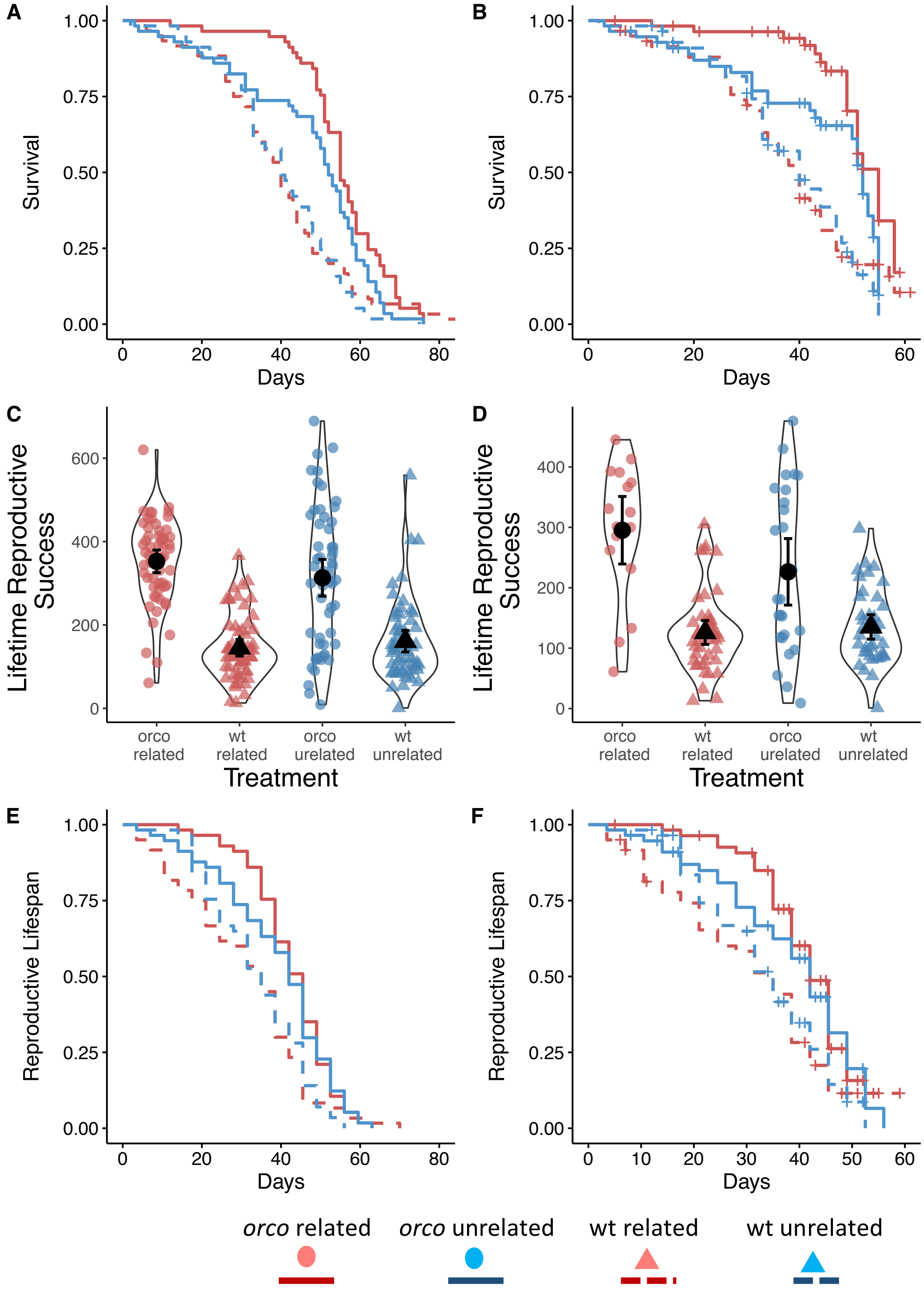


Figure 2

The relationship between genotype and relatedness, and female longevity(A-B), female lifetime reproductive success (C-D), and female reproductive lifespan (E-F). A,C&E include all groups, B,D&F include only vials in which no males died *before females. Red points illustrate related groups, blue points illustrate unrelated groups, circles illustrate ocro groups, and triangles illustrate wild-type groups, 'violin' areas represent the shape of the distribution of data.*

In all treatments, female reproduction significantly decreased throughout a female's lifespan (*orco*: $F_{1,36}$=266.4, p<0.0001; wild-type: $F_{1,36}$=220.0, p<0.0001; fig. S2a). We found no influence of relatedness on age-specific reproduction (*orco* W=207, p=0.86; wild-type: W=185.5, p=0.68) or in the rate of reproductive decline in either genotype (*orco*: $F_{1,36}$=0.03, p=0.85; wild-type: $F_{1,36}$=0.13, p=0.71; fig. S2a). We found no evidence that effects of relatedness on age-specific reproduction were masked by male death, as patterns where consistent when excluding vials in which males died before females (fig. S2b).

DISCUSSION

We investigated the role of olfaction in mediating male-male aggression, male-female harassment, and female harm in response to relatedness in the fruit fly, *Drosophila melanogaster*. Although we detected no response to relatedness in wild-type flies, contrasting with previous evidence (Carazo et al., 2014, 2015), we found some evidence consistent with reduced sexual conflict from some aspects of male behaviour and female survival in response to high relatedness in olfactory-deficient flies. Olfactory deficient males tended to show lower aggression when in related groups, although this did not reach the level of significance. Likewise, after accounting for male death, female longevity was greater in the presence of related olfactory-deficient males relative to unrelated olfactory-deficient males. However, we found no evidence that this increased longevity translated into greater reproductive lifespan or lifetime reproductive success. Kin selection theory predicts that increased relatedness should reduce sexual conflict, and our findings of reduced male-male aggression and increased female longevity in related olfactory-deficient flies are consistent with this, implying a response that doesn't rely solely on olfaction. Therefore, our results suggest that kin recognition underlying male responses to local relatedness are either independent of olfaction, or if olfaction is involved other cues make it redundant.

Olfactory deficient flies respond to relatedness

Our findings that females housed with related *orco* males experienced greater longevity, with related *orco* males trending towards decreased aggression rates, are consistent with olfactory-deficient males reducing harmful behaviours in response to relatedness. This suggests that, counter to our predictions, olfaction alone might not play a key role in behavioural responses to relatedness.

Olfaction is an important social cue in this species, involved in broad-scale species-specific and sex-specific recognition, and even in more fine-scale recognition of sexual familiarity (Billeter et al., 2009; de la Paz Fernández et al., 2010; Tan et al., 2013), making it a likely candidate mechanism for behavioural responses to relatedness. Given that Perry and colleagues (unpublished) found that genes involved in olfactory processes and functions were downregulated in response to related rivals, the lack of influence of olfaction is intriguing. It is possible that, when available, olfaction contributes towards kin discrimination in combination with a range of other sensory cues which can continue to mediate this response in the absence of olfaction. For example, male *D. melanogaster* use multiple redundant cues in the detection of rivals, with any two cues from smell, sound or touch sufficient to trigger adaptive responses in mating behaviour (Bretman, Westmancoat, et al., 2011). Many sensory cues are involved in social interactions in *D. melanogaster*. For example, olfactory and gustatory cues are important in shaping courtship behaviours (Krstic et al., 2009; Rybak et al., 2002), and females can visually discriminate between males marked with different coloured powders (Mery et al., 2009). Any of these sensory cues could allow response to relatedness, providing the cue contains sufficient variability (Breed, 2014). Indeed, there is evidence of gustatory, visual, and vocal cues signaling relatedness in insects (Lymbery & Simmons, 2020), mammals (Breed, 2014; Sebe et al., 2004), fish (Mehlis et al., 2008), and birds (Leedale et al., 2020). Therefore, because a range of sensory cues might have the potential to support kin discrimination, *D. melanogaster* might exhibit adaptive redundancy in responding to relatedness, using multiple, interchangeable sensory cues. In our experiment, olfactory-deficient flies had full use of auditory and tactile senses, and further investigation of the involvement of these cues in kin discrimination could shed light on the potential for adaptive cue redundancy in this species.

Another possibility is that responses to male relatedness could be mediated by females. Female *D. melanogaster* are capable of biasing their mate choice towards phenotypically novel males, which can be signaled through pheromonal differences (Krupp et al., 2008; Ödeen & Moray, 2008). In this study, all females possessed full olfactory capabilities, allowing them to detect such pheromonal signals. If unrelated groups represent greater mate novelty, and this increases female receptivity, this could lead to higher male aggression and courtship rates, with the potential for greater female harm. Furthermore, lower male relatedness increases the genetic diversity of the sperm and seminal fluid proteins females are exposed to, and this may increase the severity of female post-mating immune responses (McGraw & O'Neill, 2004), reducing female longevity. These hypotheses have not been ruled out by other studies of responses to relatedness in *D. melanogaster* (Carazo et al., 2014, 2015; Chippindale et al., 2015; Martin & Long, 2015). That said, although this presents an interesting hypothesis, it does not explain why such a pattern was not observed in the wild-type treatment, and further research in the role females play in the response to male relatedness would be informative.

It is important to note that although we observed reduced male-male aggression and increased female longevity with increased male relatedness in the olfactory-deficient treatment, suggesting reduced sexual conflict in response to relatedness, this did not translate into increased female reproductive lifespan or reproductive success. Inclusive fitness theory predicts that relatedness should reduce male harm to females, and thus increase both female survival and reproductive success (Hamilton, 1964; Pizzari & Gardner, 2012; Rankin, 2011; Wild et al., 2011). However, although Carazo and colleagues (2014) demonstrated increased reproductive success in female *D. melanogaster* when exposed to related male triplets, they replaced male triplets with new young triplets every week, whereas the male triplets used in this experiment co-aged with their female counterparts. As males that exhibit higher mating and harassment behaviours early in life might experience a greater age-related decline in reproductive behaviour (Sepil et al., 2020), male co-ageing likely dilutes the lifetime effects of male-female harm. This might explain why the signs of female harm in our experiment (i.e. reduced longevity) were more subtle than the reduced reproductive success observed by Carazo and colleagues (2014).

Wild-type flies did not respond to relatedness

The lack of response to relatedness in terms of social behaviour or female harm in the wild-type treatment was inconsistent with previous work, in which groups of related brothers show decreased aggression and courtship relative to unrelated groups, resulting in a higher lifetime reproductive success and slower reproductive ageing of their mates (Carazo et al., 2014, 2015). Similar responses have been reported by Martin & Long (2015) in which related males showed decreased aggression and courtship, and by Le Page and colleagues (2017), in which females housed with related familiar males had a greater lifetime reproductive success than those with unrelated unfamiliar males.

However, though these studies provide strong and consistent evidence that levels of male-male aggression and male-female harm respond to local relatedness, this effect is highly context-dependent. For example, although Carazo and colleagues found effects of relatedness on male behaviour in both 2014 and 2015 studies, the effect of male relatedness on male-male aggression reported in the 2015 study was almost double that in the 2014 study, potentially due to the addition of live yeast to vials. This demonstrates the high sensitivity of such behavioural responses to precise aspects of the experimental environment, and this sensitivity may explain why we did not observe any responses to relatedness in this study.

Furthermore, although the aforementioned studies detected significant, adaptive responses to relatedness, some previous research in *D. melanogaster* has detected no response, and the precise nature of the response differs between studies. For example, Martin & Long (2015) observed no influences of relatedness female longevity or lifetime reproductive success despite finding decreased aggression in groups of related males. Conversely, Le Page and colleagues (2017) observed no behavioural responses to relatedness, but did observe increased female longevity and reproductive success in related familiar groups. Hollis and colleagues (2015) reported increased female reproductive success when male relatedness was accompanied by high familiarity, and Chippindale and colleagues (2015) found no effect of male relatedness on female longevity or lifetime reproductive success, with high male-female harm across all treatments. Similar discrepancies have been found in the seed beetle, *Callosobruchus maculatus*: while Lymbery & Simmons, (2017) found female reproduction increased when with related familiar males, Berg and colleagues (2019) report no influence of relatedness on female lifetime reproductive success. Therefore, while the existing literature suggests that males can respond to relatedness and familiarity, this response is highly variable and context-dependent, which might result in the response sometimes being undetected, as in this study. Further research into the precise environmental cues both required for males to respond to relatedness and that may confound or override such responses is necessary for a more robust understanding of this effect.

Behaviour and fitness differed between wild-type and olfactory-deficient flies

Regardless of relatedness, olfactory-deficient males showed elevated male-male aggression, but reduced longevity, courtship, and copulation relative to wild-type males. Likewise, females exposed to olfactory-deficient males enjoyed a significantly greater lifetime reproductive success, reproductive lifespan, and longevity relative to those exposed to wild-type males. Although previous research does not suggest that the knock-out mutation of the *orco* gene, Or83b, causes major differences in male longevity or courtship (Gailey et al., 1986; Krstic et al., 2009; Tan et al., 2013; Trott et al., 2012), justifying the use of this genotype in our experimental design, it cannot be ruled out that differences between the two male genotypes might reflect either their reduced olfactory abilities or other side-effects of genotypic differences. For example, in *D. melanogaster*, olfaction is important in many social interactions, including courtship, mate choice, and the detection of potential rivals (Billeter et al., 2009; de la Paz Fernández et al., 2010; Gailey et al., 1986; Garbaczewska et al., 2013; Tan et al., 2013), and the cumulative effect of slight disruption to multiple nuances of behavioural interactions could be responsible for the behavioural and fitness differences we detected between wild-type and olfactory-deficient flies. Furthermore, it is possible that the white vs red Dahomey backgrounds of *orco* and wild-type males respectively might have resulted in slight behavioural differences between the two genotypes. Although a mini-white gene was used to rescue *orco* male eye function, previous research suggests that the mini-white gene does not fully rescue all aspects of wild-type behaviour, with males exhibiting elevated male-male courtship (Krstic et al., 2013) and reduced aggression (Hoyer et al., 2008) relative to controls. However, we observed elevated, not reduced, aggression in *orco* relative to wild-type males. Future work in which olfactory cues are removed using multiple methods such as ablation of the oenocytes responsible for expression of cuticular hydrocarbons (Billeter et al., 2009) might allow us to better disentangle the effects of olfactory loss from potential side effects of transgenic lines.

CONCLUSION

This study provides insight about the relationship between genetic relatedness and sexual conflict, highlighting the context-dependence of this relationship and suggesting that kin discrimination in *D. melanogaster* might not be mediated by olfaction alone. We hope that these results encourage future work in uncovering the sensory mechanism underlying behavioural responses to relatedness in this important model species.

