Fitness effects of evolutionary manipulations of adult sex ratio and diet in fruit flies

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Abstract

The thesis research provides insight into the evolutionary changes in response to long-term manipulation of adult sex ratio and adult diet in the fruit fly *Drosophila melanogaster.* I first synthesised understanding of how long-term manipulations of adult sex ratios have been used to test key hypotheses in responses to selection and used this to identify gaps in knowledge (Chapter 1). I then contributed to our understanding of the evolution of plasticity in female behaviour in response to the pre-copulatory social environment. The results showed that these female plasticity traits were relatively stable despite exposure to divergent levels of sexual selection and adult nutritional environment (Chapter 2). The effect of the strength of sexual selection on morphological phenotypes is not well understood and few experiments have incorporated study of the additional effects of condition dependency. In chapter 3 I provide evidence that variation in the strength of sexual selection resulted in evolved responses in important physiological traits such as wing size, body weight, and female developmental rate and that these responses were condition dependent. In chapter 4 I showed that the strength of sexual selection and dietary restriction can drive changes in reproductive morphology, for the first time demonstrating evolved responses in female spermathecal size. Finally, I contributed to the understanding of how the strength of sexual selection can act on the expression of a key seminal fluid protein 'Sex peptide'. The thesis integrates a diverse range of techniques to explore how sexual selection enacts on individuals to drive phenotypic change in a population, promoting the experimental use of experimental evolution of long-term modification of adult sex ratio and diet.

Access Condition and Agreement

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Contents

Chapter 1: Use of experimental evolution of adult sex ratios to study responses to sexual selection

Abstract

Sexual selection has been long acknowledged as a driver of evolutionary change, both through intra- and inter-sexual competition. Key to determining the strength of sexual selection is the Operational Sex Ratio (OSR), defined as the number of sexually mature adults in a population. Therefore, manipulations of OSR have been useful to demonstrate the effects of sexual selection and identify the reproductive and fitness-related traits that are most strongly shaped by it. Here I review the current literature and provide a synthesis of the effects of experimental manipulations of sexual selection via changes to the OSR. This survey showed that 13 species are represented across five orders, one third of experiments use *Drosophila melanogaster,* and the ratios of manipulation extend from 3:1 to 9:1. Additionally, the majority of studies tend to be male focussed (i.e. sperm competition, or male: male competition), which reveals gaps in our understanding of the OSR effect on female plasticity. Furthermore, few studies have investigated the effect of OSR on morphology, underpinning the important work in this thesis aimed at bridging these gaps.

Introduction

The publication of Darwin's two monumental books, "On the Origin of Species" (Darwin, 1859) and "The descent of man, and selection in relation to sex" (Darwin, 1871) are seminal pieces of work that established foundational knowledge of what we are, and where we came from. Here in this review, I focus on sexual selection as a driver of evolutionary change, and in particular how evolutionary biologists have built upon Darwin's works by using the experimental manipulation of adult sex ratio. This important technique has been used to understand the selection pressures placed on individuals and the resulting phenotypes that are shaped by selection arising from competition within and across males and females.

What is Sexual selection?

Sexual selection is a driver of evolutionary change, maximising an individual's fitness, often measured as reproductive success, in a given environment. Sexual selection is potentially distinct from natural selection in that it may produce phenotypes that are counter to adaptations essential for an individual's overall survival. Natural selection favours traits promoting survival in an environment (Darwin, 1859), whereas sexual selection operates on qualities that enhance an individual's reproductive success (Darwin, 1871, Andersson and Iwasa, 1996). Additionally, sexually selected adaptations are typically weighted towards one sex more than the other, typically the sex with the larger variation in reproductive success (generally the male) (Bateman, 1948). This arises because of the fundamental differences between the sexes in investment patterns due to anisogamy (large few female gametes and many small male gametes). The expression of sexually selected characteristics may compromise overall survival advantages. For example, the horns produced by dung beetles are typically more pronounced in males, or entirely absent from females. This male-specific or sexually dimorphic phenotype offers a sexually competitive advantage in acquiring mating opportunities for the male with larger horns, with longer horns increasing probability of mating success (Siva-Jothy, 1987). However, the expression of horns is resource-dependent (Koyama et al., 2013) and they are energetically expensive to produce (Emlen,

2001). As a result, males spend longer developing as larvae, which can increase exposure to parasitic nematodes and reduce survival probability (Hunt and Simmons, 1997). In addition, the horns can grow so large that other sensory organs may even become obstructed (Nijhout and Emlen, 1998). A well-established example of the fitness benefits and potential costliness of sexually selected male traits is represented in the peafowl's train. In peafowl, the females select males based on the morphology of their train, with larger trains being more attractive (Petrie et al., 1991). The investment in train feathers represents a fitness cost that only the fittest males can carry (Lindstrom et al., 1998). In addition to the resource cost of feather production, males with the largest trains are also subject to increased predation potential (Zahavi, 1999). Whilst the impact of train length on flight and predation remains disputed (Askew, 2014), the presence of a large ornament in one sex and not the other, represents a divergence in the pattern of resource allocation across males and females. In these examples it is evident how natural and sexual selection are intertwined but can act in different ways on individuals of each sex.

The prerequisite for sexual selection is that an individual has the opportunity to maximise their own reproductive success, and that these advantages are gained through the expression of primary or secondary sexual traits that vary within a population. Primary sexually selected traits are those associated with gamete function, size, and provisioning. For example, *Drosophila bifurca* exhibit the longest relative sperm length recorded in nature (Joly et al., 1995) and mating success has been strongly linked to sperm competition (Luck et al., 2007). Additionally, a review by Lüpold et al. (2020b) evaluated studies of sperm characteristics and fitness across four decades, and concluded there was good evidence linking sperm and testes morphology with the level of sperm competition to which males were subject.

Secondary sexually selected traits are generally defined as those involved in obtaining or ensuring fertilisations – and they offer some of the most spectacular adaptations found in nature. For example, the size dimorphism exhibited by pinnipeds, gorillas and crocodilians are largely driven by selection for increased size in order to gain increased access to females for mating (Lindenfors et al., 2002, Leigh and Shea, 1995, Warner et al., 2016), with the largest male American alligator (*Alligator mississippiensis*) measuring 4.35m (Brunell et al., 2013) and the largest female being 1m smaller (Deem et al., 2021). In addition to a large size difference,

the male Mandrill (*Mandrillus sphinx*) exhibits two large prominent canines, which display his sexual maturity and condition (Dirks et al., 2020). In the stalk-eyed fly (*Cyrtodiopsis dalmanni*), a female distinguishes between a high- and low-quality male by the length of his eye-stalks (Cotton et al., 2004a) and famously the lion's mane is associated with high reproductive fitness in males (West and Packer, 2002).

In many cases, secondary sexually selected adaptations evolved primarily in males in order to gain or maintain access to mating opportunities. I describe the two major forms of sexual selection below.

Intrasexual competition

Gaining access to mating opportunities through direct competition is also known as intrasexual competition. The archetypal example of this would be two or more males fighting for access to females, the signal this competition sends can be twofold; females identify the winner as a high-quality male, and other males recognise this individual as the gatekeeper to female access. For sexually mature males the process of maintaining access to females is resource intensive (Le Boeuf and Peterson, 1969) and acts as an honest indicator of male fitness (Emlen et al., 2012). For example, male red deer (*Cervus elaphus*) maintain a territory, which is defended from rival males throughout the breeding season, the longer this territory is maintained the more mating's the male gains (Carranza et al., 1990). However, evidence supports that there are risks associated with rutting, which can reduce overall body condition and increase the risk of death (Forsyth et al., 2005, Clutton-Brock et al., 1982). Another example is illustrated by the Northern Elephant seal (*Mirounga angustirostis*). Not only does an extreme size dimorphism exists between the sexes in this species, but males additionally develop a larger nasal turbinate, which helps prevent water loss during the mating season (Huntley et al., 1984). Males cease to eat or drink during this period and focus entirely on defending or acquiring a hareem (Huntley et al., 1984). Therefore, a male with a larger nasal turbinate has a higher potential fitness in comparison to a shorter turbinate male, due to the risk of dehydration. The direct competition between males is not limited to mammals and is seen across taxa. For example, in the Broad-horned flour beetle

(*Gnatocerus cornutus*), males, and not females, possess enlarged mandibles, which they use in fights with other males for access to mating's (Okada et al., 2006). In the Narra beetle (*Onymacris plana)*, males are typically smaller than females but possess wide elytra that show a greater allometric variation to body size (i.e. are relatively larger for a given body size; (Enders et al., 1998). The males of this species fight for mating opportunities, and individuals with the widest elytra are typically more successful (Enders et al., 1998). Male *Bolitotherus cornutus* males fight over feeding sites and control access to females - unsurprisingly, horn length is correlated with higher paternity share (Brown, 1980). Whilst physical contests clearly display an organism's potential fitness, the cost of fighting can be significant. Any injury could not only reduce success against the next challenger but may cause an infection leading to further complications or death. As a result, many animals avoid direct contests by mutual assessment. For example, male cuttlefish use elaborate and colourful displays to demonstrate size and fitness to an opponent, with smaller males altering their behaviour in response to a larger rival (Schnell et al., 2015). Another example is found in the male Satin bowerbird (*Ptilonorhynchus violaceus*) which builds elaborate structures to attract the attention of females. However, males will also actively steal valuable ornaments or decorations from other structures in order to represent themselves as high fitness males (Wojcieszek et al., 2007).

Accessing opportunities to mate is often only the first hurdle when ensuring paternity share, as many organisms mate multiply under natural conditions. This phenomenon selects strongly for the evolution of post-copulatory intrasexual competition. Strategies used to ensure reproductive success post-copulation are equally as diverse as those utilised prior to mating (Andersson and Iwasa, 1996). Mate guarding is a common tactic used by many organisms. For example, many dung beetle species exhibit intrasexual dimorphism which is directly related to their reproductive strategy (Lailvaux et al., 2005). The major morphs (those with horns) are not only better at acquiring mates (Kotiaho, 2002), but they use their horns to effectively block the tunnels in which females are housed, preventing access by rival males (Emlen, 1997). An extreme example of mate guarding can be found in the Harlequin toad (*Atelopus laetissimus*) whose amplexus (mating clasp) can last for several months (Rueda-Solano et al., 2022). Evidence suggests that this has selected for males that exhibit proportionally longer arms (Peters, 1973), and that the

mate guarding behaviour is likely a driver of this trait (Lee and Corrales, 2002). Similar to mate guarding, some male's express traits such mating or copulatory plugs that prevent remating by females. The mating plug serves as a barrier to a potential rival's sperm and is effective in preventing a second male achieving reproductive success even if the first male is sterile (Mangels et al., 2016).

Mating plugs may comprise a range of substances. For example, in *Drosophila* the mating plug is formed by the union of several distinct seminal fluid components (McDonough-Goldstein et al., 2022). In contrast in *Aranea* species, males also produce plugs with seminal fluid proteins, whilst others leave parts of their reproductive organs inside the female to act as a physical barrier (Uhl et al., 2010). It has been demonstrated that many species have evolved methods to remove the mating plug (Méndez and Eberhard, 2014) and this has been linked to the rapid diversification of male genitalia in ground beetles (Takami and Sota, 2007). In most mating systems, a female's proclivity to mate repeatedly and a male's ability to prevent females from doing so selects for the evolution of mechanisms to drive success in sperm competition (Parker, 1970, Parker and Pizzari, 2010). The mating plug is one component of this post-copulatory sperm competition.

Perhaps the most obvious component of traits that facilitate success in sperm competition is sperm itself. Sperm morphology varies greatly across taxa (Pitnick et al., 2009), and evidence shows that post-copulatory sexual selection can drive or reduce sperm trait adaptation in different systems (Morrow and Gage, 2000, Calhim et al., 2007). Sperm length in Iberian red deer, for example, has been shown to vary considerably between males, with the sperm shape determining sperm motility (Malo et al., 2006). The quantity of sperm males can transfer is often related to success in fertilisations and can thus also be strongly correlated with the strength of intrasexual competition. For example, in the fruit fly *Drosophila pseudoobscura* and the seed beetle *Callosobruchus maculatus*, males have been shown to transfer more sperm when exposed to rivals prior to mating (Price et al., 2012, Lymbery et al., 2019) However, in *C. maculatus* increased sperm transfer has little effect on fertilisation success (Eady, 1995). In addition, males of the dung fly (*Sepsis cynipsea*) exposed to potential intra-sexual competition will strategically increase their ejaculate investment to maximise reproductive success (Martin and Hosken, 2002).

Along with sperm, males transfer a suite of seminal fluid components that are linked to reproductive success (Chapman, 2001). These proteins are common across taxa (Borziak et al., 2016, Patlar et al., 2021) and not only show rapid sequence divergence amongst closely related species (Civetta and Singh, 1995), but also variation within species according to geographic separation (Cridland et al., 2023). Work on *Drosophila* has highlighted seminal fluid proteins as significant architects of male reproductive success (Chapman, 2001). One such protein 'Sex peptide' has been shown to elucidate a range of effects in females. These include responses that maximise the reproductive success of the male producer by causing an increase in a female's oviposition rate (Chen and Bühler, 1970), and by causing a reduction in a female's sexual receptivity to mating (Chen et al., 1988). For *D. melanogaster* the number of seminal fluid proteins is estimated to be around 292 (Wigby et al., 2020), whilst in humans it exceeds 2000 (Gilany et al., 2015).

Intrasexual selection amongst females is not as well documented as it is in males, though forms an important part of sexual selection. Male: male competition can produce clear physical phenotypes such as horns etc. (Emlen, D. J, 2008). However, female: female competition often revolves around competition for resources required for successful reproduction, rather than access to mating opportunities (Clutton-Brock, 2009). However, amongst social animals, their status within a colony or troop can provide a driver for female-female competition, with higher-ranking females not only accessing resources, but also mating opportunities. For example, female Chacma Baboons (*Papio hamadryas ursinus*) compete with rival females for male 'friendships' (Palombit et al., 2001) and social status, with female-female aggression increasing with number of females in the group (Cheney et al., 2012). This may provide selection for an increase in female aggression and the ability to form social bonds. Additionally, female *Eclectus roratus* compete with rival females for access to egg laying sites, and guard them for up to nine months (Heinsohn, et al., 2003).

As outlined, male-male competition clearly drives evolutionary change in male sexual traits. However, another equally potent actor on the evolution of divergent reproductive traits is intersexual selection, or mate choice.

Intersexual selection

Examples of intersexual selection can be found in both sexes; however, it is predominantly female driven, and for the synthesis of this section it will be referred to in the context of female mate choice. Female mate choice is a powerful driver of secondary sexually selected characteristics (Andersson, 1994). There is often a link between female mate choice and intersexual competition. For example, the winner of intersexual conflicts has often also demonstrated their high fitness via the expression of 'honest' sexual displays. Hence a female may choose such an individual as they will pass on high fitness characteristics to her offspring (Berglund et al., 1996). For example, the males of the rhinoceros beetle (*Trypoxylus dichotomus*) compete with other males for access to feeding sites, and there is a strong correlation between size and winning duels (del Sol et al., 2021). Additionally, size and lifetime mating success are also correlated (del Sol et al., 2021), indicating that females may perceive size an honest signal of fitness.

However, in many species a female may select an "attractive" male based on characteristics that do not immediately or obviously convey fitness (Darwin, 1871). For example, spotted bowerbird (*Chlamydera maculate*) males build structures decorated with bones and glass, and the number of decorations is associated with higher male mating success (Borgia, 1995) – but why a female chooses to mate with a male based on these metrics is difficult to discern. Additionally, many birds exhibit colour dimorphism, a trait that has evolved many times in passerines (Price and Birch, 1996), but exactly how this conveys fitness is not always immediately apparent. Some research has shown that male colouration may be linked to foraging ability, due to the quality of a male's feathers (Kodric-Brown, 1989) or to parasite resistance (Folstad and Karter, 1992), indicating some level of fitness to the female. A more modest indicator of fitness has evolved in males of species that exhibit nuptial gifting. The presentation of a nuptial gift can come in many forms and its role varied. For example, the nuptial gift can be produced 'internally' by the male and gifted to the female orally, as in the bush cricket (*Poecilimon ampliatus*) resulting in an increase in female feeding rate (Lehmann and Lehmann, 2016) or gifted in copula as occurs in the arctiid moth (*Utetheisa ornatrix*) (Iyengar and Eisner, 1999) in which the gift is associated with a range of benefits to the female, including chemical defence of eggs (Dussourd et al., 1988). Males may also provide 'external' nuptial gifts (Arnqvist and Nilsson, 2000). For example, in the spider *Paratrechalea ornata,*

males provide a gift in the form of a prey item, and the size of the gift is correlated with mating duration (Klein et al., 2014). Interestingly some species of spider have evolved an alternative and ‘deceptive’ tactic. By wrapping a non-edible gift in silk and presenting it to the female they are able secure a mating opportunity (Albo et al., 2011). Upon receipt of such gifts the female terminates mating sooner than for non-deceptive males. However, the deceptive males are still able to transfer some sperm and so potentially secure a degree of paternity (Albo et al., 2011).

Sexual selection has provided the opportunity for the evolution of many elaborate tactics in gaining paternity, and ensuring offspring are of high quality. A major facilitator acting on the strength of sexual selection is the adult sex ratio.

Sex ratios and the strength of sexual selection

Sex ratios are important population dynamics that can influence the intensity of sexual selection, competition, and conflict. Wild populations often demonstrate an adult ratio approximating 50:50 (Fisher's principle), because an imbalance would favour the underrepresented sex, resulting in a gradual return to equal representation over generations (Fisher, 1930). However, Fisher's principle assumes random mating, equal parental investment, and the lack of selective forces preferencing one sex over the other. In nature, these assumptions are not often met, and some individuals are able to acquire a higher paternity share through various sexually selected mechanisms (Andersson and Iwasa, 1996). These sexually selected traits and drivers of reproductive investment can affect the sex ratio of a population and increase or decrease selective pressures on one sex over the other. For example, sex biased predation risk due to conspicuous sexually selected traits is common across taxa (Burk, 1982, Boukal et al., 2008), resulting in an asymmetrical effect of natural selection on one sex. The division of parental investment is also disproportionately exhibited. Typically, females invest more in the care of offspring (Zeh and Smith, 2015, Queller, 1997) with 'male only' care completely absent in mammals (Clutton-Brock and Scott, 1991). This divergence in parental care can result in the temporary alteration of operational sex ratios, particularly in organisms where the female mates once per breeding season.

The asymmetry of sex ratio in relation to sexual selection is often defined by two terms. The Adult sex ratio (ASR) and the operational sex ratio (OSR). ASR is defined as the total number of adult males to females in a population (Kokko and Jennions, 2008) whilst the OSR refers to the number of sexually active individuals (or males) within a population (Kokko and Jennions, 2008). It can be difficult to separate the OSR from the ASR (Carmona-Isunza et al., 2017) and for this reason, I focus the mainly on ASR, as an indicative proxy for OSR.

OSR distortion is relatively common in the wild, and can be influenced by several factors, including sex bias predation (Burk, 1982, Boukal et al., 2008) and parental investment (Zeh and Smith, 2015, Queller, 1997). Human disruption can equally cause alterations of the OSR. For example, in wild populations of African lions (*Panthera leo*) the adult sex ratio is typically one male to 3 females (Creel and Creel,

1997). However, in areas where trophy hunting or poaching is common, the sex ratio can be distorted by 1:6 (Loveridge et al., 2016). Many species of bird display a male biased sex ratio in the wild (Donald, 2007), which has been linked to habitat decline in some species (Grüebler et al., 2008). The introduction of invasive species such as the American mink (*Neovison vison*) has additionally been linked to sex ratio distortions in European polecat (*Mustela putorius*) populations, because American mink is more successful in outcompeting female compared to male polecats (Barrientos, 2015). In many reptilian species, sex is determined by incubation temperature (Bull, 1980) and as a result climate change has been linked to a female biased sex ratio in the Hawksbill Sea turtle (*Eretmochelys imbricata*) (Kamel and Mrosovsky, 2006), whilst agriculture and climate change is linked to male bias sex ratios in the common snapping turtle (*Chelydra serpentina*) (Thompson et al., 2018). Additionally, the naturally occurring bacteria *Wolbachia* infects many species of arthropod worldwide (Hilgenboecker et al., 2008) and can cause sex ratio distortion by either killing male embryos (Jiggins et al., 2000), or converting males into functional females (Moreau and Rigaud, 2000). In addition to these factors, the OSR can be modular in time, for example males of many species arrive at breeding sites earlier than females, meaning the sex ratio will initially be male biased (Morbey and Ydenberg, 2001) and so intrasexual competition is likely to be more intense earlier in the breeding season.

The multitude of factors able to influence OSR in wild populations make it an interesting and relevant topic investigate the effect of the strength of sexual selection on trait evolution within a species. Here, I present a synthesis of research in which the OSR has been manipulated (Table 1) and describe the broad findings of these studies as a whole. I have chosen to omit studies that use the comparison of monandry versus polyandry from the main synthesis as this is distinct from OSR manipulations *per se*. Whilst monandry versus polyandry experiments investigate the mating system, they do not incorporate the complete dynamics of alterations in sex ratio across a continuum.

Manipulations of OSR

A literature search of web of science (Web of Science™, 05/12/23) using the key words “Operational Sex Ratio” reported 579 papers, the addition of “Experimental evolution” reduced it to just nine studies between 2008 & 2022. Combining “OSR” and “manipulation” reported just sixteen studies between 2005 and 2023. Therefore, the search was adapted to include “adult sex ratio”, which registered 32,036 references. However, the addition of “Experimental evolution” drastically reduced this to eighteen. The combination of “ASR” and “manipulation” recorded 176 studies that were further reduced to thirty-five with the addition of “experimental”. Further analysis removed experimental designs where only one direction was used i.e. only female biased, resulting in the twenty-nine studies represented in Table 1. Of the remaining studies just thirteen species across five orders are represented, underlining OSR manipulation as a manipulation that has been applied to date in a fairly taxonomically restricted manner.

Limitations of OSR experiments are likely due to difficulty in maintaining captive populations, and the generation time for analysis of evolved traits. This is exemplified by the finding that *D. melanogaster* represented more than one third of all studies (12). Despite the difficulties conducting OSR manipulations, these studies have produced some interesting phenotypic responses, and this synthesis demonstrates utility as a useful addition to the evolutionary biologist’s toolbox.

Several studies provide insight into the antagonistic relationship within and between males and/or females (Hosken et al., 2009, Wigby and Chapman, 2004a, Nandy et al., 2013b). Such studies that manipulate OSR typically measure female harm as an effect of male harassment on lifetime fecundity (Wigby and Chapman, 2004a, Nandy et al., 2013b), which adds to the detrimental effect on exposure to males on female lifespan shown by Partridge et al. (1987). The OSR studies highlight that Male Bias (MB) males are in general more harmful to females (Nandy et al., 2013b), whist females become more resilient to this harm in MB environments (Wigby and Chapman, 2004a). When evolved under Female Bias (FB) regimes female *D. melanogaster* become increasingly antagonistic towards each other (Bath et al., 2021). The increase in male competition in MB regimes, driving females to evolve resistance to increased male harm was present in *D. melanogaster* (Wigby and

Chapman, 2004) and *T. castaneum* (Michalczyk et al., 2011). Further analysis shows that this adaptation may be condition dependent, as resistance to male harm was compromised when nutrition was poor (Rostant et al., 2020a). The increase in male competition had a consistent effect on mating investment, with males increasing either time (mating duration) or resource (sperm investment) to ensure higher paternity (Dore et al., 2021, Gallup et al., 2019a, Rosa et al., 2017). The studies in Table 1.1 represent 13 species across five orders, a third of all experiments use *Drosophila melanogaster*, and the ratios of manipulation extend from 3:1 to 9:1. Five of the experiments manipulate the OSR for only one generation, whilst the remaining 24 were longer term manipulations ranging from 19 to 194 generations. Of the traits explored seventeen were pre-copulatory traits and 16 post-copulatory traits, with ejaculatory adaptations accounting for 7 of the 16 post copulatory experiments.

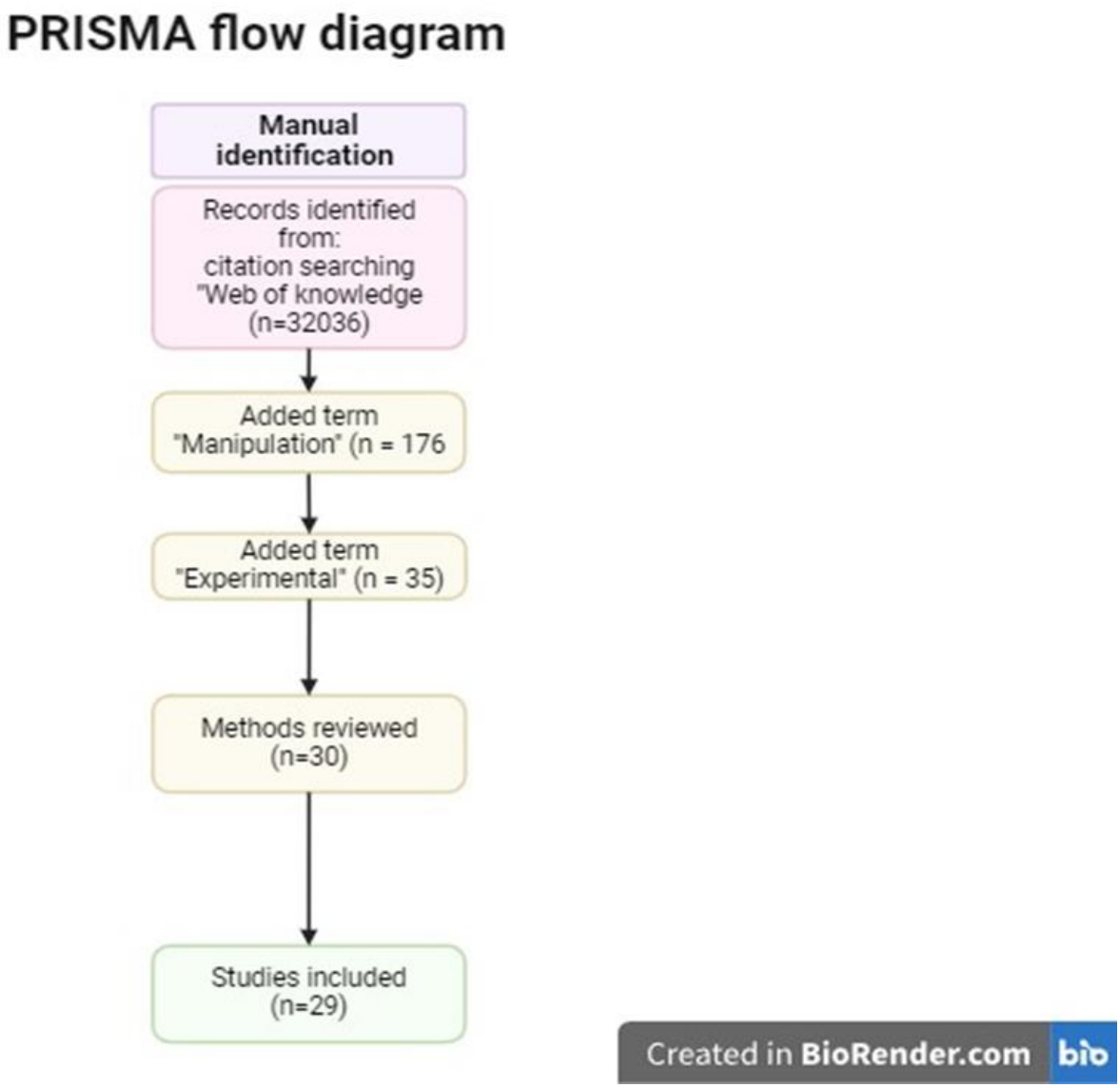

Table 1.1: Synthesis of studies manipulating OSR and their reported phenotypes. Table showing the trait studied, the species used in the study, the strength of selection (as indicated by sex ratio) and any fitness advantage or disadvantage reported within the study.

Research focus	Species	Sex ratio manipulations (Male: Female)	No. Generations	Outcome (FB = Female bias, MB = Male bias)	Study
Female resistance to male harm	*D. melanogaster*	3:1, 1:1, 1:3	32	• Females encountered an increase in mating attempts from MB males. • Females from male biased lines survived longer in the presence of males.	(Wigby and Chapman, 2004a)
Reproductive isolation	*D. melanogaster*	3:1, 1:1, 1:3	41	• Sexual conflict level did not significantly differ in response to strength of sexual conflict. • No differences in proportion of individuals mated within or between sex ratios	(Wigby and Chapman, 2006)
Sperm competition	*D. melanogaster*	2.33:1, 1:1, 1:3	140	• FB males reported to replenish ejaculates quicker than MB males. • MB males performed poorly over multiple mating's	(Linklater et al., 2007)
Behavioural and reproductive isolation	*Sepsis cynipsea & D. melanogaster*	3:1, 1:1, 1:3	41 & 35	• No evidence that sexual conflict resulted in reproductive isolation in *D. melanogaster.* • •No differences in mating latency between sex ratio treatments of *D. melanogaster* • Evidence supported reproductive isolation in S*epsis cynipsea,* with differences in mating latency	(Hosken et al., 2009)
Female resistance to male harm	*Tribolium castaneum*	1:9, 6:1	20	• MB females showed significant resistance to males. • FB females had a steeper decline in reproductive fitness and	(Michalczyk et al., 2011)

				suffered higher mortality with an increase in mating.	
Pre-copulatory sexual competition (Courtship)	*Ceratitis capitata*	1:3, 3:1	1	• FB males had higher paternity. • MB males had lower paternity despite increased investment in courtship	(Leftwich et al., 2012)
Female resistance to male harm and courtship	*D. melanogaster*	3:1, 1:1, 1:3	45	• MB line males had higher courtship frequencies, whilst FB males sired more progeny. • MB males were more harmful to females	(Nandy et al., 2013b)
Sperm competition	*Megabruchidius tonkineus*	5:1, 1:5	19	• MB males transferred larger ejaculates and received a higher paternity share. • Females with increased fertility died younger	(Booksmythe et al., 2014)
Female mate choice and male competition	*Bicyclus anynana*	3:1, 1:1, 1:3	1	• Female choosiness decreased under high MB populations. • MB increased likelihood of courtship being aborted	(Holveck et al., 2015a)
Protection against inbreeding	*Tribolium castaneum*	1:9, 9:1	45	• Increased sexual selection lines were more resilient to inbreeding. • Populations that experienced low or no sexual selection declined rapidly when inbred	(Lumley et al., 2015)
Sperm competition	*Tribolium castaneum*	1:9, 9:1	77	• High sperm competition drives evolution of sperm length, longer sperm males had 20% higher paternity. • The evolution of sperm length was condition dependent. Protein restricted lines had significantly shorter sperm	(Godwin et al., 2017a)
Sperm competition	*Callosobruchus maculatus*	1:2, 2:1	32	• Males did not diverge in testes, accessory gland, or ejaculate investment by sex ratio	(McNamara et al., 2016)

Sex role reversal	*Megabruchidius dorsalis*	1:5, 5:1	19	• FB females located males quicker than MB females and mated sooner	(Fritzsche et al., 2016)
Sexual competition	*Lethrus apterus*	3:1, 1:1, 1:3	1	• MB males increased nest attendance. • MB males sired fewer offspring	(Rosa et al., 2017)
Mate searching	*Tribolium castaneum*	9:1, 1:9	82 & 106	• MB males evolved better mate recognition. • FB males were more inaccurate at sex determination.	(Sales et al., 2018)
Mating duration	*Aquarius remigis*	1:2, 2:1	1	• Mating duration increased when exposed to males. • Only increased duration when exposed to males prior to mating	(Gallup et al., 2019a)
Age fitness effects pre and post copulation	*D. melanogaster*	2:4, 4:2	N/A	• Sex ratio did not affect lifespan of males or females.	(Sultanova and Carazo, 2019)
Sexual conflict	*Plodia interpunctella*	1:3, 3:1	110	• Female genital teeth evolved to be wider under FB lines. • No relationship between spermatophore and genital teeth.	(McNamara et al., 2019)
Inbreeding	*Tibolium castaneum*	2:1, 1:1, 1:2	46	• OSR did not affect selection against deleterious mutations. • No evidence for the role of sexual selection in relation to evolution of sex maintenance.	(Plesnar-Bielak et al., 2020)
Condition dependent resistance to male harm and lifetime fitness	*D. melanogaster*	3:1, 1:1, 1:3	47	• MB females evolved resistance to continuous male exposure, living longer compared to FB females. • MB female resistance to male exposure was compromised under low nutrition.	(Rostant et al., 2020a)
Mate choice	*Plodia interpunctella*	1:3, 3:1	133, 134, 135 & 143	• No effect of OSR on male mate choice	(Dougherty et al., 2020)
Mating duration	*D. melanogaster*	2.33:1, 1:1, 1:3	66	• MB Males increased mating duration.	(Dore et al., 2021)

				• MB males more sensitive to rivals and displayed extended mating latency.	
Condition dependent sexual selection and sexual conflict	*D. melanogaster*	2.33:1, 1:1, 1:3	35	• Condition dependent increase of FB males mating latency. Condition reduced female remating suppression.	(Sepil et al., 2022a)
Parasite resistance	*Tribolium castaneum*	9:1, 1:1, 1:9	56	• No trade-off between infection resistance and fertility	(Hangartner et al., 2013)
Reproductive trade-off with lifespan	*Tenebrio molitor*	1:3, 1:1, 3:1	1	• MB females produced more offspring but died younger. • FB males lived longer, but produced fewer offspring	(Jehan et al., 2020)
Courtship success and sperm competition	*D. melanogaster*	3:1, 1:3	190 -195	• MB males have a higher courtship success and mating frequency than FB males. • Success is higher if MB are second to mate.	(Chechi et al., 2022)
Condition dependency of fitness	*D. melanogaster*	2.33:1, 1:1, 1:3	23 & 36	• No fitness effects reported in males. • Female size responded to condition dependence in MB populations	(Bath et al., 2023)
Intrasexual aggression	*D. melanogaster*	2.33:1, 1:1, 1:3	78 & 92	• FB females were more aggressive than MB females	(Bath et al., 2021b)
Intrasexual competition	*Caenorhabditis remanei*	10:1, 1:10	30	• Females evolved larger under both FB and MB treatments and this effect was more pronounced in FB lines. • Both MB & FB directionality impacted female fecundity	(Stångberg et al., 2020)

Conclusion

Whilst experimental manipulation of the OSR clearly demonstrates a range of phenotypic effects, this experimental tool itself remains underused, and it has the potential to be used to study a wider range of fitness-related traits. The ability to effectively study the effects of OSR manipulations requires an organism that is highly fecund and has rapid development to sexual maturity – though future research should explore other suitable taxa, to gain a broader picture of conserved and novel traits across species. Additionally, studies should explore other morphological traits such as the sex combs in *Drosophila,* which show rapid evolution across species (Ng and Kopp, 2008), or the evolution of harmful male copulatory tactics as seen in the bed bug (*Cimex lectularius*) (Stutt and Siva-Jothy, 2001). As shown in Table 1.1 experimental manipulations of the OSR test female reproductive traits via fecundity or lifespan, and rarely investigate trait adaptation or morphology, with the majority focusing on male traits and reproductive fitness. Additionally, studies have not taken the opportunity to investigate an important morphological characteristic of courtship within *Drosophila* i.e. wing morphology.

In this thesis, I used populations of *D. melanogaster* subjected to long-term variation of adult sex ratios to explore how the strength of sexual selection impacts male and female traits. Studies using this system have already shown that females from lines maintained under high male bias evolved a resistance to male harm by producing more offspring in the presence of males, compared to females from female biased lines (Wigby and Chapman, 2004a), and that the strength of sexual conflict did not deter female mating (Wigby and Chapman, 2006). Additionally, this system reported the evolution of differential ejaculate allocation, with male biased (MB) males depleting their accessory glands quicker than their female-biased (FB) counterparts (Linklater et al., 2007). These populations were later restarted to include a condition dependent aspect (manipulation of adult diet), which allowed for a more holistic approach to the study of evolutionary adaptations driven by sexual selection. The resulting experiments with these newer lines demonstrated that plastic adaptations in males such as courtship and mating duration also responded to sexual selection and were apparently not costly to maintain (Dore et al., 2021). Furthermore, the additional condition dependency manipulation showed that protein restriction had an

effect on FB male mating latency, and that, in general, males held under long-term protein restrictive regimes during adulthood were less successful in supressing remating in females (Sepil et al., 2022a).

The subsequent thesis provides important additional insights into the effects of long-term sex ratio manipulation, and reveals that:

- Socially plastic responses in females are robust to evolutionary manipulations of adult sex ratio and adult nutrition (Chapter 2)
- Experimental evolutionary manipulation of adult sex ratio and adult nutrition selects for divergent development and body size-related fitness traits (Chapter 3).
- Experimental evolutionary manipulations of adult sex ratio and adult nutrition select for responses in reproductive morphology and reproductive success (Chapter 4).
- Experimental evolutionary manipulations of adult sex ratio affect the expression of a key seminal fluid protein gene, Sex Peptide, in males (Chapter 5).

These chapters provide key insight into the adaptations that respond to long-term variation in sexual selection and adult diet.

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Chapter 2: Socially plastic responses in females are robust to evolutionary manipulations of adult sex ratio and adult nutrition.

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Attribution statement

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Abstract

Background Socially plastic behaviors are widespread among animals and can have a significant impact on fitness. Here I investigated whether the socially plastic responses of female *Drosophila melanogaster* can evolve in predictable ways following long-term manipulation of adult sex ratio and adult nutrient availability. Previous reports show that female *D. melanogaster* respond plastically to their immediate same-sex social environment by altering their fecundity, laying fewer eggs after they mate if previously exposed to other females. Fecundity is also highly sensitive to a female's immediate nutritional status, being significantly reduced when dietary protein in particular is scarce. On this basis, I predicted that an evolutionary history of exposure to variation in adult sex ratio and adult nutritional environment would select strongly upon a female's plastic fecundity responses.

Results I used females that had been drawn from replicated lines that had experienced an evolutionary history of male biased, female biased or equal adult sex ratios and either standard or low-quality adult nutrition. I tested the specific predictions that a history of elevated competition among females (in female-biased regimes) would select for increasingly sensitive plastic fecundity responses to the presence of conspecifics, and that these would be magnified under poor nutritional resource regimes. In contrast to the expectations, I found that the plastic responses in females were strikingly robust to perturbations of both sexual competition and nutrient availability and did not differ significantly across any of the evolutionary regimes. The lack of response is not explained by an insufficient strength of selection. For example, among females held in isolation prior to mating, I did observe significant evolutionary responses in virgin eggs laid according to nutritional regime and in virgin egg retention to sex ratio regime.

Conclusion The lack of variation in the existence and magnitude of predicted plasticity is consistent with the idea that the costs of maintaining fecundity plasticity in females are low, benefits high, and that plasticity itself can be hard wired.

Keywords sexual selection, plasticity, mating behaviour, experimental evolution.

Background

Plastic responses expressed by individuals in response to environmental cues can be vital components of fitness across many different organisms (Bretman et al., 2011a, Wedell et al., 2002, Kasumovic and Brooks, 2011, Sheehy and Laskowski, 2023, Dingemanse and Wolf, 2013, Snell-Rood, 2013, Moczek et al., 2011, Pfennig, 2021). Such responses allow organisms to match their reproductive effort or tailor their life history to the expected or prevailing environment, and thus optimise their fitness (Price et al., 2003, Van Buskirk, 2012). Plastic responses can be manifested in many different ways. For example, they can be influenced by conditions experienced by parents, in anticipation of the environment likely to be experienced by offspring (Kasumovic and Brooks, 2011, Snell-Rood et al., 2013). They may also be set during development in anticipation for the expected adult environment (Lange et al., 2023, Yoon et al., 2023). Behavioural plasticity or allocation of resources to reproduction are two different ways that may also vary in response to the immediate conditions experienced during adulthood (Bretman et al., 2011a). Plastic responses to environmental conditions such as diet can also balance lifespan and reproductive success according to the level of resources available (Zajitschek et al., 2016, Zajitschek et al., 2013, Bretman et al., 2023) and can even change the sign of trade-offs between lifespan and reproductive effort (Collins et al., 2023).

The capacity for individuals to express plasticity will be affected by the potential fitness gains of doing so balanced against the costs of being plastic (Dingemanse and Wolf, 2013, Sheehy and Laskowski, 2023, Snell-Rood, 2013, Pfennig, 2021). The extent, tempo, and predictability of variation in expected or prevailing environments is expected to be a crucial determinant of the potential fitness benefits of plasticity. For example, if environments change very rapidly, plasticity responses may not easily be matched to them (Bretman et al., 2012). In contrast, if environments are generally stable, the fitness benefits of plasticity are expected to be minimal (Scheiner and Levis, 2021). However, much about the evolutionary drivers, pace and extent of plasticity evolution remains unclear (Lange et al., 2021). This is the topic I address here by testing key hypotheses for the evolution of reproductive plasticity in females drawn from populations subjected to long-term variation in two key factors: socio-sexual and nutritional environments.

The social and sexual environment has emerged as an important driver of plasticity. For example, male Mediterranean fruit flies that perceive elevated sexual competition due to the presence of a conspecific male in the mating arena, transfer significantly more sperm to females during mating (Gage, 1991). Similarly, male *D. melanogaster* respond to elevated sexual competition by mating for longer, transferring more of key seminal fluid proteins (Wigby et al., 2009a) and sperm (Moatt et al., 2014, Hopkins et al., 2019b, Garbaczewska et al., 2013b) - thus achieving higher reproductive success (Bretman et al., 2009b). Females also show related plastic responses to the presence of conspecifics (Bailly et al., 2021, Sarin and Dukas, 2009). For example, in *D. melanogaster*, mated females are more aggressive towards rival females than are virgins (Nilsen et al., 2004b, Bath et al., 2017) and this aggression evolves to become stronger in populations maintained over time under strong female bias (Bath et al., 2017). Female fecundity is also surprisingly plastic and varies according to the pre- and post-mating social environment (Fowler et al., 2022b) (Bailly et al., 2021). For example, females maintained in same sex conspecific groups prior to mating lay significantly fewer eggs after mating than those kept alone, with the detection of deposits left by other females being key to the assessment of the female's social environment (Fowler et al., 2022b). These findings show that both sexes can respond plastically according to the level of potential competition experienced.

Plasticity in responses to sexual competition can also be strongly determined by an individual's condition, which can vary with factors such as temperature, nutrition and population density. For example, in the moth *Plodia interpunctella*, males raised under high population density take longer to mature but have larger testes (Gage, 1995a). In *D. melanogaster,* increased availability of nutrition under lower larval density results in the production of larger females that are more aggressive (Bath et al., 2018b). However, once mated, it is smaller females that typically win physical contests (Bath et al., 2018b). In the Yellow dung fly (*Scathophaga stercoraria*), an increase in temperature is strongly correlated with a reduction in testes size (Bernasconi et al., 2002). These examples illustrate the influence of 'conditional' traits on the outcomes of sexual selection and competition (Bath et al., 2021a). Therefore, I predict that examination of the simultaneous effects of long-term manipulations of condition and the social and sexual environment, as I undertook here, should be particularly informative for identifying the primary drivers of plasticity evolution.

Manipulation of the social and sexual environment can be achieved using phenotypic engineering of the immediate number, density or sex of potential competitors, of the sensory cues available (Bretman et al., 2010b, Bretman et al., 2011c) or longer-term manipulations of the adult sex ratio (ASR, total number of adults in a population) over evolutionary time. ASR is a key variable to manipulate in the study of responses to the same and opposite sex competitive environment (Emlen and Oring, 1977, Clutton-Brock, 1988, Clutton-Brock, 2007). Sexual selection is predicted to become more intense in populations in which the ASR becomes unbalanced (Emlen and Oring, 1977, Clutton-Brock, 1988, Clutton-Brock, 2007, Pitnick, 1993, Hollis et al., 2019b). Under a male biased ASR, competition between females should decrease and between males should increase as males seek to maximise their reproductive success in the light of reduced mating opportunities (Hollis et al., 2019b, Sepil et al., 2022b). Consistent with this, experiments on *Tribolium castaneum* flour beetles have shown that males under intense competition maintained in male-biased regimes evolved more competitive sperm and gained higher paternity share in comparison to those from female-biased regimes (Godwin et al., 2017b). Similarly, female biased ASRs are expected to have opposed effects, potentially increasing competition among females for access to males or resources, while decreasing male-male sexual competition (Holveck et al., 2015b). In line with this, in South African dung beetles of the genus *Onthophagus*, species without horns are typically female biased and occur at higher densities (Pomfret and Knell, 2008). This is thought to have arisen because selection is focused on females' fecundity rather than costly horn expression in males (Simmons and Kvarnemo, 2006).

Recent work has extended evolutionary studies of the phenotypic and genetic responses to the effects of experimental variation in ASR and condition to include plasticity. This work shows that long-term manipulations of ASR can provide sufficiently strong selection for plasticity to evolve. For example, studies on the lines used here showed that males from high male-male competition lines altered their courtship repertoire by taking longer to initiate courtship. Additionally, these males also extending mating duration in response to elevated competition (Dore et al., 2020b). Here I use the same lines to test if females too exhibit plastic fecundity responses in relation to their precopulatory social environment. I asked whether the plastic responses in female fecundity (Fowler et al., 2022b) had evolved in experimental

evolution regimes selected under male-biased (MB), equal sex (EQ) and female-biased (FB) adult sex ratios maintained under high- or low-quality adult diets. The specific predictions were (i) that a history of elevated competition among females (in FB regimes) would select for increasingly sensitive plastic fecundity responses to the presence of conspecifics, which would (ii) be restricted under poor nutritional resource regimes.

Methods

Base stock maintenance and collection

All wild type non-focal flies were reared from wild-type Dahomey stock maintained at 25°C in a humidified room with a 12 h light: 12 h dark cycle. Flies were reared on a sugar-yeast-agar (SYA) medium (100g brewer's yeast, 50g sucrose, 15g agar, 30mL Nipagin (10% solution), 3mL propionic acid, 0.97L water). Flies for use in experiments were grown by allowing females to first oviposit for 24 h on agar-grape juice plate (50g agar, 600mL red grape juice, 42mL Nipagin (10% solution), 1.1L water) to acclimatise, and then on a fresh agar-grape juice plate for 4 h. Larvae were collected from the 4 h egg collection plates and reared under a controlled density of 100 larvae per vial (24 x 75 mm) each containing 7ml SYA medium. At eclosion, adults were separated by sex within 6 h of eclosion to ensure virginity and stored ten per vial in single sex groups in vials on standard SYA medium.

Experimental evolution line maintenance

Females from the experimental evolution lines were derived from those used in previous studies (Rostant et al., 2020b, Sepil et al., 2022b, Bath et al., 2021a, Dore et al., 2020b). These lines comprise three independent replicates each of Equal sex (EQ (50M: 50F)), Female Biased (FB (25M: 75F)) and 3 x Male biased (MB (70M: 30F)) lines, maintained as adults on either high or low SYA diets (100% versus 20% of the standard amount of yeast) (three sex ratio regimes x two nutritional regimes x three replicates = 18 populations). Larvae from all regimes were always reared on standard

SYA medium each generation. MB lines were maintained under a slightly less extreme adult sex ratio than were FB lines, to ensure sufficient females to easily maintain the populations. Regimes were maintained as adults within plastic boxes (12cmW x 18cmL x 8.5cmD, with gauze lid) at 25°C in a humidified room with a 12 h light: 12 h dark cycle. Adults in the high yeast lines were given access to two fresh, standard SYA medium every two or three days, whilst the low yeast lines were similarly supplied with 20% SYA medium. Nine days after setting up the adults in the boxes, each line was supplied with an agar-grape juice egg collection plate, which was replaced on day ten. Egg collection plates were maintained at 25°C following their removal from the boxes and kept within in cotton bags for two days. Four hundred larvae were then picked from the second egg collection plates and placed one hundred per vial for each line. After eclosion of the adults from these vials, flies were anaesthetised using CO_2, counted into the appropriate sex ratios, and placed again in plastic boxes. The lines have been maintained under these conditions since 23/12/2013. The flies used in the experiments were derived from generation 102 for block one, & generation 109 for blocks two & three.

Collection of experimental females from the sex ratio lines.

To minimise parental effects, and allow the detection of evolved responses, flies from the sex ratio lines for use in the experiments were reared under two generations of common garden conditions (equal sex ratio and nutritional conditions) for two generations prior to the setup of the experiments. To initiate these cultures, excess flies from the standard maintenance of the lines were transferred to 70ml bottles of standard SYA for 24 h, the adults then removed, and the deposited eggs allowed to mature to adulthood. Upon emergence the populations were transferred to an egg laying chamber (12cm diameter x 18cm high) and provided with an agar-grape juice egg collection plate for 24 h to acclimatise, which was then replaced with a fresh agar-grape juice egg collection plate for 4 h. Larvae were then picked at standard densities of 100 per vial into standard SYA vials. Upon eclosion adults were separated by sex to ensure virginity and stored ten per vial on standard SYA medium.

Effect of evolutionary manipulation of adult sex ratio and nutrition on socially plastic fecundity responses in females

Following the common garden rearing as described above, virgin females from all the lines were exposed to two social treatments and maintained either alone or grouped with non-focal conspecific standard wild type females for three days and then mated. The experiment was carried out in two blocks (replicate population one first and replicate populations two and three simultaneously). All flies used in the experiment were aged between eight -12 days old (from eclosion) at the time of mating. The grouped social treatments comprised three non-focal wild type virgin females + one focal sex ratio line virgin female, and the alone of one focal sex ratio virgin female held in social isolation. Non-focal wild type females for the grouped treatments were made identifiable by wing clipping under CO_2 anaesthesia the day before introducing them to focal females. Females were held under these social treatments for three days until the mating assay, with thirty focal flies for each treatment combination.

Standard wild type males for the mating assay were transferred into fresh vials 24 h before the mating tests began. The mating assay set ups started at 9:00am and all mating's were typically completed by 10:30am. Experimental females were each transferred into vials containing a single wild type of male for the mating assay via aspiration. Non-focal females were discarded, and the vacated vials were retained for subsequent counting of virgin eggs. Time of entry, mating latency and duration were all recorded, with any mating lasting less than 5min discarded, as they were likely to have been incomplete, with no sperm transfer (Gilchrist and Partridge, 2000c). After mating, males were removed and discarded. Focal females were retained in their mating vials for 24 h before being removed, eggs were then counted and kept at 25°C in a humidified room with a 12 h light: 12 h dark cycle for 12 days. After 12 days all the emerged progenies were frozen and counted.

Statistical analysis

Plasticity was analysed by comparing mating latency, mating duration, egg number, progeny number and virgin egg number from females from MB, EQ or FB lines, under either High or Low adult protein diets, subject to either Alone or Group conditions, prior to copulation. All statistical analysis was performed using R-4.0.2 (R Core Team, 2020). All three replicates were analysed simultaneously, with the replicates ('Population') designated as a random factor. The Shapiro-Wilk test, Q-Q plots and histograms were used to check data were normally distributed and the Levene's test to check the homogeneity of variances across treatments. Analysis of egg number and progeny were analysed using linear mixed effects models from the lme4 package (Bates et al., 2015b) and Chi-squared test were used to drop non-significant terms (supplementary material). Mating latency and duration were also analysed using the same method after being log-10 transformed. To analyse differences between group treatments, a Tukey post hoc analysis was conducted using the 'emmeans' package (Lenth, 2023). Additionally, Generalized Linear Mixed Effects Model (GLMER) with a poisson error distribution, and a negative binomial GLMER were used to check model fit versus the LMER. The GLMER with poisson structure did not fit the data well, whilst the negative binomial reported similar results to the LMER. Models were compared using Log-likelihood, Akaike's information criterion (AIC) and residual plots These data were initially analysed using the whole dataset. In subsequent analyses, zero egg counts (egg retaining females) were removed and the data for the egg laying females, as well as the number of egg retaining females were analysed separately, using a binomial generalised linear mixed model. For virgin egg data, I could not distinguish between the eggs laid by focal and non-focal females in the grouped treatments. Therefore, I analysed the data for alone versus grouped treatments separately, to avoid having to divide all the grouped data replicates by four and thus compressing the variance of those data in comparison to the alone treatment.

Results

Evolution of plasticity in females is robust to evolutionary manipulations of adult sex ratio and nutrition.

The main finding was that females from the sex ratio regimes retained plastic fecundity responses across all sex ratio and nutritional evolutionary treatments. However, counter to our predictions, this effect was not more marked in FB regimes and was also not magnified under poor resource conditions. In fact, the extent of fecundity plasticity did not vary in magnitude across any treatments. These results are presented in full, below.

(i) Mating latency, duration, post-mating fecundity and egg to adult viability

Latency to mate was significantly longer overall in grouped compared to alone females (t = 2.418, residual DF = 877.72, *p=0.0158*; Fig. 1A; Fig. S1A-C). However, mating latency was not significantly different across evolutionary sex ratio or diet regimes. There was also no significant difference in mating duration of females held alone or in groups prior to mating, across any of the sex ratio or diet regimes (t = -0.550, residual DF = 878.054 *p = 0.582*, Fig. 1B; Fig. S2A-C).

Fecundity plasticity was retained in all treatments as shown by the finding that the number of eggs laid in the 24 h period after mating was significantly affected by the pre-mating social environment (t = -2.728, residual DF = 871.1465, *p=0.00649*), with grouped treatment females consistently laying fewer eggs after mating in comparison to the females held alone prior to mating (Fig. 2A; Fig. S3A-C) as has previously been reported in wild type females (Fowler et al., 2022b). However, the magnitude of the plasticity in the fecundity responses of females to their pre-mating social environment was not significantly different across any of the sex ratio or diet regimes (table S1). Grouped females also produced fewer offspring than those held alone in the 24h after mating (t = -2.274, residual DF = 870.232, *p=0.0232*), again with no significant effect of sex ratio or diet regimes (Fig. 2B; Fig. S4A-C). There was no effect of social treatment, sex ratio or diet regime on egg-adult viability (percentage of post-mating eggs developing to adulthood after 12days) (Fig. 2C; Fig. S5A-C).

The results show that fecundity plasticity was retained in all the sex ratio regimes across both nutritional environments. However, counter to the prediction, the plasticity was not increased in females from FB regimes or magnified in females from the poor diet regimes.

(ii) Pre-mating virgin egg laying and egg retention

I also analysed the number of eggs laid, and number of eggs retained, by virgin females prior to mating in the alone versus grouped treatments. Separate analyses for the alone and grouped treatment data were conducted, as described above. Virgin egg counts for the alone treatment females differed significantly between food but not sex ratio regimes, with low food regime females held in social isolation prior to mating laying significantly higher numbers of virgin eggs than for high food regimes ($t = 2.319$, $p = 0.0204$; Fig. 3A; Fig. S6 A-C). In contrast, in the grouped treatment females there was no significant effect of sex ratio or dietary regime on the number of virgin eggs laid prior to mating (Fig. 3 B; Fig. S6D-F), though note that here I cannot distinguish those virgin eggs laid by the focal versus non focal females, which could have obscured variation among females. I separately analysed the frequency of vials with zero egg counts, to give an index of egg retention, though it is also possible that these were instances in which female ovaries contained no eggs. Among the grouped treatment females, there was again no significant difference in egg retention between females from the different sex ratios or dietary regimes. In the alone treatment females MB females from the low food regimes were significantly less likely to retain eggs in comparison to their high food counterparts ($t = -1.538$, $p = 0.0004$; Fig.4 A & B) an effect that was not observed for the other sex ratio regimes.

Overall, these results showed significant responses of virgin egg laying to nutritional regime and of virgin egg retention to the adult sex ratio regime, but only in females held alone prior to mating.

Discussion

The main aim was to investigate whether plastic fecundity responses to the presence of conspecific females had evolved in lines with an evolutionary history of difference in sexual selection and adult diet availability. To test this idea, I compared the mating behaviour and fecundity plasticity of females drawn from MB, EQ and FB sex ratio environments maintained on high and low adult diets, raised through 2 generations of common garden rearing, and then housed alone or with three conspecific females prior to mating. Female plastic fecundity responses were retained in all regimes. However,

neither plastic fecundity responses nor any pre-mating traits tested evolved in response to long-term variation in the adult sex ratio or adult diet regimes. The results also revealed consistent plasticity in mating latency, with females from all regimes mating sooner when socially isolated prior to mating. The lack of differences in plasticity across the sex ratio or nutritional regimes is not consistent with a lack of selection pressure, as I did see, among females socially isolated prior to mating, significant evolutionary responses of virgin egg laying to adult diet regime and of virgin egg retention to sex ratio regime.

Mating latency, duration, and fecundity plasticity

Mating latency was consistently plastic – females from all regimes mated significantly faster when they were held in isolation prior to mating. As neither adult diet nor sex ratio regime had any effect on this plasticity, it suggests that the response of female mating latency to the presence of conspecifics is robust across long-term perturbations of the social and nutritional environment. The majority of females mated, which also suggests low variation among virgin females about whether to mate or not mate overall and that the sexual receptivity state of females to the cues signalled by males was not altered during the experimental evolution.

A consistent effect of mating latency according to the social environment is a new finding. It is possible that the general culturing procedures used to maintain the sex ratio and nutritional regimes used here conferred consistent benefits of plasticity in mating latency. This could be due to greater predictability of the conditions experienced by the experimental evolution regimes (specified densities, timings of culturing stages and non-overlapping generations) in comparison to normal cage culture used for the wild type flies used in previous studies, in which this effect was not observed (Fowler et al., 2022b, Fowler et al., 2022c). I do not yet know the drivers of plasticity in mating latency. However, it is possible that females maintained on their own for three days prior to mating may perceive mating opportunities and competition for egg laying sites as low. This could increase a female's willingness to mate rapidly with the first prospective partner encountered. In natural settings, *D. melanogaster* females typically carry the sperm of at least two, but up to four different males (Imhof et al., 1998a). Hence, if the opportunity to mate is perceived to be low, high receptivity

to a first mating (i.e. quicker mating latency) could be more beneficial than waiting for any subsequent, even potentially fitter, male. I observed no differences in mating duration in females held alone or in groups prior to mating across any of the sexual selection or nutrition regimes. Hence, although females have the potential to influence mating duration (Lefranc and Bundgaard, 2000), whilst Eady and Brown (2017) show that both males and females are incosistent in mating duration when presented with a new parter, the results are consistent with previous reports that plasticity in extended mating duration is primarily under male control (Bretman et al., 2013b, Bretman et al., 2009b, Fowler et al., 2022c).

I set out to test whether fecundity plasticity evolved following long-term variation in adult sex ratio and nutritional regimes. The results showed that this plasticity was retained across all evolutionary regimes, with females housed alone prior to mating producing significantly more eggs after mating than did females kept in groups. This is consistent with previous research (Fowler et al., 2022b). Egg-to-adult viability did not differ across any regimes and hence, owing to their higher fecundity, females that were socially isolated prior to mating also produced significantly more offspring after mating than did those held in groups. Therefore, the differences observed in egg laying were wholly attributable to manipulations of the same sex social environment prior to mating, and not to the evolutionary lineage from which the focal females were drawn. Hence, counter to the main prediction, the plastic fecundity responses of females when exposed to conspecifics did not evolve according to variation in sexual selection or resource levels.

The benefits of fecundity plasticity are not yet clear. However, I suggest females held in groups prior to mating might perceive higher levels of resource competition, and thus lay fewer eggs after mating to reduce it. Following this reasoning, I had expected plasticity to evolve in response to elevated variation in sexual selection across the regimes (Rosvall, 2011). Specifically, I expected the increased level of female-female competition in FB regimes leading to enhanced plasticity in response to the same sex environment. The lack of response to sex ratio regime in reproductive output was surprising, given reported plasticity effects in males (Bretman et al., 2009b, Dore et al., 2020a, Dore et al., 2020b) and in other traits in females (Holland and Rice, 1999b, House et al., 2019, Wigby and Chapman, 2004b, Rostant et al., 2020b). The results are consistent with the idea that the maintenance of this type of plasticity may carry

low fitness costs, or that any such cost is accumulated across lifespan (Holland and Rice, 1999b, Nandy et al., 2013a, Tilszer et al., 2006, Wigby and Chapman, 2004b, Chapman et al., 1995), which would not have been captured here.

Fecundity plasticity was also expected to respond differentially to the nutritional regimes. Our hypothesis was that it would be costly for females to oviposit eggs in an area in which other females were doing the same, particularly under food limitation. Under these conditions, females should retain eggs or search for less densely populated oviposition sites. This was not observed, as the extent of fecundity plasticity was unaffected by nutritional regimes. Given that protein restrictive diets can also reduce protein content in eggs (Kutzer and Armitage, 2016) and that low protein in adult diets generally significantly reduces female fecundity (Dick et al., 2011, Zajitschek et al., 2019), I expected that the lines maintained under an evolutionary history of restrictive adult diets (20% protein) would evolve to produce fewer eggs or alternatively to become more efficient in nutrient acquisition leading to higher fecundity when measured in a common garden. There was little evidence that this was the case, with no significant differences in post-mating fecundity attributable to evolution under the different adult dietary regimes. This may indicate that the females are either gaining sufficient protein from the carry over effects of resources gained during larval development (Aguila et al., 2013), at least for an initial batch of eggs (Bowman and Tatar, 2016, Aguila et al., 2013) or that the restricted adult diet lines have evolved to cope with limited protein availability in adulthood without reducing egg quality (Kutzer and Armitage, 2016).

Evolutionary responses of virgin egg laying and egg retention

The lack of predicted responses in fecundity plasticity could be explained by lack of sufficient selection pressure. However, arguing against this were the evolutionary responses to nutritional and sex ratio regimes that I did observe in virgin egg laying and egg retention. Interestingly, these responses were evident only among females that were socially isolated prior to mating.

The number of virgin eggs laid by focal females kept alone was significantly higher for females drawn from the low adult food regimes but was unaffected by adult sex ratio

regime. This effect could result from elevated selection for food utilisation efficiency (Bowman and Tatar, 2016) among females exposed over the long-term to a poor adult nutritional environment. Why it would be evident only in females held in isolation prior to mating is not clear. However, I note that it was not possible to designate the virgin eggs of the focal females when they were held in groups, which could have obscured any variation in egg laying among focal females. Females held in groups also have the potential to learn from each other regarding oviposition decisions (Sarin and Dukas, 2009), which would be interesting to test further.

Among females held in isolation prior to mating, there was also a significant effect of the sex ratio regime on virgin egg retention, with MB females from the low food regimes being more likely to retain their eggs, or to have no eggs to lay, in comparison to MB females from the high food regimes. No such differences were seen for females from the other sex ratio regimes. The result showed that virgin egg retention was contingent on both the long-term sex ratio and nutritional regime. The response of increased egg retention in MB females on low in comparison to high food could indicate that virgin egg production for these females in particular is potentially costly. However, the significance of virgin egg production overall is not well understood, and costs of virgin egg production are generally assumed to be low (Tatar and Promislow, 1997).

Overall, consistent with previous works, I show that females adjust their post-mating fecundity according to the social environment. This plasticity was unexpectedly robust to long-term evolutionary manipulations of sexual selection and resource levels. The results show how such plasticity can be fairly hard wired to evolutionary perturbations.

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Author’s contributions

T.C., W.H., M.J.G.G and N.M conceptualised the study. N. M. conducted the lab work and the data analyses and wrote the first draft of the manuscript. All authors contributed to writing and revising the manuscript and approved the final version.

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Declarations

Ethics approval and consent to participate.
Protocols were approved by the appropriate animal welfare and ethical review board of the university. Formal ethical approval was not required. All methods were incompliance with relevant guidelines.

Consent for publication.
Not applicable

Competing interest
The authors declare that they have no competing interests.

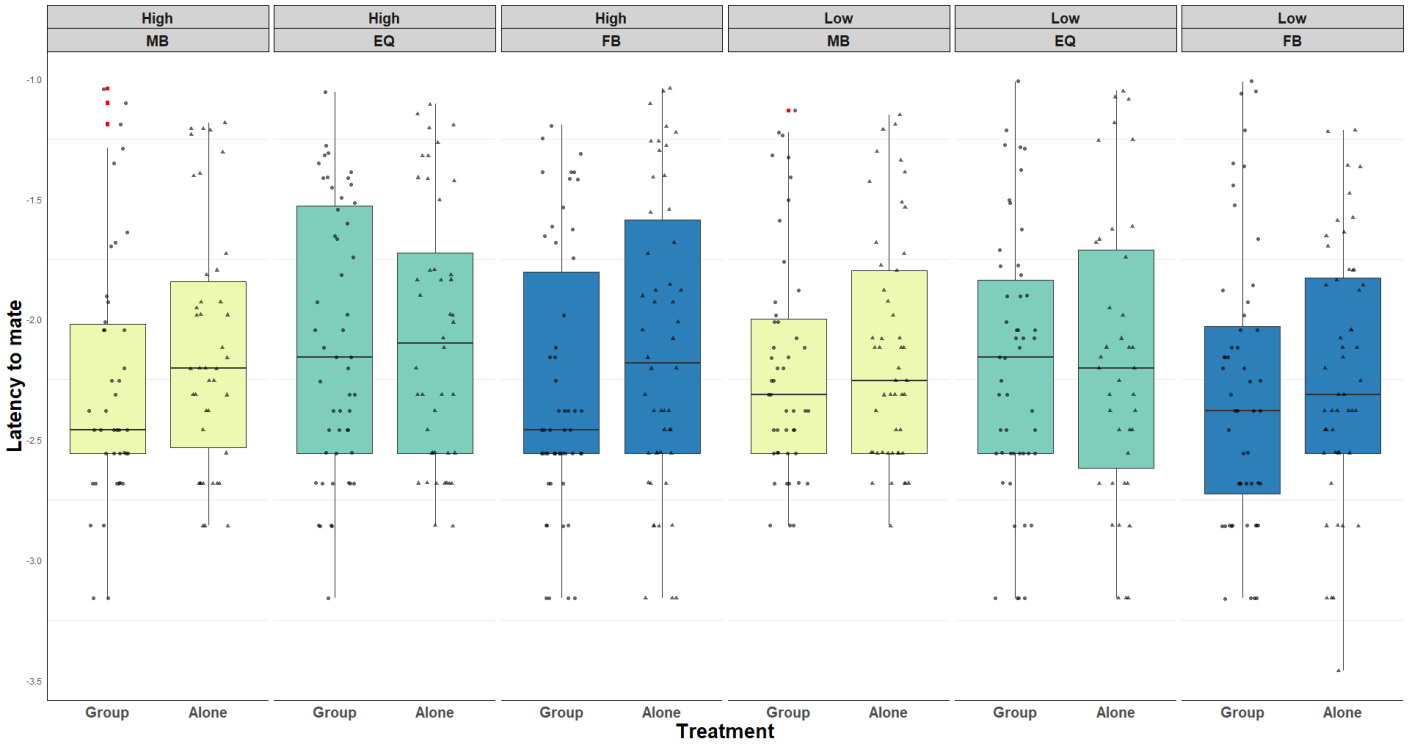
Supplementary material

Table S1. Egg number LMER drop terms.

Code	Term	DenDF	F-Value	P-Value
drop1(Data1Check, test='Chisq')	Social:Sex.Ratio:Food	870.47	0.021	0.9792
drop1(Check2, test='Chisq')	Social:Sex.Ratio	872.44	1.1159	0.3281
	Social:Food	872.45	2.4953	0.1145
drop1(Check3, test='Chisq')	Social:Sex.Ratio	872.55	1.1084	0.3306
	Social:Food	872.60	2.5074	0.1137
drop1(Check4, test='Chisq')	Sex.Ratio	13.86	1.3819	0.2836
	Social:Food	874.59	2.6862	0.1016
drop1(Check5, test='Chisq')	Social:Food	874.62	2.6394	0.1046
drop1(Check6, test='Chisq')	Food	15.78	2.5365	0.1311
drop1(Check7, test='Chisq')	Social	875.62	44.933	3.65e-11

Figure 1

(A)


High
MB
High
EQ
High
FB
Low
MB
Low
EQ
Low
FB
Latency to mate
-1.0
-1.5
-2.0
-2.5
-3.0
-3.5
Group
Alone
Treatment

(B)

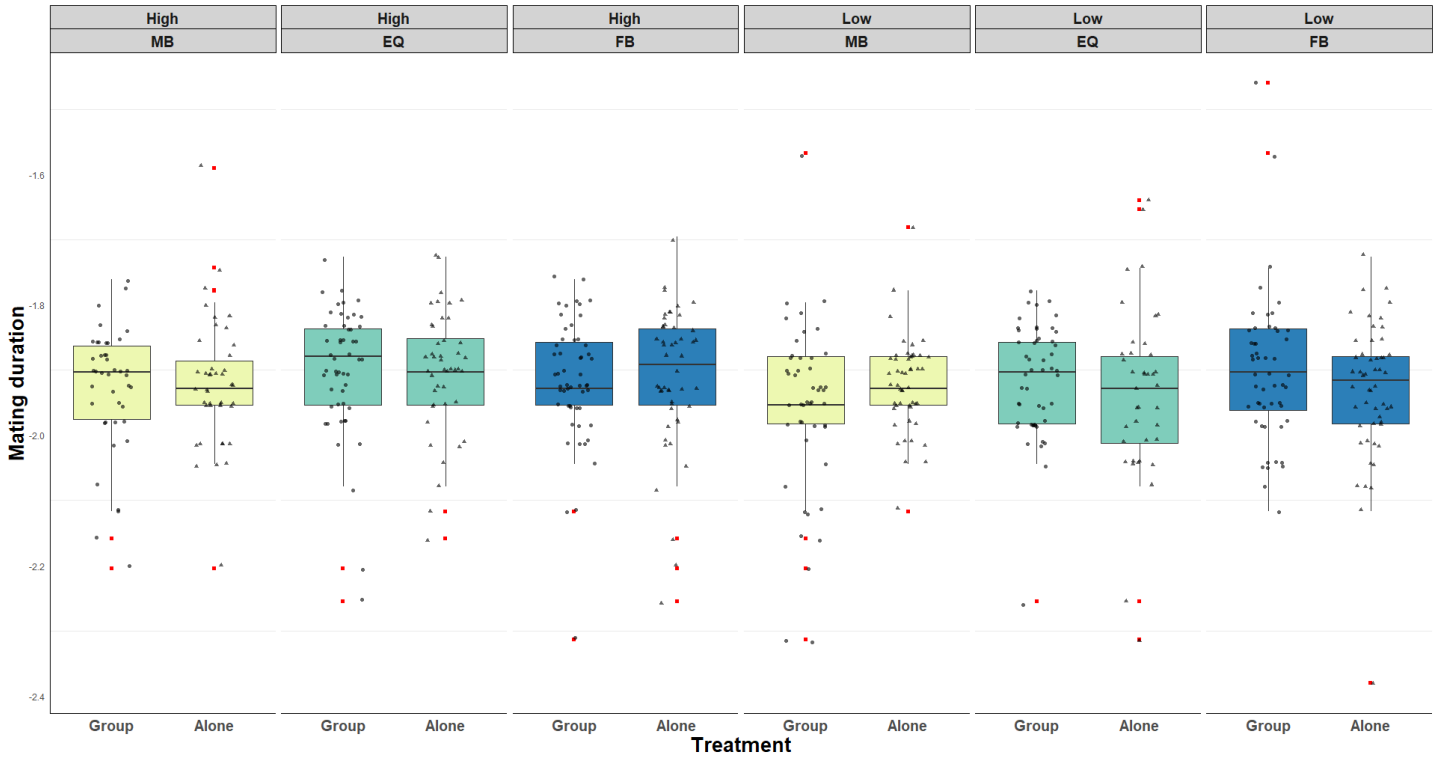
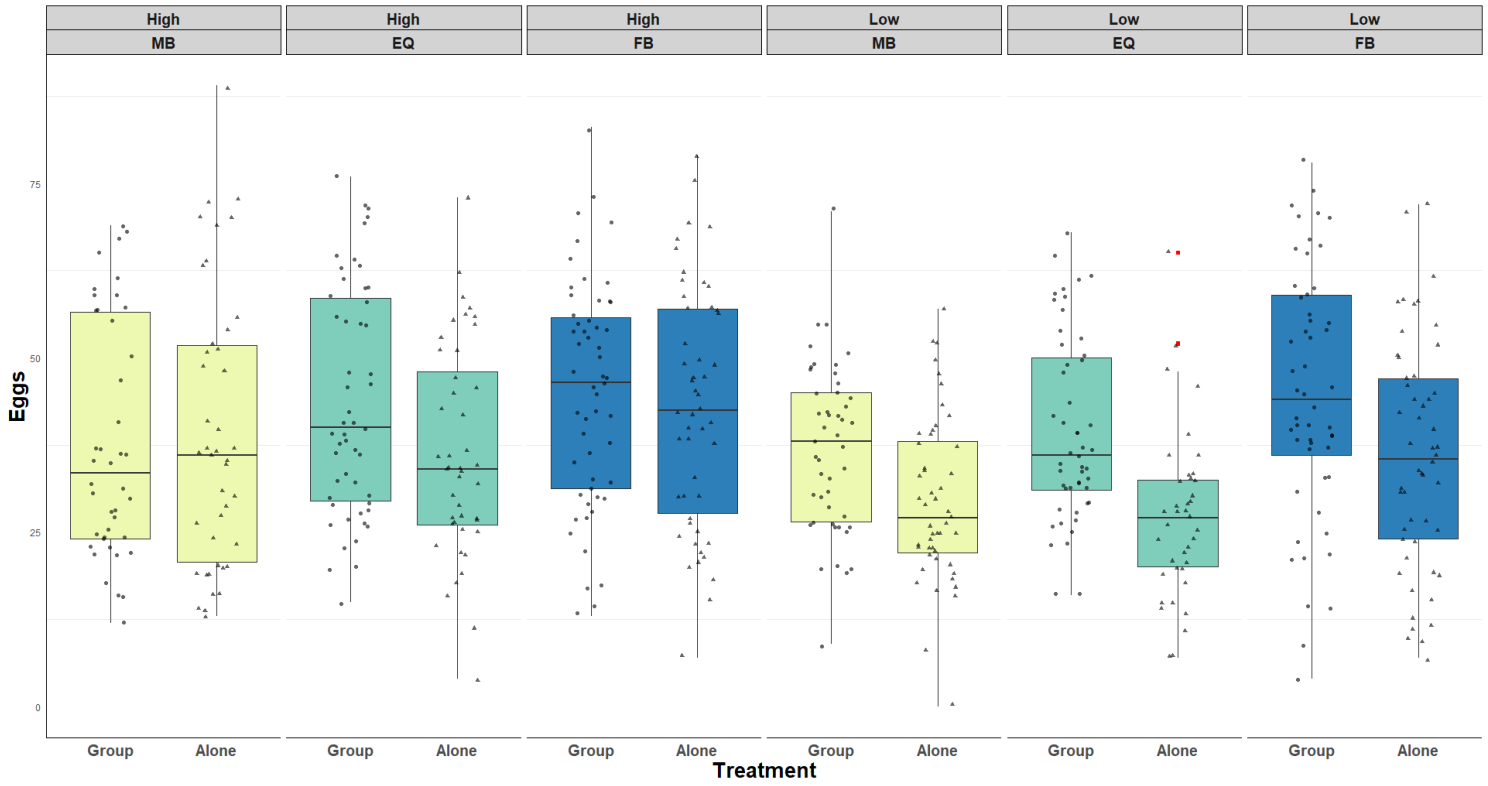