SUPPLEMENTARY MATERIAL

Supplementary table 1

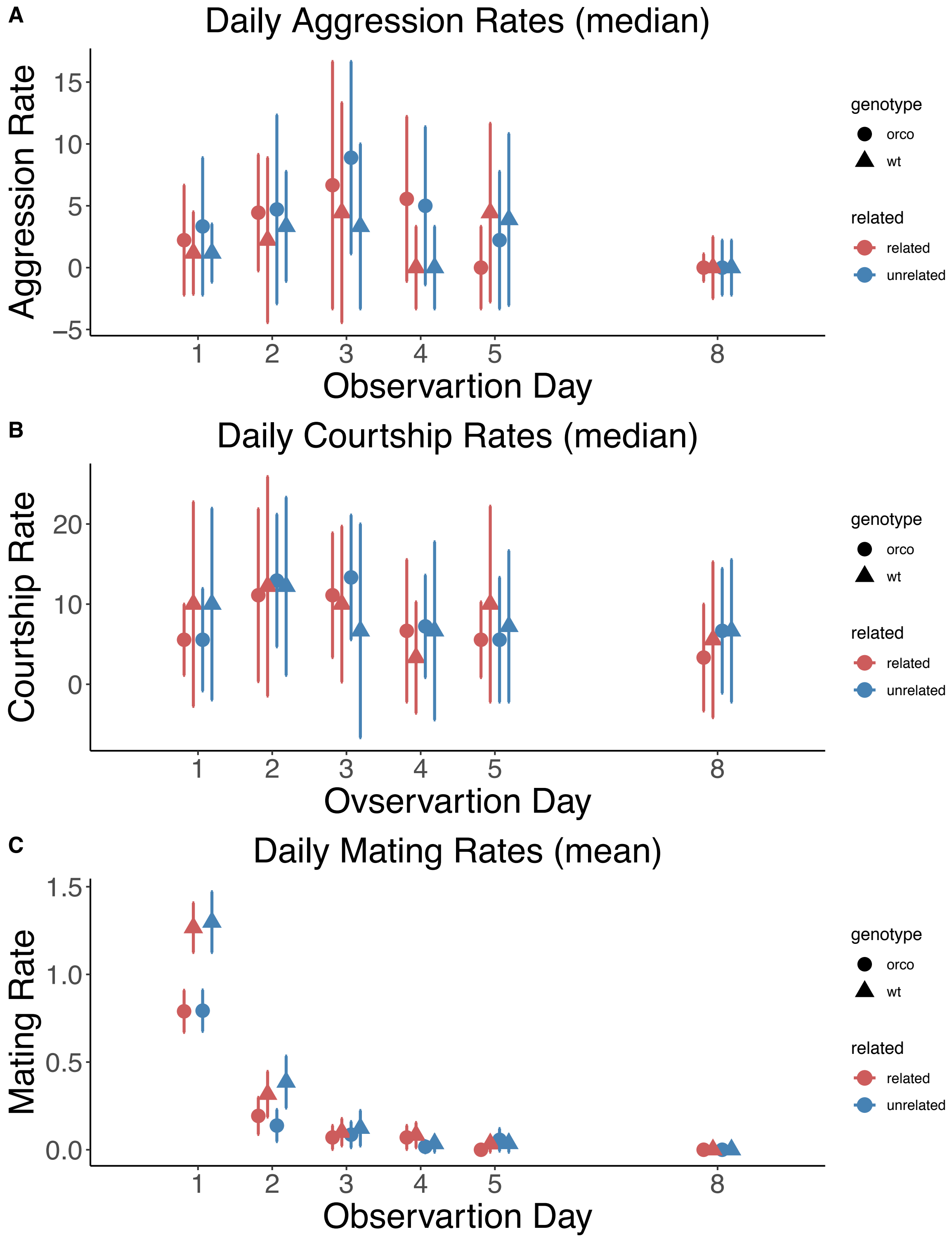
The number of vials in which 0, 1, 2, or 3 males died before the female for each treatment. Genotype significantly influenced the percentage of males that died (χ^2_3=28.1, p<0.0001), but within each genotype, there was no effect of relatedness (orco: χ^2_3=6.4, p=0.09; wt: χ^2_3=3.6.1, p=0.31).

	Orco Related		Orco Unrelated		WT Related		WT Unrelated	
	n	%	n	%	n	%	n	%
No males died before female	17	29.8	26	45.6	45	75	39	68.4
1 male died before female	12	21.1	9	15.8	7	11.7	9	15.8
2 males died before female	8	14.0	12	21.1	4	6.7	7	12.3
All males died before female	20	25.1	10	17.5	4	6.7	2	3.5
total	57		57		60		57	

Supplementary table 2

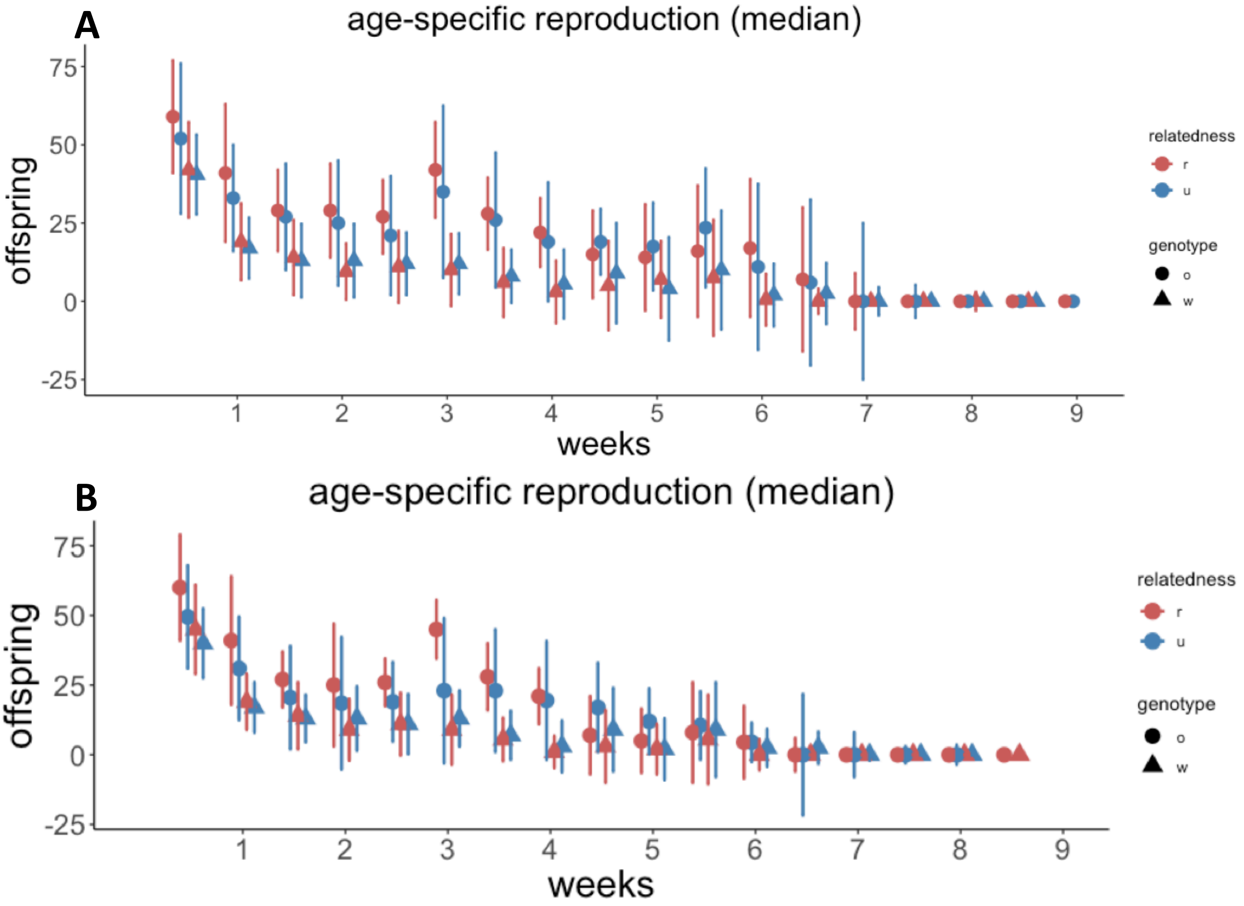
The influence of early male death on female fitness. Models were run to test the effects of the percentage of a female's life spent with all three males, at least two males, and at least one male on longevity, reproductive lifespan (survival models) and lifetime reproductive success (linear model). Model statistics correspond to the percentage of a female's life spent with all three males.

Female fitness parameter	Effect of early male death	
	direction	Model statistics
Longevity	increased	χ^2_1=21.6, p<0.0001
Lifetime Reproductive Success	increased	χ^2_1=6.7, p=0.010
Reproductive Lifespan	increased	χ^2_1=4.95, p=0.026



Supplementary Figure 1

Daily behavioural rates. A) shows median aggression rates, B) shows median courtship rates, and C) shows mean mating rates. Red points illustrate related *groups, blue points illustrate unrelated groups, circles illustrate ocro groups, and triangles illustrate wild-type groups*



Supplementary Figure 2

Age-specific median female reproductive success. A) includes data from all vials, B) includes data from just vials in which no males died before females. Red points illustrate related groups, blue points illustrate unrelated groups, circles illustrate ocro groups, and triangles illustrate wild-type groups.

Chapter 5: A poor larval diet reduces adult aggression in male *Drosophila melanogaster*

ABSTRACT

Aggressive behaviours occur throughout the animal kingdom, with agonistic contests governing access to resources. Nutrition experienced during development has the potential to influence aggressive behaviours in adults through effects on growth, energy budgets and an individual's internal state. In particular, poor-quality developmental nutrition might decrease adult aggression through limiting growth and energy budgets, or alternatively might increase adult aggression through enhanced motivation to compete for resources. However, the direction of this relationship – and effects of developmental nutrition experienced by rivals - remains unknown in most species, limiting our understanding of how early life environments contribute to variation in aggression. We investigated these alternative hypotheses by assessing male-male aggression in adult fruit flies, *Drosophila melanogaster,* that developed on low, medium or high protein diets. We found that low protein developmental nutrition reduced adult aggressive lunging, as well as threat displays against rivals that developed on low protein diets. These effects appeared to be independent of nutritionally-determined differences in body size. Males performed relatively more aggression on a central food patch when facing rivals of poor nutrition, suggesting that developmental nutrition affects aggressive interactions through social effects in addition to individual effects. Our finding that poor developmental nutrition reduces aspects of male-male aggression in *D. melanogaster* is consistent with the idea that resource budgets mediate

aggression and in a size-independent manner. This study opens the potential for further exploration of this model system to improve understanding of the links between nutrition and aggression.

INTRODUCTION

Aggression is widespread among animals (e.g., mammals, Sinn et al., 2008; birds, Johnsen & Zuk, 1995; fish, Neat et al., 1998; Seebacher et al., 2013; and invertebrates, Brown et al., 2007; Elias et al., 2010), including humans, where aggressive behaviours can have detrimental effects on societies (Blanchard & Blanchard, 2003; Georgiev et al., 2013; Sluyter et al., 2003). Success in aggressive contests can provide superior access to critical reproductive resources such as food, territories and mates (Belenioti & Chaniotakis, 2020; Clutton-Brock & Albon, 1979; Georgiev et al., 2013; Hoffmann, 1987b; Huntingford et al., 2012), but contests come with costs, including physical damage, time and energy expenditure, and increased predation risk (Briffa & Sneddon, 2007; Haley, 1994; Neat et al., 1998). Individuals often display temporary, reversible changes in aggressive behaviours throughout life (Georgiev et al., 2013; Huntingford et al., 2012), and much of this variation is likely to result from differences in an individual's environment (Dochtermann et al., 2015; Han & Dingemanse, 2017). Understanding the ecological factors that determine aggressive behaviour can help to elucidate its evolution and consequences.

Nutrient availability and quality are key components of an individual's environment and can shape behavioural strategies expressed throughout life

(Lihoreau et al., 2015). In many species, early life is key for nutrient acquisition, and the balance of nutrients in this critical period can have profound effects on body size, resource allocation to adult traits, and internal state (Amitin & Pitnick, 2007; Han & Dingemanse, 2017; Lihoreau et al., 2015; Pillay et al., 2016; Royle et al., 2005; Zikovitz & Agrawal, 2013). These effects can determine the relative ability (i.e., resource-holding potential) and motivation (i.e., resource valuation) to invest in aggressive contests and the fitness pay-offs from doing so. Thus, individuals should benefit from moderating aggression adaptively in response to nutritional environments experienced in early life (Scharf, 2016).

However, the net effect of developmental nutrition on aggressive ability and motivation – and hence the direction of the relationship between developmental nutrition and aggression – remains unclear. High-quality developmental nutrition often increases adult body size, and larger individuals are more likely to initiate and win aggressive contests in many species (Bath et al., 2018; Briffa & Sneddon, 2007; Brown et al., 2007; Hoffmann, 1987b; Shackleton et al., 2005). Likewise, high nutrient availability during development can increase relative resource allocation to traits (such as weapons) that enhance aggressive ability (Monaghan, 2008; Colasurdo et al., 2009). Alternatively, a high-quality developmental diet might decrease aggressive motivation and the fitness benefits an individual gains from attaining a resource through effects on internal state (Arnott & Elwood, 2008; Bath et al., 2018; Elias et al., 2010).

The few studies that have investigated the relationship between developmental nutrition and aggression report contrasting responses across species. In the

African striped mouse, *Rhabdomys dilectus chakae*, early-life protein deficiency leads to increased aggressive behaviour (Pillay et al., 2016), whereas in the Southern field cricket, *Gryllus bimaculatus*, a high-protein developmental diet increases aggression (Han & Dingemanse, 2017). Furthermore, variation in developmental diet among individuals can influence aggressive interactions by generating asymmetries in fighting ability among rivals (Asahina, 2017; Briffa & Sneddon, 2007; Parker, 1974), but few studies have evaluated effects of developmental nutrition on both focal and rival individuals. Hence, the strength and direction of early life diet effects on adult aggression remain largely unknown.

We used the fruit fly, *Drosophila melanogaster*, to investigate how nutrition experienced during development influences adult male aggressive strategies. *Drosophila melanogaster* serves as an important model organism for both aggression and nutrition. Male *D. melanogaster* engage in frequent contests over mates and territories, and success in contests influences mating success (Kravitz & Fernandez, 2015). Aggressive behaviours range from wing threat displays and fencing spars with forelegs to lunging, the principal aggressive behaviour, in which a male rears up and thrusts his upper body at his opponent (Andrews, 2016; Hoffmann, 1987; supplementary table 1). As a holometabolous insect with a juvenile food-acquiring stage distinct from the adult stage, the diet received during early life is critical to development in *D. melanogaster* (Boggs, 1981). For example, early-life diet strongly impacts viability, body mass and post-copulatory reproductive traits (e.g. Bross et al., 2005; Gebhardt & Stearns, 1993; McGraw et al., 2007). However, despite the wealth of knowledge on both diet and agonistic contests in this species, the direct link between developmental nutrition and adult aggressive behaviours has not been fully explored. Understanding the relationship between developmental nutrition and aggression would help to establish a system for in-depth investigation of the underlying functional mechanisms linking nutrition and aggression.

We subjected male flies to low, medium or high protein diets during larval development and then measured their adult aggressive behaviours. To test the effects of both an individual's diet and the diet of its social partners, we set up contests between pairs of males from all combinations of diet treatment in a fully factorial experimental design. Protein availability is a key component of developmental nutrition (Harrison et al., 2017; Lihoreau et al., 2015) and can be limiting under natural conditions (Han & Dingemanse, 2017). We predict that if developmental nutrition influences aggression through effects on growth and allocation to traits that mediate aggression, then high protein larval diets will increase aggression, whereas if developmental nutrition influences aggression through effects on motivation, then low protein larval diets will increase aggression.

METHODS

Experimental Flies

All flies were derived from an outbred wild-type Dahomey stock population that has been maintained since 1970 in cages with overlapping generations (Carazo et al., 2015). Stock populations were maintained on standard laboratory medium (supplementary table 2). Fly husbandry was carried out at 25^{o}C on a 12:12h light:dark cycle.