High
MB
High
EQ
High
FB
Low
MB
Low
EQ
Low
FB
Mating duration
-1.6
-1.8
-2.0
-2.2
-2.4
Group
Alone
Treatment

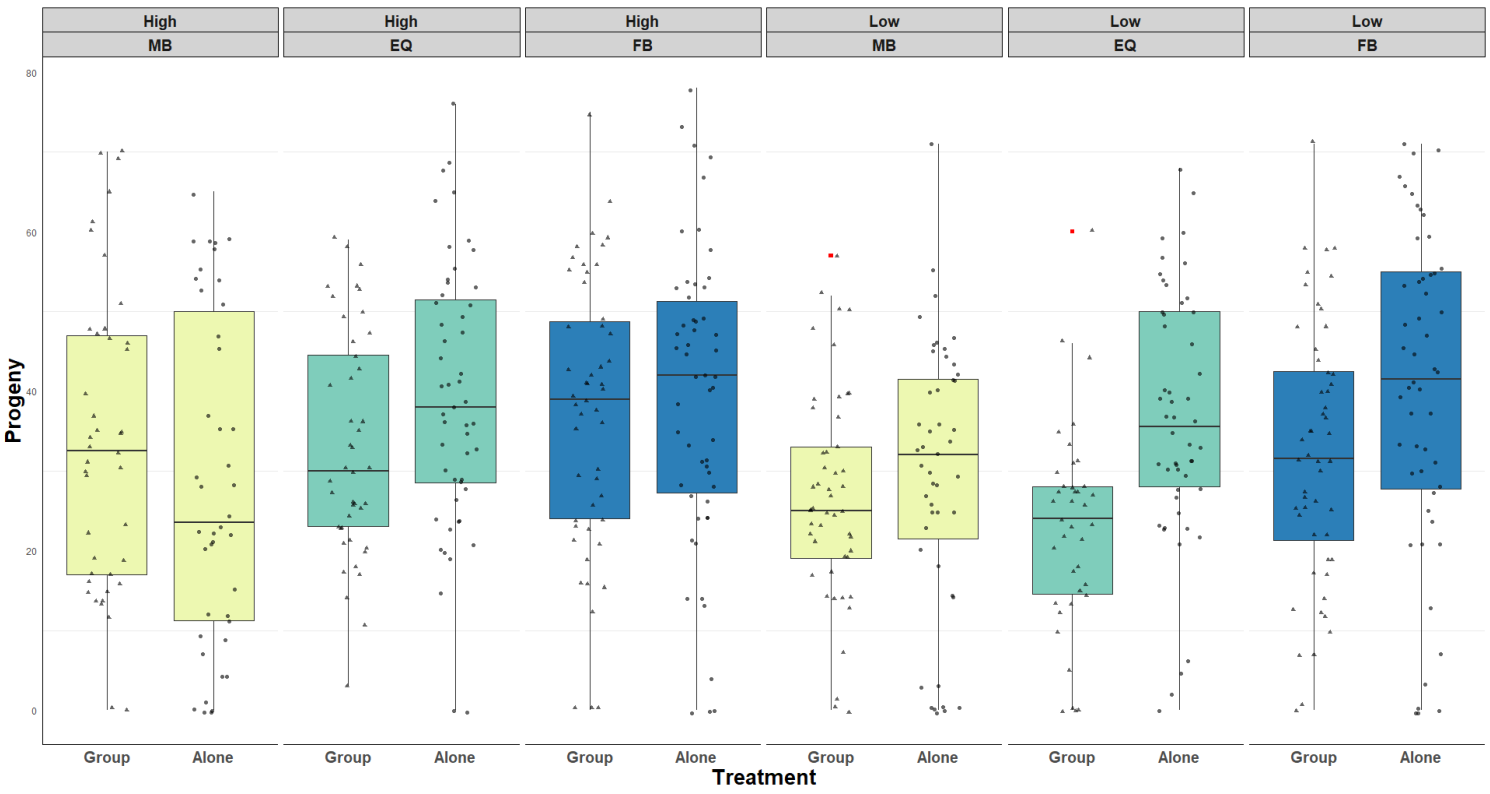
Fig 1. (A) Latency to mate ('loglat' in log_{10} minutes), and (B) mating duration ('logdur' in log_{10} minutes) of females from the sex ratio and diet regimes exposed for 3 days prior to mating to conspecifics or left alone. Females from the Female-biased (FB), Equal sex (EQ) or Male-biased (MB) sex ratios and standard 100% protein (High) or 20% protein (Low) diet regimes were tested. Females were either housed alone (A) or grouped with three rivals (G) for 3 days prior to mating. All conspecific non focal females and males were standard wild type. Boxplots show median line, with boxes representing upper and lower 25% quartile and whiskers representing the range, and points representing individual records, outliers highlighted in red.

Figure 2

(A)


High
MB
High
EQ
High
FB
Low
MB
Low
EQ
Low
FB
Eggs
75
50
25
0
Group
Alone
Treatment

(B)


High
MB
High
EQ
High
FB
Low
MB
Low
EQ
Low
FB
Progeny
80
60
40
20
0
Group
Alone
Treatment

(C)

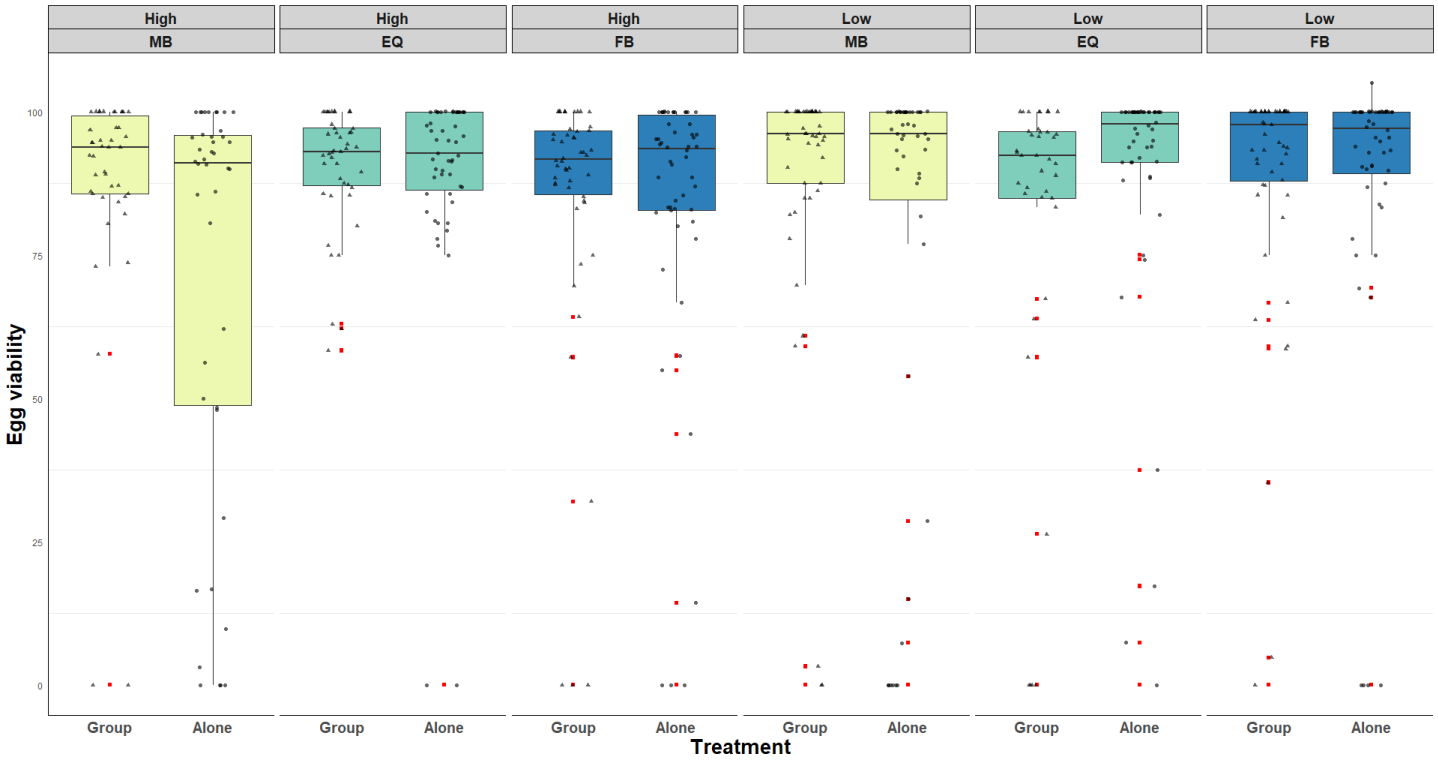
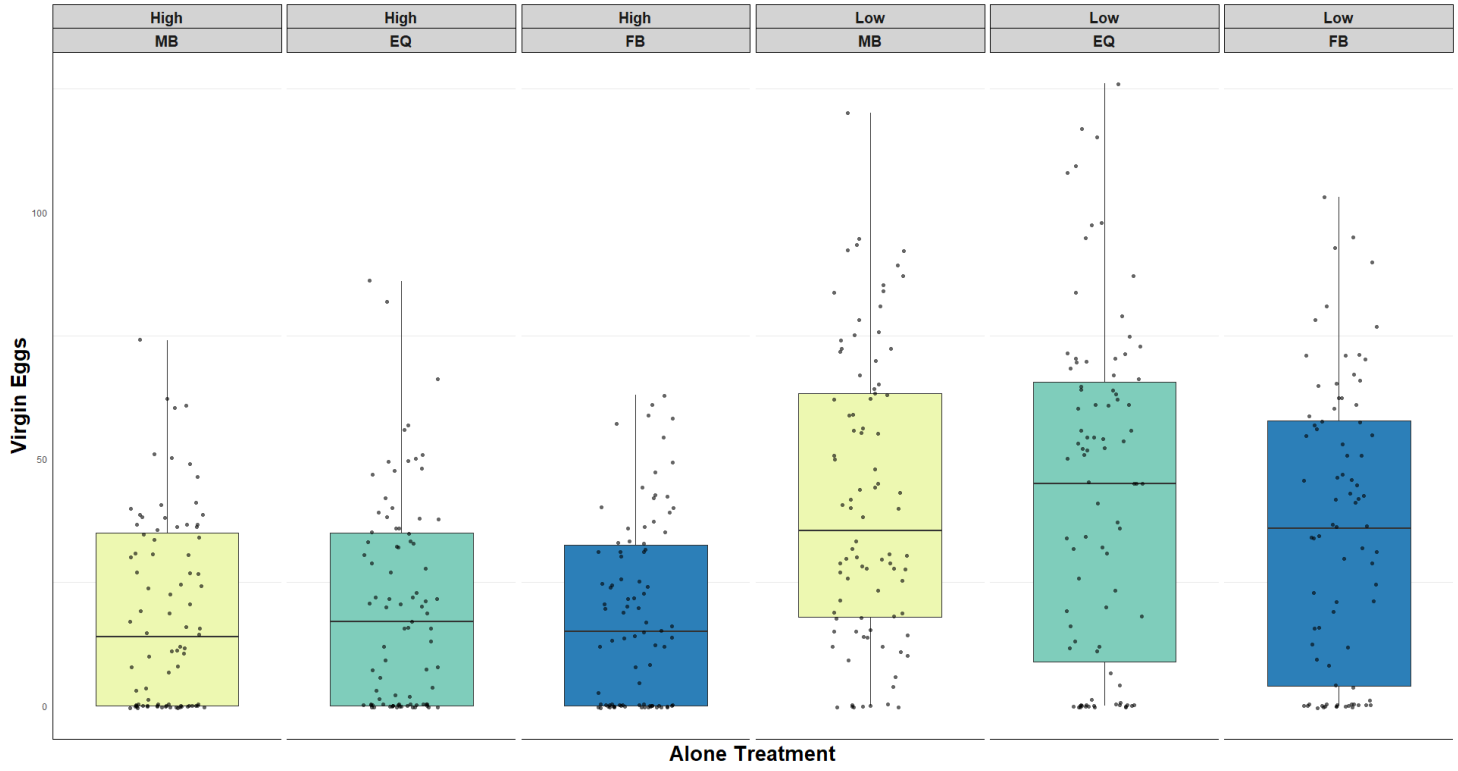


Fig.2. (A) Post-mating fecundity ('Eggs', number of eggs per female per 24h following mating), (B) Progeny production ('progeny', number of progeny emerging from the eggs laid in (A) per female per 24h following mating), (C) Egg to adult viability ('Egg viability', number of progeny / eggs per female per 24h following mating) of females from the sex ratio and diet regimes exposed for 3 days prior to mating to conspecifics or left alone. Females from the Female-biased (FB), Equal sex (EQ) or Male –biased (MB) sex ratios and standard protein (High) or 20% protein (Low) diet regimes were tested. Experimental females were either housed alone (A) or grouped with three rivals (G) prior to mating assay. All conspecific non focal females and males were standard wild type. Boxplots as per Fig.1.

(A)


High
MB
High
EQ
High
FB
Low
MB
Low
EQ
Low
FB
Virgin Eggs
100
50
0
Alone Treatment

(B)

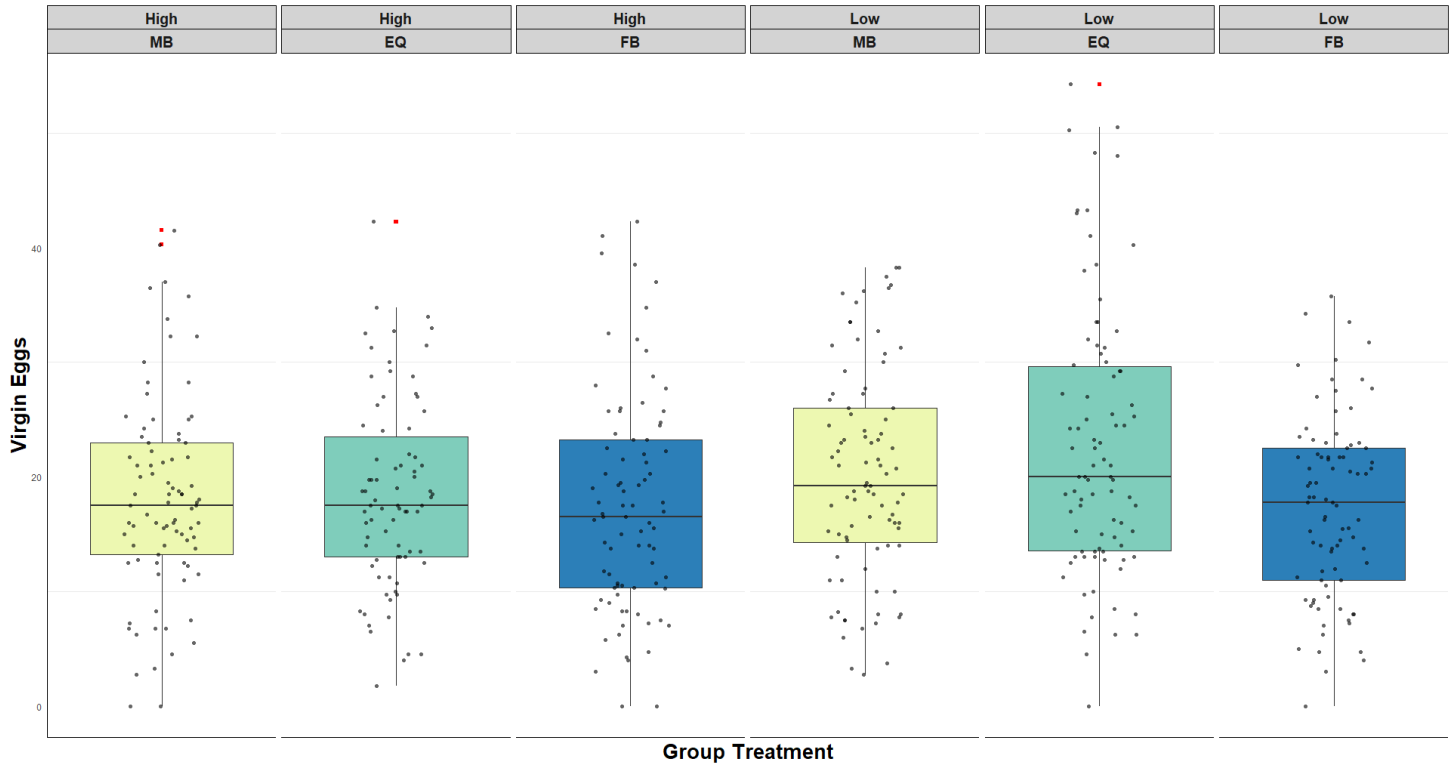
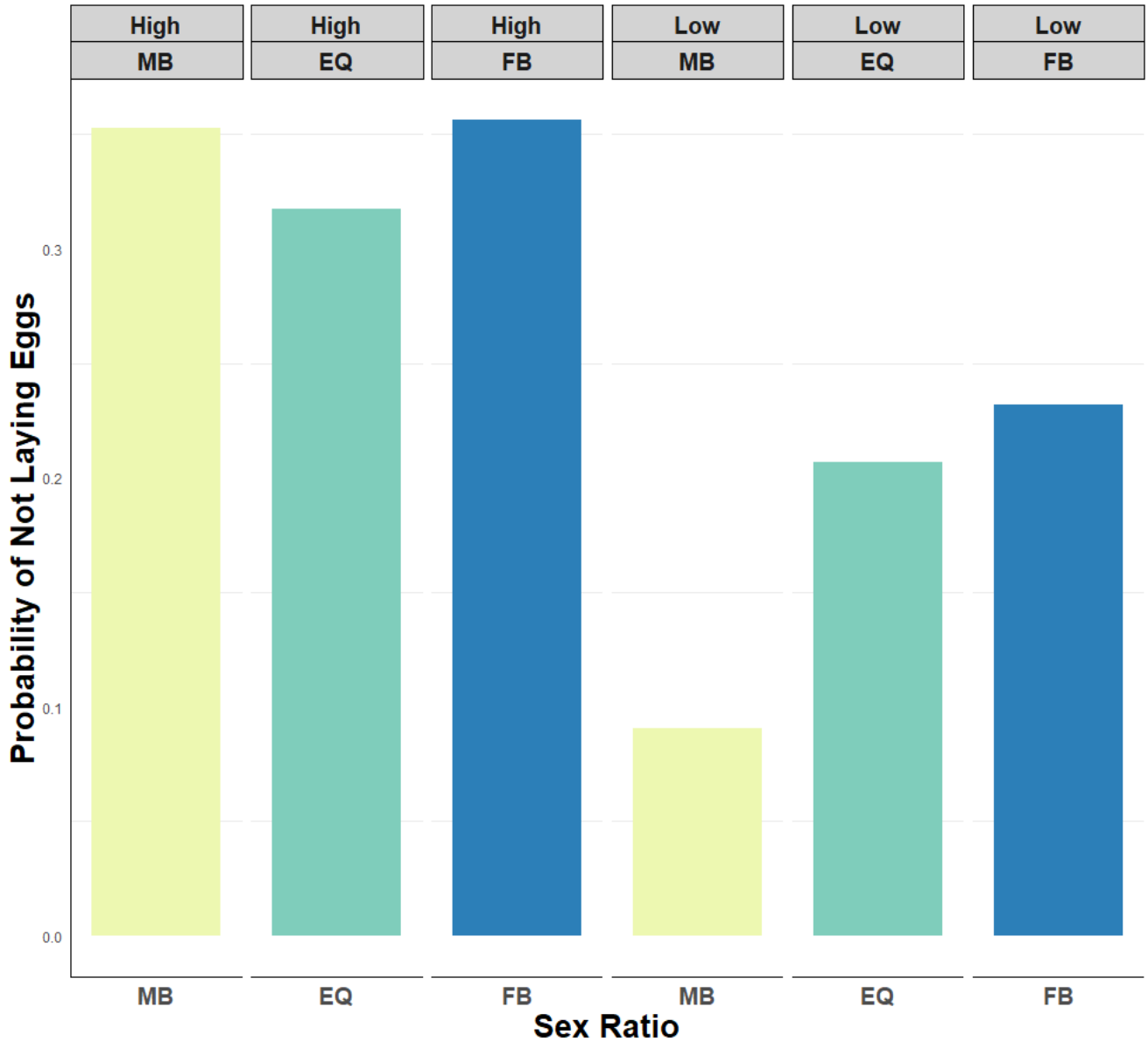

High
MB
High
EQ
High
FB
Low
MB
Low
EQ
Low
FB
Virgin Eggs
40
20
0
Group Treatment

Fig. 3 Virgin female fecundity for females from the sex ratio and diet lines held (A) alone and (B) in Groups. Virgin egg counts over 3 days prior to mating for females from the sex ratio and diet lines exposed for 3 days to conspecifics or left alone. Equal (EQ), Female-biased (FB), or Male–biased (MB) sex ratios from standard protein (High) or 20% protein (Low) diet regimes. Females were either housed alone (A) or grouped with three rivals (G). Boxplots showing median line, with boxes representing upper and lower 25% quartile and whiskers representing the range, and points representing individual records, outliers highlighted in yellow.

(A)


High
MB
High
EQ
High
FB
Low
MB
Low
EQ
Low
FB
Probability of Not Laying Eggs
0.3
0.2
0.1
0.0
MB
EQ
FB
MB
EQ
FB
Sex Ratio

(B)

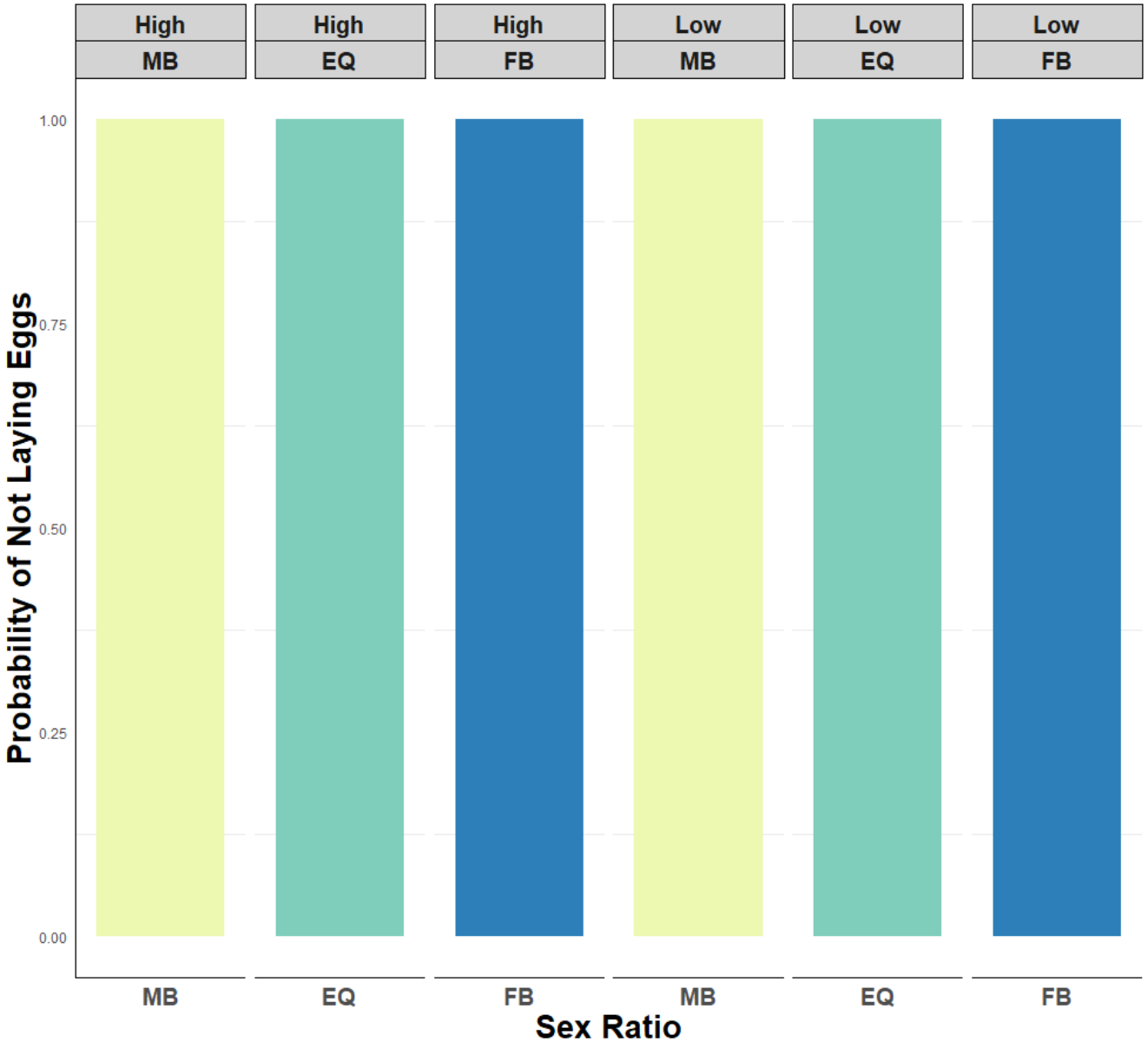


Fig.4 Probability of females from the sex ratio lines retaining virgin eggs held (A) alone and (B) in groups. Probability of not laying eggs forfocal females exposed for 3 days to conspecifics (G) or left alone (A). Equal (EQ), Female-biased (FB), or Male –biased (MB) sex ratios from standard protein (High) or 20% protein (Low) diet regime. All conspecific non focal females were standard wild type.

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Appendix 1

Behavioural response to the experimental manipulation of female pre-copulatory environment

The social environment has been shown to shape an individual's behaviours in many species. Male *Drosophila melanogaster* alter their mating behaviours in response to their pre-copulatory social environment (Bretman et al., 2009a). Females also show plastic responses to the pre-copulatory social environment in terms of their egg laying patterns, with females held alone prior to mating laying more eggs than individuals kept in groups (Fowler et al., 2021). As shown in chapter 2, this response was conserved amongst females subjected to long-term variation in their adult sex ratio regimes. Here I attempted to understand the behavioural differences that are associated with divergent pre-copulatory social treatments (females kept alone or in groups prior to mating). Using wild type virgin females, I conducted 20-minute sweeps over an 8h period, scoring a range of behaviours and conducting egg counts at 8h and 24h timepoints. As expected, based on previous work, females maintained alone in social isolation laid more eggs than did females maintained in groups, though this was not significant. Interestingly, at 8h, the group treatment females had produced a similar number of eggs. However, overnight the alone treatment females produced more eggs, indicating the possibility of a circadian effect on egg laying behaviour, which is consistent with recent research (Bailly et al., 2023). The most striking difference was observed in two of the behavioural phenotypes scored, with alone treatment females spending a significant amount of time "off the food, moving", compared to group treatment females who spent a significant amount of time "on the food, not feeding". This experiment clearly demonstrates a significant effect of the pre-copulatory social environment on a female's subsequent movement and feeding behaviours.

Methods

Stock maintenance and collection.

All wild type flies were reared from wild-type Dahomey stock maintained at 25°C in a humidified room with a 12 h light: 12 h dark cycle. Flies were reared on a sugar-yeast-agar (SYA) medium (100g brewer's yeast, 50g sucrose, 15g agar, 30mL Nipagin (10% solution), 3mL propionic acid, 0.97L water). Flies for use in experiments were grown by allowing females to first oviposit for 24 h on agar-grape juice plate (50g agar, 600mL red grape juice, 42mL Nipagin (10% solution), 1.1L water) to acclimatise, and then on a fresh agar-grape juice plate for 4 h. Larvae were collected from the 4 h egg collection plates and reared under a controlled density of 100 larvae per vial (24 x 75 mm) each containing 7ml SYA medium. At eclosion, adults were separated by sex within 6 h of eclosion to ensure virginity and stored ten per vial in single sex groups in vials on standard SYA medium.

Experimental set up

Virgin females were exposed to two social treatments and maintained either alone or grouped with non-focal conspecific standard wild type females for three days and then mated. All flies used in the experiment were aged between 7-8 days old (from eclosion) at the time of mating. All aspects of this study were conducted at 25°C in a humidified room with a 12 h light: 12 h dark cycle. The grouped social treatments comprised four wild type virgin females + of which one was selected at random to represent the group treatment, and the alone of one wild-type virgin female held in social isolation. Females were held under these social treatments for three days until the mating assay, with twenty focal flies for each treatment combination. After three days, focal females were transferred into a vial with a single wild-type virgin male and mated. Once mating had occurred males were removed, and the behavioural assay began.

Behavioural assay

Spot checks were taken of each vial in 20 min sweeps over an 8 h period and the behaviour recorded was categorised as follows:

A = feeding
B = on food not moving, not feeding
C = on food moving
D= grooming
E= off food, not moving
F = off food, moving
G= ovipositing
H =probing

After 8 h egg counts were conducted, and flies were left for 24 h at 25°C in a humidified room with a 12 h light: 12 h dark cycle and egg counts scored once again.

Statistical analysis

All statistical analysis were performed using R-4.0.2 (R Core Team, 2020). The Shapiro-Wilk test was used to check data were normally distributed and the Levene's test to check the homogeneity of variances across treatments. Where data were not normally distributed, they were $\log_{10}$ transformed. Analysis of egg number, progeny number, latency and mating duration were performed using linear mixed effects models using the lme4 package (Bates et al., 2015a) and behaviour was analysed using glmer with a negative binomial distribution from the glmmTMB package (Brooks et al., 2017). The Akaike's Information Criterion (AIC) was used to check for model fit. Post-hoc analysis where appropriate was performed using the Tukey's Test.

Results

Analysis was performed on each behaviour separately. Behaviours D, G & H were removed from the analysis due to very low frequencies of observations.

Behaviour	z-value	P-value
A = Feeding	0.855	0.392
B = On food, stationary, not feeding	4.009	< 0.001***
C = On food moving	-1.755	0.079
E = Off food, not moving	1.10	0.269
F = Off food, moving	-5.616	< 0.001***

Table S2. Results of glmer comparing counts of each behaviour for singly mated 'Alone' or 'group' treatment females.

Figures 1 and 4 show the significant behavioural differences between 'Alone' and 'Group' treatment females. 'Group' females were significantly more likely to be recorded on the food, stationary, not feeding compared to the 'Alone' treatment females (Table S2). Comparatively, 'Alone' females were significantly more likely to be off the food, moving (Table S2).

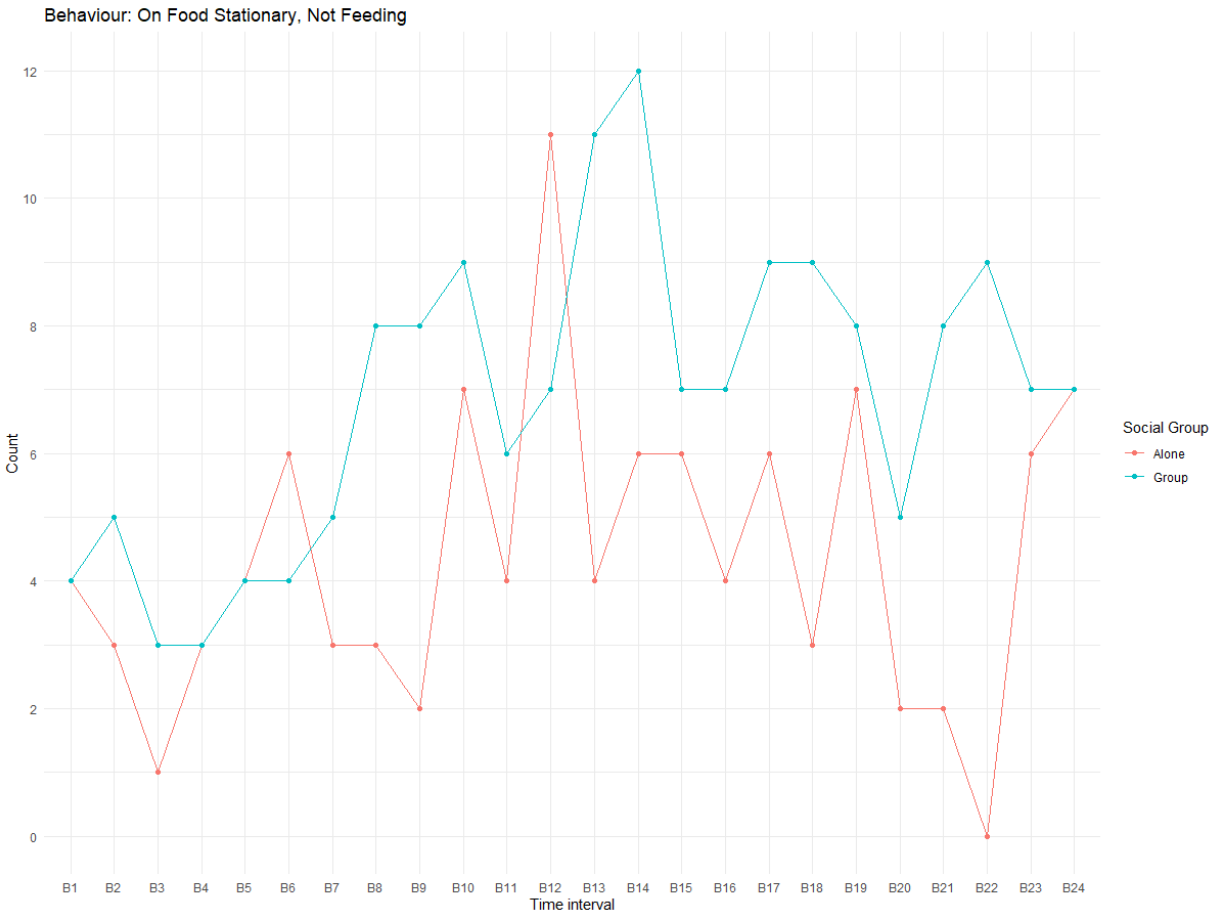

Fig 1. Total count data of Behaviour B "On food, stationary, not feeding" for females held alone or in groups prior to mating.

Line graph of counts for behaviour B for wild type females held alone (red) or in groups (blue) for 72h prior to mating, recorded at 20min intervals (B1-24) over the 8h sampling period.