To generate experimental males, we collected eggs from the stock population using grape-agar plates smeared with yeast, transferred them at a standard density (50 eggs/vial) to vials containing food media (low, medium or high protein), and incubated them until eclosion. We modified standard sugar-yeast-agar medium (0.5 sugar, 1.0 yeast) to create a ‘low’ (L; 10% standard yeast), ‘medium’ (M; 20% standard yeast), and ‘high’ protein media (H; 120% standard yeast; supplementary table 3). Preliminary tests showed that these differences in protein level were sufficient to affect fly development, generating differences in developmental duration and adult body size. Developmental duration was extended as protein quantity was reduced, so egg collections for each treatment were staggered to synchronise adult eclosion. Developmental duration can also influence body size, which might therefore influence aggression. Although the implications for adult behaviour arising from variation in developmental duration in response to the nutritional environment are interesting, this is beyond the scope of our experiment. Therefore, to reduce size variation resulting from developmental duration within treatments, we excluded extreme early or late emerging adults from the experiment (developmental windows included: L=14-16 days, M=11-12 days, H=10 days). We note that future work investigating the interaction between nutrition and developmental duration on aggression would help to further refine how developmental conditions influence adult behaviours.

We collected adult males using ice anaesthesia within 6 h of eclosion to ensure virginity, and transferred them to vials containing standard laboratory medium, housing them individually to prevent the formation of social hierarchies that might influence aggressive behaviour (Penn et al., 2010; Trannoy et al., 2016). To differentiate males in behavioural trials, we painted each male with a small dot of red or white acrylic paint on the dorsal thorax between 1 and 4 days post-eclosion. Similar paint treatments had no detectable effect on behaviour in previous studies (Morimoto et al., 2016; Nilsen et al., 2004).

Behavioural Trials

Before trials, we deprived flies of food for two hours in vials containing moist cotton wool to prevent desiccation. We then transferred pairs of males via gentle aspiration into observation chambers (20 mm diameter, 5 mm depth) containing a food patch of standard food media combined with yeast paste (5 mm diameter). In each pair, we arbitrarily designated one male as the focal male and the other the rival male. We paired males in all possible combinations of treatments in a fully factorial design (supplementary table 4). To avoid confounding effects of paint colour, we painted half of the focal males red and the other half white. After a 5 minute acclimatisation period, we recorded behaviour using a video camera (Toshiba Camileo X400) for 15 minutes. We conducted behavioural trials between 2-7h Zeitgeber time over four days.

A single observer blind to the larval nutritional treatment scored the videos using JWatcher (v. 1.0,Blumstein & Daniel, 2007), recording the duration and frequency of five aggressive behaviours (fencing, chasing, tussling, lunging and wing threat; supplementary table 1) for each focal and rival male. We also recorded the total locomotion duration and whether behaviours were performed on or off the food patch to allow testing for differences in locomotion and food patch access respectively.

After trials, we froze and weighed males to assess the influence of diet on body mass. We weighed flies immediately after freezing to assess their mass during trials (wet mass), and weighed them again after they were dried for 48 hours at 60^{o}C (dry mass).

Statistical Analysis

We performed analyses in R, version 3.6.2 (2019-12-12). We expressed behavioural data as total durations (in seconds) of each behaviour, and also as count data for lunges. To test the influence of focal male nutrition, rival male nutrition, and their interaction on focal male aggression, we conducted general linear models for the number of lunges and duration of fencing, chasing and wing threat independently and for total aggression duration summed over all five recorded behaviours. Because both lunging and chasing data were zero-inflated (101 and 58 out of 187 focal males lunged or chased respectively), we analysed the influence of nutrition on the likelihood of these behaviours being displayed in binomial general linear models, and then on lunge frequency or chase durations in the subset of flies that did display these behaviours, using negative binomial and gaussian general linear models respectively. As there were only two incidents of tussling, we did not analyse tussling separately. Because nutrition might affect locomotion, which might impact aggressive encounters, we assessed the influence of focal and rival nutrition on locomotion in a linear model.

To assess the interdependence of behaviour of the two individuals in a pair, we assessed the influence of rival total aggression duration on focal total aggression duration in linear models. To test whether this relationship was influenced by larval nutrition, we ran an additional model including focal and rival nutrition and their interaction with rival aggression on focal total aggression.

In male *D. melanogaster*, food patches provide access to mates and males will aggressively defend these sites (Hoffmann & Cacoyianni, 1990; Lim et al., 2014; Markow, 1988). To test for difference in food patch occupancy, we analysed the influence of focal and rival larval nutrition and their interactions on the amount of time focal individuals spent on the food patch using linear models. To evaluate how diet affects aggressive behaviour around food patches, we assessed the influence of focal and rival larval nutrition on the proportion of aggressive behaviour that a focal individual performed on relative to off the food patch using general linear models fitted with quasibinomial distributions.

We used diagnostic plots to assess model fit, and where data were over-dispersed, we used square root or log transformations to avoid violating the assumptions of parametric statistics. We included day as a fixed factor (Harrison et al., 2018) and Zeitgeber time as a covariate in all models to account for temporal variation in behaviour. We initially assessed models using type II ANOVA (analysis of variance) tests, and, when significant effects of developmental nutrition were detected, we further explored the effect of nutrition using post hoc Tukey tests. Because developmental nutrition might influence behaviour through effects on body mass, we first analysed the influence of developmental nutrition on wet and dry body mass using linear models. For aggressive behaviours that showed a significant response to focal or rival developmental nutrition, we further explored whether body mass had an effect on aggression above and beyond that of developmental nutrition, using simplified models including only focal or rival nutrition (depending on which had shown an effect in original models), and the corresponding mass, conducting sequential sum of squares analysis (type I ANOVA) to test the effect of mass after the main effects of developmental nutrition had been accounted for. For these latter two analyses, we used dry mass, as a measure of body size independent of recent water intake.

RESULTS

Developmental nutrition influenced adult mass

As expected, we found that developmental nutrition influenced adult mass (wet mass: $F_{2,373}=56.4$, $p<0.0001$; dry mass: $F_{2,370}=60.2$, $p<0.0001$; fig. 1). Post hoc tests revealed that low nutrition males were significantly lighter than medium and high nutrition males, which didn’t differ in mass, with a 12% reduction in both wet and dry mass between high and low nutrition males. These results demonstrate that males responded to the diet treatments.

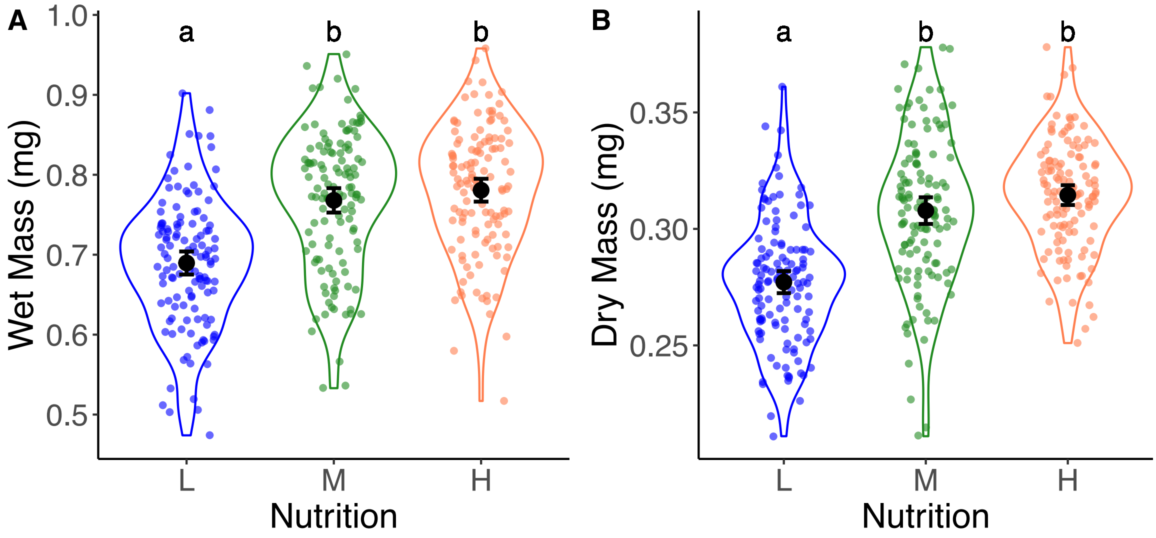


Figure 1

The wet mass (A) and dry mass (B) of adult males depending on their developmental nutrition. Black points show means; black bars show 95% confidence intervals, 'violin' areas represent the shape of the distribution of data.

Poor developmental nutrition reduced some aspects of aggression

A focal male's developmental nutrition influenced its likelihood of lunging (fig. 2a; $\chi^2_{2,174}$=8.1, p=0.018). Post hoc analyses revealed that high nutrition males were more likely to lunge than low and medium nutrition males (M-H comparison: z=-2.4, p=0.048; L-H comparison: z=-2.5, p=0.030). However, in the subset of males that displayed lunging, focal male diet did not influence the number of lunges displayed (fig. 2b; $\chi^2_{2,88}$=4.1, p=0.132). We detected no effect of a rival male's developmental nutrition on focal male lunging (probability of lunging: $\chi^2_{2,174}$=4.1, p=0.130; lunge frequency in those that lunged: $\chi^2_{2,88}$=2.1, p=0.335), nor evidence of an interaction between a male's developmental nutrition and that of his rival (probability of lunging: $\chi^2_{4,174}$=1.1, p=0.895; lunge frequency in those that lunged: $\chi^2_{4,88}$=6.5, p=0.162).

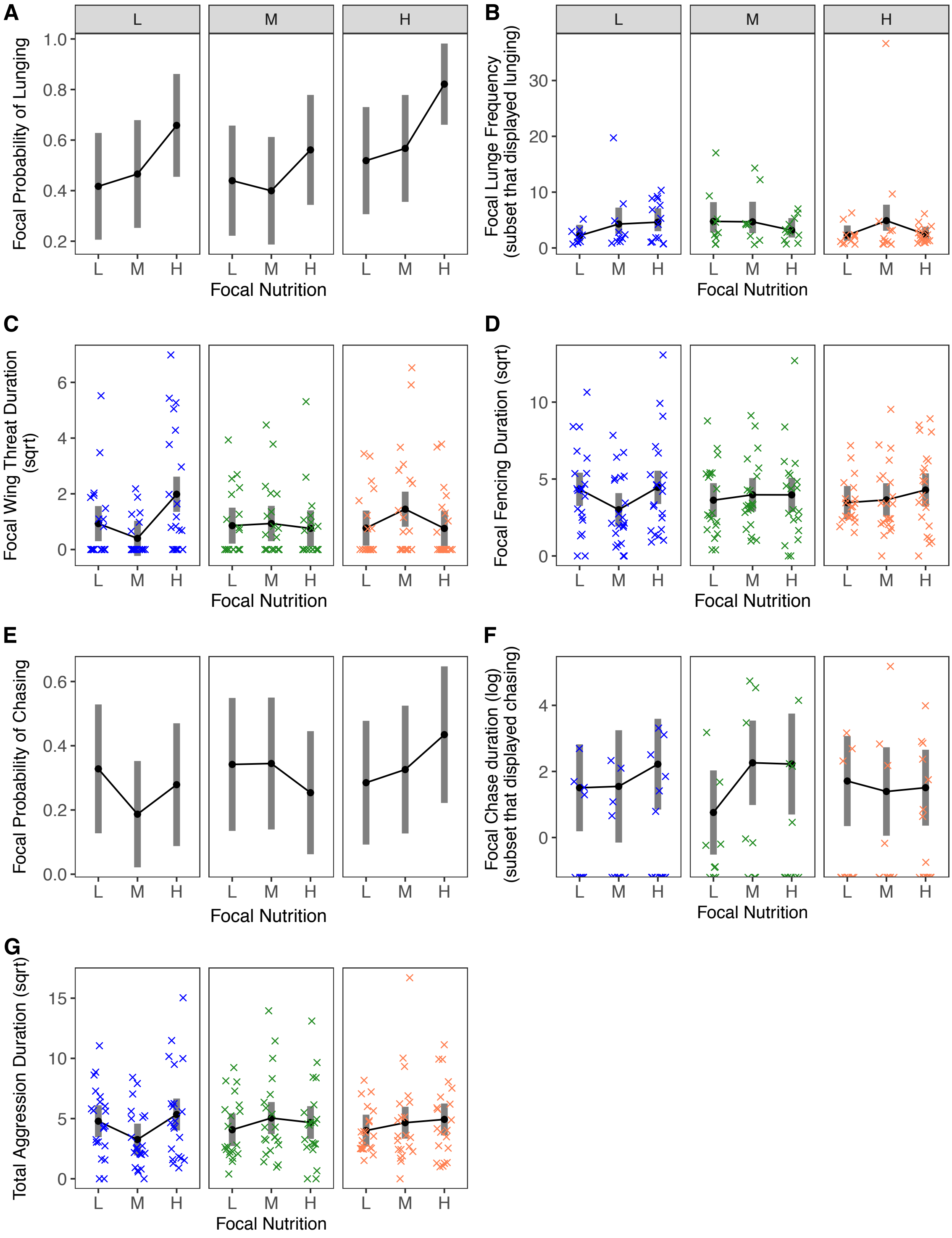


Figure 2

The influence of focal and rival developmental nutrition on focal male lunging probability (A), lunging frequency of those that displayed lunging (back-

transformed from negative binomial, B), wing threat duration (C), fencing duration (D), chase probability (E), chase duration of those that chased (F), and total aggression duration (G). The x-axis shows focal nutrition, and panels show rival nutrition. Grey bars represent 95% confidence intervals.

Our data do not support the hypothesis that high nutrition males lunge more often solely as a consequence of their larger body size, as focal male lunging probability was not related to focal male mass (χ^2_1=0.02, p=0.886), and sequential sums of squares analysis revealed no further effect of focal body mass after focal male developmental nutrition was accounted for (wet mass $F_{1,179}$=0.2, p=0.653; dry mass $F_{1,179}$=1.91, p=0.168).

Males of high-protein developmental nutrition showed increased wing threat compared with males that developed on lower protein, but only when against rivals of low protein (interaction: $F_{4,174}$=3.6, p=0.008; focal male nutrition $F_{2,174}$=6.4, p=0.002; rival nutrition $F_{2,174}$=0.06, p=0.938; fig.2c). This pattern was not explained by differences in mass, as we found no effects of focal or rival male mass on wing threat duration (focal male mass: $F_{1,176}$=0.04, p=0.816; rival male mass: $F_{1,176}$=0.10, p=0.748) and no interaction between focal and rival mass ($F_{1,176}$=0.16 p=0.691). Furthermore, sequential sums of squares analysis revealed no interaction between focal and rival body mass after focal and rival developmental nutrition were accounted for (interaction between focal and rival wet mass $F_{1,170}$=0.089, p=0.766; interaction between focal and rival dry mass: $F_{1,169}$=0.87, p=0.352). Threat displays can represent strategies to settle contests without costly escalation. However, we found no evidence that wing threat reduced escalated fighting, as there was a positive correlation between lunging duration and wing threat duration (Kendall's rank correlation τ=0.3, z=5.1, p<0.0001; supplementary fig.1).

The influence of larval nutrition on lunging and wing threat could not be explained by differences in locomotion, as we detected no differences in locomotion duration related to developmental nutrition (focal nutrition $F_{2,174}$=1.4, p=0.240; rival nutrition $F_{2,174}$=2.6, p=0.079). We found no detectable effect of the developmental nutrition of focal and rival males on other forms of male aggression (fencing: focal nutrition: $F_{2,174}$=1.2, p=0.291, rival nutrition: $F_{2,174}$=0.1, p=0.954, interaction: $F_{4,174}$=0.8, p=0.504; chasing probability: focal nutrition: $\chi^2_{2,174}$=0.3, p=0.879, rival nutrition: $\chi^2_{2,174}$=1.1, p=0.592, interaction $\chi^2_{4,174}$=2.5, p=0.645; chasing duration in those that chased: focal nutrition: $F_{2,45}$=0.9, p=0.431, rival nutrition: $F_{2,45}$=0.1, p=0.892, interaction: $F_{4,45}$=0.7, p=0.619; total aggression; focal nutrition: $F_{2,174}$=1.0, p=0.364, rival nutrition: $F_{2,174}$=0.03, p=0.968, interaction: $F_{4,174}$=1.3, p=0.273; fig.2).

The developmental diet of competitors influenced aggression performed on the food patch

We investigated how aggression related to access to the food patch because food patches represent valuable breeding sites for male *D. melanogaster*. We detected no effect of developmental nutrition on the time focal males spent on the food patch (focal nutrition: $F_{2,174}$=0.6, p=0.552, rival nutrition: $F_{2,174}$=0.1, p=0.867, interaction: $F_{4,174}$=0.8, p=0.519). However, focal males performed relatively more of their aggression on the food patch (as opposed to off the food patch) when competing against rivals of low developmental nutrition ($\chi^2_{2,168}$=18.9, p<0.0001; fig.3), but neither focal nutrition nor the interaction between focal and rival nutrition had a detectable effect (focal nutrition: $\chi^2_{2,168}$=3.4, p=0.182; interaction: $\chi^2_{4,168}$=8.1, p=0.089). Although focal males displayed relatively more of their aggression on the food patch as rival mass decreased ($\chi^2_{1,172}$=5.3, p=0.021; supplementary fig. 2), sequential sums of squares analysis revealed no further effect of rival mass after rival developmental nutrition was accounted for (rival wet mass $F_{1,178}$=0.8, p=0.474; rival dry mass $F_{1,176}$=0.2, p=0.849).

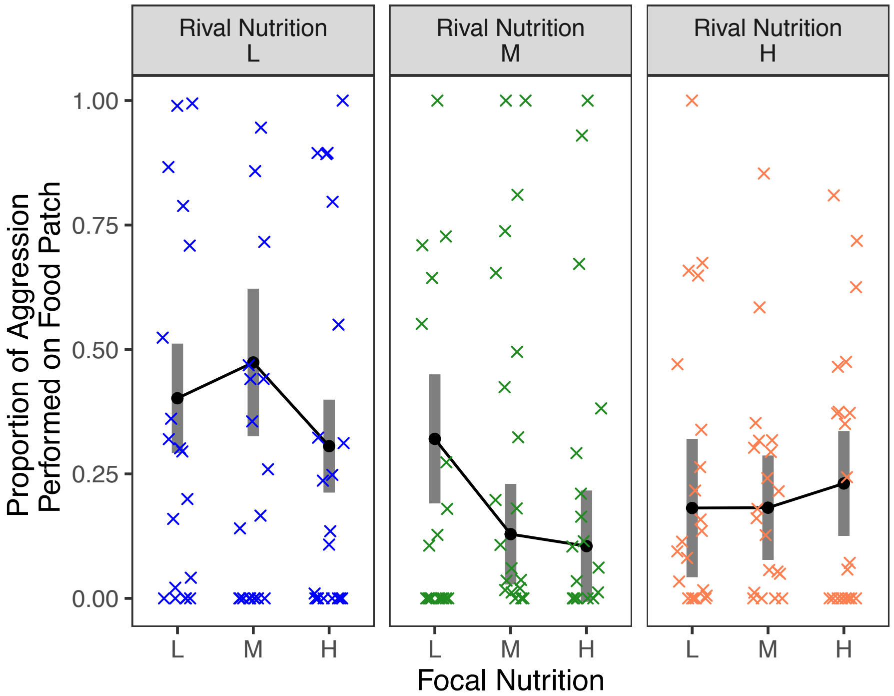


Figure 3

The influence of focal and rival larval nutrition on the proportion of aggression the focal male performs on the food, relative to off the food. Grey bars represent 95% confidence intervals.

Aggression levels are not correlated within pairs

Because rival behaviour might influence focal male behaviour, we examined the relationship between the two. The total duration of focal male aggression was not influenced by the total duration of rival aggression ($F_{1,181}$=1.2, p=0.277; supplementary fig. 3). We found no evidence that the relationship between focal and rival male aggression was influenced by differences in developmental nutrition (interaction with focal nutrition $F_{2,165}$=0.2, p=0.855, interaction with rival nutrition $F_{2,165}$=0.3, p=0.724).

DISCUSSION

We found that developmental nutrition influenced adult male aggression in *D. melanogaster*: low protein developmental nutrition reduced the likelihood of aggressive lunging (against all rivals) and the duration of wing threats (against rivals of low protein nutrition). Although decreased developmental nutrition reduced adult mass, the influence of developmental nutrition on aggression appears above and beyond any influence of body size. The developmental nutrition of rival males did not influence direct physical aggression rates, but did influence where males performed aggression and the use of threat displays. Focal males concentrated their aggression to the food patch when competing with rivals of low-protein developmental nutrition, suggesting that males may be better able to access food resources against lower-quality rivals. Our findings suggest that adult *D. melanogaster* alter aggressive tactics in light of their own developmental nutrition, through pathways distinct from body size, and that the nutritional environment of social partners also impacts contest characteristics.

The hypothesis that poor developmental nutrition would restricted growth and allocation to traits that mediate aggression (Monaghan, 2008) predicts that poor developmental nutrition should decrease adult aggression. The reduced lunging probability of males subjected to poor developmental nutrition supports this prediction. However, although low larval protein levels reduced adult body size, in line with previous findings (Monaghan, 2008; Zikovitz & Agrawal, 2013), differences in body size did not explain the relationship with aggression, over and above the effect of developmental nutrition. This suggests that the relationship is mediated through size-independent effects of nutrition, such as changes to the internal state and energy budget. The absence of size effects in our study might be explained by our experimental design, which minimised size variation within treatments; however, there remained substantial size variation among treatments, suggesting that insufficient variation did not explain the absence of size effects beyond nutritional effects. Our results suggest that underlying variation in the developmental environment might explain the positive association between size and aggression found in both male and female *D. melanogaster* (Bath et al., 2018; Hoffmann, 1987b; Hoyer et al., 2008; Markow, 1988) as well as other invertebrates (Brown et al., 2007; Shackleton et al., 2005) and vertebrates (reviewed in Briffa & Sneddon, 2007).

Developmental nutrition might play a larger role in determining aggression than body size per se does because nutrition can influences a range of physiological factors including resource allocation, energy levels, and the relative growth of different traits. Indeed, in male *Drosophila*, developmental nutrition can have wide-ranging impacts, with poor developmental nutrition reducing a male’s ability to transfer sperm and induce a refractory state in mates (McGraw et al., 2007), reducing his courtship success (Morimoto et al., 2016; Sharp & Agrawal, 2009; Wigby et al., 2016), and reducing his success in post-copulatory sperm competition (Bangham et al., 2002; Morimoto et al., 2016). These effects can also be independent of dietary influence on size (McGraw et al., 2007). Furthermore, in other species, diet-induced correlates of condition, such as resting metabolic rate and energy reserves, can better predict aggression than body size does (e.g., in the freshwater prawn *Macrobrachium rosenbergii*, Brown et al., 2003, swordtail *Xiphophorus helleri*, Royle et al., 2005, and damselfly *Calopterya splendens xanthostama*, Plaistow & Siva-Jothy, 1996). If developmental nutrition does cause differences in physiology, then males that develop on poor nutrition might adopt alternative strategies to maximize fitness returns from their limited energy reserves, rather than engaging in contests they are likely to lose.

We found that different aspects of male aggression - including chasing and fencing - did not show the same response to developmental nutrition as did lunging. The high intensity of lunging might make it more sensitive to nutrition than less intense aggressive behaviours. Furthermore, while developmental nutrition influences the likelihood of lunging, it did not influence lunge frequency amongst those males that did lunge, suggesting focal developmental nutrition influences the propensity to engage in high-intensity lunging, but has less influence on the number of lunges required to resolve a contest. These results highlight the importance of measuring multiple aspects of aggressive contests, as measuring a single aggressive behaviour might not capture the full picture of how ecological factors influence aggression (Alekseyenko et al., 2010; Certel & Kravitz, 2012; Chen et al., 2002). Our finding that larval nutrition has varying influences on different aspects of aggression is not unusual. For example, in *D. melanogaster* the developmental environment influences several male sexual traits (as described above; Bangham et al., 2002; McGraw et al., 2007; Wigby et al., 2016), but other sexual traits such as sperm length (Amitin & Pitnick, 2007) and mating rate, duration and latency (Edward & Chapman, 2012; Lefranc & Bundgaard, 2000) show little or no sensitivity. Thus, different aspects of multifaceted behaviours, such as aggressive and sexual behaviours, might be free to change independently, allowing fine-tuned responses to ecological cues.

Nutrient quality and quantity can signal the nature of the prevailing social environment, providing information about mates, rivals, and the costs and benefits of adult behavioural strategies (Elias et al., 2010; Enquist & Leimar, 1987). Such information can influence behavioural motivation and resource valuation, with the developmental environment priming individuals to cope with similar conditions as adults (Wigby et al., 2016). Our finding of decreased aggressive lunging after development on low protein nutrition does not support the prediction that a poor quality developmental diet increases aggression through increased resource valuation and motivation to compete aggressively for food (Bath et al., 2018; Elias et al., 2010). The benefit of flexibility in behavioural strategies in accordance with environmental factors, such as food availability, decreases as the duration between cue detection and the performance of the behavioural strategy increases (Bretman, Westmancoat, et al., 2011; Fusco & Minelli, 2010). For example, male *D. melanogaster* extend mating duration in response to cues of increased competition experienced as adults, but not in response to cues experienced during development (Bretman et al., 2016). In a similar way, changes in resource valuation in response to adult nutritional conditions might override any larval nutritional experiences. Further studies that consider the influence of adult food deprivation on aggression would enhance our understanding of how nutrition impacts resource valuation in this species.

Surprisingly, males did not vary their level of direct physical fighting in response to the developmental nutrition of their rival. Contest theory suggests that physical fighting should be used sparingly against rivals of superior condition (Bishop & Cannings, 1978; Briffa & Sneddon, 2007; Enquist & Leimar, 1983; Hammerstein & Parker, 1982; Leimar & Enquist, 1984; Maynard & Parker, 1976). Previous studies report that both male and female *D. melanogaster* regulate their aggression in response to opponent body size (Bath et al., 2018; Hoffmann, 1987b). However, the nutritional-induced variation in body mass in this study was relatively small and more representative of the natural variation (e.g., a 12% difference in mass between low- and high-larval nutrition males in these experiments, versus a 50% difference in Hoffmann, 1987). Because receiving aggression in *D. melanogaster* seldom results in direct physical damage to important body structures such as the wings (Guo & Dukas, 2020), fighting behaviour might be less sensitive to small differences in rival condition in this species. Our results support the view that fighting is instead primarily determined by an individual’s own developmental nutrition.

However, focal individuals did respond to the developmental nutrition of their rival in the relative amount of aggression performed on the food patch and in threat behaviour. Food patches can represent breeding sites (Lim et al., 2014; Markow, 1988), so this result suggests that larval nutrition and subsequent adult mass of a rival might influence a male's ability to dominate access to breeding sites. Threat displays can allow individuals to assess or intimidate rivals without engaging in costly fights (Clutton-Brock & Albon, 1979; Logue et al., 2010). However, our results suggest increased wing threat did not reduce engagement in lunging, as males that developed under high protein performed both more wing threats and more aggressive lunging. It is possible that in natural settings, or in the large population cages in which these males recently evolved, threat displays by high-quality males might cause low-quality rivals to flee, avoiding escalated conflict, but this was not possible in these observation chambers. Further research into the relationship between threat displays and escalated aggression in other settings is necessary to improve understanding of the adaptive value of threat displays in this species.

Previous research has demonstrated that early life nutrition can influence post-copulatory male-male competition in *D. melanogaster* (McGraw et al., 2007), and our results provide evidence that this influence extends to pre-copulatory male-male competition. Our results demonstrate that, under these experimental conditions, an individual's direct aggression is primarily determined by its own developmental nutritional, but the nutritional experience of the rival influences threat behaviour and the defense of food resources during contests. Our findings contribute to the view that early life experiences, particularly nutritional experience, shape behaviour throughout life (Gluckman et al., 2016; Monaghan, 2008). In humans, the balance of nutrients received during childhood can influence aggressive behaviours later in life (Galler et al., 2012; Liu et al., 2004). Uncovering the ecological factors that determine aggression can help us understand variation in antagonistic behavioural strategies and predict social dynamics, and, in a human context, might help to limit the negative consequences of aggression.

SUPPLEMENTARY MATERIAL

Supplementary table 1

A description of male aggressive behaviours (Nilsen et al, 2004, Dow & von Schilcher, 1974, Andrews, 2017). Lunging, chasing, tussling and fencing all represent aggressive fighting, whereas wing threat represents an aggressive threat display.