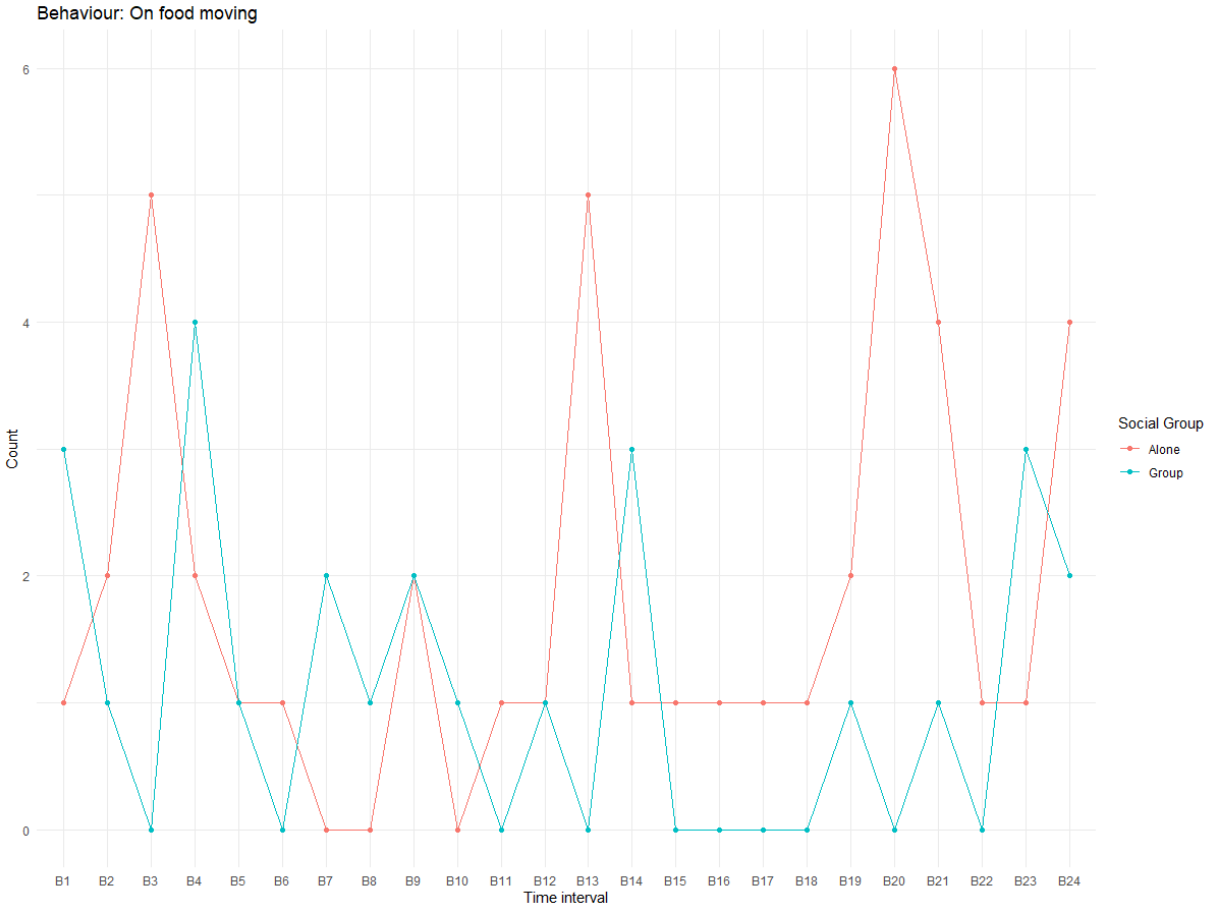

Fig 2. Total count data of Behaviour C "On food, moving" for females held alone or in groups prior to mating.

Line graph of counts for behaviour C for wild type females held alone (red) or in groups (blue) for 72h prior to mating, recorded at 20min intervals (B1-24) over 8h sampling period.

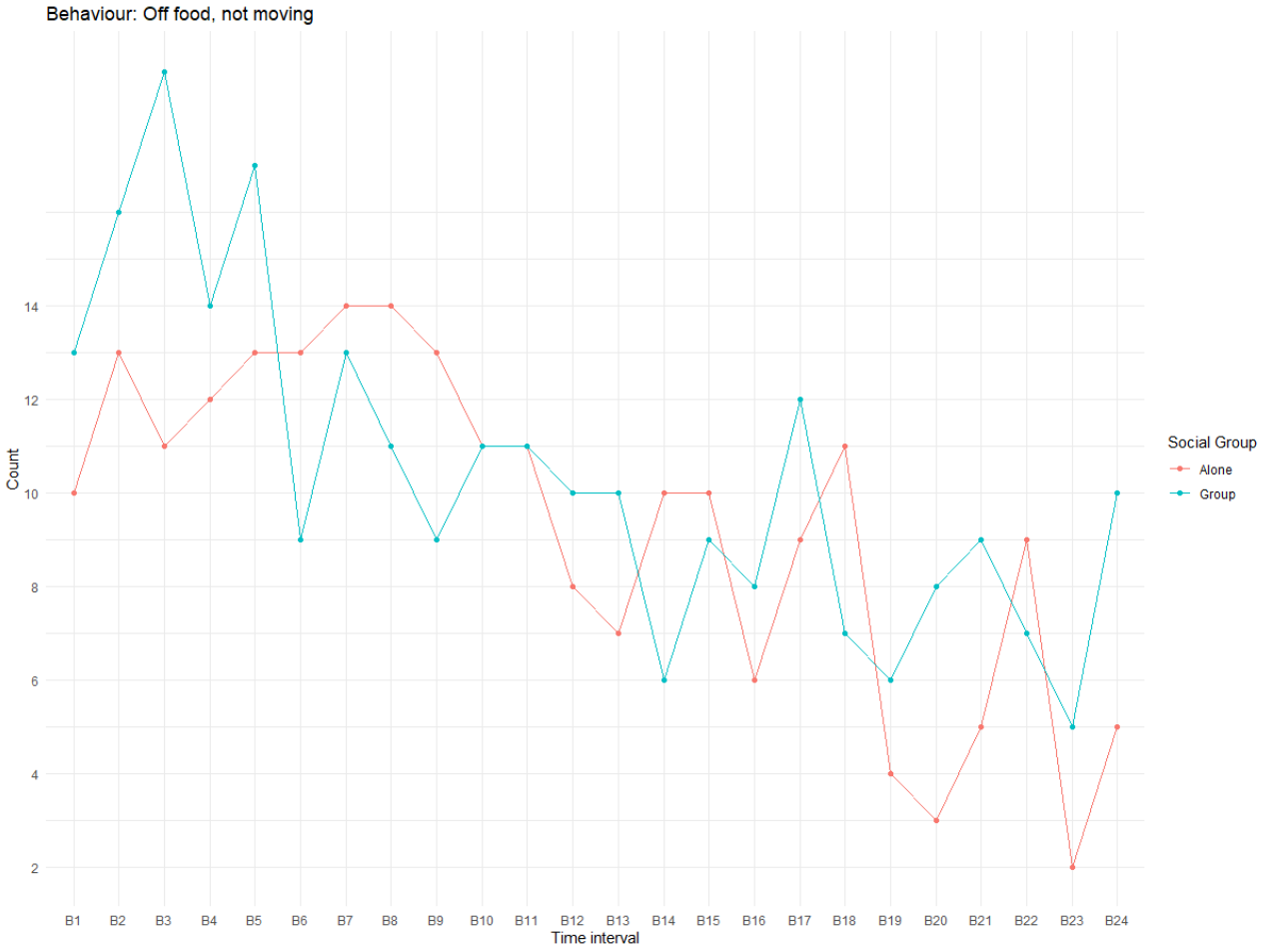

Fig 3. Total count data of Behaviour E "Off food, not moving" for females held alone or in groups prior to mating.

Line graph of counts for behaviour E for wild type females held alone (red) or in groups (blue) for 72h prior to mating, recorded at 20min intervals (B1-24) over 8h sampling period.

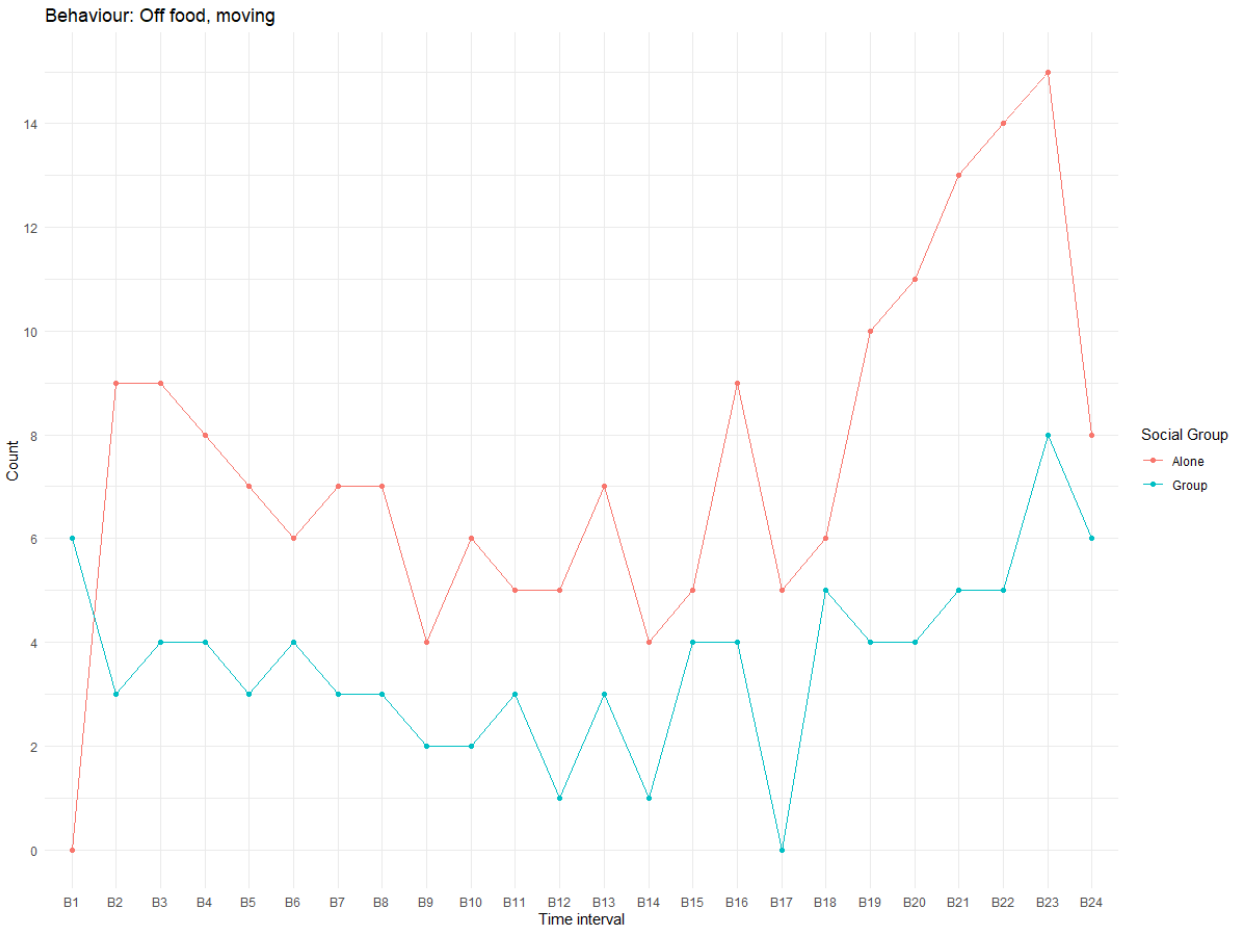

Fig 4. Total count data of Behaviour F "Off food, moving" for females held alone or in groups prior to mating.

Line graph of counts for behaviour F for wild type females held alone (red) or in groups (blue) for 72h prior to mating, recorded at 20min intervals (B1-24) over 8h sampling period.

Conclusions

The findings of movement differences between females subjected to different social environment treatments highlights potential mechanisms that could mediate differences in the allocation of reproductive effort. Future experiments should focus on attaining a 24hr record of behavioral differences, and incorporate recent research by investigating juvenile hormone activity between the social treatments. Experimentally manipulating day length and/or light intensitiy may highlight an important interaction between ciradian rhythm, sociality and oogenesis.

Appendix References

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Chapter 3: Effect of experimental evolutionary manipulation of adult sex ratio and adult nutrition on development and body size-related fitness traits in *Drosophila melanogaster*

Abstract

The effects of evolutionary manipulations of sexual selection have been documented in many organisms. Previous research shows that both male and female *D. melanogaster* selected under evolutionarily distinct adult sex ratio and nutritional

regimes evolve differential plastic behavioural responses to the same or opposite sex. For example, male *D. melanogaster* evolving under male biased sex ratios express an alternate courtship repertoire and extended mating duration, and females evolving in a female biased environment become more aggressive towards other females. Here I investigated whether such responses to variation in sexual selection and nutrition might be mediated by, or traded off with, developmental traits or body size. To do this, I examined egg size, development time, developmental survival, body weight and wing morphometry in males and females drawn from lines selected for 110 generations under male- (MB), female-biased (FB) or equal (EQ) adult sex ratios experiencing either standard or poor-quality adult diets. I found that egg size was significantly larger in flies drawn from the MB standard nutrition regimes, and that both sexes of adults from this regime had significantly larger wing areas than did the corresponding FB flies. No detectable sex ratio effect on wing area was found in flies from the restrictive poor diet, though all flies from all the restricted diet regimes had significantly larger wings than those evolving under standard dietary conditions. Overall, these results show that variation in sex ratio can select for changes to size - related fitness traits, but that the impact of this selection is curtailed under conditions of nutritional stress. The lack of variation exhibited under a restrictive diet lends additional support to the role of condition in the evolution of sexually selected traits in males.

Attribution statement

I would like to thank Professor Tracey Chapman for their help with collecting progeny.

Introduction

Sexual selection can broadly be defined as the process by which choice is exerted by an individual of one sex about whether to mate and who to mate with, and the process of competition between members of the same sex for the opportunity to mate (Darwin, 1871). It is a potent selective evolutionary force that can drive rapid phenotypic change (Petrie et al., 1991). Sexual selection continues to be a well-studied and vibrant field of research within evolutionary biology as a whole. Recent

research has emphasised that the effects of sexual selection on genomes as a whole can be far reaching (Rowe and Houle, 1996). The ever-increasing feasibility and power of large-scale genome or transcriptome sequencing experiments is now also revealing with unparalleled resolution, the underlying basis of responses to sexual selection itself (Fan et al., 2012).

Long-term evolutionary experiments have been particularly powerful in showing the effects of varying sexual selection and documenting the resulting responses at the phenotypic and genomic levels, in real time (Michalczyk et al., 2011, Wiberg et al., 2021). For example, long-term manipulation of adult sex ratio over many tens of generations have been shown to influence the degree of sexual competition experienced (Leftwich et al., 2012, Bath et al., 2021b). These types of studies can then illustrate the broad-ranging impacts of such manipulations. For example: changes in ejaculate investment (Linklater et al., 2007), female resistance to male-induced reproductive costs (Wigby and Chapman, 2004a), male cognition (Baur et al., 2019) and mating duration (Gallup et al., 2019b) are all documented evolutionary responses to variation in sexual competition and illustrate the broad spectrum of traits that can respond. Sexually selected traits are often considered costly (Daniel, 1992, Morbiato et al., 2023) and their expression, and indeed an individual's overall condition, can be affected by nutritional availability in the short (Cotton et al., 2004a) and long-term. The fact that variation in sexually selected phenotypes appears to be highly nutritionally sensitive indicates the potential for strategic allocation of resources, due to nutrient limitation, and strong overall effects of condition dependence. However, how such responses might trade-off with developmental characteristics (e.g. speed of development, eventual adult size) remains relatively unknown and is the topic I explore in this chapter.

The body of research summarised above supports the idea that investigations of the effects of simultaneous long-term manipulations of both the potential for sexual selection and dietary variation should be very useful in identifying ecologically relevant responses, including those that show condition-dependency. I used such a set of lines here to test for developmental and size-related responses to long-term (>110 generations) variation in adult sex ratio and diet. This also allowed me to test whether there was evidence that previously reported responses were traded off against developmental speed and eventual adult body size, in either sex.

Studies of the developmental phase in invertebrates have revealed that a variety of physiological traits including morphological characteristics are potentially shaped by the strength of sexual selection (Gray et al., 2018, Gage, 1995b, Bonduriansky, 2007). However, these studies have typically assessed the impact of larval diet or population density on the development on these traits. Whilst larval diet is a major factor for determining body size (Nijhout, 2003), adult diet is also able to influence body weight, and can mitigate the negative impact of a poor larval diet (Poças et al., 2022). It is possible that the restriction of adult diet could also impact the quality of female reproduction (Partridge et al., 2005b) and so reduce the opportunity for sexually selected adaptations to be expressed. Conspecific aggression is reported to be lower when nutritional availability is restricted (Ueda and Kidokoro, 2002, Bath et al., 2021b, Edmunds et al., 2021), (Duxbury and Chapman, 2019) and so pre-copulatory sexual selection within these groups may be reduced. These findings motivate the study described in this chapter of the effects of long-term variation in adult diet on fitness-related traits with potential impacts upon success in reproduction such as body size.

A second focus of study of the work described in this chapter was to document wing size and shape. The wings of *Drosophila* are not only important for flight but serve as an essential element of communication for both sexes (Jonsson et al., 2011, Kerwin et al., 2020). There is significant variation in courtship song within and between species of *Drosophila* (Hoikkala and Kaneshiro, 1997). Additionally, there is good evidence for sexual dimorphism and variation in shape between populations (Cowley et al., 1986, Gilchrist et al., 2000). During courtship, the males perform a courtship routine composed of a “song” and “dance” in which the wings are an important component (Bastock and Manning, 1955, Bennet-Clark and Ewing, 1970). The courtship song of male *Drosophila melanogaster* comprises two distinct parts, a species-specific “pulse” and a “sine” song, which increases female receptivity (Schilcher, 1976). Females respond to male courtship with their own song, which has been linked to the extent of seminal fluid investment made by the male (Kerwin et al., 2020). Studies that have measured wing size found that males with larger wings are more successful in winning fights and accessing females (Partridge et al., 1987a, Menezes et al., 2013, Partridge and Farquhar, 1983). Additionally larger females are subjected to more wing vibrations than are smaller females, although this is relative

to the size of the male (Turiegano et al., 2013). The disparity in acquiring access to females for mating's gives the opportunity for selection to act upon wing size when competition is high, but additionally, in female biased populations, a larger female could be more successful in attracting potential partners. Of key relevance to the experiments described here, wing size is determined during the larval stage (Parker and Struhl, 2020). Hence variation in adult diet does not directly influence this phenotype, although parental condition is expected to have significant impacts (Lee et al., 2023). Females also use their wings as a threat displays to other females (Nilsen et al., 2004a) and larger females win more fights (Bath et al., 2018a). An increase in competition for resources and mating opportunities for females, could increase conspecific aggression and select upon overall body size, which is correlated with wing size (Reeve and Robertson, 1953, Stillwell et al., 2011).

Wing size variation is manifested during larval development (Parker and Struhl, 2020) and has the potential to influence development time, either because of the time needed to harvest the appropriate amount of resources (potentially evident in the egg to pupariation stage) or to express the full adult phenotype itself (potentially evident in the duration of pupation). In *D. melanogaster*, time to pupariation is negatively associated with larval weight (i.e. as larval weight increases, time to pupariation decreases), but shows large variation across populations (De Moed et al., 1999). Any phenotypic differences influenced by sexual selection have the potential to affect critical weight thresholds required for pupation. Additionally, a lower critical weight predicts that smaller flies should pupariate more quickly and can result in lower adult weight (David and Clavel, 1967). Egg size and embryonic development speeds vary significantly across closely related *Drosophila* species (Markow et al., 2009) and within *D. melanogaster*, eggs size also shows a positive correlation with development time, but not adult weight (Azevedo et al., 1997).

Though the effects of wing and / or body size on male and female reproductive success have been fairly well established, exactly how variation in the intensity of sexual selection influences the evolution of these phenotypes, how this variation impacts upon developmental traits and how adult diet can influence sexually selected, condition-dependent phenotypes is not well known. Here, I addressed these important gaps using lines of *D. melanogaster* evolved for 110 generations under fixed adult sex ratios of male bias (MB), female bias (FB) or equal sex and

differing adult diets of standard SYA (High) or a protein restricted diet containing 20% of the standard SYA protein. I tested the effect of these long-term manipulations on adult dry body weight, wing size (length and area), egg size, and development time (both egg to pupariation, and egg to eclosion times). The specific predictions are laid out in the table below.

Table 3.1. Predicted responses of developmental differences in both sexes to long-term variation in sexual selection and adult diet.

N	Traits	Expectation	Rationale
1	Egg size – by adult sex ratio	Egg size will be larger in MB lines	Females in the MB regimes may be selected for increased fecundity owing to their fewer numbers of individuals. I would expect larger females to produce larger eggs, hence larger eggs in the MB regimes (Yanagi and Tuda, 2012).
2	Egg size – by diet	Egg sizes will be smaller in low food regimes	Poor adult diet will reduce condition of females and be evidenced by smaller eggs (Oberhauser, 1997)
3	Development time – by adult sex ratio	Development time will be slower in MB lines	Stronger selection for large body size in MB lines will result in slower development for MB males as they need for time to accrue the nutrients to build a bigger body (Poças et al., 2022).
4	Development time – by diet	Development time will be slower in the Low diet regimes	Poor adult nutrition will also select for longer larval feeding to counter the effects off a poor adult diet (Krittika et al., 2019).
5	Body weight – both sexes	Flies from the High food regimes will be heavier in general than those for the low food lines	Poor adult diets will reduce condition of parents and be evident in smaller body size (Poças et al., 2022).
6	Wing size – both sexes	Wing size in low adult dietary regimes will be smaller than those in the high dietary regime	Poor adult diets will reduce parental condition and be evident in smaller wings (Krittika and Yadav, 2022).
7	Wing size - males	Wing size will be larger for males in	Increase in male-male competition experienced in MB lines will select for

		MB populations compared to FB males	larger wing sizes in the most successful males (Menezes et al., 2013).

Methods

Experimental evolution line maintenance

Experimental evolution flies are represented by 18 Sex Ratio lines consisting of three independent replicates of the Equal Sex (EQ (50M: 50F)) regimes, three x Female Biased (FB (25M: 75F)) and three x Male biased (MB (70M: 30F)) lines, maintained as adults on either High or Low protein diets. Individuals were maintained within plastic boxes (12cmW x 18cmL x 8.5cmD, with gauze lid) at 25°C in a humidified room with a 12 h light: 12 h dark cycle. High protein lines were given access to two fresh, standard SYA medium vials (25 x 75 mm) every 2-3 days, whilst the Low protein lines had similar access to vials containing 20% SYA medium (i.e., 20% of the standard amount of yeast in the diet, all other ingredients remained unchanged). Nine days after setting up the adults in the boxes, each line was supplied with an initial agar-grape juice egg collection plate, this was repeated on the 10^{th} day. Egg collection plates were maintained at 25°C following removal from the boxes and kept in cotton bags for two days until all eggs had hatched. Four hundred larvae were then picked from these egg collection plates in densities of one hundred per vial for each line. After eclosion from these cultures, flies were anaesthetised using CO_2 and counted into their specified sex ratios and placed in the plastic boxes as described. The lines have been maintained under these conditions since 23/12/2013. The flies used in the experiments came from generation 110.

Experimental protocol testing the effect of evolutionary manipulation of adult sex ratio and nutrition on D. melanogaster development.

A sister population of each experimental evolution treatment was set up and each line was supplied with an agar-grape juice egg collection plate, four hundred larvae were then picked from these egg collection plates in densities of one hundred per vial for each line. To standardise for parental effects, virgin flies were collected from

the vials nine Days later using CO_2 and separated into same sex cohorts of ten individuals per vial. After 48 h fifty males and fifty females selected from each population and placed in a plastic box (12cmW x 18cmL x 8.5cmD, with a gauze lid) at 25°C in a humidified room with a 12 h light: 12 h dark cycle and supplied with two agar-grape juice egg collection plates and left for 4 h. After 4 h males were removed by using CO_2 and females were allowed to lay for 24 h on the egg collection plates. After 24 h one egg collection plate was frozen and used for egg measurement data. The second egg collection plate was kept under the same conditions as stock lines and all eggs allowed to hatch. Larvae were then picked from this plate and transferred into fresh SYA vials at densities of fifty per vial (ten vials per treatment). After four days, each vial was checked at 8 h intervals, any pupariations were marked and number recorded per interval. Eclosion of adults was recorded at 12 h intervals, where adults were removed and frozen for further measurements.

Measurements taken included egg width, egg length and egg volume. Egg length and width were measured using ToupView (RRID:SCR_017998) and a micrometre to calibrate scale. Egg volume was approximated using an ellipsoid formula ($V = \frac{\pi}{6}$ x L x H^2). The development time was recorded from the day eggs were oviposited to pupariation, and the day from egg oviposition to eclosion. To measures adult dry weight, flies were dried at 60°C for 36 h and measured in µg using a 5 -decimal place analytical balance. Wings were carefully removed from flies and placed in a drop of ethanol: glycerol mix on a glass slide and measured under a compound microscope using ToupView and a micrometre for calibration. Four measurements were taken using consistent landmarks (Fig 3.1). To attain wing length and area measurements. Wing area was calculated using Heron's formula (Area = $\sqrt{p(p-b)(p-c)(p-d)}$, $P = \frac{b+c+d}{2}$) with the landmarks indicated in Fig 3.1.

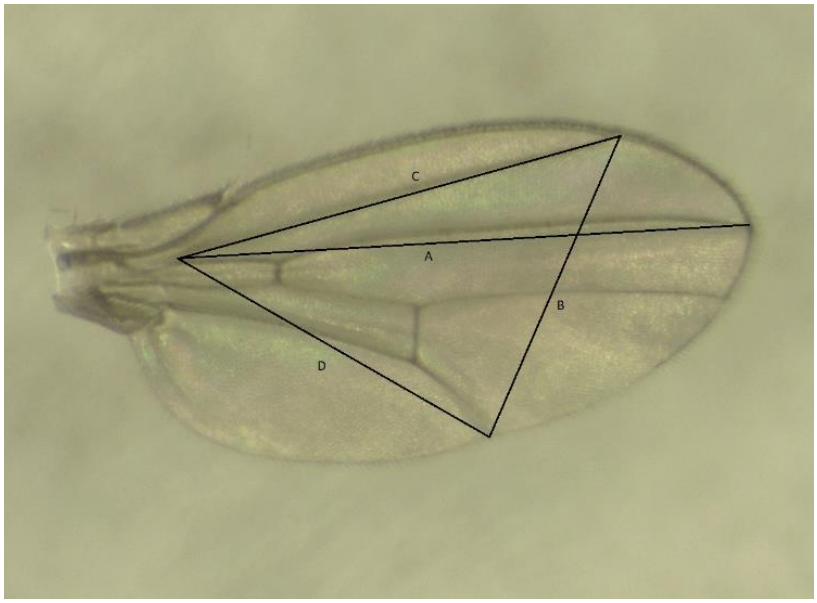

Fig 3.1. Landmarks used for wing measurement (A = Length, B, C and D = Wing area). Scale bar?

Statistical analysis

All statistical analysis were performed using R-4.0.2 (R Core Team, 2020). The Shapiro-Wilk test was used to check data were normally distributed and the Levene's test to check the homogeneity of variances across treatments. Replicate populations were incorporated into the model as a random grouping variable. Analysis of wing size and adult dry weight were log transformed and analysed using linear mixed effects models from the lme4 package (Bates et al., 2015), Egg volume was analysed using a glmer with a Gamma distribution from the lme4 package. Development time was analysed using a hazards model (Therneau, 2012) for event data, and separately using generalised linear mixed effect models from lme4 package with a Poisson distribution, censored data was removed. The Akaike's Information Criterion (AIC) was used to check for model fit. Post-hoc analysis were performed using the Tukey's Test and estimated marginal means provided from the Emmeans package (Lenth, 2023).

Results

Evolution of developmental and body size traits in the sex ratio regimes

The prediction that developmental traits would differ amongst the sex ratio regimes (Table 3.1) was upheld. The impact of long-term restricted adult diets across generations also had a significant effect. These effects are outlined below.

1. *Response of egg size to long-term variation in sexual selection and adult nutrition*

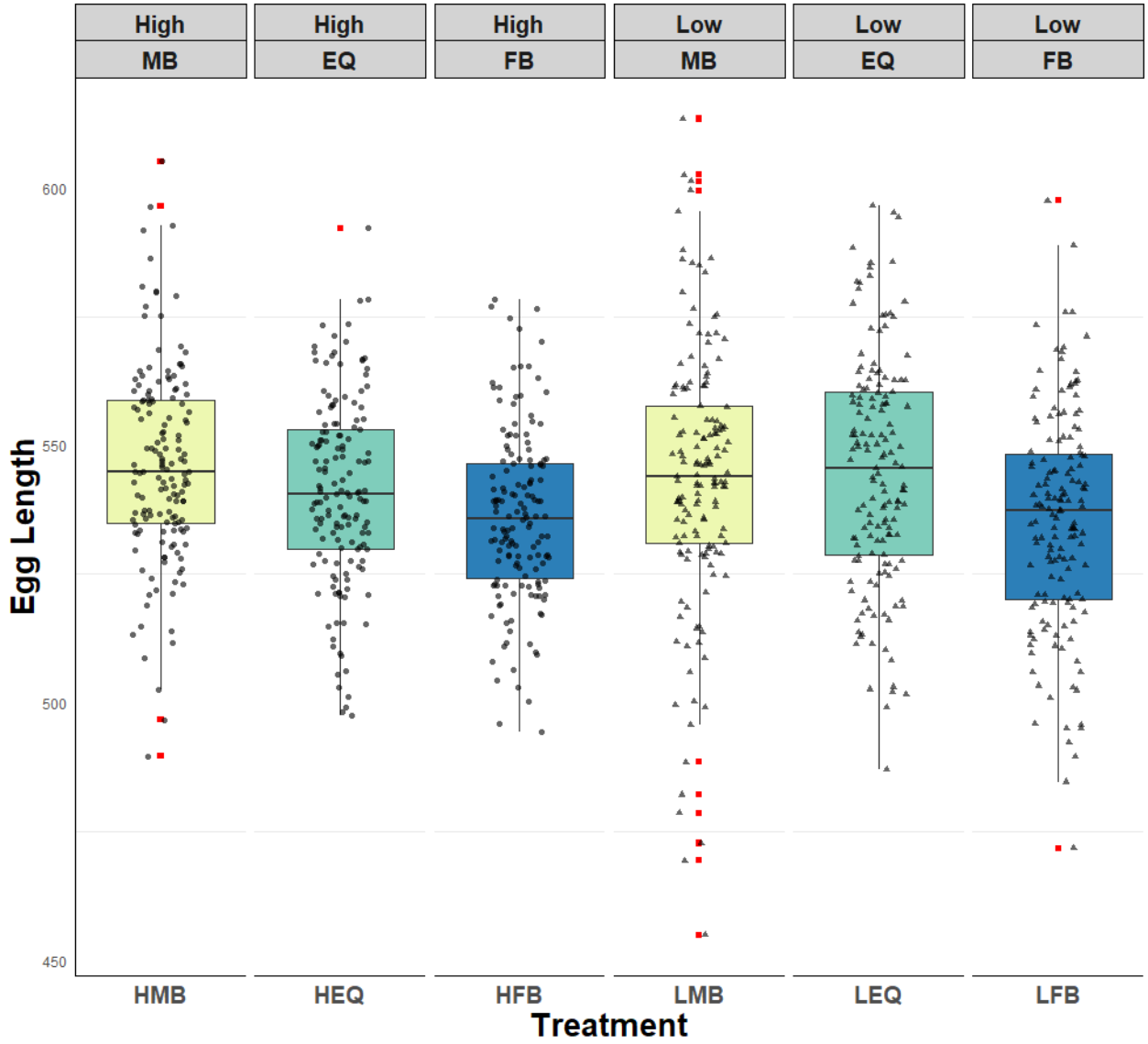

Fig 3.2. Egg length (µm) produced by females from the sex ratio regimes. N =50 per replicate from female-biased (FB), Equal sex (EQ) or Male –biased (MB) sex

ratios and standard protein (High, H) or 20% protein (Low, L) diet regimes (N = 150 eggs per sex ratio/diet). Boxplot showing median line, with boxes representing upper and lower 25% quartile and whiskers representing the range, and points representing individual records, outliers highlighted in red.

In line with our predictions (table 3.1), the egg length reported significant differences by adult sex ratio in egg. Further analysis using a Tukey post hoc reported a significant difference between sex ratios MB & FB for egg length ($z = 2.420, p = 0.041$). However, in contrast to predictions in Table 3.1 no difference between diet was detected ($z = 0.939, p = 0.338$) Fig 3.2. (Table S3.6a & S3.6b)

Egg volume was significantly different among sex ratio regimes, in line with predictions (Table 3.1), reporting significant differences between MB ($z = 2.118, p = 0.0483$) & FB ($z = -2.264, p = 0.0362$) but not adult diet (Table 3.7a) regimes. Additional Tukey post hoc contrast show the significant difference is between HMB (High Male Bias) and HFB (High Female Bias) treatments, but not between any other comparisons (Table 3.7b) ($t = -3.578, p = 0.0151$) Fig 3.3.

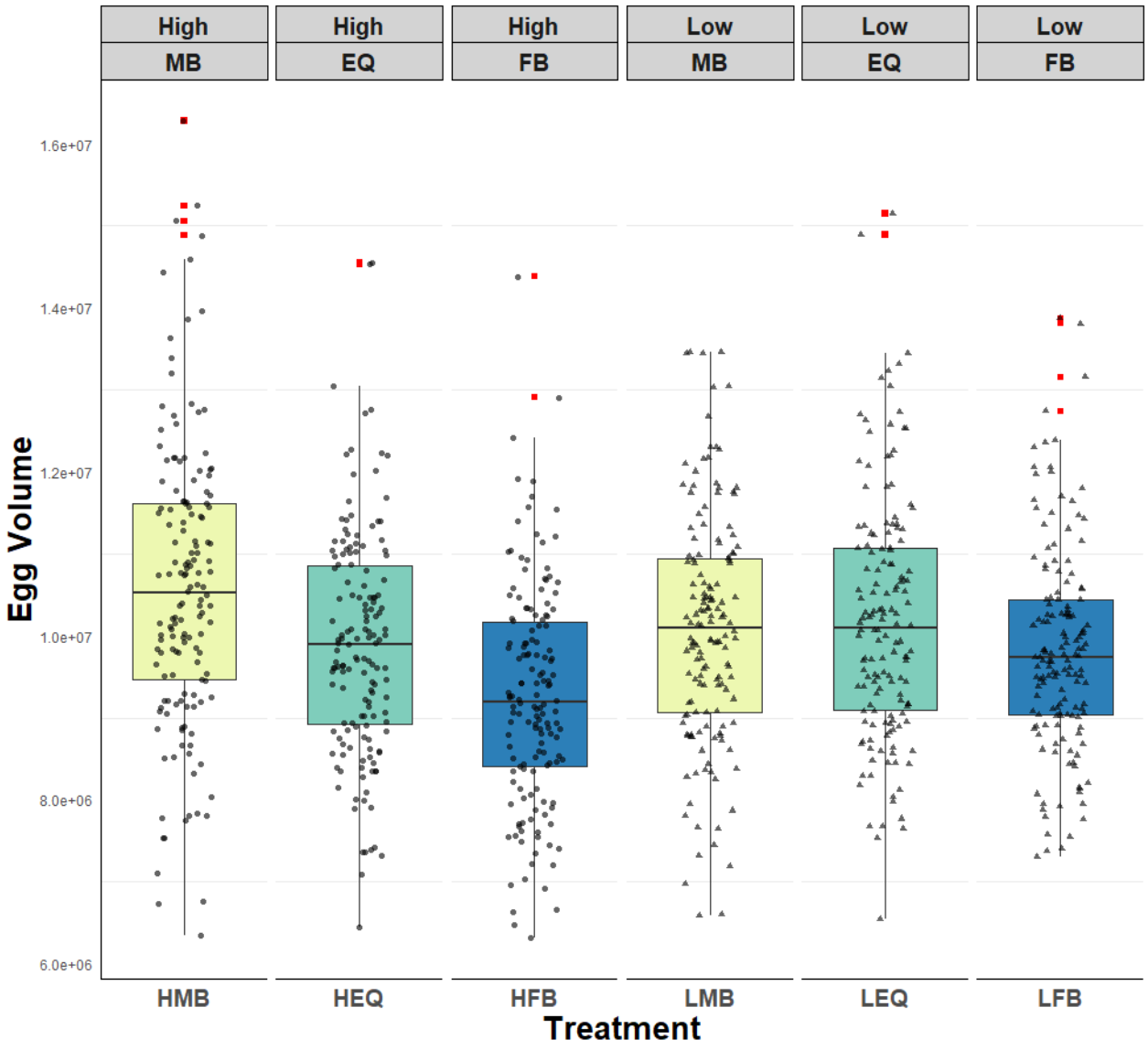

Fig 3.3. Egg volume (µm) produced by females from the sex ratio regimes. N =50 per replicate from female-biased (FB), Equal sex (EQ) or Male –biased (MB) sex ratios and standard protein (High, H) or 20% protein (Low, L) diet regimes (N = 150 eggs per sex ratio/diet). Boxplot showing median line, with boxes representing upper and lower 25% quartile and whiskers representing the range, and points representing individual records, outliers highlighted in red.