Aggressive behaviours	Description
Lunging	*The male raises up on hind legs and rapidly thrusts his upper body at his opponent*
Chasing	*One male rapidly pursues his opponent, remaining in close proximity. Contact and even aggressive lunging may occur during chasing*
Tussling	*Both opponents raise up on hind legs and become interlocked in a prolonged aggressive struggle*
Fencing	*A male uses his forelimbs to bat his opponent. It includes front-on and side-on action with any of the two front or middle legs, and can be performed by one or both opponents, alone or in combination with other behaviours.*
Wing Threat	One male faces the rival and raises both wings in unison at an angle of ~45 degrees for a duration of >1s

Supplementary table 2

standard laboratory medium for adult flies

Ingredients	**Amount Per Litre**
Agar (g)	6.923
Maize (g)	69.231
Soya (g)	8.308
Yeast (g)	14.077
Malt (g)	69.231
Molasses (ml)	23.077
Nipagin (ml)	30.000
Acid mix (propionic acid + orthophosphoric acid, ml)	5.385

Supplementary table 3

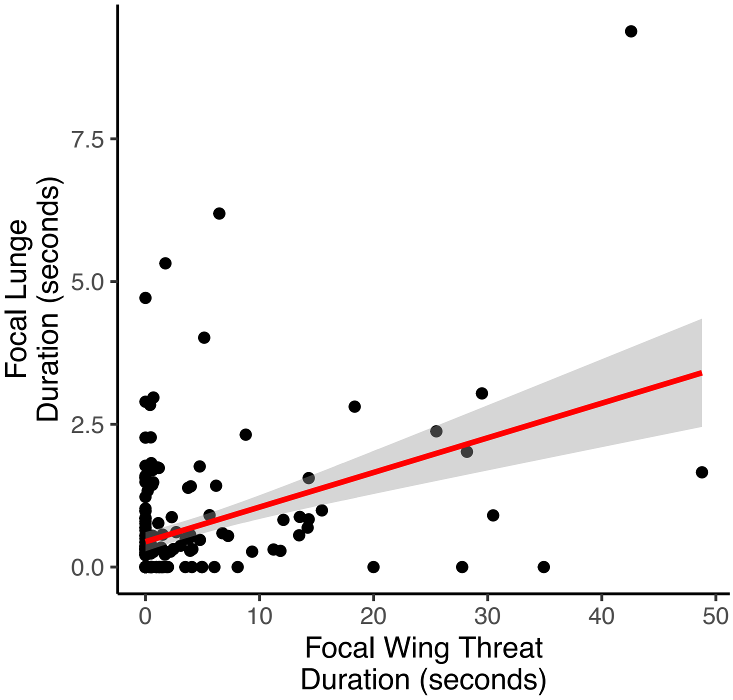
larval food recipes (values per litre media)

Ingredients	Amount Per Litre		
	High Yeast	Medium Yeast	Low Yeast
Water (ml)	960	960	960
Agar (g)	15	15	15
Sugar (g)	50	50	50
Yeast (g)	120	20	10
Nipagin (ml)	30	30	30
Propionic acid (ml)	3	3	3

Supplementary table 4

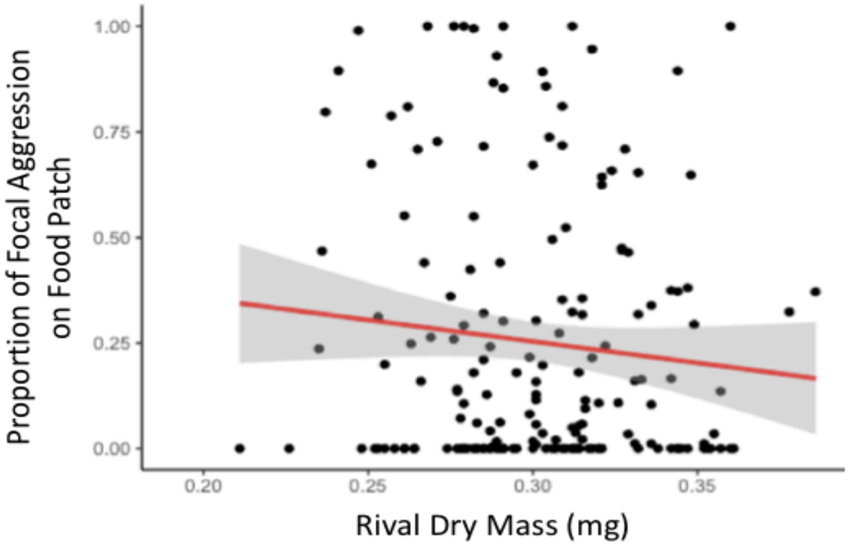
the sample sizes for each treatment

Focal Nutrition	Rival Nutrition	N
L	L	21
L	M	20
L	H	21
M	L	21
M	M	21
M	H	21
H	L	21
H	M	20
H	H	21



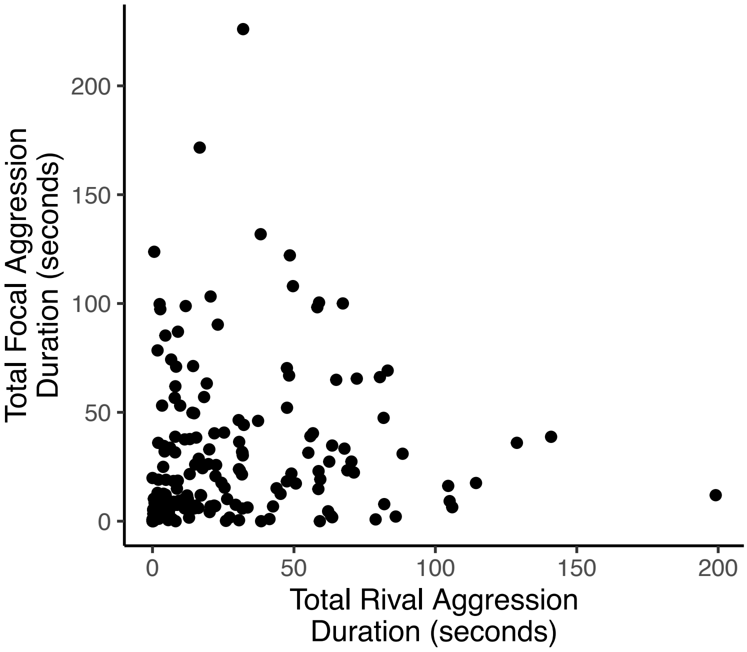
Supplementary Figure 1

The relationship between focal male wing threat duration and lunging duration. Points represent the raw data, the red line shows the correlation with confidence intervals represented by the shaded grey area



Supplementary Figure 2

The relationship between rival male dry mass and the proportion of aggression the focal male performed on the food patch, relative to off the food patch. Points represent the raw data, the red line shows the correlation with confidence intervals represented by the shaded grey area



Supplementary Figure 3

The relationship between rival male total aggression duration and focal male total aggression duration, pooled for all diet combinations. Points represent the raw data.

Chapter 6: 'Hangry' *Drosophila*: food deprivation increases male aggression

ABSTRACT

Aggressive interactions are costly, such that individuals should express aggression strategically in response to environmental stresses. Many organisms experience frequent periods of food deprivation, which can influence an individual's capacity and motivation to engage in aggression. However, because food deprivation can simultaneously decrease resource-holding potential and increase valuation of food resources, its net impact on aggression is unclear. Here, we tested the influence of increasingly prolonged periods of adult food deprivation on inter-male aggression in the fruit fly, *Drosophila melanogaster*. We found that males displayed increased aggression following periods of food deprivation longer than 24 hours. Increased aggression in food-deprived flies occurred despite their reduced mass. This result is likely explained by an increased attraction to food resources, as food deprivation increased male occupancy of central food patches, and food patch occupancy was positively associated with aggression. Our findings demonstrate that aggressive strategies in male *D. melanogaster* are influenced by the nutritional environment, highlighting the need to consider past nutritional stresses to understand adaptive variation in aggression.

INTRODUCTION

Aggressive contests occur throughout the animal kingdom and involve a wide range of agonistic behaviours, from non-contact threat displays to escalated physical fights (Briffa et al., 2015; Briffa & Sneddon, 2007). Aggressive contests typically occur over resources – such as food, territories and mates – that are critical for reproduction (Chen et al., 2002; Clutton-Brock & Albon, 1979; Dow & von Schilcher, 1975; Hoffmann, 1987b; Hoffmann & Cacoyianni, 1990; Watson & Parr, 1981). Aggression is costly, carrying the risk of physical damage and predation, along with time and energy expenditure (Briffa & Sneddon, 2007; Haley, 1994; Neat et al., 1998). Contest theory suggests that aggression should be expressed according to an individual's relative fighting ability (resource-holding potential) and perceived value of the contested resource (resource valuation) (Briffa & Sneddon, 2007; Enquist & Leimar, 1987).

A key factor shaping resource-holding potential and resource valuation is an individual's nutritional environment. Access to nutritional resources varies, and animals often experience periods of food deprivation (Droney, 1996). Food deprivation can have long-term impact on internal state, determining an individual's ability to invest in life history traits (Rol & Houle, 1996; Zikovitz & Agrawal, 2013) and affecting size, physiology and behaviour (Han & Dingemanse, 2015, 2017; Harrison et al., 2017; Lihoreau et al., 2015). Furthermore, food limitation can signal information about the physical and social environment, such as the characteristics of potential mates (Zikovitz & Agrawal, 2013) and rivals (Fricke et al., 2008; McGraw et al., 2007) and the future environment for potential offspring (Kotiaho et al., 2001; Tudor et al., 2018).

However, because food deprivation can simultaneously decrease resource-holding potential and increase the value of food resources, the net impact on aggression remains unclear (Stocker & Huber, 2001). Resource-holding potential is influenced by body size, weapon-like appendages, and energy reserves (Briffa & Sneddon, 2007; Plaistow & Siva-Jothy, 1996), and food deprivation can compromise these traits (Baker et al., 2003; Poças et al., 2020), reducing fighting ability (Marden & Waage, 1990; Plaistow & Siva-Jothy, 1996). Resource valuation is influenced by the potential fitness gains of winning contested resources, and food deprivation might increase the need to attain resources, increasing motivation to engage in escalated and persistent aggression (Elias et al., 2010; Enquist & Leimar, 1987; McNamara & Houston, 1989; Stocker & Huber, 2001). Furthermore, the balance between the opposing influences of food deprivation on fighting capacity and motivation might vary with the severity of food deprivation. As yet, we do not know how the severity of food deprivation might affect aggressive behaviours, and, because food deprivation in nature can span brief to prolonged periods, it is important to understand how aggression changes along a continuous gradient of food deprivation.

We tested how adult food deprivation influences male aggression and food patch occupancy in the fruit fly, *Drosophila melanogaster*. Aggression is a key social behaviour for male *D. melanogaster* with an important function in mate acquisition (Hoffmann, 1987b, 1987a; Kravitz & Fernandez, 2015). Contests often occur over food sources, which represent not only nutrition, but also high-value mating sites where males locate mates (Hoffmann & Cacoyianni, 1990; Markow, 1988). Because *D. melanogaster* consume decaying fruits, which are seasonally and spatially variable, nutritional quality and quantity varies in natural settings (Chng et al., 2017; Markow, 1988). Adult nutrition affects male post-copulatory reproductive success (Fricke et al., 2008), but nutritional effects on pre-copulatory interactions via inter-male aggression are unknown. We hypothesised that exposure to food deprivation might decrease aggression by reducing male resource-holding potential, or might increase aggression by increasing resource valuation and motivation, and that these alternative outcomes might depend on the duration of food deprivation.

METHODS

Experimental Flies

Flies were derived from an outbred Dahomey stock population (Carazo et al., 2015). Fly husbandry and experiments were carried out at 25°C on a 12:12h light:dark cycle. Experimental flies were reared at a density of 200/bottle. We collected virgin males within 6h of eclosion using ice anaesthesia. We randomly assigned males to one of five treatments: food deprivation from eclosion (120-144h; n=24) or for 72h (n=58), 48h (n=59), or 24h (n=62), or no food deprivation (n=62).

We placed males assigned to food deprivation from eclosion in individual vials lacking nutritional substances but containing agar for moisture. We placed all other males in individual vials containing standard food medium (supplementary table 1), and transferred them to agar vials at the assigned number of hours before trials. As a handling control, we transferred males assigned to ‘no food deprivation’ to new food vials 24h before trials.

Behavioural Trials

We transferred pairs of flies from each treatment into vials containing agar with a central 0.2cm diameter patch of food media combined with yeast paste, with 1.5cm between the agar and cotton bung for flies to interact. We allowed flies 10 minutes acclimatisation before trials. During trials, an observer blind to treatment scanned vials for 3 seconds in turn, recording the number of lunges and tussles and the number of flies chasing, fencing, and occupying the food patch in each scan (supplementary table 2). We conducted 3 blocks and carried out trials for 5h from lights-on. We froze males immediately after trials and weighed them before and after drying for 48h at 60°C.

Statistical Analysis

We performed analyses in R, version 3.6.2 (2019-12-12). We converted spot check data into both bouts per minute (‘behaviour rates’) and binary responses (when data were zero-inflated) to describe whether the behaviour occurred.

We assessed treatment effects on the total aggression rate (lunging, fencing, chasing and tussling) in linear models. We assessed treatment effects on the occurrence of each behaviour using binomial general linear models. We did not analyse the occurrence of tussling alone, as it was only observed in 2 pairs. We analysed treatment effects on mass, and the relationship between mass and total aggression, in separate linear models. We conducted sequential sum of squares analysis to test for effects of mass after food deprivation had been accounted for.

We analysed treatment effects on food patch occupancy rates in a linear model. We tested the correlation between aggression rates and food patch occupancy using Spearman’s rank correlation because the data were not normally distributed (Shapiro-Wilk: aggression: $W=0.74$, $p<0.0001$; food patch occupancy: $W=0.88$, $p<0.0001$). We conducted sequential sum of squares analysis to test for effects of food patch occupancy on aggression after food deprivation had been accounted for.

To test for the potential influence of outliers, we re-fitted models using winsorized data, and, as this had minimal impact on results, we reported all statistics for non-winsorized data (Carazo et al., 2015). We included block as a fixed factor in all models and used post-hoc Tukey tests to explore the results.

RESULTS

Food deprivation increased aggression

Food deprivation increased aggression ($F_{4,258}= 6.4$, $p<0.0001$; figure 1). We observed a trend of increasing aggression with longer food deprivation, with significant differences between satiated males and those experiencing ≥48h food deprivation (figure 1a). Likewise, the likelihood of lunging and fencing increased with food deprivation duration (lunging:$\chi^2_{4,258}=13.3$, $p=0.010$; fencing:

$\chi^2_{4,258}$=17.5, p=0.002), though chasing was unaffected ($\chi^2_{4,258}$=7.4, p=0.115; figure 1b-d).

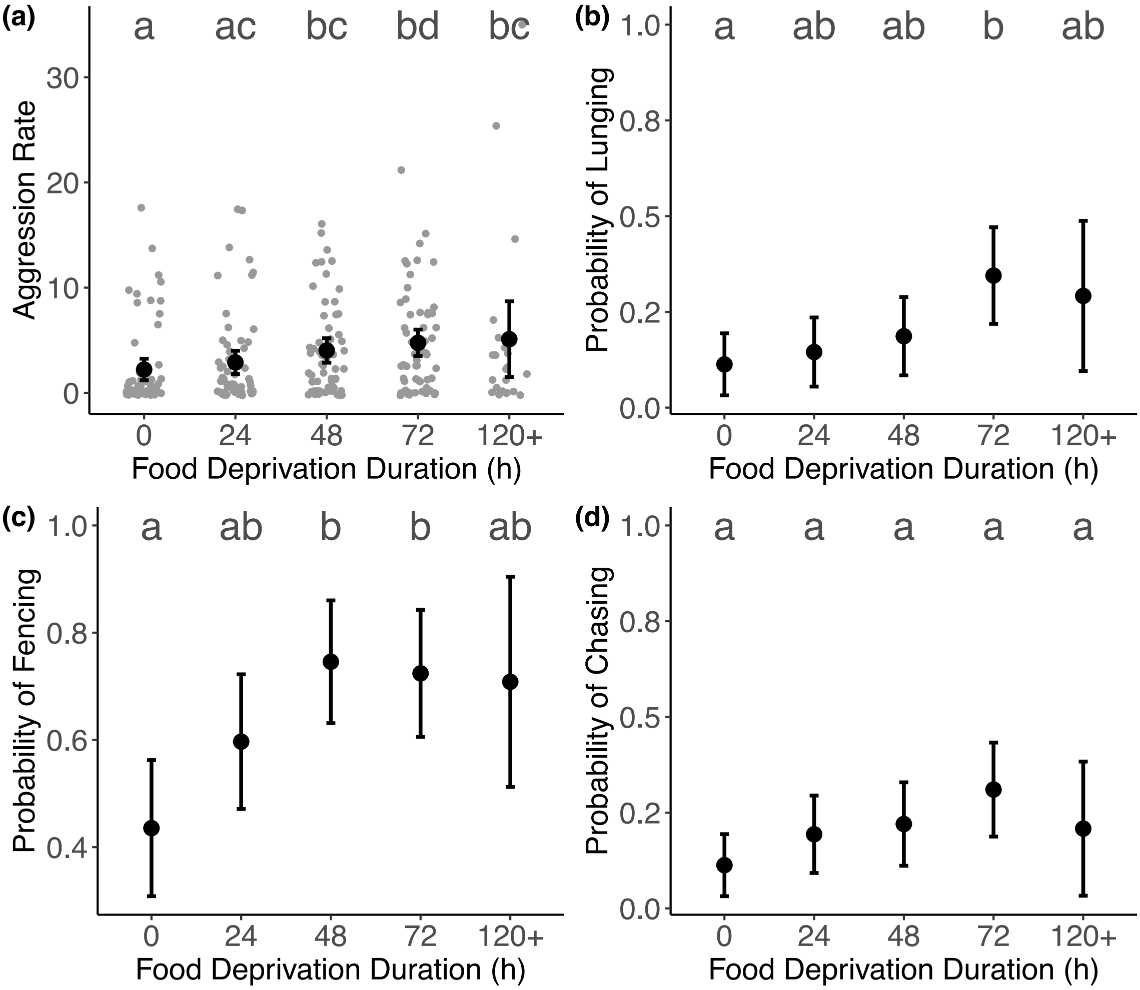


Figure 1

Relationships between food deprivation and total aggression rate (A; aggressive bouts/minute), and the probability of lunging, fencing, and chasing (B-D, respectively). Of 260 pairs, 54 performed lunging, 167 performed fencing, and 55 performed chasing. Black points show means with 95% confidence intervals. Letters denote significant differences among groups by post-hoc tests.

We observed a reduction in wet mass after 24h of food deprivation ($F_{4,257}$=7.9, p<0.0001), with no further reduction with longer food deprivation, whereas dry mass decreased further after 48 and 72h ($F_{4,252}$=189.9, p<0.0001; figure 2). Total aggression was negatively related to mean dry mass for a pair ($F_{1,255}$=14.4, p=0.0002), but we detected no relationship with wet mass ($F_{1,260}$=0.7, p=0.397; supplementary figure 1). However, sequential sum of squares analysis revealed that the negative relationship between dry mass and aggression was no longer detectable after accounting for food deprivation: dry mass was positively related to the variation in aggression that was not explained by food deprivation ($F_{1,251}$=4.0, p=0.047, slope=3.01±3.56).

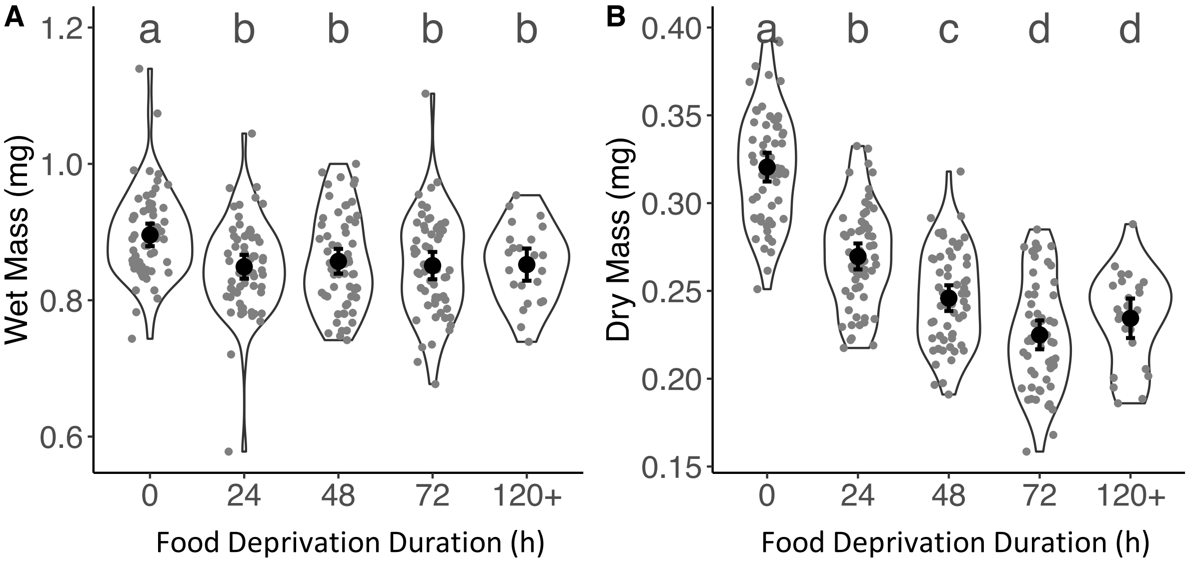


Figure 2

The effect of food deprivation duration on wet (A) and dry mass (B). Black points show means with 95% confidence intervals, 'violin' areas represent the shape of the distribution of data.. Letters denote significant differences among groups by post-hoc tests.

Food deprivation increased food patch occupancy

Males experiencing any food deprivation spent more time on the food patch than satiated males, with further increases in food patch occupancy with prolonged food deprivation ($F_{4,258}$=23.4, p<0.0001; figure 3a). Food patch occupancy was positively correlated with aggression (ρ=0.38, p<0.0001; figure 3b). Sequential sum of squares analysis revealed that the positive relationship between food occupancy and aggression remained after accounting for the influence of food deprivation, with food occupancy positively correlating with the variation in aggression that was not explained by food deprivation ($F_{1,257}$=12.9, p=0.0004).

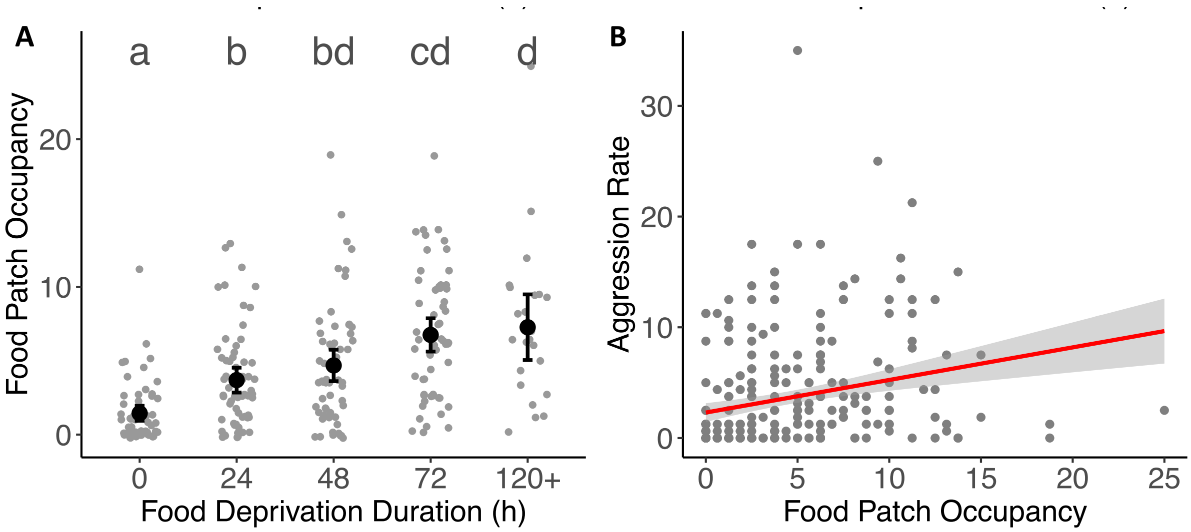


Figure 3

Relationships between food deprivation and food patch occupancy (A; bouts/minute), and the relationship between food patch occupancy and aggression rate (B; both in bouts/minute. In A, black points show means with 95% confidence intervals. In B, points represent the raw data, the red line shows the correlation with confidence intervals represented by the shaded grey area Letters denote significant differences among groups by post-hoc tests.

DISCUSSION

Periods of food deprivation are common in many animals, and so plastic behavioural strategies to mitigate the negative consequences of nutritional stress are also common (Steinberg, 2018). We found that prolonged food deprivation increases both male aggression and food-patch occupancy in *D. melanogaster*, and that these behaviours are positively correlated. Increased aggression following food deprivation occurred above and beyond effects of the reduced body mass resulting from food deprivation. These findings support the hypothesis that prolonged food deprivation increases aggression through increasing resource valuation and motivation. Our results demonstrate that males modify their aggression in response to information gleaned from the nutritional environment (Bretman et al., 2016; Fusco & Minelli, 2010). In popular parlance, food-deprived male fruit flies get ‘hangry’.

Increased resource valuation

Increased aggression by food-deprived males could be explained by increased valuation of nutritional resources, and hence increased motivation to access those resources. Consistent with this hypothesis, we observed increased food patch occupancy with extended food deprivation, and this was correlated with increased aggression. Attraction to food resources is influenced by nutritional status in many organisms, including humans (Aimé et al., 2007; Farhadian et al., 2012; Uher et al., 2006). Although male *D. melanogaster* gain most of their lifetime nutrition in larval development (Edgar, 2006), adult feeding is necessary to develop internal reproductive structures and to maximise mating success (Baker et al., 2003; Fricke et al., 2008). Sexual maturation occurs in the days following eclosion (Eastwood & Burnet, 1977; Markow & O’Grady, 2008), and if food deprivation slows this process, then food-deprived males might increase their food valuation to support completion of development.

Our results suggest that at least part of the increase in aggression following food deprivation could have resulted from greater male-male proximity with increased occupation of the food patch. Increased food patch occupancy might have resulted from the heightened sensitivity to food odours after food deprivation in *D. melanogaster* (Edgecomb et al., 1994; Farhadian et al., 2012). Greater food patch occupation might increase inter-male aggression in *D. melanogaster* via the action of Gr5a+ gustatory receptor neurons and octopamine signalling (Andrews, 2016; Lim et al., 2014). Thus, the increased aggression displayed by food-deprived males appears coupled with increased occupation of food patches, triggered by an increased attraction to food odours, allowing increased access to food following deprivation.

Increased motivation to access mating sites

If prolonged periods of food deprivation signal a reduced likelihood of survival (Good & Tatar, 2001; Tigreros, 2013), then males should invest more in immediate reproductive effort (i.e., terminal investment; Clutton-Brock, 1984; Krams et al., 2015; Moatt et al., 2016). Aggression in male *D. melanogaster* can occur over access to mates (Hoffmann, 1987b; Kravitz & Fernandez, 2015; Nilsen et al., 2004) and food patches are important for access to females, which are attracted to nutritionally-rich oviposition sites (Hoffmann & Cacoyianni, 1990; Lim et al., 2014; Markow, 1988). Thus, increased aggression by food-deprived males might be a strategy to maximise short-term reproductive output in environments where survival is uncertain. Further investigation into how aggression influences the reproductive output of food-deprived males could shed light on this hypothesis.

No strong support for decreased resource-holding potential

Our findings provide no strong evidence that adult food deprivation decreases resource-holding potential. Body size is a common correlate of resource-holding potential (Asahina, 2017; Kemp & Alcock, 2003; Stockermans & Hardy, 2013), and larger mass can increase aggressive initiation, escalation and success in *D. melanogaster* (Asahina et al., 2014; Bath et al., 2018; Hoffmann, 1987b; Hoyer et al., 2008) and other species (DiMarco & Hanlon, 1997; Kelly, 2008; McCann, 1981; Schuett, 1997). We found that adult food deprivation decreased body mass, with reduced dry mass suggesting the depletion of fat or structural protein (Kristensen et al., 2011; Robinson et al., 2000). However, these lighter, food-deprived males displayed elevated aggression. Thus, increased resource valuation caused by dietary restriction might override any reduction in resource-holding potential (e.g., Nosil, 2002). Alternatively, increased aggression in food-deprived males might result from a 'desperado' effect, in which individuals of poor condition engage in fights even when likely to lose, because they cannot gain fitness benefits by not engaging at all (Elias et al., 2010; Grafen, 1987).

A monotonic relationship between food deprivation and aggression

We speculated that the direction of the relationship between food deprivation and aggression might depend on the severity of food deprivation. Food deprivation can cause a reallocation of resources from reproduction to survival, delaying reproduction until conditions improve (Shanley & Kirkwood, 2000), and brief food deprivation might result in individuals decreasing aggression to conserve resources. However, severe food deprivation that reduces survival might trigger a terminal investment in reproduction (Shanley & Kirkwood, 2000), increasing aggressive motivation to attain resources before death. Conversely, starvation might render individuals too weak to fight, while brief food deprivation might increase aggressive motivation before decreased resource-holding potential occurs. These processes would generate a non-linear relationship between food stress and aggression. Similar non-linear responses have been reported for male post-copulatory success: male *D. melanogaster* siring success is maximised under intermediate levels of adult nutrition (Fricke et al., 2008). Likewise, previous evidence suggests aggression peaks at intermediate food patch size (Lim et al., 2014). Our results did not reveal a U-shaped or inverse U-shaped relationship between food deprivation and aggression, but a continuous decrease in aggression as food deprivation duration extended beyond 24 hours. This suggests that increased resource valuation might be the strongest consequence of adult food deprivation, resulting in increased aggressive motivation despite any reduction in fighting capacity. Alternatively, our food deprivation treatments might not have been severe enough to capture a switch-point driven at which resource-holding potential becomes critically low; indeed, no experimental males died following our treatments. This, combined with our observation that food deprivation longer than 24 hours was necessary to decrease aggression, suggests that 24 hours without food is well-tolerated and that up to 120 hours without food does not compromise survival in adult male *D. melanogaster*.

Our findings that adult food deprivation increases aggression and food patch occupancy in male *D. melanogaster* demonstrate that behavioural strategies critically depend on the nutritional environment, even in adult insects with low food requirements. These results highlight the need to consider the environmental stresses experienced in the recent past in order to understand adaptive variation in behaviour.

SUPPLEMENTARY MATERIAL

Supplementary table 1

Standard food medium (values per litre media)

Ingredients	**Amount Per Litre**
Agar (g)	6.923
Maize (g)	69.231
Soya (g)	8.308
Yeast (g)	14.077
Malt (g)	69.231
Molasses (ml)	23.077
Nipagin (ml)	30.000
Acid mix (propionic acid + orthophosphoric acid, ml)	5.385

Supplementary table 2

A description of male aggressive behaviours (Nilsen et al, 2004, Dow & von Schilcher, 1974, Andrews, 2017)

Aggressive behaviours	Description
Lunging	*The male raises up on hind legs and rapidly thrusts his upper body at his opponent*
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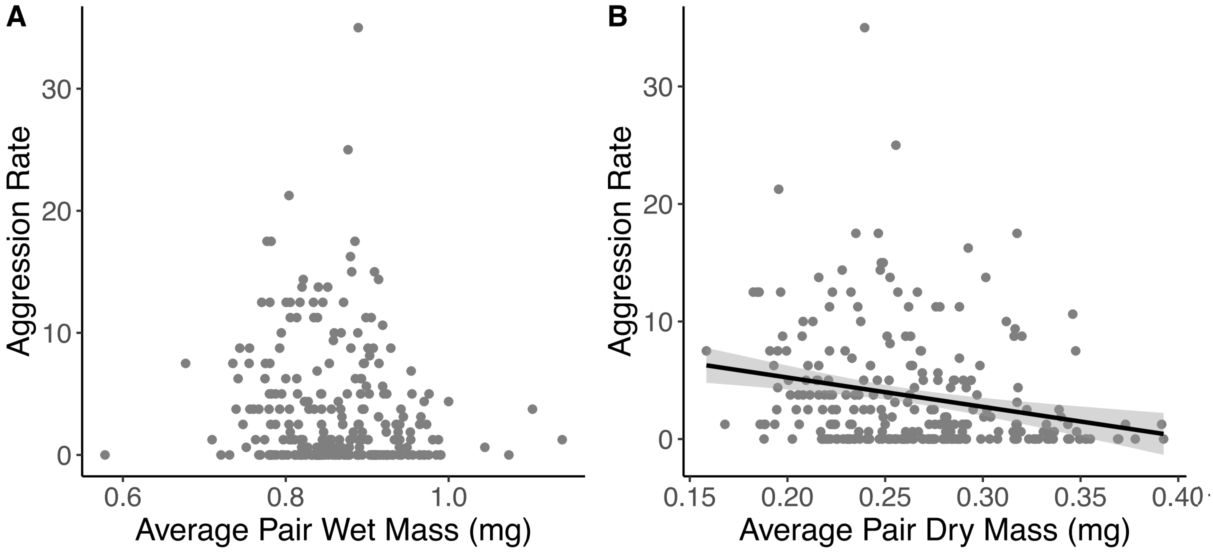
Tussling	*Both opponents raise up on hind legs and become interlocked in a prolonged aggressive struggle*
Fencing	*A male uses his forelimbs to bat his opponent. This includes front-on and side-on action with any of the two front or middle legs, and can be performed by one or both opponents, alone or in combination with other behaviours.*



Supplementary Figure 1

The relationship between the mean wet mass (A) and dry mass (B) of a pair of males and the observed rate of aggression, measured in bouts per minute, Points represent the raw data, the black line shows the correlation with confidence intervals represented by the shaded grey area.