Our first prediction (Table 3.1) that egg size would vary by sex ratio, and that in particular the larger females (MB regimes) produced bigger eggs was upheld, but only in the high food regimes. In contrast to our second prediction, egg sizes in low diet females were not smaller in size comparative to high diet regimes.

Response of development time to long-term variation in sexual selection and adult nutrition

There was no significant effect of sex ratio or diet for time from egg to pupariation. However, there was a significant interaction for time to pupariation for MB lines from the low diet regimes in comparison to all other regimes (z = 3.203, *p = 0.00136*) (Table S3.1. Post hoc analysis indicated that there was significant variation in pupariation time between LMB & HMB (*z = -3.243*, *p = 0.0150*), LMB & LEQ (*z = -3.888, p = 0.0014*) and LMB & LFB (*z = -3.169*, *p = 0.0191*) treatments, Fig 4.

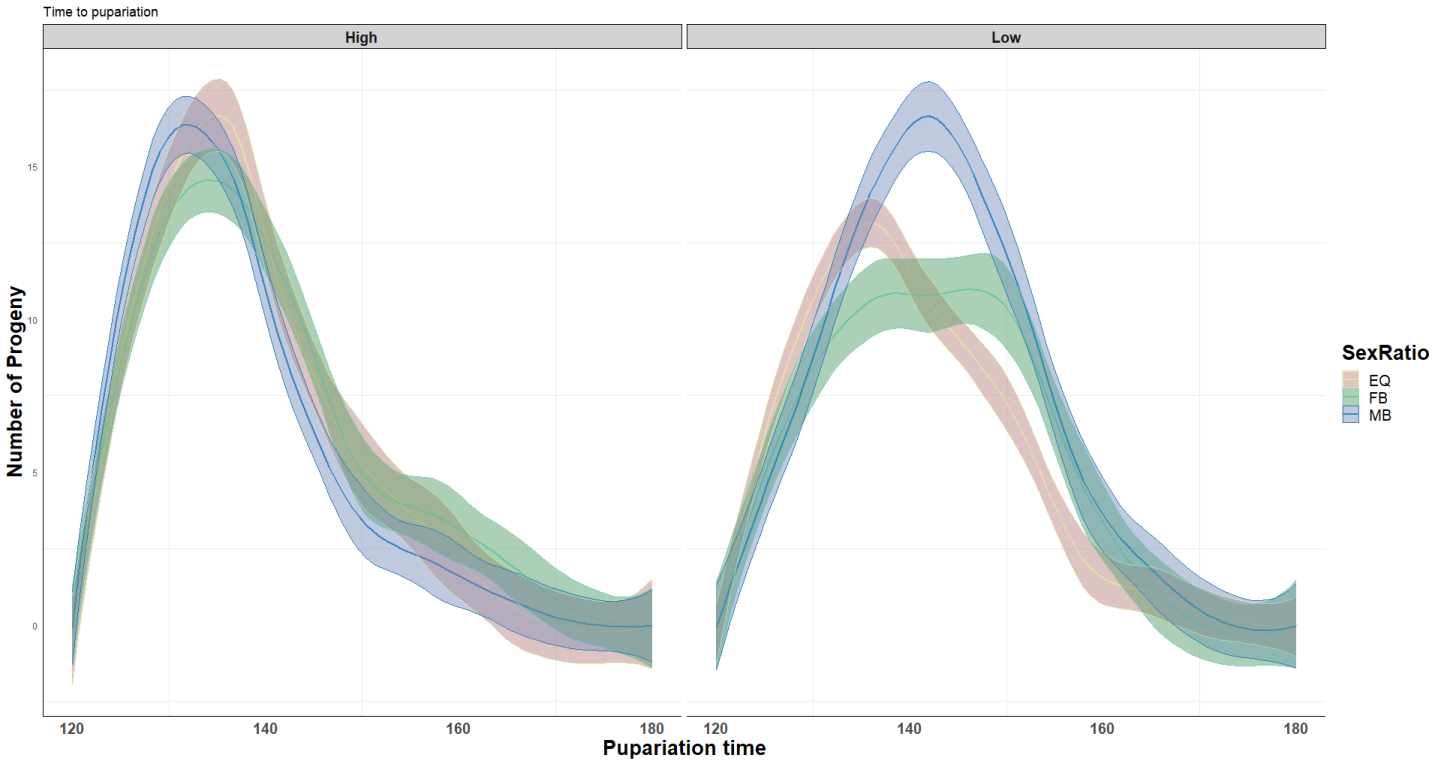

Fig 3.4. Time (h) to pupariation in individuals of both sexes drawn from the sex ratio regimes. The pupariation of Female-biased (FB), Equal Sex (EQ) or Male – Biased (MB) sex ratios and standard protein (High) or 20% protein (Low) diet regimes. Pupariation events were recorded at 8h cycles (10 vials per replicate (n =

50 flies per vial). Lines represent average time to pupariation across the replicate's, shaded areas are the 95% confidence intervals.

As with pupariation time, there was no significant effect of sex ratio or diet. However, an interaction for time to eclosion was significantly different for the LMB (*z = 3.068*, *p = 0.002*) treatment, and the analysis showed that, overall, Low food regimes were significantly slower to eclose (*z = -2.411*, *p = 0.015*) Fig S5a. When separated by sex the effect of diet remained significant for females (*z = -2.725, p = 0.006*) but not males (*z = -1.643, p = 0.100)*, (Fig 3.5a & 3.5b). Whilst the result was non-significant LMB males were slower in general to develop than males from the other regimes (*z = 1.759, p = 0.0786*). However, post hoc analysis did not report any significant effects overall of sex ratio or diet (Table S3.4). LMB females had significantly slower times to eclosion compared to other treatments (*z = 3.673, p = < 0.001*) (Table 3.3b). A separate analysis of the time to eclosion using a cox survival model suggested LMB females were slower to eclose, however, it was not significant (Table S3.5a & S3.5b). AIC was significantly lower for the glmer (10372.82) compared to the cox model (70834.51).

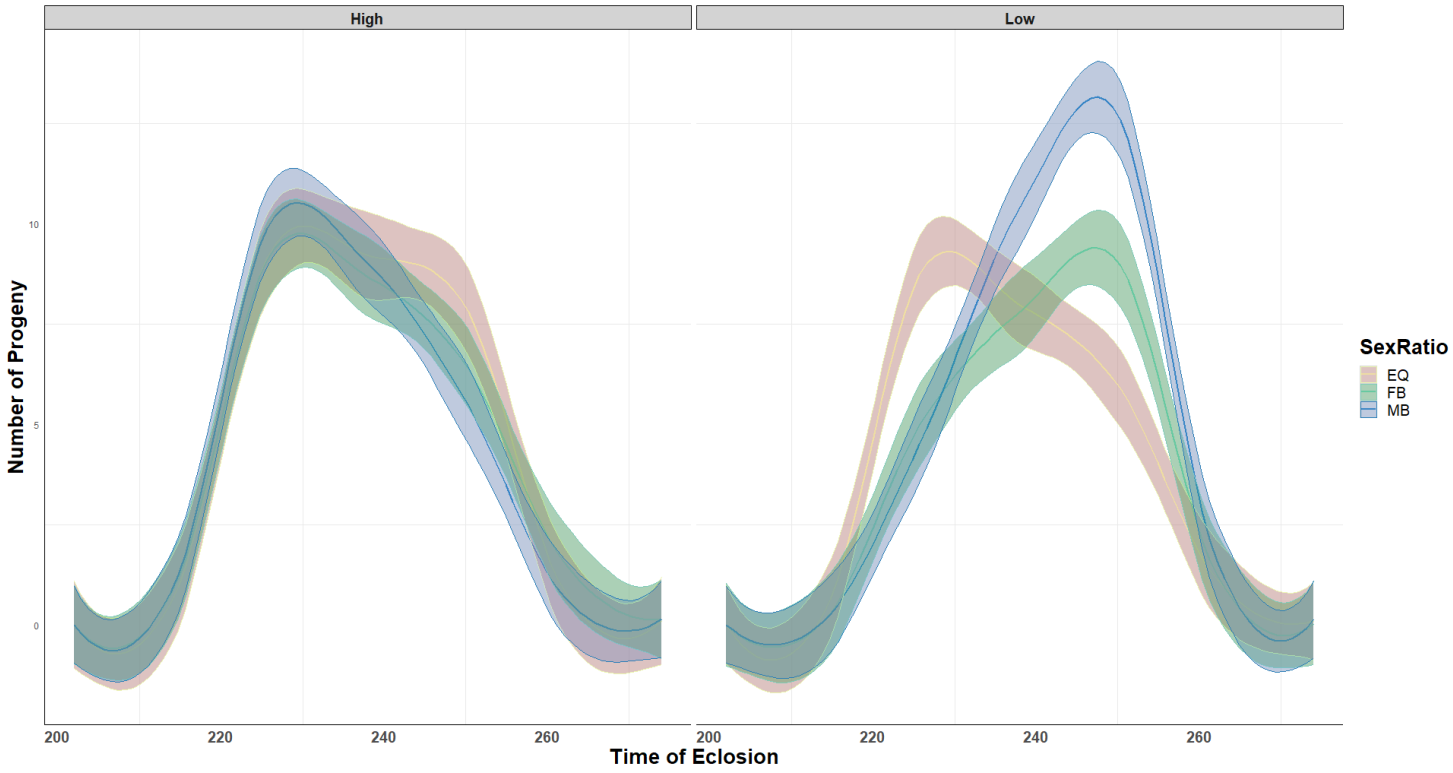

Fig 3.5a. Time (h) to eclosion of females from the sex ratio regimes. The eclosion time of female *D. melanogaster* from Female-Biased (FB), Equal Sex (EQ) or Male –Biased (MB) sex ratios and standard protein (High, H) or 20% protein (Low, L) diet regimes. Eclosion events were recorded in 12h cycles where flies were removed and sexed. 10 vials per replicate (n = 50 flies per vial,). Lines represent average time to eclosion, shaded areas are the 95% confidence intervals.

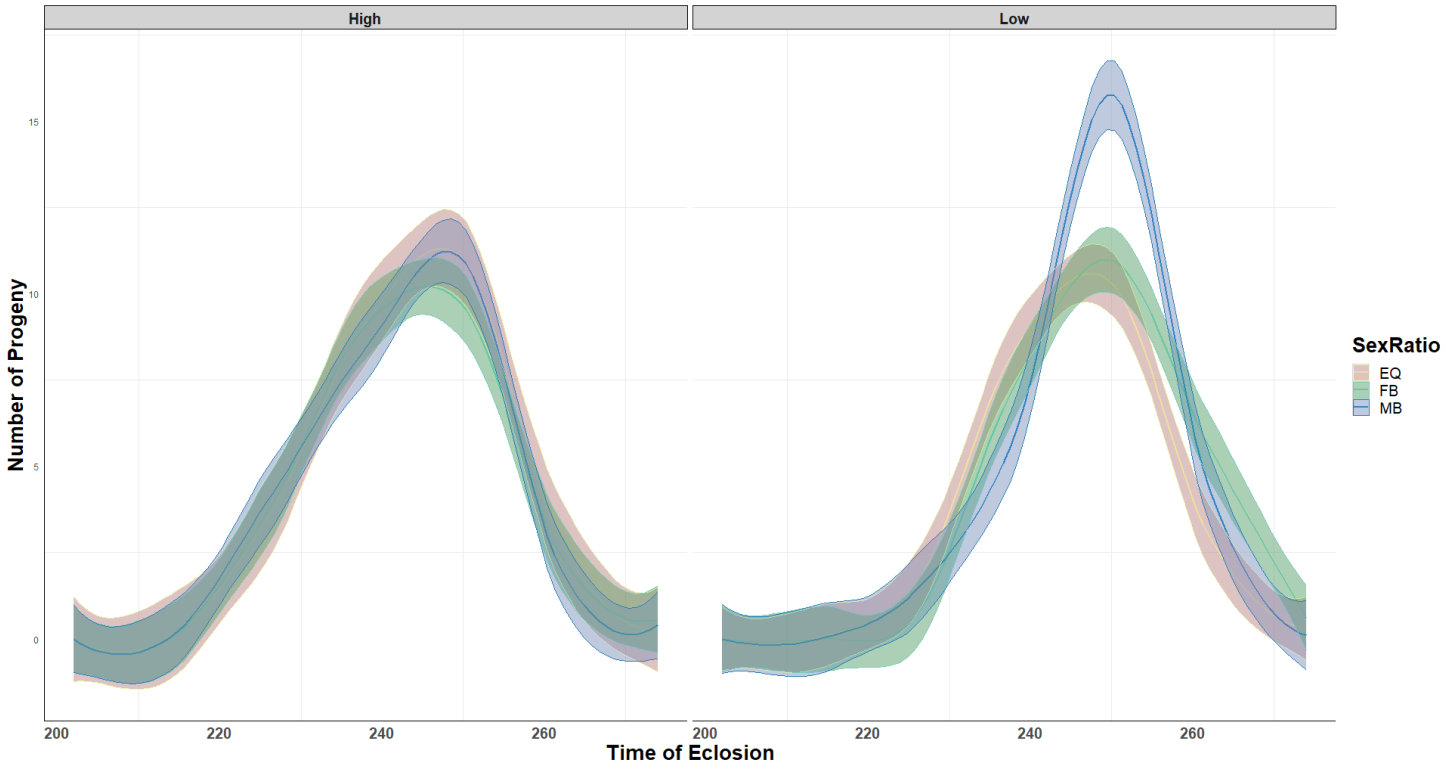

Fig 3.5b. Time (h) to eclosion of males from the sex ratio regimes. The eclosion time of male D. melanogaster from Female-Biased (FB), Equal Sex (EQ) or Male –Biased (MB) sex ratios and standard protein (High, H) or 20% protein (Low, L) diet regimes. Eclosion events were recorded in 12h cycles where flies were removed and sexed. 10 vials per replicate (n = 50 flies per vial,). Lines represent average time to eclosion, shaded areas are the 95% confidence intervals.

The prediction that sex ratio would select for longer time from oviposition to eclosion in MB lines was not upheld (Table 3.1). There was an interaction with sex ratio and diet with low MB flies being slower to develop comparatively to other treatments (Table 3.1). When separated by sex female *D. melanogaster* from the low dietary

regimes were much slower to develop compared to those from high dietary regimes. Further breakdown of these results indicated that only under the effects of increased or decreased sexual selection was this apparent, as LEQ females' development time corresponded with those from high food regimes.

Response of adult dry weight to long-term variation in sexual selection and adult nutrition

In males, there was an interaction between food and sex ratio for LMB males (*z = -2.647, p = 0.014*), however post hoc analysis shows no significant differences between the groups (Table S3.8a) counter to our predictions (Table 3.1). (Fig 6a).

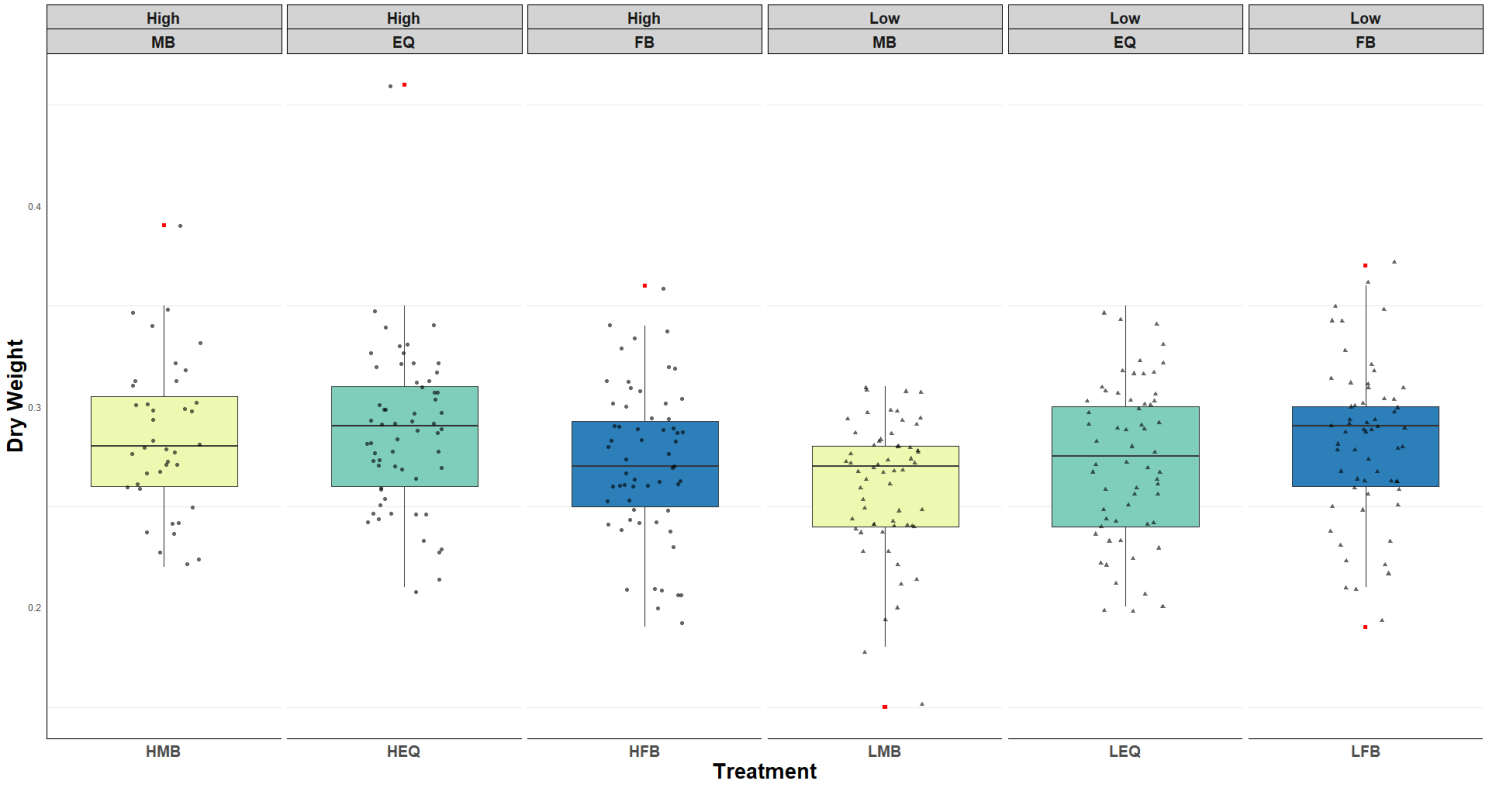

Fig 3.6a. Adult dry body weight (µg) for males from the sex ratio regimes. N =2 Males per vial from Female-Biased (FB), Equal Sex (EQ) or Male –Biased (MB) sex ratios and standard protein (High, H) or 20% protein (Low, L) diet regimes (10 vials per replicate, 3 replicates per sex ratio/dietary regime, N = 60 males per sex ratio/diet). Measurements were taken after 36h in 60°C. Boxplot showing median line, with boxes representing upper and lower 25% quartile and whiskers

representing the range, and points representing individual records, outliers highlighted in red.

Female adult dry weight differed significantly across the sex ratio treatments, with MB females being significantly heavier than FB females (*z = 2.410, p = 0.042*). There was also a significant effect of diet regime (*z = -3.305, p = <0.001*). Pairwise comparisons (table S3.9) showed significant differences between HMB and LMB (*z = -2.898, p = 0.043*) female weight and HEQ and LEQ (*z = -3.305, p= 0.012*) with the high diet regimes being heavier. However, the difference between HFB & LFB was not significant (*z = -0.656, p = 0.986)* Fig 6b.

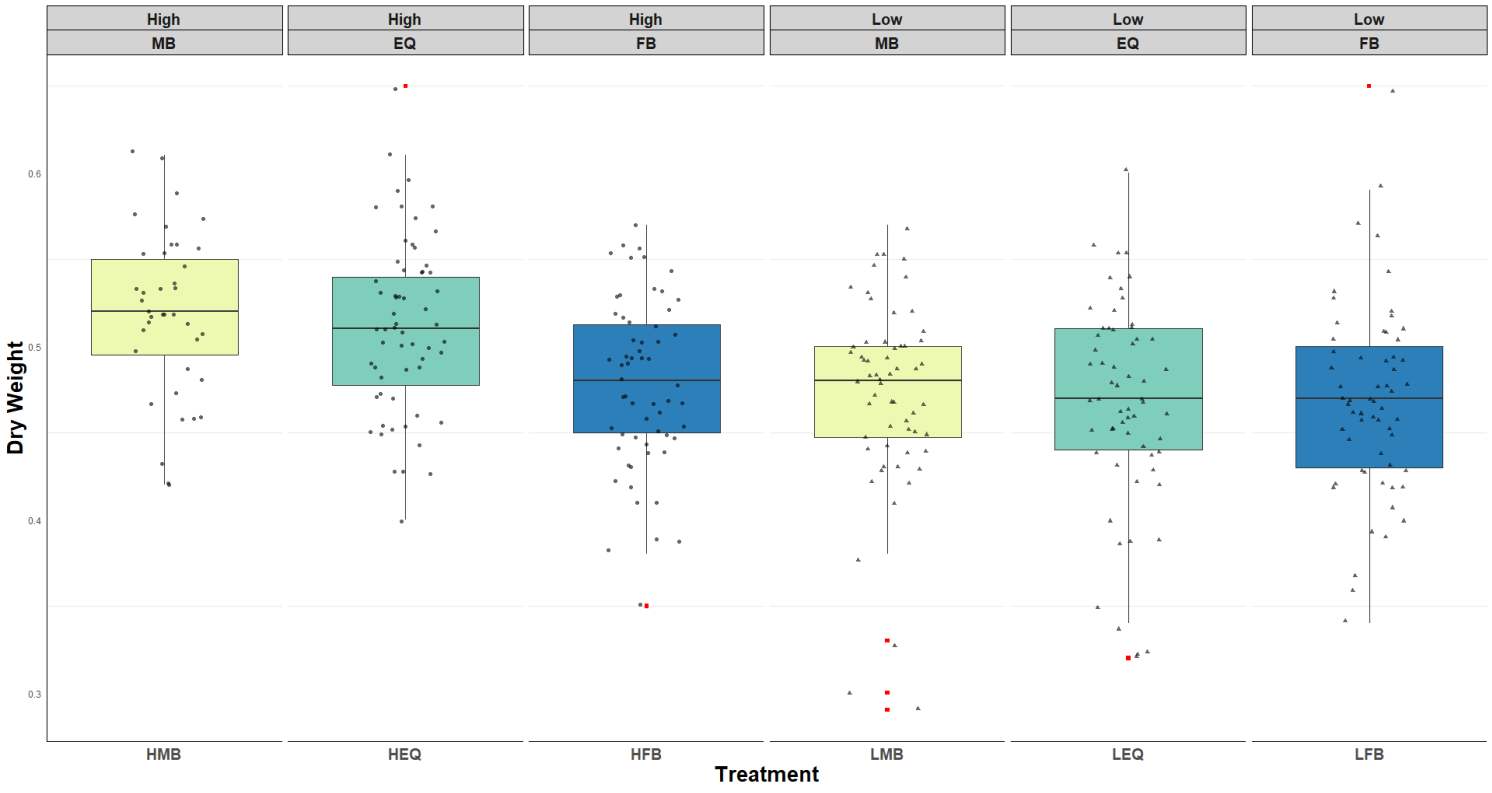

Fig 6b. Adult dry body weight (µg) for females from the sex ratio regimes. N =2 females per vial from Female-Biased (FB), Equal Sex (EQ) or Male –Biased (MB) sex ratios and standard protein (High, H) or 20% protein (Low, L) diet regimes (10 vials per replicate, 3 replicates per sex ratio/dietary regime, N = 60 females per sex ratio/diet). Measurements were taken after 36h in 60°C. Boxplot showing median line, with boxes representing upper and lower 25% quartile and whiskers

representing the range, and points representing individual records, outliers highlighted in red.

Our predictions (Table 3.1) were met in females but not in males, reporting a significant difference between dietary regimes. In the high nutrition lines, our predictions of variation by sex ratio were also met in females, but not under low nutrition. Counter to our prediction's males did not diverge in body weight by sex ratio.

Response of wing size to long-term variation in sexual selection and adult nutrition

Male wing area was significantly different between dietary regimes and sex ratios, as predicted in table 3.1. Interestingly, counter to our predictions males from the restricted diet (Low) treatments had larger wings than those from the high diet (*z = 3.355, p = <0.001*). In general MB wing area was significantly larger than FB males (*z = 3.959, p = <0.001*) and FB wing area was much smaller than for EQ males *(z = -2.783, p = 0.014)*. Post hoc analysis showed significant differences (table S3.10) between HMB and HFB males *(z = 3.215, p = 0.0348)* and highlighted a large divergence in wing size between HFB & LFB males (*z = -4.053, p = 0.004*) Fig 7a, matching our predictions in table 3.1.

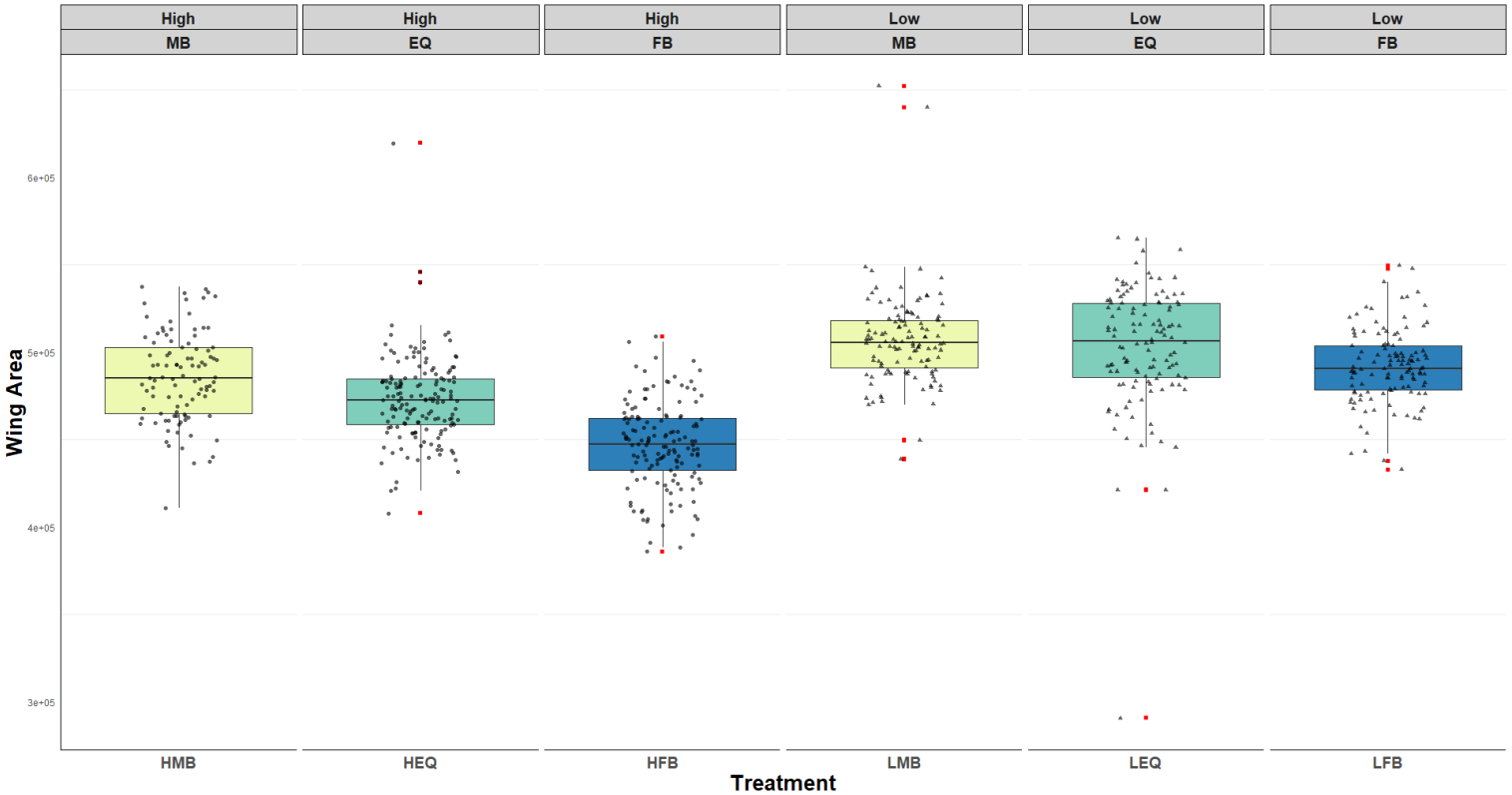

Fig 7a. Average wing area (µm) for males from the sex ratio regimes. Wing size was an average of both wings from males (N =2, per vial) from Female-Biased (FB), Equal Sex (EQ) or Male –Biased (MB) sex ratios and standard protein (High, H) or 20% protein (Low, L) diet regimes (10 vials per replicate, 3 replicates per sex ratio/dietary regime, N = 60 males per sex ratio/diet). Measurements calculated using Heron's formula. Boxplot showing median line, with boxes representing upper and lower 25% quartile and whiskers representing the range, and points representing individual records, outliers highlighted in red.

Male wing length showed a similar trend across sex ratios, with MB wings being significantly longer than for FB males (*z = 2.536, p = 0.03*), and with again a significant effect of diet (*z = 2.262 p = 0.023*). However, unlike wing area the largest reported difference was between HMB and LMB male wings (*z = 3.304, p = 0.012*) (Fig 7b).

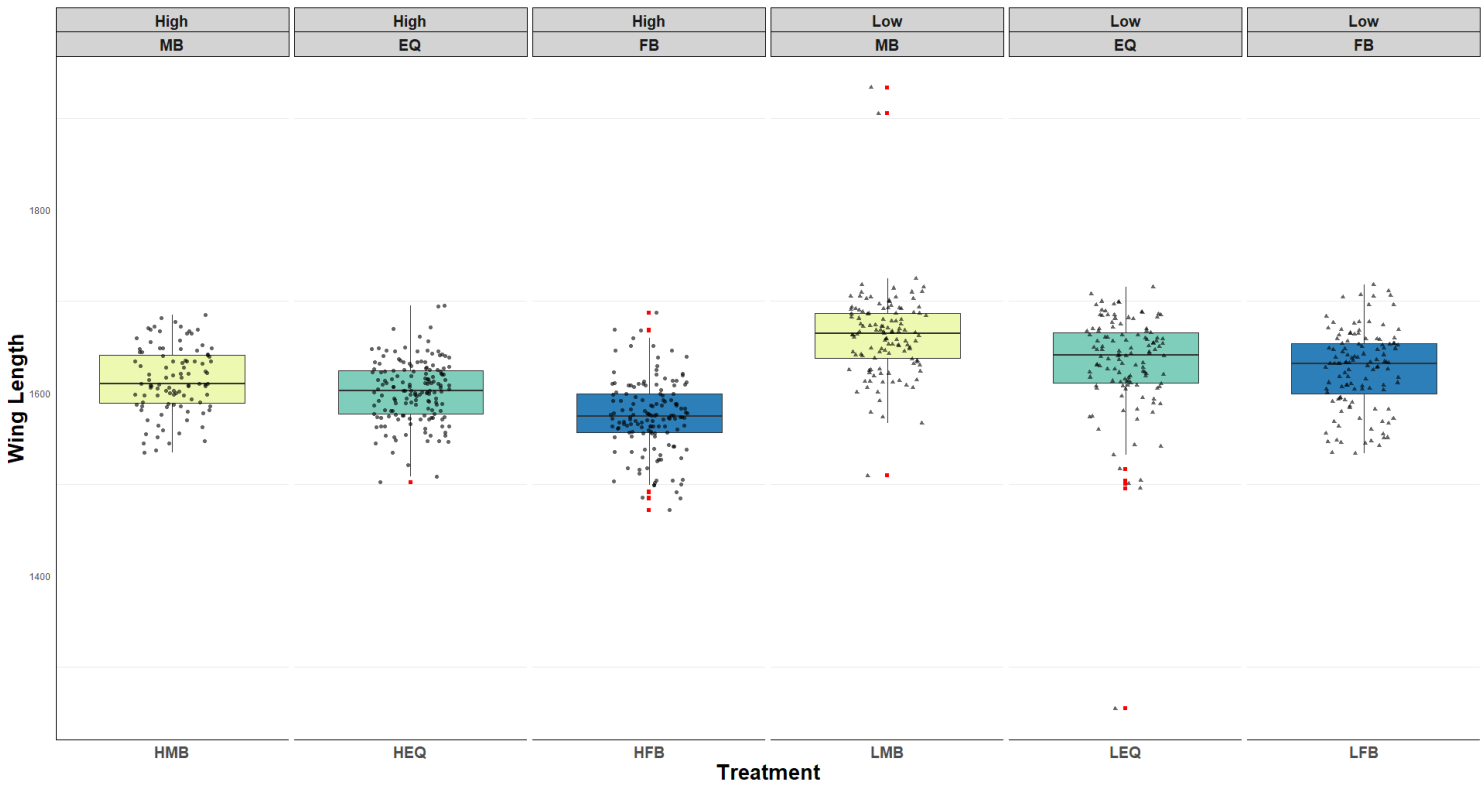

Fig 7b. Average wing length (µm) for males from the sex ratio regimes. Wing size was an average of both wings from males (N =2, per vial) from Female-Biased

(FB), Equal sex (EQ) or Male –Biased (MB) sex ratios and standard protein (High, H) or 20% protein (Low, L) diet regimes (10 vials per replicate, 3 replicates per sex ratio/dietary regime, N = 60 males per sex ratio/diet). Measurements calculated from landmarks Identified line A. Boxplot showing median line, with boxes representing upper and lower 25% quartile and whiskers representing the range, and points representing individual records, outliers highlighted in red.

Female wing area was also significantly different between dietary regimes in line with our predictions (Table 3.1). However, as with the males, the low adult diet treatment females had significantly larger wing areas than those from the high diets (*z = 3.4, p = <0.001*). The differences in wing area between the sex ratios followed a similar pattern to that observed in the males, with MB female wing area being significantly larger than FB females (*z = 3.540, p= 0.001*) and FB female wing area being significantly smaller than EQ line females (*z = -2.386, p = 0.044*). Pairwise analysis indicated significant differences between HFB and HMB (*z = -3.540, p = 0.00531*) HFB and LFB treatments (*z = 4.419, p = < 0.001*) and HEQ and LEQ (*z = 3.400, p = 0.008*) (Fig 8a).