Chapter 7: General Discussion

In chapter 2 I find empirical support for the long-held assumption that sexual selection is sensitive to the operational sex ratio in males, with a male-biased sex ratio strengthening sexual selection on males. This response was largely due to increased variance in mating success, accompanied by an increased maximum potential strength of post-copulatory sexual selection amongst those successful in mate acquisition. However, in females, variance in mating success and reproductive success was consistently low, with no evidence for a correlation between these two parameters. I concluded that the sex ratio is a reliable measure of the potential for sexual selection in males, but the reproductive biology of both sexes needs to be considered, and adoption of a framework of social selection to better capture how intra-sexual competition operates in females might allow more accurate exploration of how ecology shapes selection in females.

In chapter 3, I found that in male *D. melanogaster*, evolution under female-biased conditions reduced the proportion of aggression performed on food patches, but the evolutionary sex ratio did not influence the frequency and intensity of male aggression. In females, evolution under a female-biased sex ratio increased the magnitude by which aggression is elevated post-mating, but this only occurred after mating with co-evolved males. Furthermore, there was evidence consistent with genetic correlation between the sexes in high-intensity aggressive behaviours. I concluded that the sex ratio can influence the evolution of some aspects of aggression in both males and females, but the nature and extent of this influence differs between the sexes, potentially reflecting the differences in intra-sexual competition in males and females.

In chapter 4, our findings suggested that olfaction might not be the sole mechanism by which male *D. melanogaster* reduce aggressive and harmful behaviours in response to high relatedness. When olfactory-deficient males were placed in related or unrelated groups, there was evidence of lower male-male aggression and male-female harm in related groups. However, I found no evidence of an influence of relatedness on any behavioural or fitness traits in wild-type groups. I concluded that while the potential for relatedness to reduce sexual conflict in *D. melanogaster* might be highly sensitive to the environmental conditions, olfaction is unlikely the sole mechanism for this response.

In chapter 5, I showed that nutritional stress during development reduced adult aggressive lunging in male *D. melanogaster*. Furthermore, males performed more aggression on food patches when against rivals that had developed under nutritional stress. Nutritional deprivation during development might reduce resource holding potential and aggressive capability, and I concluded that early life nutrition can have long-lasting impacts on aggression.

In chapter 6, I demonstrated that nutritional stress during adulthood increased aggression in male *D. melanogaster*. This pattern contrasts with the response to developmental nutritional stress found in chapter 5, and I suggest that nutritional deprivation during adulthood might increase resource valuation and motivation to fight. I concluded that aggressive strategies are influenced by the past nutritional environment, and the nature of the relationship between nutrition and aggression is sensitive to the life history stage at which nutritional stresses are experienced.

These findings add to our understanding of how ecology shapes aggression, both through long-term evolutionary change, and short-term behavioural plasticity. Although aggression has long been of interest to biologists, it remains a rapidly progressing field. As scientific technology progresses, new possibilities open up to explore aggression and address outstanding questions that have previously been impractical to test empirically. Simple video-recording technologies allow detailed behavioural analysis and the current development in automated video analysis offers the potential of mass behavioural screening on a large scale (Branson et al., 2009; Dankert et al., 2009; Drosophila activity monitor). Advances in proteomics and genomics allow mass screenings to reveal changes in gene expression accompanying behavioural changes (e.g., Perry et al, unpublished), and this motivated our investigation of the role of olfaction in responding to relatedness in chapter 4. A fine-tuned understanding of the genetic and neural pathways underlying aggression allows manipulated lines of *D. melanogaster* in which aggression is artificially elevated or suppressed to be used to explore the costs and benefits of expressing different levels of aggression (e.g., Asahina et al., 2014, although these lines have not yet been used for this purpose). Furthermore, as the field of aggression has progressed, research in female aggression has grown rapidly, but our understanding of female aggression still lags behind males. In chapters 2 and 3, I investigated the selective forces driving aggression in both sexes simultaneously, highlighting possible genetic correlations and co-evolution between males and females, and illustrating the potential insights that can be gained by future research that incorporates both sexes. Thus, the fruit fly remains a highly fruitful (pardon the pun) system in which to investigate the fundamental properties determining how aggression responds to prevailing environmental conditions both plastically and through long-term evolution. Findings from *D. melanogaster* can reveal conserved behavioural patterns generalisable to a broader range of species, potentially allowing a deeper understanding of antisocial behaviours in human society.

Here, I discuss the significance of my findings to the wider field of aggression, and highlight exciting potential future directions in each of the areas of my thesis: how features of the population structure shape the evolution of aggression through sexual and social selection; the responsiveness of aggression to relatedness and kin selection in the light of sexual conflict; and the relationship between nutrition and aggression.

Population sex ratio and the evolution of aggression through sexual selection

Males typically display more exaggerated secondary traits and contest behaviours than females, which has previously resulted in the fields of sexual selection and aggression being largely dominated by research on males. Recently, there has been a wave of interest in analogous processes in females (Bath et al., 2017, 2018; Cain & Ketterson, 2013; Cain & Rosvall, 2014; Clutton-Brock & Huchard, 2013; Clutton-Brock & Huchard, 2013; Rosvall, 2011), promising a greater understanding of the role of selection arising female-female competition for reproductive resources in shaping female behaviours such as aggression. Our findings from chapters 2 and 3 add to this growing body of interest in female-female competition and its evolutionary consequences. However, because much traditional sexual selection theory was constructed in the context of male-male competition, there is a discrepancy between sexual selection literature and the nature of female-female competition, and this discrepancy is reflected in chapter 2 and 3. In chapter 3, I found evidence for female aggression evolving in response to the sex ratio, illustrating not only that aggression is a common female behaviour, but that this behaviour might be shaped by the intensity of intra-sexual competition, resembling forces of sexual selection traditionally associated with male sexual traits and behaviours. While these findings are insightful, our knowledge of the operation of such selection and the functional and mechanistic properties of aggression in females still lags behind that for males, highlighted in chapter 2 where I find no influence of the sex ratio on the operation of sexual selection in females, and show that female mating success doesn’t translate into a reproductive advantage as it does in males.

There is a growing notion that the framework of sexual selection might require modification to better reflect the operation of analogous processes in females (Cain & Rosvall, 2014; Clutton-Brock & Huchard, 2013; Clutton-Brock & Huchard, 2013). Because female reproductive success is typically limited by the availability of resources required for reproduction, as opposed to the number of mates, regressing mating success against reproductive success (i.e. Bateman gradients) might fail to capture the most important components of reproductive success in females (Cain & Rosvall, 2014; Rosvall, 2011). Modifying these metrics to incorporate the reproductive outcomes resulting from variability in resource acquisition over an ecologically relevant timescale could better capture the nature of intra-sexual competition in the context of reproduction in females (Cain & Rosvall, 2014; Clutton-Brock & Huchard, 2013; Clutton-Brock & Huchard, 2013). That said, because the reproductive consequences of resource acquisition might accumulate over a female's reproductive lifespan (Clutton-Brock & Huchard, 2013; Clutton-Brock & Huchard, 2013), and because female resources are allocated into both offspring quantity and quality (Rosvall, 2011), the 'ecologically relevant timescale' for capturing the consequences of female intra-sexual competition could be lengthy, potentially spanning multiple generations.

This type of work is plausible with *D. melanogaster*. Due to their short lifecycles and their amenability to controlled laboratory environments, it is possible to manipulate the level of female-female competition, measure female feeding, and record the fitness impacts in both their lifetime reproductive success, and the lifetime reproductive success of their offspring. Recent advances in nutritional geometry have led to the development of techniques that make it possible to record food intake in *D. melanogaster* via capillary feeders (Diegelmann et al., 2017), facilitating very precise measurement of female food acquisition. Thus, the relationship between female-female competition over nutritional resources and reproductive success could be measured by modifying the design used in chapter 2: groups of flies at each sex ratio could be kept under food-limited conditions with nutrients only available through capillary feeders, and reproductive success could be measured over a female's lifetime, retaining offspring to record transgenerational measures of offspring quality such as size, longevity and fecundity. Replacing classic measures of standardised variance in mating success with standardised variance in nutrient acquisition could better reflect the opportunity for selection arising from pre-copulatory intra-sexual competition in females, and regressing variance in lifetime reproductive success or offspring quality against variance in nutrient acquisition could better capture the intensity of such selection processes in females. If the force of selection arising from intra-female competition for reproductive resources increases with the intensity of female-female competition (analogous to male sexual selection), then the variance in nutrient acquisition, reproductive success and offspring quality should increase as the population becomes more female-biased, and this should be accompanied by a stronger positive relationship between nutrient acquisition and offspring quantity and quality.

Secondly, while female traits that confer an advantage in the context of reproductive success might be shaped by processes analogous to sexual selection, whether and how female aggression translates into reproductive success remains largely unresolved. For example, despite strong evidence that mating increases aggression in female *D. melanogaster* (Bath et al., 2017, 2018; Nilsen et al., 2004), and a range of other species (lizards, *Egernia whitii*, Sinn et al., 2008; mosquitofish, *Gambusia holbrooki*, Seebacher et al., 2013; house mice *Mus Musculus Domesticus*, Mainardi et al., 1996), our understanding of the associated benefits and adaptive functions driving the evolution of this post-mating increase in aggression remains relatively limited. Resolving these gaps is essential for a fundamental understanding of how prevailing environmental conditions select for different aggressive strategies. There is some evidence from the whites skink, *Egernia whitii*, that elevated female aggression is linked to higher early-life survival rates in offspring (Sinn et al., 2008). Likewise, in the dark eyed junco, *Junco hyemalis*, more aggressive females are more likely to produce successful nests in stressful conditions (Cain & Ketterson, 2013). Post-mating processes such as oviposition, lactation and gestation are energy-intensive and can increase a female's nutritional requirements, potentially increasing aggressive competition for food (Barnes et al., 2008; Stockley & Bro-Jørgensen, 2011). In female *D. melanogaster*, egg-production increases protein requirements, (Jensen et al, 2015; Lee et al, 2008), and feeding increases post-mating, potentially as a direct response to egg production (Barnes et al., 2008) or mating cues (Bath et al., 2017). However, strong evidence linking aggressive attainment of increased maternal nutrition to reproductive success is lacking (Stockley & Bro-Jørgensen, 2011). *D. melanogaster* could offer an excellent opportunity for such empirical work. By monitoring female-female post-mating aggression (using techniques similar to those used in chapter 3), monitoring female feeding behaviour, and recording resultant reproductive success, it would be possible to assess the relationship between aggression, feeding and reproduction, testing whether female aggression does in fact increase female reproductive success by increasing access to nutritional resources.

Understanding the adaptive value of increased post-mating aggression in females can allow understanding of why and under which conditions it might be selected for, but knowledge of the underlying mechanisms is needed to understand what selection acts on, and can reveal potential sexual conflict, evolutionary constraints or genetic correlations. Across many of the species in which female post-mating aggression has been observed, the underlying mechanisms remain largely unresolved. Progress in this area has been made in *D. melanogaster* by Bath et al., (2017), demonstrating that female aggression is stimulated by the receipt of sperm at mating, and partly by the seminal fluid protein ‘sex peptide’. However, after these ejaculate components are transferred to females, the downstream mechanisms leading to increased aggression remain elusive.

One potential hypothesis is that increased feeding post-mating could be directly linked to increased aggression. Female *D. melanogaster* increase feeding post-mating (Barnes et al., 2008; Bath et al., 2017), and I found evidence consistent with this in chapter 3. In male *D. melanogaster*, the presence of food can increase aggression via the action of Gr5a+ gustatory receptor neurons and octopamine signaling (Andrews, 2016; Lim et al., 2014), and in chapter 5 I found food occupation to be positively correlated with male aggression. Thus, it is plausible the increased feeding rates associated with egg production post-mating could promote female aggression, either through increased motivation to access food, or through increased proximity accompanying aggregation on food patches.

Another hypothesis is mating might stimulate female aggression through the production of juvenile hormone. The receipt of sex peptide stimulates juvenile hormone production in female *D. melanogaster* (Fan et al., 2000; Moshitzky et al., 1996; Soller et al., 1999). Juvenile hormone plays an important role in the control of female aggression in other insect species such as paper wasps (Tibbetts et al., 2013) cockroaches (Kou et al., 2009), and burying beetles (Scott, 2006), displaying similar properties in stimulating aggression as testosterone does in vertebrates (Scott, 2006). Much progress has been made into the effects of juvenile hormone by Tibbetts and colleagues (2013) who developed an assay in which female paper wasps, *Polistes dominulus*, were treated with the synthetic juvenile hormone analogue ‘methoprene’ prior to aggression trials. It is possible that juvenile hormone plays a similar role in *D. melanogaster*, and similar techniques could be adopted, testing whether applied methoprene can elevate aggression in this species, and whether it can raise virgin aggression levels to those displayed by mated individuals, simulating the effect of mating.

While female *D. melanogaster* provide an excellent system to begin to bring our understanding of female aggression closer to that in males, many aspects of the evolutionary ecology of male aggression in *D. melanogaster* remain unresolved. In chapter 3, I found that the sex ratio influenced the evolution of aggression levels in females, but not males, with males showing evolution only in the location of fights in relation to food. I speculated that female aggression might be more sensitive to the prevailing environment due to higher associated costs: because reproduction has higher energy demands in females than males (Vargas et al., 2010), females might suffer greater costs of diverting energy to aggression, and thus express aggression more economically (Cain & Ketterson, 2013). However, the nature of potential costs of aggression in male *D. melanogaster* is less clear.

Aggression in animals can carry a wide range of potential costs, from energy expenditure and physiological stresses to extensive physical damage or ultimately death (reviewed by Briffa & Sneddon, 2007; Georgiev et al., 2013; Guo & Dukas, 2020). These costs are dependent on species morphology, physiology, life-history and environment (Georgiev et al., 2013). In species with highly developed weapons and fighting structures, the injury costs of aggression are high. For example, in the musk ox, *Ovibos moschatus*, the large horns and powerful body mass of males mean fighting can result in death (Georgiev et al., 2013; Wilkinson & Shank, 1976). Likewise, male elephant seals, *Mirounga angustirostris*, strike each other with dangerous blows (Haley, 1994), and mouth-wrestling, biting and chasing causes significant scale damage in the African cichlid, *Tilapia Zillii* (Neat et al., 1998). In species with no specialised fighting structures, energy expenditure costs can be great, especially when contests are elongated or escalated (Briffa & Sneddon, 2007; Georgiev et al., 2013; Marden & Waage, 1990; Plaistow & Siva-Jothy, 1996).

Drosophila melanogaster have no specific fighting structures, and the precise nature of the costs of aggression are largely unknown in this species. Early work of Hoffmann and colleagues suggests some evidence for costs: males selected for territoriality might suffer reduced longevity (Hoffmann & Cacoyianni, 1989), and males were occasionally observed performing increased preening, ‘limping’ or becoming stuck on their backs after contests (Hoffmann, 1987a). Recently, much progress has been made in our understanding of the costs of aggression in male *D. melanogaster* by Guo & Dukas (2020). By exposing focal male flies to either ‘hyper-aggressive’ rivals, juvenile non-aggressive rivals, or no rivals, and recording their longevity, they demonstrated that increased aggression reduces longevity. While this is exciting progress, demonstrating costs of aggression in a species without weapons, many questions remain.

Firstly, the proximate mechanisms by which longevity is reduced are unclear. Findings from Guo & Dukas (2020) and Bretman et al, (2013) do not support the hypothesis of physical damage resulting from aggression, suggesting that direct body damage is unlikely to represent important costs. Possibly, the energetic expenditure involved in engaging in aggressive contests could direct energy away from somatic maintenance and survival. However, experiments in which flies have been subjected to forced exercise report positive effects on endurance and longevity as opposed to costs (Sujkowski et al., 2015). Metabolic changes resulting from aggression could incur costs. For example, in many species, aggression is associated with elevated levels of testosterone (e.g. lizards *Sceloporus jarrovi*, Marler & Moore, 1988; Japanese quail, *Coturnix coturnix*, Ramenofsky, 1983; Bank voles *Clethrionomys glareolus*, Mills et al., 2007; reviewed in Briffa & Sneddon, 2007), and this can result in suppression of the immune system (Georgiev et al., 2013). It is possible that similar hormonal or metabolic changes accompanying aggression in *D. melanogaster* could carry fitness costs. Furthermore, in some species, aggression can be associated with damaged social relationships (Georgiev et al., 2013) and, in humans, might be related to anxiety (Andrews et al., 2019). Recent research has demonstrated anxiety-like behaviours in *D. melanogaster* (Bath, Thomson, et al., 2020; Perry & Baciadonna, 2017), and it is possible that aggression could induce an ‘anxious’ state, which might carry detrimental costs.

Secondly, as the work of Guo & Dukas (2020) involved exposing focal males to ‘hyper-aggressive’ rivals, observed costs likely stem from receiving high levels of aggression. However, expressing high levels of aggression can also be costly, and disentangling the costs of the receipt and expression of aggression could further our understanding of what governs the level of aggression individuals display. Future work in which costs of aggression are measured in flies displaying elevated aggression, such as hyper-aggressive strains used by Guo & Dukas (2020) or genetically manipulated flies (e.g. Asahina et al., 2014), or in which the relative costs of winners and losers are compared, could advance our understanding of the regulation of aggression in male *D. melanogaster*.

Kin structure, social environment and the response of aggression to relatedness

Since the work of Carazo and colleagues (2014), the ability of *D. melanogaster* to reduce male-male aggression and male-female harm in response to relatedness has attracted attention and prompted interesting hypotheses about the potential for kin selection in relatively unstructured laboratory-adapted populations (Carazo et al., 2015; Chippindale et al., 2015; Hollis et al., 2015; Le Page et al., 2017; Martin & Long, 2015). There have been multiple attempts to replicate these findings from different research groups using numerous strains of *D. melanogaster*, with varying degrees of success. Some studies support the ability of relatedness and familiarity to reduce sexual conflict in *D. melanogaster*, although the behaviours and life-history traits in which this response is detected vary (Carazo et al., 2014, 2015; Hollis et al., 2015; Le Page et al., 2017; Martin & Long, 2015), whereas other studies report no effect of relatedness (Chippindale et al., 2015). This is reflected in our findings in chapter 4, where despite finding no effect of relatedness in wild-type flies, olfactory-deficient flies showed some ability to respond to relatedness as predicted. Thus, despite sufficient evidence to demonstrate that *D. melanogaster* can modify aggressive and harmful behaviours in response to relatedness, these inconsistencies highlight how little we know about how this behaviour occurs. Future research focusing on the underlying mechanisms of behavioural responses to relatedness in *D. melanogaster* might firstly, help resolve discrepancies when testing for this response, secondly, further our understanding of how responses to kin might have evolved in this species, and, finally, might allow us to make inferences about conserved mechanisms of kin recognition relevant across a broader range of species.

These mechanistic questions prompted my research in chapter 4, and although I detected no evidence of olfaction alone mediating aggression and female harm in response to relatedness, there is exciting potential for future research into other possible mechanisms. My interest in olfactory mechanisms in the response to relatedness was stimulated by the recent work of Perry et al., (unpublished), in which they analysed the gene expression of groups of related or unrelated male *D. melanogaster*. While large responses to relatedness were found in genes related to olfaction, the transcription levels of other genes also changed in response to relatedness. One gene of particular interest is yolk protein 1, which was down-regulated in males in response to unrelated, unfamiliar rivals. Yolk protein1 is involved in yolk deposition in oocytes in females, but is also expressed in low levels in males, and there is evidence of differential RNA regulation of yolk protein 1 in males in response to rivals in this species (Mohorianu et al. 2017). Yolk protein 1 is an analog of the gene vitellogenin, which plays a role in the regulation of social behaviour in eusocial Hymenoptera (Amdam et al., 2007; Nelson et al., 2007). The prospect that an analog of a gene that plays an important role in social regulation in eusocial insets may be involved in social behaviours in the solitary fruit fly is intriguing, posing implications for evolutionarily conserved gene functions. The role of yolk protein 1 in male *D. melanogaster* is not fully understood, but its potential involvement in mediating the response to relatedness is interesting. My experimental design used in chapter 4 could be adapted to allow research into the role of yolk protein 1 in the response to relatedness in *D. melanogaster* by substituting the olfactory-deficient males with males with artificially upregulated or downregulated levels of yolk protein 1 (e.g., via temperature sensitive mutations, Bownes & Hames, 1978). Such work could reveal exciting possibilities about the conserved genetic control of social behaviour across eusocial and solitary insects.

Furthermore, my results from chapter 4 do not rule out the possibility that a response to male relatedness might in fact be mediated by female behaviour. Existing research into the role of relatedness in reducing sexual conflict has focused on male-mediated behavioural responses, with hypotheses that males might be selected to reduce harm when competing with brothers to prevent inclusive fitness costs (Carazo et al., 2014; Pizzari & Gardner, 2012; Rankin, 2011). However, male mating success is not solely controlled by male-male competition, courtship, and coercion: female choice and resistance also plays an important role (Delcourt & Rundle, 2011; Jirotkul, 1999; Krupa & Sih, 1993;

Lauer et al., 1996; Thornhill, 1992). Despite this, the role of female choice in response to male relatedness has been largely overlooked in *D. melanogaster*. Recently, work in the red junglefowl, *Gallus gallus*, has addressed the potential relationship between male relatedness and female choice (Tan et al., 2017). This revealed that, when presented with one unrelated and two related males, females bias mating towards the unrelated male, and this choice plays an important role in determining male mating success (Tan et al., 2017). It is possible that female *D. melanogaster* might also adjust their response to male mating efforts depending on genetic composition of groups of males, and this might drive the differences in male-male aggression and male-female harm observed in response to relatedness. For example, female *D. melanogaster* exhibit a higher mating frequency in groups composed of males from multiple strains (Billeter et al., 2012), and bias their mate choice towards unfamiliar males (Ödeen & Moray, 2008). Such behaviour could reflect an adaptation to prevent inbreeding or to avoid re-mating with the same mate, observed across multiple species (i.e., the rare male effect; Bateman, 1998; Hughes et al., 2013; Ödeen & Moray, 2008; Singh & Sisodia, 2000; Zeh et al., 1998). Furthermore, the greater diversity of male odours in unrelated groups might signal a greater male density (Carazo et al., 2007), and females might lower their resistance in response (i.e., convenience polyandry; Rowe, 1992; Wigby & Chapman, 2004). Thus, it is possible that female *D. melanogaster* could display higher mating rates when mate relatedness is lower, which could lead to increased female harm in terms of reduced longevity and lifetime reproductive success (Arnqvist & Rowe, 2005; Carazo et al., 2014; Chapman et al., 1995; Wigby et al., 2009; Wigby & Chapman, 2005).

In chapter 4, while I used olfactory-deficient males, all females had full olfactory abilities. Modifications to the experimental techniques by either using olfactory-deficient females, or odourless males, and comparing the effects of relatedness on male-male aggression and male-female harm to groups with wild-type individuals, could allow investigation as to whether or not females drive previously reported effects of male relatedness by responding to male odours.

Thus, while kin selection theory suggests that relatedness should reduce aggression and harm, the presence of this response in laboratory-adapted populations of *Drosophila melanogaster*, in which there is likely a low degree of genetic structure within the population in their recent evolutionary past, is an exciting development. The ability of such populations to respond to relatedness suggests the possibility of highly conserved mechanisms of kin discrimination, and the genetic tools available with the study of *D. melanogaster* offer exciting potentials to explore the proximate mechanisms of kin discrimination and subsequent responses.

Resource availability, nutrition, and aggression

In chapter 5 and 6, I found that the nutritional environment experienced by male *D. melanogaster* influences their aggressive behaviours, but the directions of this effect depends on the life history stage at which nutritional stresses are experienced, suggesting that the mechanisms underlying nutritional influences vary throughout life. These findings pose important implications for the study of the ecology of aggression, highlighting the need to consider the stage in an individual's life course at which environmental stresses are experienced in order to understand context-dependent impacts on future behaviours.

An interesting next step would be to assess the relative strength of and potential interactions between nutritional stresses at different life stages by simultaneously manipulating nutrition at both developmental and adult stages in a fully factorial design. Potentially, nutritional stresses experienced early in life might result in fixed changes in the developmental trajectory (Bretman, Gage, et al., 2011; Royle et al., 2005), such as smaller body size, and the subsequent effects on aggression might be irreversible, regardless of adult nutrition. For example, in tephritid fruit flies, a good adult diet cannot compensate for the impact of a poor larval diet on stress resistance (Weldon et al., 2019), and similar patterns might be seen in aggression. Alternatively, if the past larval environment is a poor predictor of the prevailing environmental conditions during reproductive periods, adult male aggression might be most sensitive to environmental cues later in life, allowing flexible behavioural responses to current conditions (Bretman et al., 2016). Furthermore, good conditions early in life might allow individuals to better cope with stresses later in life (i.e., the silver spoon effect; Grafen, 1988; Monaghan, 2008), or stressful conditions early in life might prime individuals to cope with such stresses later in life (Monaghan, 2008), and both of these hypotheses can influence the interaction between nutritional stress early and later in life.

Furthermore, my results contribute to a fast-growing field of research into the effects of nutrition on behaviour. Over recent years, there has been a growing focus on the different macro- and micro-nutrients that make up the diet (i.e. nutritional geometry, Han & Dingemanse, 2015; Harrison et al., 2017; Nestel et al., 2016), and the ways in which these nutritional elements influence development, behaviour and fitness (Nestel et al., 2016). Individuals need a wide array of varying nutrients consumed in the correct balance to maximise their fitness (Han & Dingemanse, 2015) and it would be interesting to explore the effect of diet on aggression in *D. melanogaster* from the perspective of nutritional geometry. In chapter 5, I focused on manipulations of protein levels in development. Protein is of major importance to herbivorous and omnivorous insects (Han & Dingemanse, 2017; Matavelli et al., 2015; Poças et al., 2020), and my results reveal that the quantity of developmental protein influences adult aggression. However, it is possible that the quality of protein could also have important behavioural implications. For example, the form and quality of yeast can affect larva-to-pupa development in *D. melanogaster*, with long-term effects on adult survival, mating behaviour, pheromones and food preferences (Grangeteau et al., 2018). Similarly, the composition of essential amino acids in protein sources can influence fecundity and lifespan in *Drosophila*, with methionine alone having a large effect (Grandison et al., 2009). Further refining my experimental framework to vary the quality of developmental protein, and testing the resultant effects on adult aggression, could allow a more refined understanding of the mechanisms by which developmental protein influences aggression. Likewise, carbohydrates also represent key nutrients, serving not only as an energy source but also as a substrate for the biosynthesis of other macromolecules (Chng et al., 2017; Poças et al., 2020), and form a major component of the diet of *Drosophila* in the wild (Chng et al., 2017). Thus, future work comparing the effects of developmental carbohydrate levels on adult aggression with those of protein could reveal exciting results as to the relative functions of different macronutrients in *Drosophila* behaviour and development.

Furthermore, in chapter 6, I focused on adult nutritional deprivation. However, the consequences of dietary imbalance can be more complex than and distinct to the effects of dietary restriction (Shingleton et al., 2017). As different traits require specific quantities of proteins and carbohydrates, the relative proportions of these nutrients may play a larger role in trait expression than simply the overall caloric intake (Poças et al., 2020), and future work in which adults are deprived of specific nutrients as opposed to total deprivation might allow a more fine-scale understanding of how adult nutrition influences aggression.

With rapidly increasing interest in nutrition from a geometric framework, there has been a significant advancement in the tools and technologies available to monitor nutrition (Lihoreau et al., 2015). For example, marking nutrients with dyes or radioactive labels (Buffin et al., 2009; Wong et al., 2009), along with enhanced technologies for tracking and monitoring animals (Lihoreau et al., 2015; Pérez-Escudero et al., 2014) mean it is now possible to monitor long-term nutritional strategies with fine-tuned detail and accuracy, allowing the influence of nutrition on behaviour to be studied in ever greater depth (Lihoreau et al., 2015). Furthermore, with a growing awareness of the impact of the nutritional quality of food on human antisocial behaviours (Gesch, 2017; Iribarren et al., 2004; Oddy et al., 2009; Schroeder & Higgins, 2017), investigating the fundamental nature of the relationship between nutrition and behaviour using model organisms such as the fruit fly can have important implications for society.

Conclusion

Aggression is an important component of reproductive competition in males and females alike, and optimising the expression of aggression in response to the environment can result in fitness gains. In this thesis, I aimed to use the fruit fly, *Drosophila melanogaster*, to explore how the social environment, population structure and nutritional availability shape the aggressive behaviours of both males and females. The study of aggression in both sexes simultaneously demonstrates differences in the selection for and role of aggression in males and females, and can shed light on the potential for the role of genetic correlations and sexual conflict in shaping aggression. Understanding how ecological factors influence aggression in this model organism can reveal fundamental properties of this behaviour, which have the potential to be relevant over a wider taxonomic scale.

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