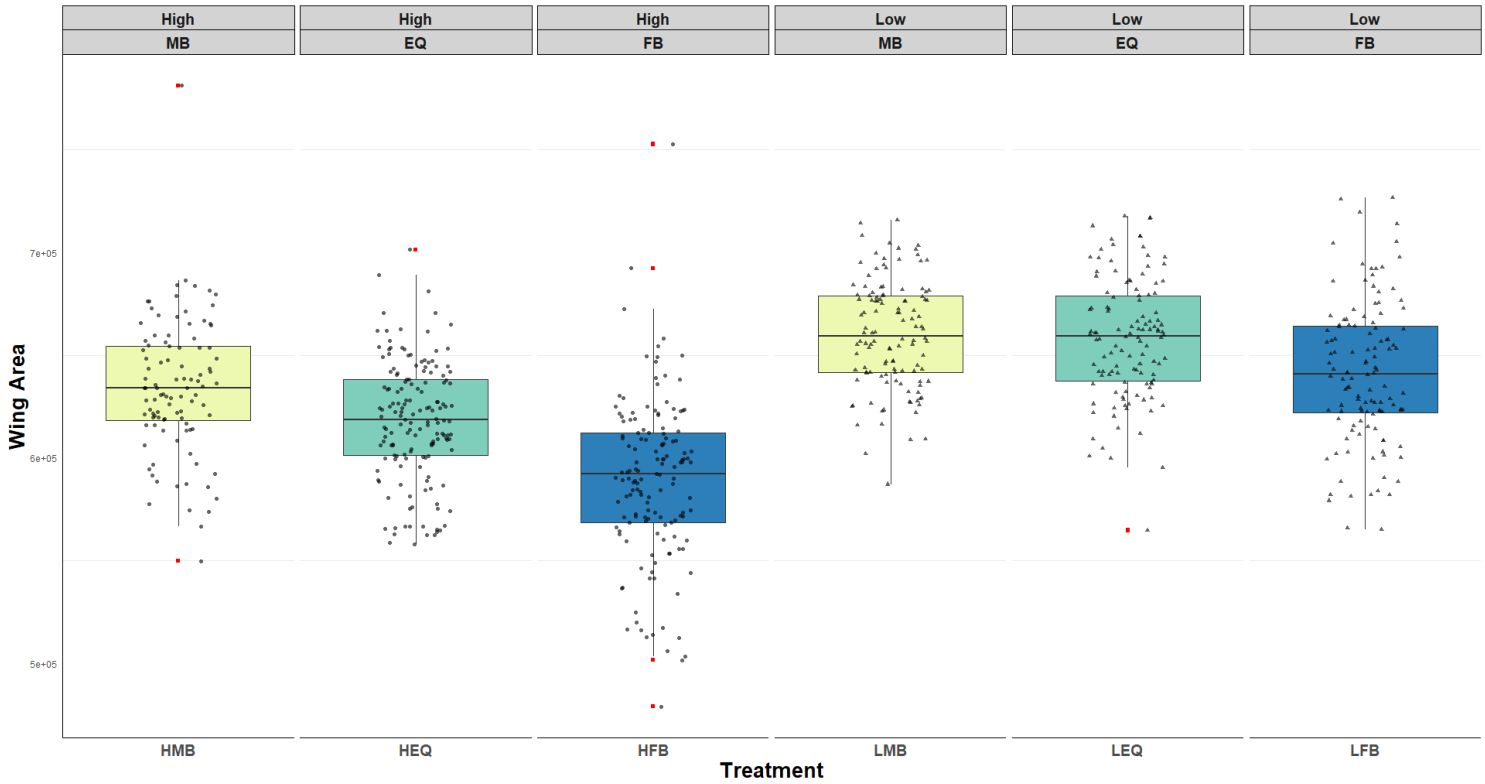

Fig 8a. Average wing area (µm) for females from the sex ratio regimes. Wing size was an average of both wings from females (N =2, per vial) from Female-Biased

(FB), Equal Sex (EQ) or Male –Biased (MB) sex ratios and standard protein (High, H) or 20% protein (Low, L) diet regimes (10 vials per replicate, 3 replicates per sex ratio/dietary regime, N = 60 females per sex ratio/diet). Measurements calculated using Heron's formula. Boxplot showing median line, with boxes representing upper and lower 25% quartile and whiskers representing the range, and points representing individual records, outliers highlighted in red.

Female wing length was significantly different between dietary regimes, with low diet females having considerably longer wings overall (*z = 2.442, p = 0.014).* Whilst the pattern for wing size by sex ratio followed that found in males, with the longest wing length being in females from the MB lines, though this was not a statistically significant effect (Fig 8b).

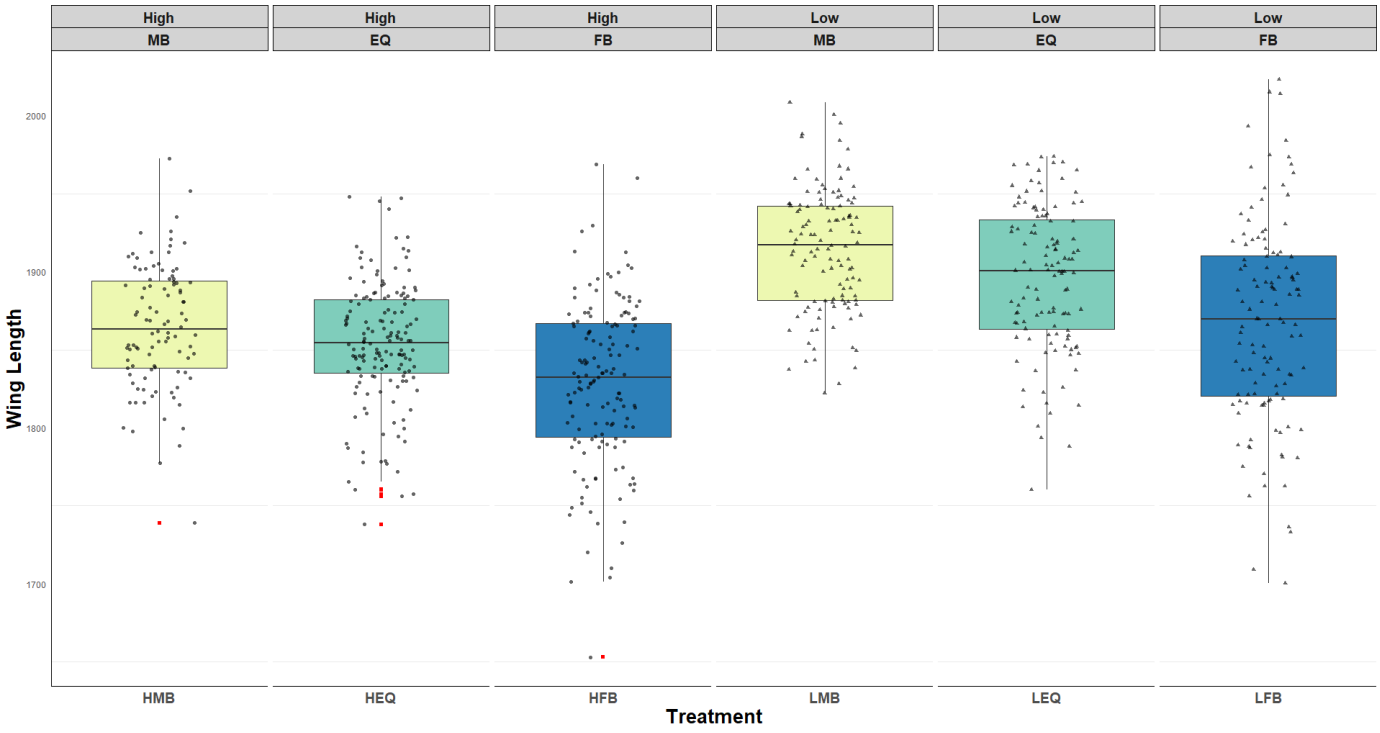

Fig 8b. Average wing length (µm) for females from the sex ratio regimes. Wing size was an average of both wings from females (N =2, per vial) from Female-Biased (FB), Equal Sex (EQ) or Male –Biased (MB) sex ratios and standard protein (High, H) or 20% protein (Low, L) diet regimes (10 vials per replicate, 3 replicates per sex ratio/dietary regime, N = 60 females per sex ratio/diet). Measurements calculated from landmarks Identified line A. Boxplot showing median line, with boxes

representing upper and lower 25% quartile and whiskers representing the range, and points representing individual records, outliers highlighted in red.

Overall, the wing size results show that as predicted (Table 3.1), MB males evolved larger wings, however, this response was only significant in high adult dietary regimes. Counter to our predictions affecting wing size by adult diet, low food regime flies in both sexes reported significantly larger wings than flies under high diet regimes.

Discussion

The main question addressed in this chapter was whether exposure to long-term variation in adult sex ratio differentially selected on developmental and body size traits in males and females, and whether these effects were condition dependent, responding differently in the contrasting adult dietary regimes. To test this, I analysed the development time from egg to pupariation, egg to eclosion, egg size, body weight, and wing size in flies that were standardised for parental effects and drawn from lines selected for over 110 generations. These lines were held at fixed adult sex ratios of MB, FB, or EQ in stable population densities under either standard SYA adult diet (high) or a protein restrictive diet (low). The results showed a significant increase in development time linked to adult dietary restriction in female *D. melanogaster,* with low regime flies developing slower. An interaction between sex ratio and adult dietary restriction, increased development time for LMB selection lines, comparative to the other lines. In addition to this, I found a significant effect of sex ratio on wing size, with MB populations demonstrating larger wings in both length and area comparative to FB lines. There was an indication that wing size selection was condition dependent and was not as strongly evidenced in low dietary regime flies as in the high treatment. I also report differential wing morphology demonstrated consistently across sex ratios in both male and female *D. melanogaster* in the low food treatments. Flies from these regimes despite being significantly lighter in adult weight, they demonstrated larger wing sizes across sex ratios.

Evolution of larger egg size in 'high, male bias lines (HMB).

The predictions that egg size would be larger in MB lines was supported (Table 1), but only in the high treatment food lines. Contrastingly, our prediction that eggs would be smaller in low nutrition lines was not supported. Egg volume was significantly larger in HMB lines than HFB lines, with HEQ regimes situated intermediately. It is interesting to note that although the length of the egg was influenced by sex ratio within both nutritional regimes, a significant pairwise difference in egg volume was only observed between HMB and HFB treatments. Previous studies have found evidence that larger females produce larger eggs (Yanagi and Tuda, 2012). My results were consistent with this in terms of larger egg volumes produced by females from the high diet regimes. However, the significant adult weight differences between the dietary regimes did not correlate with egg size by length or volume differences. In other experiments using these lines (chapter 2 & 4) female fecundity did not differ between the lines suggesting that females are able to compensate for any protein restriction (Vijendravarma et al., 2010) and sustain egg volume.

Evolution of extended development time in low dietary regimes under divergent sex ratios.

Development time from egg to pupariation did not differ significantly across the sex ratio regimes. However, I did detect an effect of the LMB line flies being slower to initiate pupariation than other lines, with the largest difference between HMB and LMB lines. This result suggests that long-term restricted adult diet impacts upon the time spent as larvae, but under a long-term male biased scenario. There was also a reduced time to pupariation in LFB lines however this was less pronounced than in LMB lines.

In addition, the time to eclosion was slower for flies from the LMB lines. When separated by sex, the development time from egg to eclosion was only significant for females. A study investigating immediate protein restriction on development time found that 20% restrictive protein is still suitable for maintaining standard

development time (Krittika et al., 2019). Therefore, our results suggest that the developmental time for females is impacted by the interaction with protein restriction and long-term exposure to male biased populations. Understanding the nature of possible interactions between long-term effects of sex ratio and adult diet will require additional experimental studies. Perhaps females are spending longer as larvae in order to maximize body size at eclosion, or it may be a result of the larger wings seen in these lines, and this difference is magnified by the size dimorphism between the sexes.

Females evolved to be heavier in high nutrition, male bias populations, but males did not.

Male dry body weight did not evolve as a direct result of long-term manipulations of adult sex ratio or dietary regimes, counter to our initial predictions (Table 1). This perhaps should be expected, as it has been demonstrated that adult males do not consume as much protein as do females, instead preferring carbohydrates (Kubli, 2010), which were not restricted in this experiment. However, in LMB males I did observe a significant reduction in adult dry weight compared to the other regimes. One potential explanation for this effect is a potential trade-off with absolute body weight and seminal fluid protein (SFP) production/replenishment. In a highly competitive environment, it has been suggested that males invest more SFPs with each mating (Wigby et al., 2009b). The replenishment of SFPs invested would be a potential drain on resources and as such a trade-off with overall bodyweight might occur over time.

The differences in female adult dry body weight indicated not only a significant difference in body weight between dietary regimes but also a significant effect of sex ratio. Females from ‘low’ (protein restricted) regimes were significantly smaller than their ‘High’ diet conspecifics. This was in line with our prediction that flies would be heavier in high diet regimes (Table 3.1). Females typically prioritise protein-based diets (Kubli, 2010) and when diet is restricted overall body weight has been reduced (Poças et al., 2022). An interaction between sex ratio and diet was also recorded for focal females. Males typically prefer larger females (Byrne and Rice, 2006, Andersson and Iwasa, 1996) and so in a male biased environment this could potentially incur significant costs from male mating attempts, and harassment (Wigby

and Chapman, 2004a). Female size may be a response to this increase in harassment, male *D. melanogaster* take longer to initiate courtship with large females and deliver more wing vibrations in comparison to small ones (Turiegano et al., 2013). In addition, large females are more likely to be moving (Turiegano et al., 2013). This could be an indicator of female resistance to male courtship, and a signal to males of fitness.

Overall, my results suggest that for a female, restricted protein as an adult exerts much greater selection than is true in males. Additionally, females became larger when experiencing a high male biased environment, but only in environments in which resources were abundant.

Wing size increased when flies were held in Male Biased populations.

The analysis of wing size showed a significant effect of sex ratio and diet, as was predicted (Table 1). However, the increase in wing size for protein restrictive regimes was unexpected, with significantly larger wings seen in both sexes of all sex ratio regimes in the low diet treatments in comparison to their high food counterparts. Whilst it is difficult to identify the precise driving factors involved without further experiments, adult sex ratio and adult diet are known to play an important role in divergence of wing size and would repay further study in this context.

Wings are central to male courtship behaviours and the series of pulses and hums produced in the courtship song (Schilcher, 1976). It has been shown empirically that males with longer wings are more competitive (Menezes et al., 2013), so it could be expected that under high male-male competition longer/larger wings might convey a fitness advantage. Male wing size could also be a response to the increase in female body size. Previous research has shown that HMB lines are slower to mate (Dore et al., 2021) and that larger females receive more courtship song (Turiegano et al., 2013). In this experiment I show that in HMB regimes male weight did not increase, but females in HMB regimes did. If adult weight represents the disparity in adult size between HMB males and females, then proportionally the difference in size between the sexes is largest in HMB lines. Resulting in a longer courtship routine for HMB flies. and longer wings in response to female selection (Menezes et al., 2013). Male success in competition has also been linked to the responding courtship song

produced by females as well as seminal fluid modulation (Kerwin et al., 2020). Hence, females may be responding to the potential increase in SFP allocation (Wigby et al., 2009b) by increasing their own wing size in response. The effect of adult sex ratio on wing size was observable in the low food regimes. However, flies from the Low adult diet regimes showed smaller differences in wing area, suggesting a decreased ability to respond appropriately to the competitive environment in both sexes. The divergence in wing size for low food regimes found in this study is interesting. HEQ lines are expected to most closely resemble a well-fed control population, with LEQ to represent a population under greater nutritional stress. A recent study found *D. melanogaster* flies under parental protein restrictive diets had reduced wing size after 20 generations (Krittika and Yadav, 2022). This is counter to what I found and although it is not clear what the population densities were in their study, those results suggest another mechanism acting upon these regimes in our experiment. A potential driver for wing size may be linked with aggressive or defensive behaviours. A common aggressive behaviour 'Wings erect' (Ueda and Kidokoro, 2002), under a regime of limited food resource (low) a larger wing size may convey a competitive advantage for acquiring or defending resources. Females have been shown to elicit aggressive behaviour in the presence of yeast (Ueda and Kidokoro, 2002, Nilsen et al., 2004a) so resource limitation may select for increased aggression. It is worth noting that it was absolute, not relative, wing size that was analysed in this experiment. Hence, the wings for females were on average significantly larger in low regimes relative to their average body size. Therefore, it is possible that the minimal responses of wing size to adult sex ratio observed might have been masked to some extent in the protein restricted regimes.

The overall wing size results suggested that in both male and females, the response to an increase in male sexual selection is to increase wing size, whilst a decrease in male-male competition drives wing size down. This effect was somewhat degraded in lines evolving under adult protein restrictive diets, but trends relating to sex ratio in divergence of wing size are still observable in 'low' diet regimes. The effect of dietary restriction extending wing length and area seems counter to available research and suggests another unmeasured characteristic of the population maintenance. Females typically congregate on food resources. However, it has been observed during maintenance of these populations that the flies do not mass on the protein

restrictive food. It remains unclear what benefits larger wings might provide in this context and further studies can shed light on these phenotypes by measuring flight strength, or courtship behaviour. Additionally, it is uncertain what influence sex ratio has on development time but the interaction between dietary restriction and male bias population (LMB) is interesting.

Conclusion

In summary, these results show that long-term variation in sex ratios did not select strongly on development time but did drive divergence in wing size, a trait which is key to the successful acquisition of mates. Additionally, it was clear that, when maintained under protein restriction as adults, wing size differences were reduced, indicating the existence of potential fitness costs. The variation in wing size produced by all restrictive adult protein flies was interesting and additional studies of wing morphology, musculature and performance may help to elucidate its adaptive functions.

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Supplementary material

Table S3.1. Output of GLMER for time to pupariation

Pupariation time	Estimate	Std. Error	z value	Pr(>|z|)
SexRatioFB	0.03370	0.07084	0.476	0.63423
SexRatioMB	-0.04673	0.07127	-0.656	0.51206
FoodLow	-0.09310	0.07151	-1.302	0.19295
SexRatioFB:FoodLow	0.01810	0.10081	0.180	0.85753
SexRatioMB:FoodLow	0.32153	0.10039	3.203	0.00136 **

Table S3.2. Output of GLMER for male & female time to eclosion

Eclosion time	Estimate	Std. Error	z value	Pr(>|z|)
SexRatioFB	-0.04688	0.04810	-0.975	0.32978
SexRatioMB	-0.04785	0.04811	-0.995	0.31995
FoodLow	-0.11715	0.04859	-2.411	0.01590
SexRatioFB:FoodLow	0.04518	0.06893	0.655	0.51216
SexRatioMB:FoodLow	0.20921	0.06818	3.068	0.00215

Table S3.3a. Output of GLMER for time to eclosion for males only

Eclosion time	Estimate	Std. Error	z value	Pr(>|z|)
SexRatioFB	-0.04955	0.07517	-0.659	0.5099
SexRatioMB	-0.03437	0.07504	-0.458	0.6470
FoodLow	-0.12467	0.07586	-1.643	0.1003
SexRatioFB:FoodLow	0.11256	0.10716	1.050	0.2935
SexRatioMB:FoodLow	0.18741	0.10653	1.759	0.0786 .

Table S3.3b. Output of GLMER for time to eclosion for females only

Eclosion time	Estimate	Std. Error	z value	Pr(>|z|)
SexRatioFB	-0.040176	0.049351	-0.814	0.41560
SexRatioMB	-0.061520	0.049622	-1.240	0.21506
FoodLow	-0.137974	0.050627	-2.725	0.00642 **
SexRatioFB:FoodLow	-0.001788	0.072345	-0.025	0.98028
SexRatioMB:FoodLow	0.258573	0.070406	3.673	0.00024 ***

Table 3.4 Post hoc comparisons of development time differences between adult sex ratios

Sex Ratio	Estimate	Std. Error	z value	Pr(>|z|)
FB - EQ	-0.04955	0.07517	-0.659	0.787
MB - EQ	-0.03437	0.07504	-0.458	0.891
MB - FB	0.01518	0.07546	0.201	0.978

Table S3.5a Cox model output for female likelihood of eclosion

Term	Z-value	P-value
SexRatioFB	-0.169	0.865
SexRatioMB	0.645	0.519
FoodLow	-0.031	0.976
SexRatioFB:FoodLow	-0.559	0.576
SexRatioMB:FoodLow	-1.454	0.146

Table S3.5b Cox model output for male likelihood of eclosion

Term	Z-value	P-value
SexRatioFB	0.539	0.590
SexRatioMB	-0.052	0.959
FoodLow	-0.598	0.550
SexRatioFB:FoodLow	-0.245	0.807
SexRatioMB:FoodLow	-0.120	0.905

Table 3.6a Output of lmer for log10 transformed egg length.

Egg length	Estimate	Std. Error	Residual DF	z value	Pr(>|z|)
SexRatioFB	-0.009020	0.007729	17.999988	-1.167	0.258
SexRatioMB	0.009687	0.007729	17.999988	1.253	0.226
FoodLow	0.007013	0.007729	17.999988	0.907	0.376
SexRatioFB:FoodLow	-0.008712	0.010931	17.999988	-0.797	0.436
SexRatioMB:FoodLow	-0.012139	0.010931	17.999988	-1.111	0.281

Table 3.6b Output for Post hoc Tukey analysis log10 transformed egg length.

Sex Ratio comparison	Z-value	P-value
FB - EQ	-1.167	0.4730
MB - EQ	1.253	0.4219
MB - FB	2.420	0.0409 *

Table 3.7a Output for lmer for log10 Egg volume

Egg volume (log10)	Estimate	Std. Error	Residual DF	z value	Pr(>|z|)
SexRatioFB	-0.06706	0.02963	18.00000	-2.264	0.0362 *
SexRatioMB	0.06275	0.02963	18.00000	2.118	0.0483 *
FoodLow	0.02840	0.02963	18.00000	0.959	0.3505
SexRatioFB:FoodLow	0.03366	0.04190	18.00000	0.803	0.4322
SexRatioMB:FoodLow	-0.07317	0.04190	18.00000	-1.746	0.0978.

Table 3.7b Output for Tukey post hoc analysis for log10 transformed Egg volume contrast.

Sex Ratio contrast	t-ratio	P-value
EQ High - FB High	1.848	0.4536
EQ High - MB High	-1.729	0.5253
EQ High - EQ Low	-0.783	0.9681
FB High - MB High	-3.578	0.0151***
FB High - FB Low	-1.710	0.5371
MB High - MB Low	1.234	0.8165
EQ Low - FB Low	0.921	0.9377
EQ Low - MB Low	0.287	0.9997
FB Low - MB Low	-0.633	0.9874

Table 3.8a Post hoc contrast of male dry body weight

Sex Ratio comparison	Z-value	P-value
FB - EQ	-1.703	0.204
MB - EQ	-0.095	0.995
MB - FB	1.482	0.299

Table 3.8b Post hoc contrast of treatments of male dry body weight

Sex Ratio contrast	t-ratio	P-value
EQ High - FB High	1.385	0.7351
EQ High - MB High	0.079	1.0000
EQ High - EQ Low	1.236	0.8151
FB High - MB High	-1.229	0.8191
FB High - FB Low	-0.909	0.9408
MB High - MB Low	2.228	0.2561
EQ Low - FB Low	-0.760	0.9719
EQ Low - MB Low	1.207	0.8297
FB Low - MB Low	1.966	0.3876

Table 3.9 Tukey post hoc contrast of treatments of female dry body weight

Sex Ratio contrast	Z value	P-value
HEQ - HMB	-0.249	0.9999
HFB - HMB	-2.410	0.1526
LMB - HMB	-2.898	0.0436 *
HFB - HEQ	-2.313	0.1885
LEQ - HEQ	-3.305	0.0122 *
LFB - HFB	-0.656	0.9865
LEQ - LMB	-0.469	0.9972
LFB - LMB	-0.134	1.0000
LFB - LEQ	0.335	0.9994

Table 3.10 Pairwise comparison of treatments of male wing area

Sex Ratio contrast	t- ratio	P-value
HMB - HEQ	0.988	0.9178
HMB - HFB	3.215	0.0348
HMB - LMB	-2.092	0.3201
HEQ - HFB	2.250	0.2486
HEQ - LEQ	-2.715	0.1043
HFB - LFB	-4.053	0.0045
LMB - LEQ	0.390	0.9987
LMB - LFB	1.289	0.7881
LEQ - LFB	0.899	0.9435

Table 3.11 Pairwise comparison of treatments of female wing area

Sex Ratio contrast	t- ratio	P-value
HMB - HEQ	0.964	0.9253
HMB - HFB	2.883	0.0733
HMB - LMB	-1.917	0.4133
HEQ - HFB	1.936	0.4034
HEQ - LEQ	-2.760	0.0954
HFB - LFB	-3.590	0.0145
LMB - LEQ	0.142	1.0000
LMB - LFB	1.238	0.8145
LEQ - LFB	1.096	0.8786

Chapter 4: Effect of experimental evolutionary manipulations of adult sex ratio and adult nutrition on reproductive morphology and reproductive success in *Drosophila melanogaster*

Abstract

The experimental manipulation of adult sex ratio has proven to be a useful tool for testing the short- and long-term effect of varying the intensity of sexual selection. Here I investigated the impact of sexual selection on the reproductive morphology of *Drosophila melanogaster* by artificially manipulating adult sex ratio and adult diet for over 110 generations. Previous studies have shown that *D. melanogaster* respond to long-term changes in adult sex ratio by modifying their courtship behaviour and mating investment as well as their investment in morphological traits such as body weight and wing size. Such morphological characteristics have also been reported to vary as a function of protein restriction in the adult diet. Here, I predicted that modifying the strength of sexual selection and adult nutrition simultaneously would select strongly on the reproductive morphology of both male and female *D. melanogaster* to optimise reproduction. I used *D. melanogaster* populations that had evolved for over 110 generations under either male biased, female biased or equal adult sex ratios at either a standard or low-protein adult nutrition. I predicted that a history of high or low sexual competition would select for differences in investment in reproductive morphology. Specifically, the females from male biased regimes would be exposed to higher levels of male ejaculates, due to increased number of males, and therefor mating events, in comparison to females from female biased regimes. I predicted that this would select for larger sperm storage organs in females from the male biased lines. Conversely males from male biased lines were expected to be exposed to a higher risk of sperm competition, selecting for longer sperm and larger testes. These predictions were both expected to be affected by the adult nutritional regimes, with fewer adult resources available expected to reduce the extent of the overall morphological responses to selection. The results showed that, in line with the prediction, females from male biased lines had significantly larger spermathecae, but not seminal receptacles, with no differences seen according to adult dietary

regime. This increase in sperm storage organ size did not translate to a higher fecundity over 15 days, following a single mating. However, variation in progeny production by females from male biased selection lines over this time was significantly influenced by adult dietary regime. An increase in testis size was evident, as predicted, in males from the male biased sex ratio. However, this difference was not significant, nor was it affected by adult diet regime. Males from the female biased regimes had significantly larger accessory glands, suggesting that frequent mating's by males selects for larger non sperm ejaculates. The results show that there are significant effects of manipulating sex ratio on reproductive morphology due to levels of sperm competition. Females may adapt larger spermatheca to endure increased exposure to ejaculate components. Meanwhile, males may have evolved larger accessory glands in populations with greater access to mating opportunities. The lack of response of reproductive morphology to adult diet supports the idea that larval not adult nutritional regimes determine the relative size of these key reproductive characteristics.

Attribution statement

I would like to thank Mabel Sydney and Alex Siddall for their help with the mating assays.

Introduction

Sexual selection is focussed on the various traits that ensure success in reproduction (Darwin, 1871). Many studies on this topic have been directed towards the behaviours, strategies, or adaptations that lead to success in securing an opportunity to mate (pre-copulatory competition) (Jennions and Petrie, 2000). Variation in the success of mating and reproduction determines the strength of selection on the relevant suites of reproductive traits involved (Hosken and House, 2011, Trivers, 1972). Pre-copulatory sexually selected traits can represent signals to a potential partner that an individual is not only ready to mate but is also of a high quality (Petrie et al., 1991). Pre-copulatory strategies are varied and can be linked in many instances to the evolution of elaborate phenotypes that serve in obtaining mates and mating's (Darwin, 1871). Such traits have long fascinated evolutionary biologists because, in some cases, pre-copulatory morphological or behavioural traits may interfere with the expression of other ecologically important functions such as sight, smell, or locomotion (Emlen, 2001, Goyens et al., 2014, Goyens et al., 2015). Competition through physical contest may be the most obvious signal of a partner's quality. For example, male Rhinoceros beetles (*Trypoxylus dichotomus*) compete against other males for feeding territories, and there is a clear correlation between longer horns and winning fights (Karino et al., 2005). Males that hold territories gain access to the females that come to feed in their territories (McCullough et al., 2012). Hence the phenotype for long horns, which is associated with winning fights and holding territories is strongly selected. However, in the many species in which males' mate with multiple partners, pre-copulatory selected traits may become more costly either because they are expensive to maintain (Siva-Jothy, 1987), or because they trade-off with post-copulatory investment required to obtain fertilisations (Kvarnemo and Simmons, 2013).

The evolution of post-copulatory strategies can be equally as diverse as that seen in pre-mating traits involved in acquiring access to mating's. For example, direct sperm competition is a key way in which males attempt to acquire paternity over their rivals. The ability to produce more sperm to become more competitive can result in a larger investment in a single mating or permit multiple mating's (Lupold et al, 2020). Additionally, the capacity to replenish sperm rapidly allows more frequent, effective

mating's (Lüpold et al., 2020a). It is not only the production of numerous sperm that can be subject to strong selection, but also sperm morphology (Simmons and Garcia-Gonzalez, 2021). Longer sperm can be associated with faster swimming speeds and such sperm may be better able to dislodge smaller sperm, resulting in a higher fertilization rate (LaMunyon and Ward, 1998). Moreover, in *Drosophila* sperm length has been shown to evolve relatively rapidly, compared to other phenotypes (Fitzpatrick et al., 2020) and is strongly correlated with evolution of female sperm-storage organs (Miller and Pitnick, 2002), and that size may play a role in sperm precedence (Miller and Pitnick, 2002). Additionally, seminal fluid proteins can play an important role in post-copulatory fertilisation success (Chapman, 2001). Male *Drosophila* produce a diverse array of seminal proteins (Zeender et al., 2023, Sepil et al., 2019), which have a range of functions (Chapman and Davies, 2004) and perform essential biological roles when transferred into females (Neubaum and Wolfner, 1999, Chapman et al., 2000). Key seminal fluid proteins are produced in the accessory gland of *D. melanogaster* and their expression is sensitive to the prevailing levels of sperm competition (Hopkins, et al, 2019). For example, males selected for larger accessory glands show a competitive advantage in fertilization events (Wigby, et al., 2009). These seminal fluid proteins can increase the likelihood of paternity for a male by decreasing the recipient female's likelihood of remating (Bretman et al., 2010c) or increasing the efficiency of sperm storage (Avila and Wolfner, 2009, Tram and Wolfner, 1999).

The mechanisms by which these diverse post-copulatory strategies evolve are less well characterised. In particular, the role that post-copulatory sexually selected traits in males can have on female phenotypes, or indeed the effect that selected female phenotypes have on a male's reproductive morphology, remain obscure. *D. melanogaster* has proven to be a valuable model for evolutionary studies of the significance of sexual selection over many decades. The mating system and biology of this species allows for investigation of both pre- and post-copulatory phenotypes that are subject to sexual selection. Here I addressed some key gaps in knowledge by using populations of *D. melanogaster* held at female bias (FB), equal sex (EQ) or male bias (MB) adult sex ratios under a standard or a reduced protein diet. After 110 generations of selection under these regimes I measured the effect of increased or decreased levels of sexual selection on *D. melanogaster* male and female

reproductive morphology (male testes, seminal vesicle, and accessory gland, and female spermathecae and seminal receptacle).

The observation that females can reject, retain, or select favoured sperm appear to be common across taxa but the mechanisms are not well known. Such cryptic female choice allows females to selectively utilise a “preferred” male’s sperm (Albo and Peretti, 2015) or eject unwanted sperm from an undesirable male (Sato et al., 2017). Across many taxa, diverse strategies in females have evolved to maximise the fitness advantages of limited mating opportunities and the efficient use of sperm. The effective storage of sperm by females is highly specialised in insects and there are a wide variety of different types and numbers of sperm storage organs (Pitnick et al., 1999). Even within the *Drosophila* fruit fly the genus*,* the morphology and relative size of the paired spermathecae are surprisingly diverse across species (Pitnick et al., 1999). Some species of *Drosophila* (including in *D. melanogaster*) also possess a second sperm storage organ - the seminal receptacle - which varies tremendously in length (0.41mm to 81.67mm) across species (Pitnick, et al., 1999). These organs are key to both male and female reproductive success. For example, it has been shown that fertilization success can be linked to female reproductive morphology, and that selection for an increase in seminal receptacle length correlates with sperm length (Miller and Pitnick, 2002). Previous studies have looked at adult sex ratio effect on the rate of depletion in male testes and accessory glands after repeated mating’s (Linklater et al., 2007). It was found that after 53 generations males from male biased (MB) lines showed significantly greater depletion in their accessory gland, but not testis, volume upon serial mating’s, in comparison to FB lines. This suggested that there were divergent patterns of investment in reproductive strategies in response to levels of male competition.

Here, I built upon this previous work by testing whether there were any corresponding phenotypes evident in the female sperm storage organs (seminal receptacle length and spermathecal size). Previous studies have shown that the secretory molecules produced by the female sperm storage organ can facilitate the storage and viability of stored sperm (Wolfner, 2011, Schnakenberg et al., 2011). The sperm stored in different sperm storage organs are also stored and used in a temporally different manner (Fowler, 1973). For example, fluorescent microscopy techniques show that the sperm stored in the seminal receptacle are used first in

fertilizations (Laturney et al., 2018). This and many other studies (Miller and Pitnick, 2002, Pitnick et al., 1999, Fitzpatrick et al., 2020) provide evidence for discrete functions of the different sperm storage organs, which could facilitate female cryptic choice of some sperm over others.

The male testes, seminal vesicle, accessory gland, and sperm size are all key elements of male reproductive morphology directly linked to fitness. They could be subject to variation in sexual selection independently or in concert. For example, sperm competition in the dung beetle *Onthophagus taurus* is associated testes size, and males with larger testes have a fertilization advantage (Simmons and Garcia-Gonzalez, 2021). The seminal vesicle is a distinct region at the basal end of *D. melanogaster* testes, which is used to store mature sperm. The ability to store more mature sperm allows for an individual to capitalise on multiple mating opportunities or transfer an increased investment in a single mating (Laturney et al., 2018). One study detected an increase in size of the seminal vesicle of male *D. melanogaster* when exposed to a rival male (Moatt et al., 2014), so it is possible that long-term exposure to a male biased sex ratio could select for the evolution of larger seminal vesicles. Additionally, the size of the sperm head could also respond to selection arising from variation in the level of sperm competition experienced (Simmons et al., 2021). Sperm are diverse and respond rapidly to levels of sexual selection (Ward, 1998). However, there is currently no consensus on whether longer sperm are necessarily the most competitive (Simpson et al., 2014, Pitnick and Markow, 1994, Morrow and Gage, 2001, Godwin et al., 2017a).

Varying the degree of sexual selection under experimental evolution has been an effective tool to show the fitness impact of pre- and post- copulatory traits (Godwin et al., 2017a). In addition to modifying the strength of sexual selection I also investigated the effect of adult diet on the evolution of reproductive phenotypes. For example, dietary restriction has been shown to impact fecundity in female butterflies (Jaumann and Snell-Rood, 2019), somatic maintenance in *D. melanogaster* (Zajitschek et al., 2018) and body condition in *D. grimshawi* (Droney, 1998). However, few if any of these studies simultaneously assessed the impact of adult diet (potential condition dependency) with sexual selection.

Here I focused on testing the impact of long-term variation in sexual selection and adult diet on reproductive morphology of both sexes. Using *D. melanogaster* exposed to evolutionary divergent adult sex ratios of male bias (MB), equal sex (EQ) and female bias (FB) and adult dietary regimes maintained on standard SYA diets or SYA containing only 20% of the protein in comparison to the standard control, I tested the impact of adult sex ratio and adult diet on reproductive and morphological phenotypes. That these populations retain sufficient genetic variation to respond to the experimental manipulation of sexual selection and adult diet is highlighted by previous studies in which distinct pre- and post-copulatory responses have been documented (Sepil et al., 2022a, Dore et al., 2021). Additionally, I also tested whether any morphological differences were associated with the rate and duration of offspring production in females. I predicted that reproductive traits would change in response to manipulation of the intensity of sexual selection and diet as follows:

Table 4.1. Predicted responses of reproductive morphology in both sexes to long-term variation in sexual selection and adult diet.

Traits	Expectation	Rationale
Female spermathecae & seminal receptacle size	Evolve to be larger in females from male biased populations	Females from male-biased lines will be exposed to higher numbers of more diverse sperm and ejaculate components (Wedell et al., 2002, Eberhard, 1996). To increase the opportunity for cryptic female choice, or store more sperm overall, females could evolve larger sperm storage organs (Pitnick et al., 1999, Hosken et al., 2001).
Female spermathecae, seminal receptacle size and fecundity	Evolve to be smaller in females evolved under protein restrictive adult diets	Trade-offs between different traits may be more acute under nutritional stress (Partridge et al., 2005a), leading to lower resources available for sperm storage organs. Females held with males from male-biased lines should also produce more offspring over a longer period (Collin and Ochoa, 2015).
Male testes, seminal vesicles, and	Evolve to be larger in male biased populations	Potential to gain fitness advantages in male-biased regimes from increased investment in ejaculates and sperm numbers (Lymbery et al., 2019, Wedell et al., 2002, Pitnick et al., 2001).

accessory glands		
Male testes, sperm size and accessory glands	Evolve to be smaller in males evolved under protein restrictive adult diets	Trade-offs between different traits may be more acute under nutritional stress (Kolss and Kawecki, 2008), leading to lower resources available to invest in reproductive traits (Gray et al., 2018).
Sperm Head size	Evolves to be larger in male-biased regimes	Should be selection for increased sperm size in male-biased regimes, assuming this gives a fitness advantage in sperm competition (Gomendio and Roldan, 2008).

Methods

Experimental evolution line maintenance

Experimental evolution lines comprised 18 Sex Ratio lines consisting of three independent replicates of each of Equal Sex (EQ (50M: 50F)), Female-Biased (FB (25M: 75F)) and Male-Biased (MB (70M: 30F)) lines, maintained as adults on either high or low protein diets (see below). Populations were maintained within plastic boxes (12cmW x 18cmL x 8.5cmD, with gauze lid) at 25°C in a humidified room with a 12 h light: 12 h dark cycle. High protein lines were given access as adults to two fresh, standard SYA medium vials every two or three days (100g brewer's yeast, 50g sucrose, 15g agar, 30mL Nipagin (10% solution), 3mL propionic acid, 0.97L water). The Low protein lines had access as adults to 20% SYA medium (i.e. 20% of the standard amount of yeast in the diet, all other ingredients remaining unchanged). All regimes were raised at standardised densities (one hundred larvae per vial) and on standard SYA medium during development. Nine days after setting up the adults in the boxes, each line was supplied with an initial agar-grape juice egg collection plate (50g agar, 600mL red grape juice, 42mL Nipagin (10% solution), 1.1L water), this was repeated on the 10th day.

Egg collection plates were maintained at 25°C following removal from the boxes and kept in cotton bags for two days to allow egg hatching. Four hundred larvae were then picked from each of these egg collection plates and placed in densities of one hundred per vial for each line (four replicates vials of one hundred larvae each), on standard SYA for all regimes. After eclosion, flies were anaesthetised using CO_2 and

counted into their specified sex ratios and placed in the plastic boxes as described above to initiate the next generation. The lines had been maintained under these conditions since 2013 until COVID-19 restrictions began in March 2020. Maintenance for these lines was then relaxed whereupon cultures were maintained in bottles on SYA medium (two bottles per replicate) and maintained at 18°C. This reduced the number of generations of relaxed selection. All flies used in this experiment had experienced 110 generations of selection under the sex ratio and dietary regimes as described above. Flies used in this experiment were from generations 145 and 146, having spent a total of 35 and 36 generations under subsequent relaxed selection imposed by COVID-19 restrictions.

The effect of evolutionary manipulation of adult sex ratio and nutrition on the reproductive morphology.

The rearing environment of the experimental flies was standardised. To do this, equal sexes (50 males and fifty females) from all populations were isolated using CO_2 and transferred to the standard rearing plastic boxes (12cmW x 18cmL x 8.5cmD, gauze lid) and supplied with two agar-grape juice egg collection plates for 24h. One hundred larvae from each population were collected and transferred to fresh SYA medium vials (10 larvae per vial; SYA: 100g brewer's yeast, 50g sucrose, 15g agar, 30mL Nipagin (10% solution), 3mL propionic acid, 0.97L water). Thirty males and thirty females from each population and replicate were collected as virgins as they eclosed and isolated for five days to ensure all flies were sexually mature at the point of the dissections. At five days old, all the experimental flies were transferred to 1.5ml microtubes, flash frozen in liquid nitrogen, then transferred to storage at -80°C until the dissections were done.

Measurement of male reproductive morphology.

Samples were removed from the -80°C freezer and allowed to thaw on ice for 30 minutes before dissection. Both wings were removed, and the thorax photographed using a Zeiss Discovery V.12 stereomicroscope at 4x magnification. A drop of 1 x Grace's medium was placed on a glass slide and, using a pair of fine forceps, the male was held in on its side by the thorax. A small incision was then made into the distal end of the abdomen using a second pair of sharpened fine forceps. The

aedeagus was pulled gently away from the body until the testes and accessory glands emerged, or the testes could also be partly pushed out from the abdomen cavity, if needed, using the first pair of forceps. Once separated from the body, any extraneous tissue surrounding the testes and accessory glands was gently teased away and the testes were straightened. The testes and accessory glands were then photographed using Zeiss Discovery V.12 stereomicroscope at 4x magnification. All images measured in ImageJ (N = 15 individuals per replicate) and calibrated at the corresponding magnification using a micrometre. Both testes and accessory glands were measured, and an average used for analysis.

Preparation of sperm for measurement of sperm head size using fluorescence microscopy.

The seminal vesicle contains mature, individualised sperm, and is located at the proximal end of the testes. This part of each testis was dissected and placed into a fresh drop of Grace's medium. The seminal receptacle was then gently disrupted, and the mature sperm allowed to disperse into the medium. Samples were air dried for 4-6hr to ensure sperm cells were fixed to the glass slide. 1x Phosphate Buffered Saline (PBS) was applied to the edge of the slide at an angle, washing away any additional debris. Sperm were then stained by flooding the slide with a 1:1000 concentrate of 4′,6-diamidino-2-phenylindole (DAPI), and then placed into a dark chamber for 30 minutes. After staining excess DAPI was removed by washing the sample with 1xPBS. The DAPI-stained sperm heads were then photographed using a Zeiss AxioStar Plus fluorescent microscope at 350nm with DAPI filter and measured in ImageJ (N = 10 sperm heads per individual, N = 15 individuals per replicate).

Measurement of female reproductive morphology.

Samples were removed from the -80°C freezer and allowed to thaw on ice for 30 minutes before dissection. Both wings were removed, and the thorax was photographed using a Zeiss Discovery V.12 stereomicroscope at 4x magnification. Adding a drop of 1 x PBS to a glass slide, the female was secured in place by

grasping the thorax with a pair of fine forceps. A second pair of sharpened fine forceps was then used to grip the apical end of the abdomen, which was then gently pulled away from the body to remove the entire reproductive tract. Care was taken to keep the reproductive tracts intact by gentle removal of the ovaries from the abdomen. The spermathecae were isolated from the reproductive tract dissections and removed to be photographed separately under 20 x magnification. The width of the spermatheca was the metric used to represent overall size. Leaving the seminal receptacle attached to the uterus provided anchorage, submerging the reproductive tract in crystal violet stain, and washing with 1 x PBS aided in the identification and removal of the connective tissue holding in the seminal receptacle in a coiled position. Once this was done, the seminal receptacle could be uncoiled on a glass slide within a shallow pool of 1 x PBS using fine mounted needles. Excess 1x PBS was removed and the seminal receptacle photographed (20x magnification) before any desiccation occurred. All images measured in ImageJ (N = 15 individuals per replicate) and calibrated at the corresponding magnification using a micrometre. Both spermatheca were measured, and an average used for analysis.

Experimental protocol testing the effect of evolutionary manipulation of adult sex ratio and nutrition on female offspring output.

To test for correlates of differences in reproductive morphology associated with offspring production, I measured the reproductive output of once mated females from all lines raised under standardised conditions (N = 10 females per replicate). Each population was transferred to an egg laying chamber (12cm diameter x 18cm high) and provided with an agar-grape juice egg collection plate for 24 h as stimulus, before providing a fresh agar-grape juice egg collection plate for a further 24 h. Larvae were then picked at densities of 20 into standard SYA vials. Upon eclosion, adults were separated by sex to ensure virginity and females then stored individually in standard SYA vials. All females were mated to standard Dahomey wild type (WT) males that were collected from cultures raised at standard densities (from agar-grape juice egg collection plates placed into wild type population cages for 24 h).

Experimental females from the lines and wild type males were all allowed to mature for 5 days before mating's occurred. The mating assay was conducted by transferring

WT males into each of the vials containing single females using an electronic pooter. I recorded time of entry, start and end of mating, equating to latency to mate and mating duration. Any mating's lasting less than 10 minutes were discarded as complete sperm transfer may not have occurred (Gilchrist and Partridge, 2000a). After the mating's, males were discarded, and females transferred over to new food vials every 24 h. Females were anaesthetised for transfers by exposing them to a brief puff of CO_2 for 3 seconds. The vacated vials were cultured for a 12-day period and all emerging progeny then counted.

Statistical analysis

All statistical analysis were performed using R-4.0.2 (R Core Team, 2020) on both absolute measurements and measurements normalised to body size. The three replicates of each sex ratio and diet regime were analysed simultaneously, with the replicates (indicated by Population) designated as a random factor. Where possible individual data were nested within Population as an additional random factor. The Shapiro-Wilk test was used to check data were normally distributed and the Levene's test to check the homogeneity of variances across treatments. Where data were not normally distributed, they were $\log_{10}$ transformed. Analysis was performed using mixed effects models using the lme4 package (Bates et al., 2015). Where data could not be transformed an appropriate family distribution was added to the model. The Akaike's Information Criterion (AIC) was used to check for model fit. Post-hoc analysis was performed using the Tukey's HSD Test. For egg count data, generalised linear mixed effect models with a Poisson distribution where used. To investigate the likelihood of an individual not laying, the results were converted into a binary operator and analysed using a GLM with a binomial distribution. To reduce the likelihood of a type I error Bonferroni's correction was used to adjust p values.

Results

Evolution of female reproductive morphology in response to long-term variation in adult sex ratio and diet regimes

The prediction that female reproductive morphology would evolve in response to differing degrees of sexual selection and adult dietary variation was upheld for the spermathecae but not for seminal receptacle size. There was no evidence for any effect on morphology of long-term variation in adult diet. The results are explained in more detail below.

Thorax size

The thorax length of females from divergent lines of ancestral levels of sexual selection did not differ significantly according to sex ratio (Table 4.1) or adult dietary regime (Fig 4.1; Table S4.2).

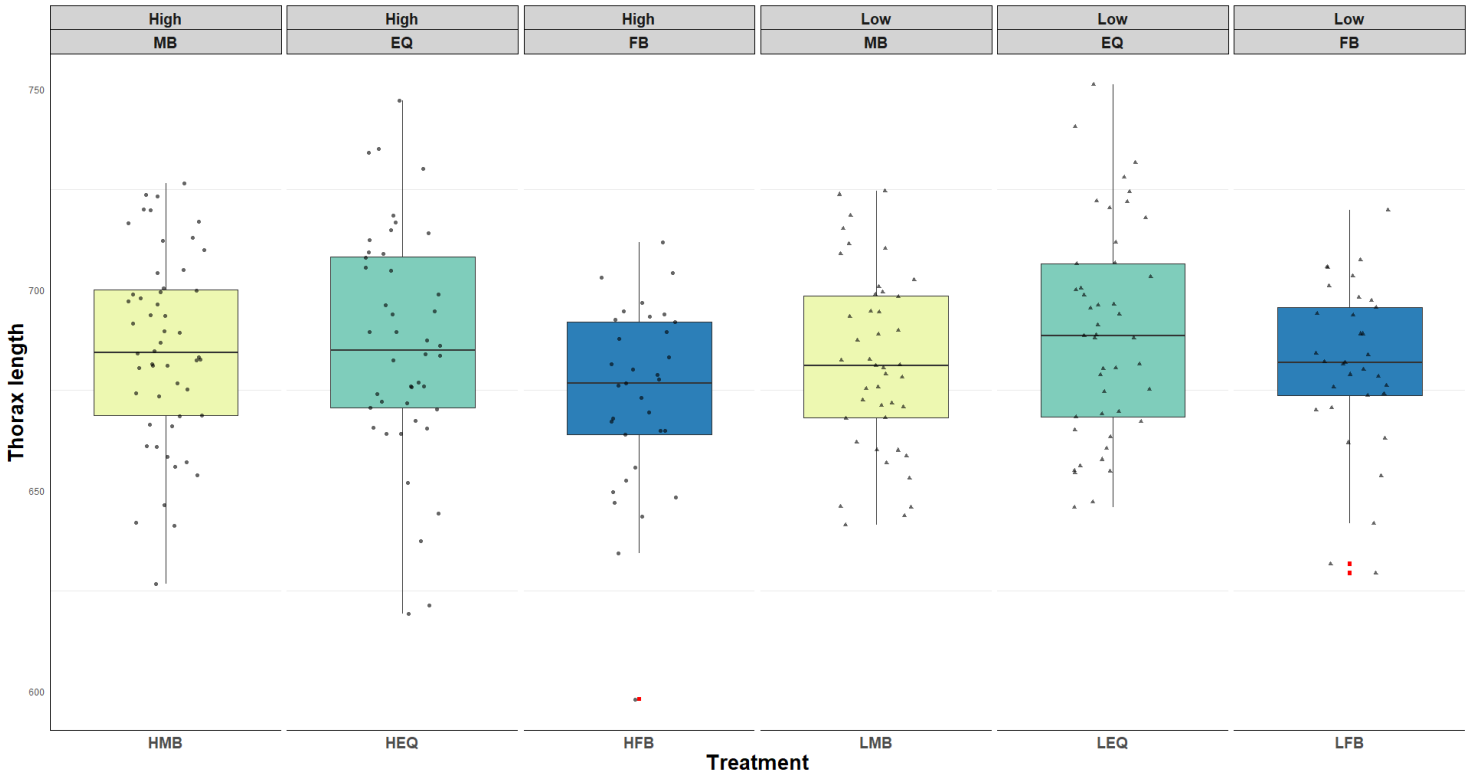

Fig 4.1. Average thorax length (μm) for females from the sex ratio regimes. Thorax measurement was taken from 5-day old virgin females from female-biased (FB), Equal sex (EQ) or Male –biased (MB) sex ratios and standard protein (High, H) or 20% protein (Low, L) diet regimes. Boxplot showing median line, with boxes representing upper and lower 25% quartile and whiskers representing the range, and points representing individual records, outliers highlighted in red.

Spermathecal size

As predicted (Table 4.1), absolute spermathecal diameter was significantly larger in females from male-biased populations (*t = 2.144, residual DF 17.86, p= 0.046), Fig 4.2a)*. However, there were no significant differences associated with adult dietary regime (Table S4.3a), which ran counter to the prediction that spermathecae would be smaller in diet-restricted regimes. When the data were normalised to body size, spermathecal diameter remained significantly larger in females from male-biased populations *(t = 3.015, residual DF 17.87, p= 0.007),* (Fig. 4.2b). A Tukey post hoc analysis reports significant differences between MB and EQ (*z = 3.015, p = 0.007*) and MB and FB *(z = 2.433, p = 0.039)* sex ratios. There were no other significant differences associated with sex ratio or adult dietary regime when normalised to body size (Table S4.3b).

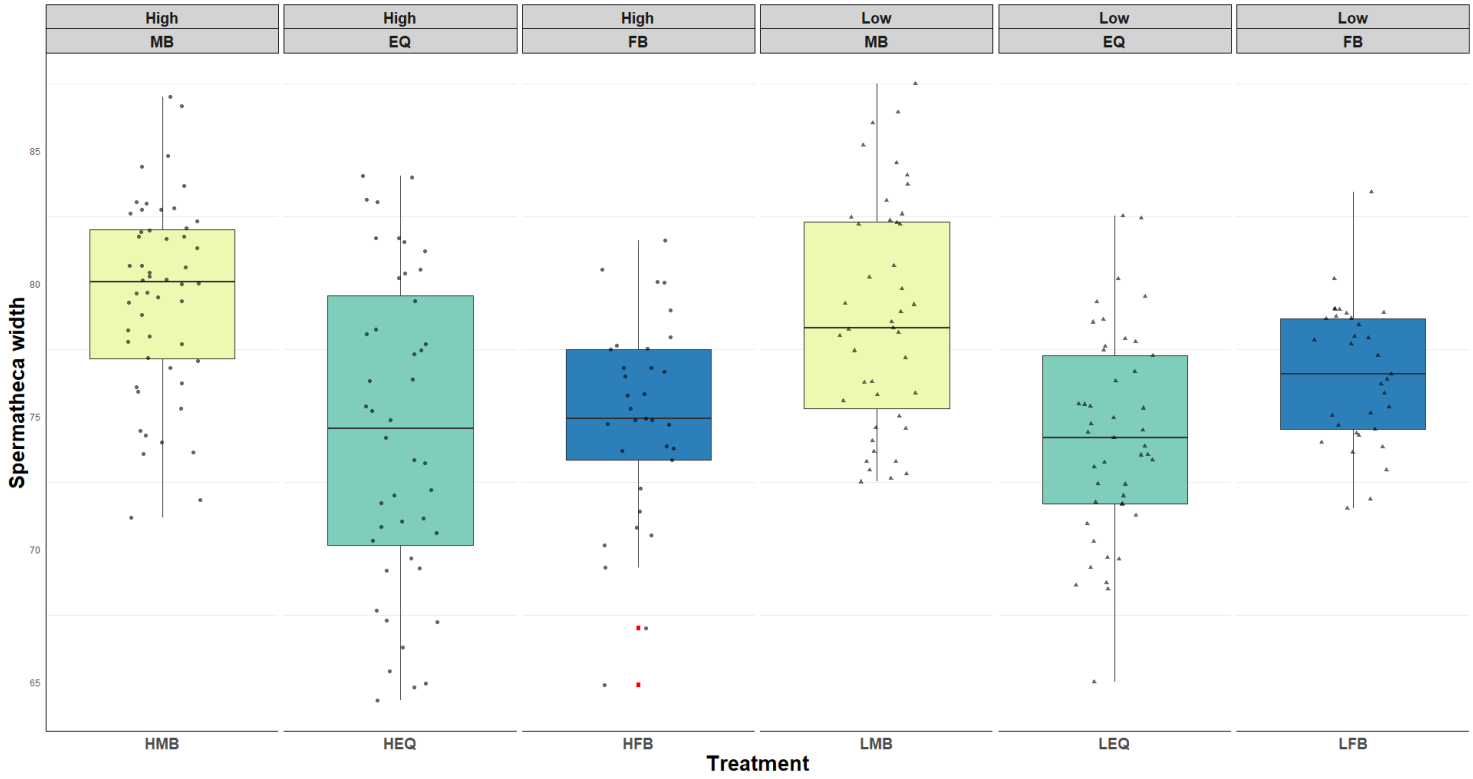

Fig 4.2a. Average absolute spermathecal diameter for females (µm) from the sex ratio and adult diet regimes. Spermatheca size measurement was taken from 5-day old virgin females from female-biased (FB), Equal sex (EQ) or Male –biased (MB) sex ratios and standard protein (High, H) or 20% protein (Low, L) diet regimes. Boxplot showing median line, with boxes representing upper and lower 25% quartile

and whiskers representing the range, and points representing individual records, outliers highlighted in red.

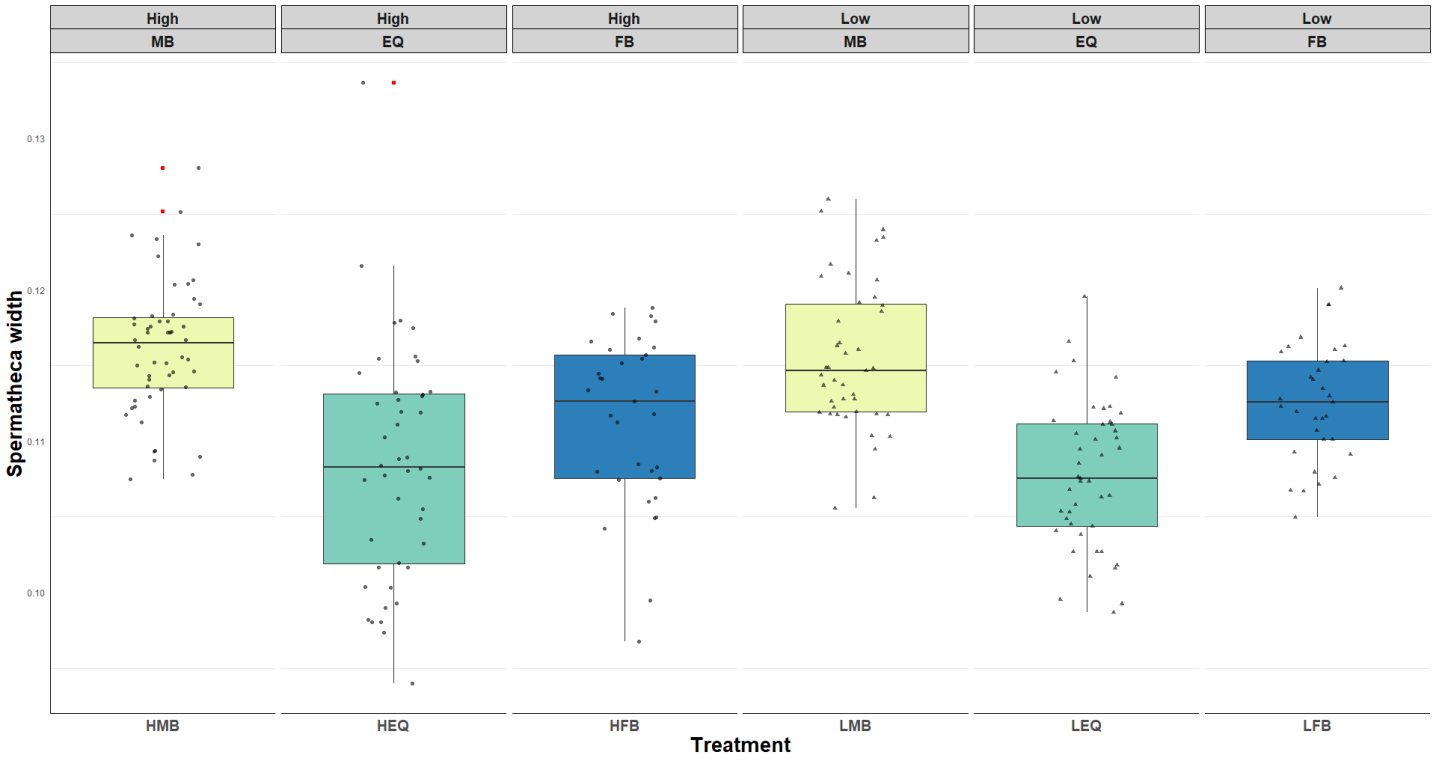

Fig 4.2b. Normalised Average Spermatheca diameter (µm) for females from the sex ratio regimes. Spermatheca size measurement was taken from 5-day old virgin females from female-biased (FB), Equal sex (EQ) or Male –biased (MB) sex ratios and standard protein (High, H) or 20% protein (Low, L) diet regimes. Measurements were then normalised to body size using thorax length as a proxy. Boxplot showing median line, with boxes representing upper and lower 25% quartile and whiskers representing the range, and points representing individual records, outliers highlighted in red.

Seminal receptacle size

In contrast to the prediction (Table 4.1), female seminal receptacle length did not differ significantly according to sex ratio or adult dietary regime (Table S4.4a, Fig 3A;

Fig 4.3a). This did not differ when the data was normalised to body size (Table S4.4b, Fig 4.3b).

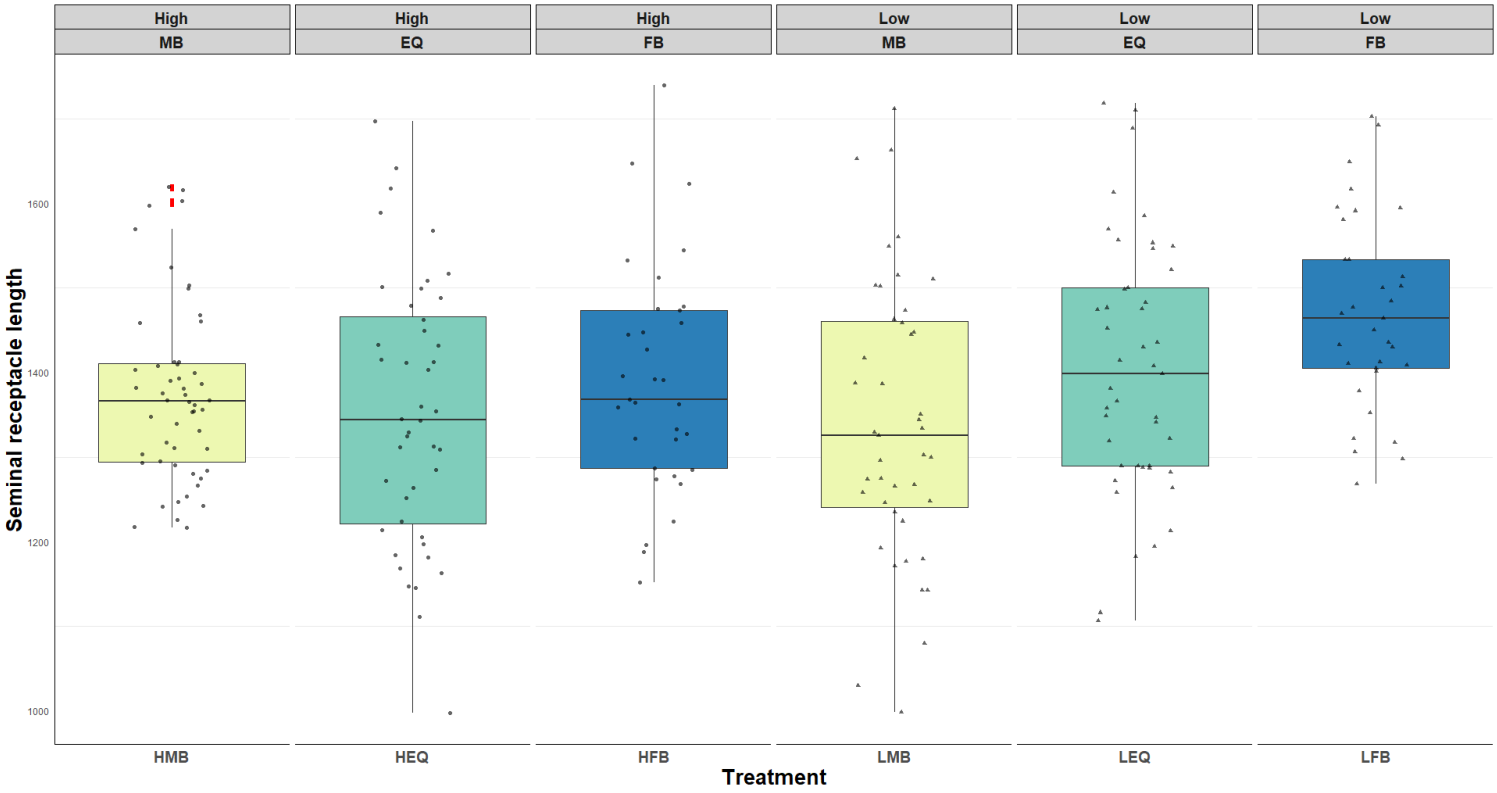

Fig 4.3a. Average Seminal receptacle length (µm) for females from the sex ratio regimes. Seminal receptacle length measurement was taken from 5-day old virgin females from female-biased (FB), Equal sex (EQ) or Male –biased (MB) sex ratios and standard protein (High, H) or 20% protein (Low, L) diet regimes and transformed using log10. Boxplot showing median line, with boxes representing upper and lower 25% quartile and whiskers representing the range, and points representing individual records, outliers highlighted in red.

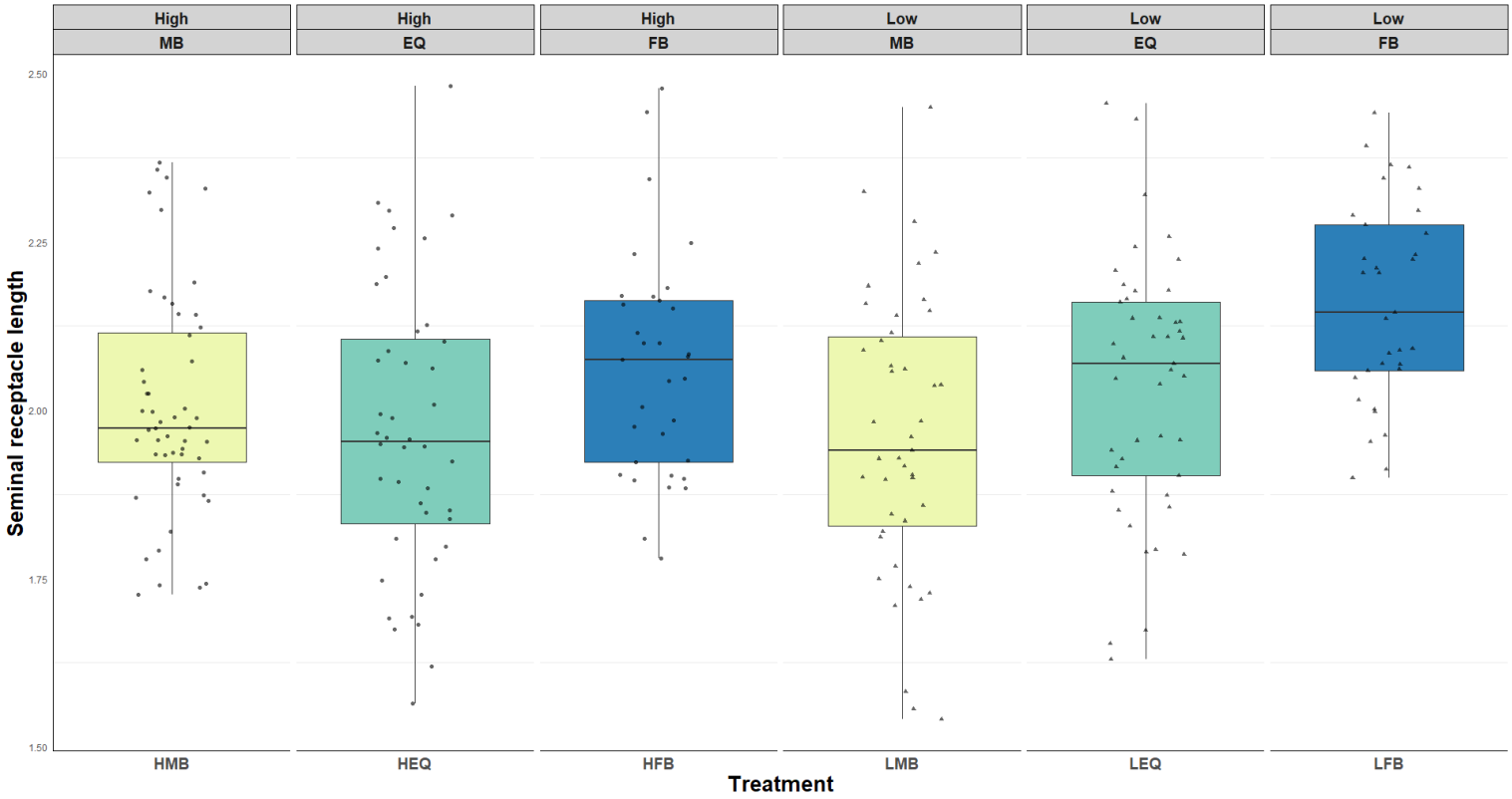

Fig 4.3b. Normalised average seminal receptacle length (μm) for females from the sex ratio regimes. Seminal receptacle measurement was taken from 5-day old virgin females from Female-Biased (FB), Equal Sex (EQ) or Male –Biased (MB) sex ratios and standard protein (High, H) or 20% protein (Low, L) diet regimes and transformed using log10. Measurements were normalised to body size using thorax length as a proxy. Boxplot showing median line, with boxes representing upper and lower 25% quartile and whiskers representing the range, and points representing individual records, outliers highlighted in red.

Evolution of male reproductive morphology in response to long-term variation in adult sex ratio and diet regimes

The prediction that male reproductive morphology would evolve in response to long-term variation in sexual selection was also upheld. However, not all size differences observed were significant, and some were not explained by sex ratio or dietary

regime. There was no evidence that the selection regimes had any effect on sperm head size.

Male thorax length

Male thorax length did not differ significantly across by sex ratio (Table S4.5) or adult diet regimes *(t = 0.293, residual DF = 17.681, p = 0.773)* (Fig 4.4).

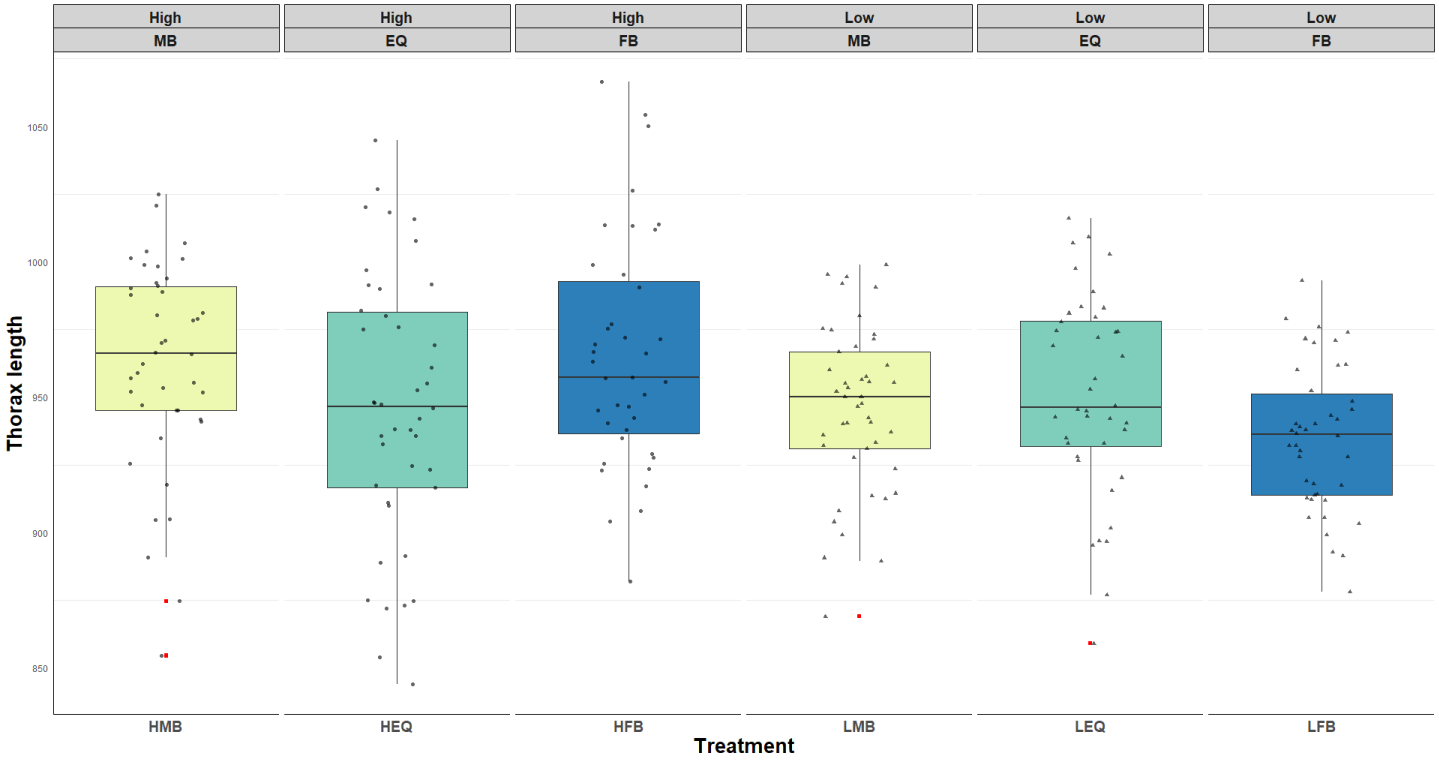

Fig 4.4. Average thorax length (µm) for males from the sex ratio regimes. Thorax measurement was taken from 5-day old virgin males from Female-Biased (FB), Equal Sex (EQ) or Male –Biased (MB) sex ratios and standard protein (High, H) or 20% protein (Low, L) diet regimes. Boxplot showing median line, with boxes representing upper and lower 25% quartile and whiskers representing the range, and points representing individual records, outliers highlighted in red.

Testis Length

Overall, counter to the predictions (Table 4.1), there was no significant main effect of sex ratio or diet on testis length (Table S4.6a). However, a significant interaction between sex ratio and diet on testes length was significant in FB low males compared to other treatments (*t = 2.538, residual DF = 18.385 p = 0.020*), with these males having longer testes compared to other males (Fig 4.5). This was counter to the prediction that it would be male-biased and / or standard diet regime males that would have larger reproductive structures. No other significant differences were detected in the reproductive morphology measures when normalised to body size (Table S4.6b).

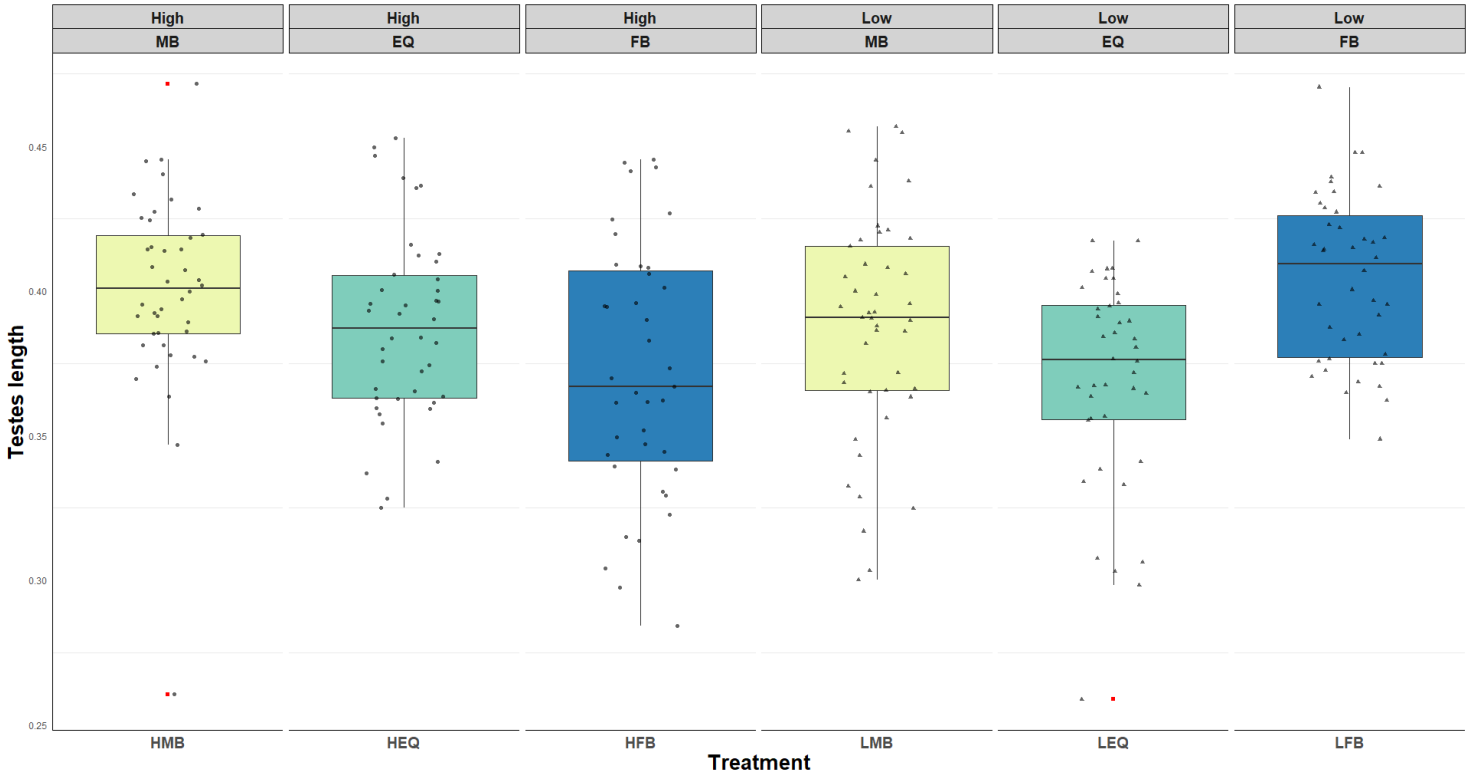

Fig 4.5. Normalised testis length (µm) for males from the sex ratio regimes. Testes length measurement was taken from 5-day old virgin males from Female-Biased (FB), Equal Sex (EQ) or Male –Biased (MB) sex ratios and standard protein (High, H) or 20% protein (Low, L) diet regimes. Measurements were normalised to body size using thorax length as a proxy. Boxplot showing median line, with boxes representing upper and lower 25% quartile and whiskers representing the range, and points representing individual records, outliers highlighted in red.

Testes Area

In line with the prediction (Table 1), the testes of MB males were generally larger by area *(t= 2.039, residual DF = 17.822, p = 0.056*). However, this trend was not statistically significant (Table S4.7a) and when normalised to body size the results were non-significant (Table S4.7b). There were no other significant differences by diet or sex ratio whether normalised or not (Table S4.7a & S4.7b), (Fig. S4.6a & Fig S4.7b).

Seminal vesicle length

Counter to expectations (Table 1), the length of the seminal vesicle did not significantly differ between sex ratio or dietary regime (Table S4.8a) (Fig.4.7). This did not differ when data was normalised to body size (Table S4.8b) (Fig. S4.8).

Seminal vesicle area

Similarly, the seminal vesicle area did not significantly differ between sex ratio or dietary regime (Table S4.9a), and this did not change when normalised to body size (Table 4.6b).

Accessory gland length.

There was no evidence that absolute accessory gland length differed significantly between any sex ratio or dietary regimes (Table S4.10a) (Fig S4.9b), and this did not change when normalised to body size (Table S4.10b).

Accessory gland area

I found that accessory gland area was significantly larger in males drawn from the female biased lines (t = 2.223, residual DF = 18.23, p = 0.039) (Table S4.11a); normalised to body size (*t = 2.469, residual DF = 18.225, p = 0.023)* (Fig.9B). This

ran counter to any of the original predictions (Table 1), no dietary effect was found in either normalised (Table S4.11a) or non-normalised data (Table S4.11b).

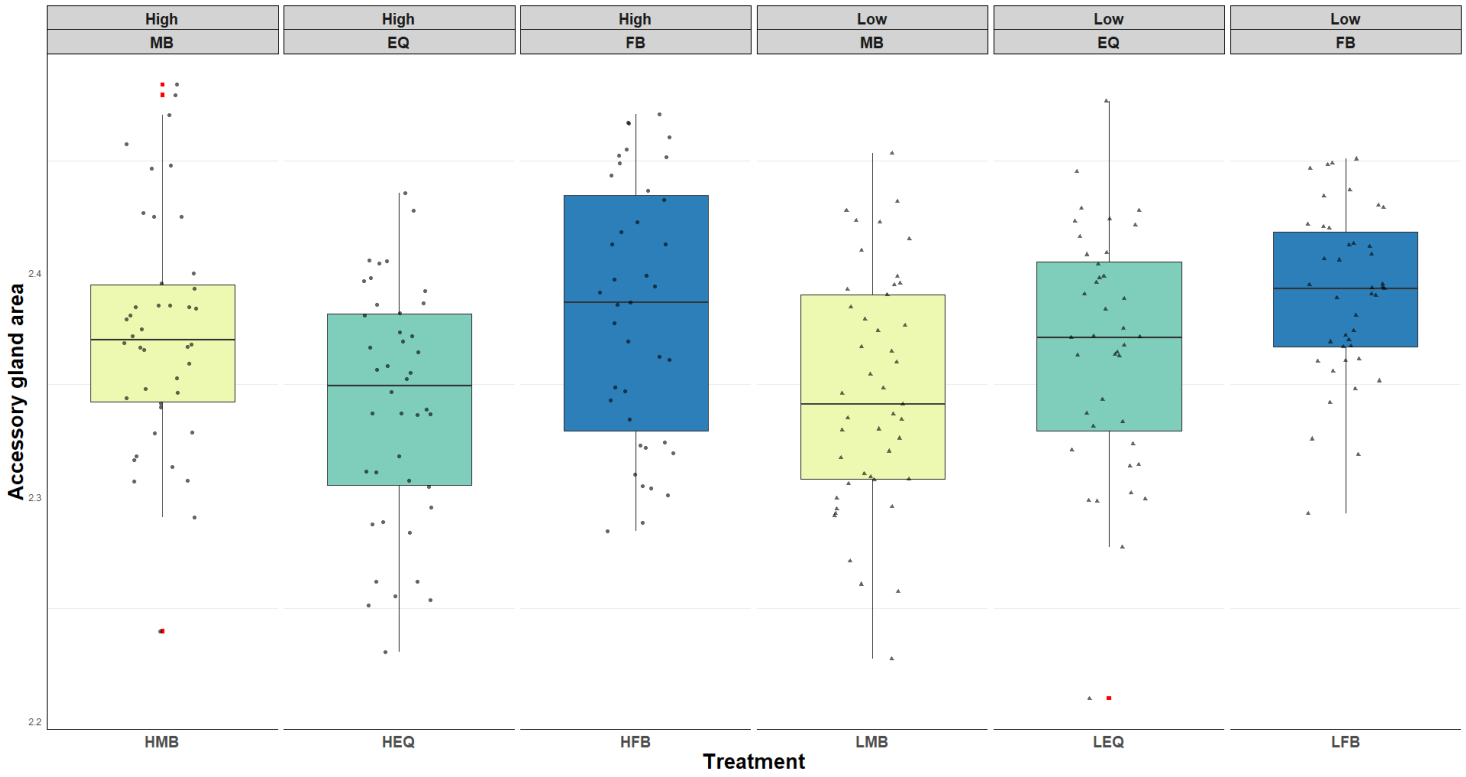

Fig 4.6. Normalised Accessory gland area (µm²) for males from the sex ratio regimes. Accessory gland area measurement was taken from 5-day old virgin males from female-biased (FB), Equal sex (EQ) or Male –biased (MB) sex ratios and standard protein (High) or 20% protein (Low) diet regimes. Measurements were normalised to body size using thorax length as a proxy. Boxplot showing median line, with boxes representing upper and lower 25% quartile and whiskers representing the range, and points representing individual records, outliers highlighted in red.

Sperm head length

Counter to expectations, there was no evidence for any significant differences in sperm head size across sex ratio or adult diet regimes (Fig S4.10).

Mating latency

A significant interaction between sex ratio and diet was reported in latency to mate (*t = 2.159, residual DF = 17.875, p = 0.044*). The results show that females from LMB regimes were slower to mate when compared to females from other regimes. In addition, a significant main effect of diet was detected, showing longer latencies to mate for females from low diet regimes. *(t = -2.103 residual DF = 17.846, p = 0.049).* A Tukey post-hoc analysis confirmed there was a significant effect of diet on latency to mate (z = -2.107, *p* = 0.035) but no significant effect of sex ratio alone (Table S4.13a) (Fig 4.7).

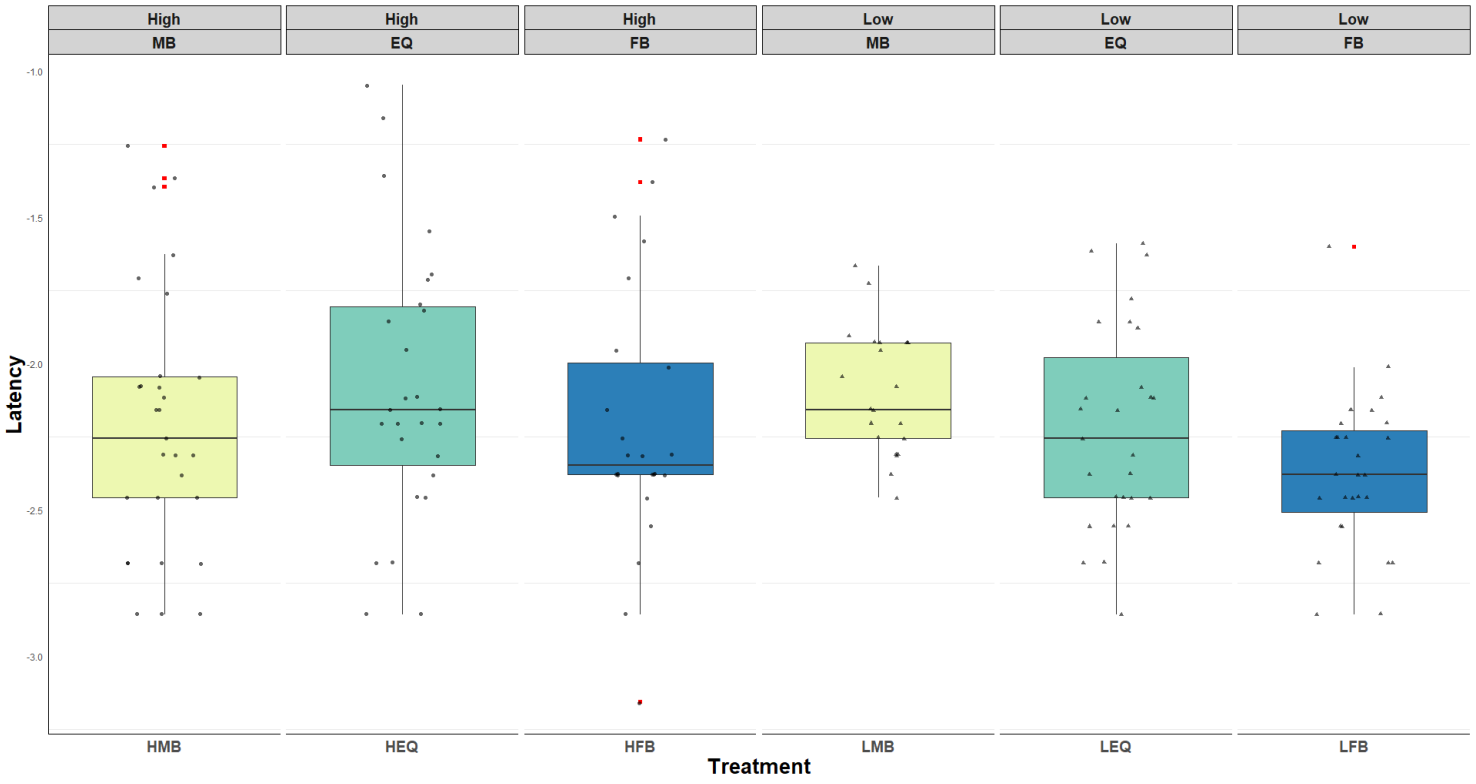

Fig 4.7. Latency to mating for females from the sex ratio regimes mated to wildtype males. Latency to mate was from 5-day old virgin wild type males mated to females from either female-biased (FB), Equal sex (EQ) or Male –biased (MB) sex ratios and standard protein (High) or 20% protein (Low) diet regimes. Measurements were Log10 transformed for better fit. Boxplot showing median line, with boxes representing upper and lower 25% quartile and whiskers representing the range, and points representing individual records, outliers highlighted in red.

Mating Duration

There was a significant effect of sex ratio on mating duration (F value = 3.467, p = 0.033). with male bias males mating significantly longer than EQ or FB regimes. No main effect of diet was detected.

Post-hoc analysis showed a significant difference between MB and FB, with females from MB regimes mating for significantly longer (Z = -2.454, p = 0.0376) (Fig 4.8). Mating duration was also longer in MB compared to EQ (z = -2.076, p = 0.0949) however, this was not statistically significant) (TableS4.13b).

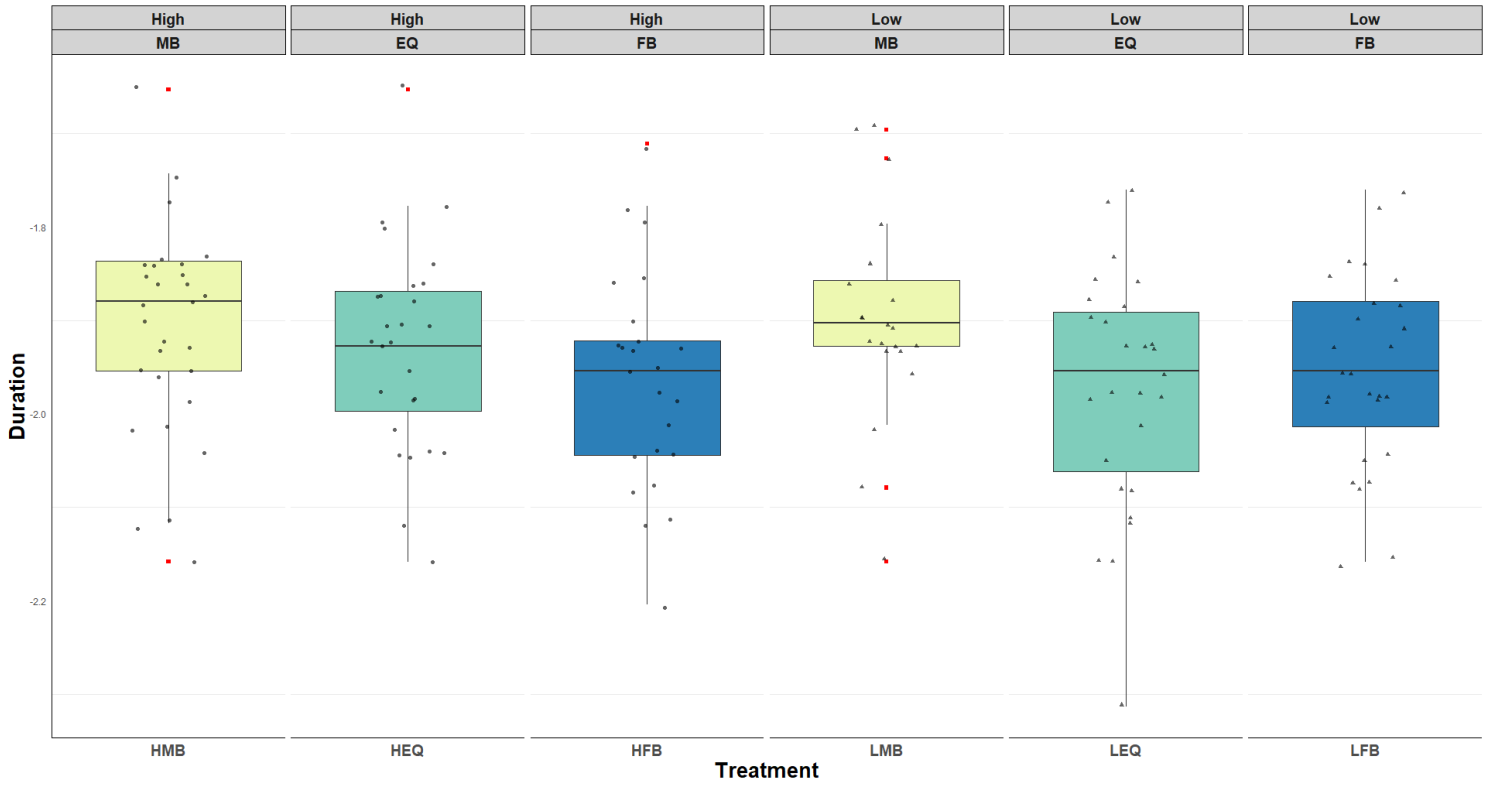

Fig 4.8. Duration of mating for females from the sex ratio regimes mated to wildtype males. Duration of mating using 5-day old virgin wild type males mated to females from either female-biased (FB), Equal sex (EQ) or Male –biased (MB) sex ratios and standard protein (High) or 20% protein (Low) diet regimes. Boxplot showing median line, with boxes representing upper and lower 25% quartile and whiskers representing the range, and points representing individual records, outliers highlighted in red.

Female offspring production over time

Overall, the total offspring production of females from the sex ratio and diet regimes over the 15-day period did not differ significantly (Table S4.14) (Fig 4.13). However, there was significant temporal variation in reproductive output per day over the test period, with a significant effect of diet ($z = 2.087$, $p = 0.036$*).* Females from Low FB and Low MB regimes produced significantly more eggs on five of the fifteen days, whereas the High food treatments did not vary significantly on any day (Fig 4.9). On day 15 the likelihood of a female not producing offspring was slightly higher in MB lines (25%) than FB lines (18%), and EQ (23%) but this effect was not significant (*p = 0.09*). Thus, the results ran counter to the prediction that reproductive output would be higher overall in females from male-biased lines.

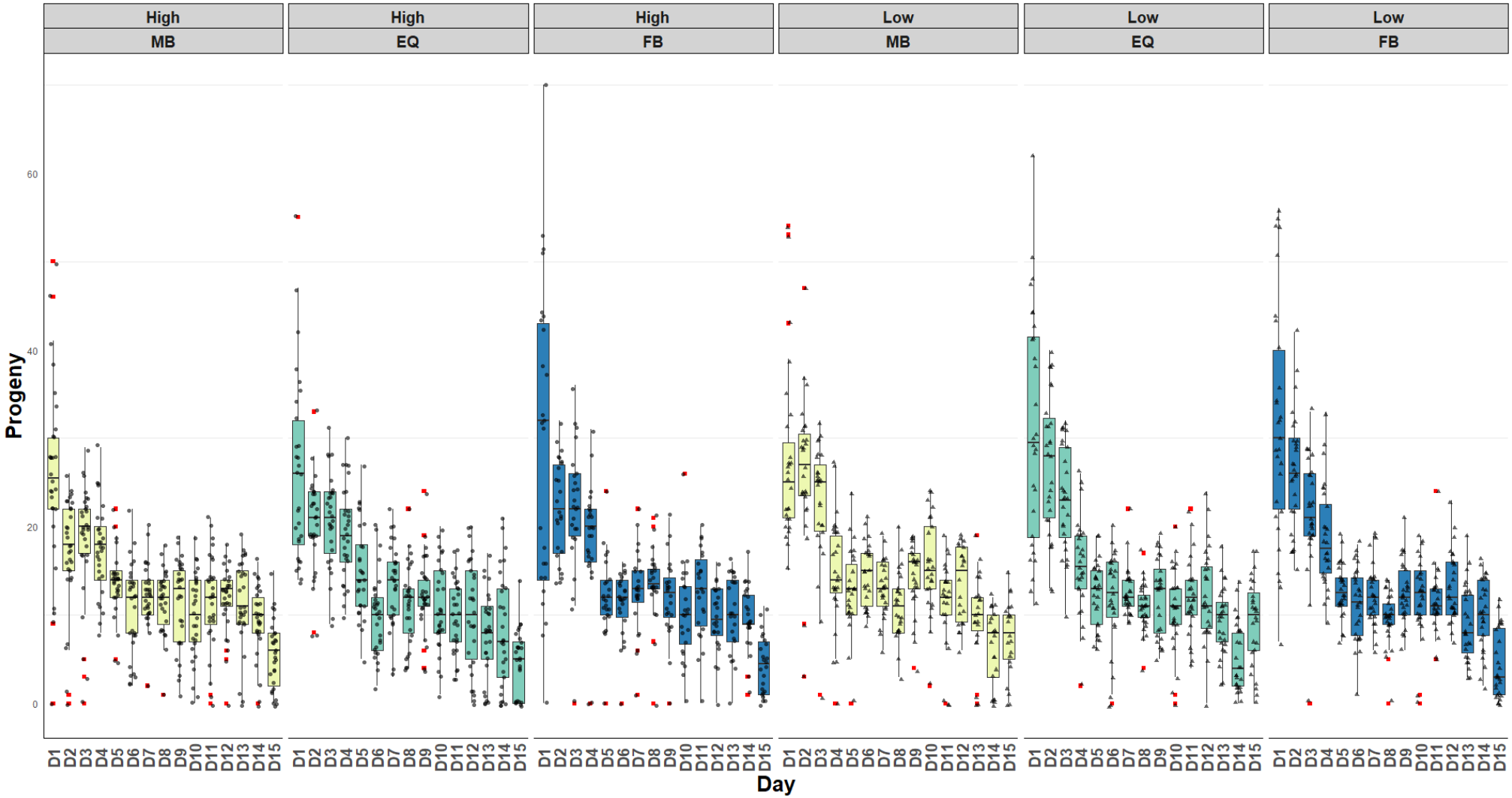

Fig 4.9. Progeny produced by females from the sex ratio regimes over 15 days. Total progeny of females from either female-biased (FB), Equal sex (EQ) or Male –biased (MB) sex ratios and standard protein (H) or 20% protein (L) diet regimes mated to wild type males. “D” represents day1 -15 on the x axis, plotted against number of progenies. The solid line represents a smooth trendline fitted to the data using the loess method with a shaded ribbon around the trendline indicating the confidence interval

Discussion

The main research question posed in this study was whether populations of *Drosophila melanogaster* exposed to an evolutionary history of variation in sexual selection and adult resource availability exhibited divergent reproductive morphologies. To test this, I measured a range of reproductive morphology data for males and females from each line. Our analysis detected evolved morphological differences between the female spermatheca with MB females having larger spermatheca than FB lines. In addition, evolved differences between FB and EQ regime males were reported, with FB males having larger accessory glands by area, in comparison to EQ males. The second question addressed whether any differences in reproductive morphology affected the reproductive fitness of females from these lines. I found significant differences between reproductive output over time due to adult nutritional regime, and an interaction for diet and sex ratio on latency to mate. However, overall numbers of offspring produced did not differ by sex ratio or adult nutritional history. Only mating duration responded directly to evolutionary variation in sex ratio, with MB females mating for significantly longer. These patterns are discussed in more detail, below.

Female sperm storage organs and fecundity

The prediction that sperm storage organs would evolve to become larger in females exposed to more mating's and thus more sperm (Table 1) was somewhat upheld. Females from male-biased (MB) lines had significantly larger spermatheca than FB females, though with no differences in seminal receptacle size across any regimes. The increased size of the spermathecae in MB females was even more apparent when normalised to body size. These findings strongly suggest an effect of adult sex ratio on this phenotype. I detected no differences in the length of the seminal vesicle. The evolution of larger seminal receptacles is strongly correlated with sperm length (Pitnick et al., 1999). However, studies have shown that spermathecae are capable of rapid evolution (Pitnick et al., 1999, Prokupek et al., 2010, Prokupek et al., 2008). Both the seminal receptacle and spermathecae are used in sperm storage. The

seminal receptacle is the recipient of the majority of sperm (Gilbert, 1981) and in *D. melanogaster* the sperm stored here take precedence in fertilization events (Laturney et al., 2018, Fowler, 1973). Correspondingly, the spermathecae are believed to be more important for long-term sperm storage (Fowler, 1973). However, studies have shown that they also produce secretions essential for sperm storage in general, and that these elements additionally work in the seminal receptacle (Schnakenberg et al., 2011). Females without spermathecae or the ability to produce spermathecal secretions suffer from reduced sperm storage (Allen and Spradling, 2008). It is possible that the larger spermathecae seen in MB females is a response to an increase in exposure to sperm from multiple males rather than the ability to store sperm for longer term. The results of the female progeny production assay did not indicate a higher overall number of offspring for MB females. Females were not dissected and checked for remaining sperm, so it is possible that the MB females could have continued producing offspring for even longer than was tested. However, the pattern of offspring produced over the 15 days and the number of females that had ceased egg laying at the end of the assay did not indicate a difference by sex ratio in favour of MB females. Further to this, studies in *D. melanogaster* mutant females with an additional spermatheca similarly showed no significant difference in offspring produced over a 20-day period (Dhillon et al., 2020). However, females with three spermathecae increased and decreased offspring production at faster rates than females with the normal two, indicating divergent sperm allocation patterns (Bangham et al., 2003). The standard maintenance of the lines used here when under selection does not extend beyond 12 days, and so any advantage in long-term sperm viability is likely to be irrelevant to fitness. Whilst the overall number of offspring produced did not respond to levels of sex ratio or diet, the pattern of investment by females did indicate an effect of adult dietary regime on egg laying pattern. This may indicate that flies from these populations allocate resources differently due to long-term exposure to low protein environments.

Female mating latency

The mating assay performed at the start of the reproductive output test was performed using five-day old virgin males drawn from the wildtype (WT) Dahomey

stock. Whilst five-day old virgin females were drawn from their respective sex ratio regimes. Latency was influenced by diet, with low food females being slower to mate than females from the high food adult diet regimes. It is possible that the WT males, perceived these females as low quality and so took longer to initiate mating. Previous work in these lines has shown that MB males take longer to initiate mating (Dore et al., 2021) and that this was affected by diet (Sepil et al., 2022a). Recent research by Fowler et al. (2022a) reports a significant effect of social environment on mating latency, with grouped females taking longer to mate (Fowler et al., 2022a). However, here I found no effect of sex ratio but instead a significant interaction, with Low MB females taking longer to start mating, compared to the other treatments. Previous experiments have shown divergence in wing length for females and this effect was greater in low food females (McConnell, Chapter 3). The observed effect on latency to mate could be attributed to female responses to male courtship or other possible cues being more difficult to maintain under a low dietary regime with higher male attention (Antony and Jallon, 1982).

Female mating duration

I observed a significant effect of sex ratio, with WT males mating with females from MB lines for significantly longer than they did with females from the other regimes. Mating duration is often viewed as a type of male reproductive investment and can be influenced by a number of factors including the male's social environment (Wigby et al., 2009b), female size, age, (Lüpold et al., 2010) mating status (Singh and Singh, 2004) and cuticular hydrocarbons (CHCs) detected during copulation (Everaerts et al., 2010). All males in this study were housed in vials with nine other male competitors prior to mating, hence their perception of sperm competition should have been similar. Additionally, all flies in this experiment were 5-day old virgins, and whilst my data from chapter 3 for these populations show a difference in female body weight, this was linked to dietary regime and not sex ratio, thorax measurements taken as a proxy for body size in the research described in this chapter showed no significant differences. The difference in mating duration here appeared to be influenced by the female encountered and their evolutionary history. This adds complexity to previous research that showed an interaction between mating duration

and male pre-copulatory social environment (Bretman et al., 2010a, Bretman et al., 2013a). Our results could indicate that females from the MB lines do not initiate decamping behaviour as readily as those from EQ or FB lines, or that they produce signals to the male, increasing their investment. One candidate signal would be the copulatory female song which is dependent on seminal fluid components in the male ejaculate (Kerwin et al., 2020). However, elevated transfer of seminal fluid components such as Sex Peptide and Ovulin have been shown to increase oviposition (Heifetz et al., 2000b, Heifetz et al., 2001, Wigby et al., 2009b), yet I did not observe an increase in progeny in relation to the manipulation of the adult sex ratio. This suggests that the wild type males did not transfer more ejaculate during mating, and that the duration of copulation was due to a different mechanism. Another candidate for the mechanism by which mating is extended could be divergent or diminished CHCs present on the females. It is known that females produce CHCs attracting male courtship (Antony and Jallon, 1982) and when olfactory cues are supressed, male *D. melanogaster* extend mating duration (Bretman et al., 2017). In male biased populations, females are exposed to an increase in male harassment, and have evolved to minimise the resulting deleterious effects (Wigby and Chapman, 2004a, Holland and Rice, 1999a). In *Drosophila serrata,* CHC expression was found to be strongly affected by sex ratio, with CHC expression increasing with female bias (Gershman and Rundle, 2017). Alternatively, a decrease in receptivity to male CHCs intended to promote female receptivity (Grillet et al., 2006) could be brought about by extended exposure to these compounds in the male biased regimes. Chemical analysis of the compounds produced by flies under these sex ratio and dietary regimes may help to shed light in the effect of sex ratio on mating duration. Mating duration was only weakly correlated with increased offspring production for high MB and high EQ lines, whilst a slight negative correlation with low EQ lines (Fig. 12b). This indistinct relationship between duration and offspring, and evidence that sperm transfer happens begins almost immediately (Gilchrist and Partridge, 2000b), suggests another function of mating duration. It is possible that mating duration could be linked to spermatheca size. MB females recorded the longest mating duration and possessed the largest spermatheca. A longer duration linked to a sperm storage organ would imply an increased investment in sperm. However, in this experiment sperm stored was not recorded. Additionally, we did not see a higher reproductive output in MB females

over the 15 days, however, this could be related to egg production and not sperm limitation.

Male reproductive morphology

Male reproductive morphology did not exhibit marked variation across the sex ratio or diet regimes. However, one difference that was observed was an effect of sex ratio on accessory gland area, with males from female biased lines having significantly larger accessory glands than was found for males from MB or EQ lines. The accessory gland (AG) in *D. melanogaster* provides an assortment of essential seminal fluid proteins that elicit a wide range of responses in the mated female (Chapman and Davies, 2004). It has been shown that males with larger AGs have a competitive advantage (Wigby et al., 2009b) and so in populations in which sperm competition is higher (MB biased) I would expect to see larger AGs. However, experimental data have shown that there is a positive correlation between mating frequency and AG size (Bangham et al., 2002) as could be experienced in the female biased regimes. The benefit to having larger AGs in conditions in which high sperm competition is experienced, and the association with larger AGs with an increase in mating frequency, may offer an explanation as to why I did not see a significant difference between MB and FB lines, but instead between FB and EQ flies. To understand what fitness advantages may be conferred, more experiments are needed to determine the impact of large AGs such as seminal fluid protein expression, and male sperm investment patterns.

Overall, these results show that whilst the evolutionary pressures of sex ratio can drive changes in male and female reproductive morphology. However, the fitness consequences of these divergent traits, such as the larger spermathecae in females may be difficult to detect over a single mating. This may in part due to male reproductive investment typically containing significantly more sperm than a female can store (Kaufman and Demerec, 1942) and the proclivity for male and female *D. melanogaster* to mate multiply (Imhof et al., 1998b). There is also a clear effect of adult dietary regime having evolutionary effects on the morphology of both male and females and upon the pattern of progeny generated by the female. Further studies could investigate sperm investment patterns by extending the number of days of egg laying over lifetime or using mutant males with fluorescent sperm. It could also be

useful to investigate the males by performing crosses with conspecifics from opposing sex ratios. There is also an opportunity to investigate cuticular hydrocarbons produced by flies under divergent sex ratio and adult dietary regimes as this would contribute to knowledge gaps in this area.

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Supplementary material

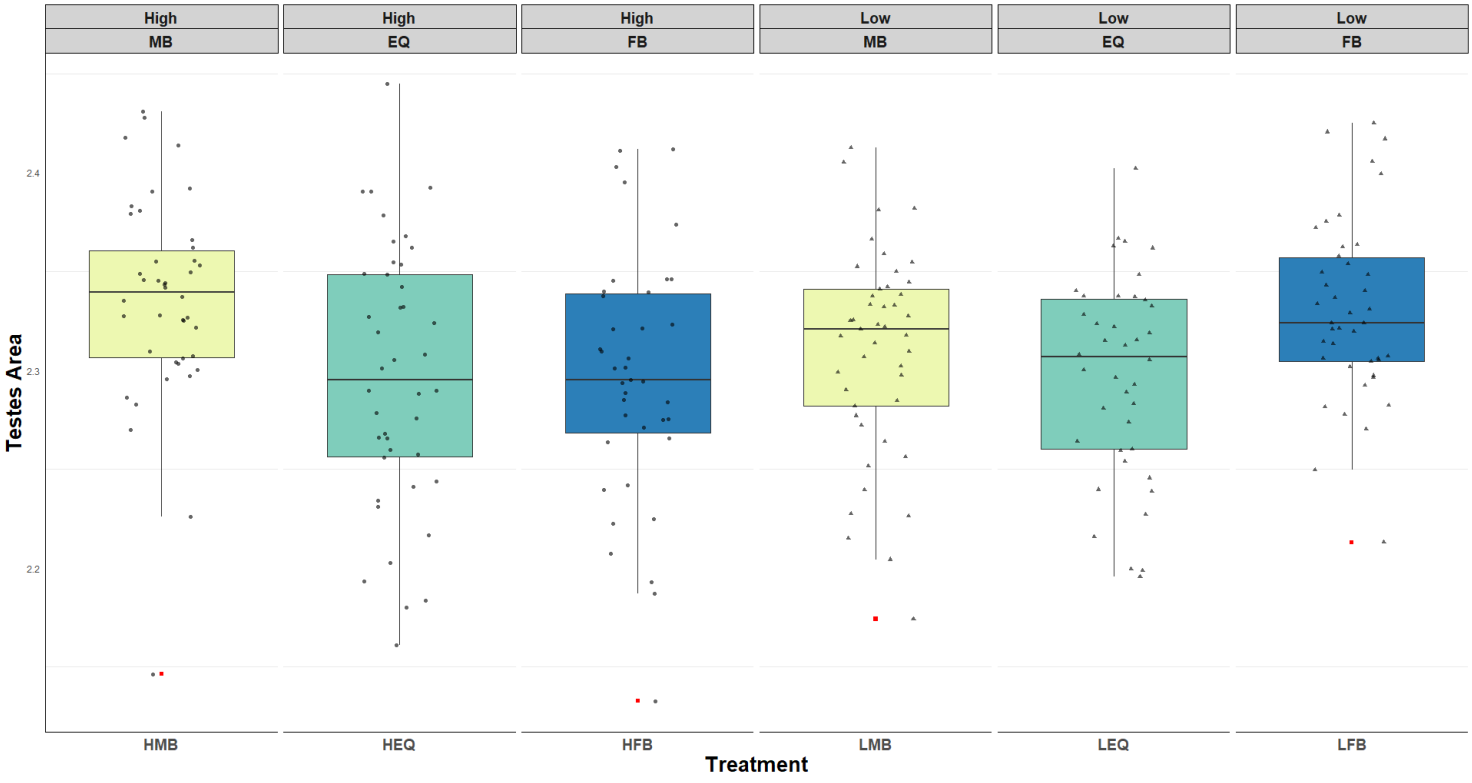

Fig S4.6. Normalised testes area (µm²) for males from the sex ratio regimes. Testes area measurement was taken from 5-day old virgin males from female-biased (FB), Equal sex (EQ) or Male –biased (MB) sex ratios and standard protein (High) or 20% protein (Low) diet regimes. Measurements were normalised to body size using thorax length as a proxy. Boxplot showing median line, with boxes representing upper and lower 25% quartile and whiskers representing the range, and points representing individual records, outliers highlighted in red.

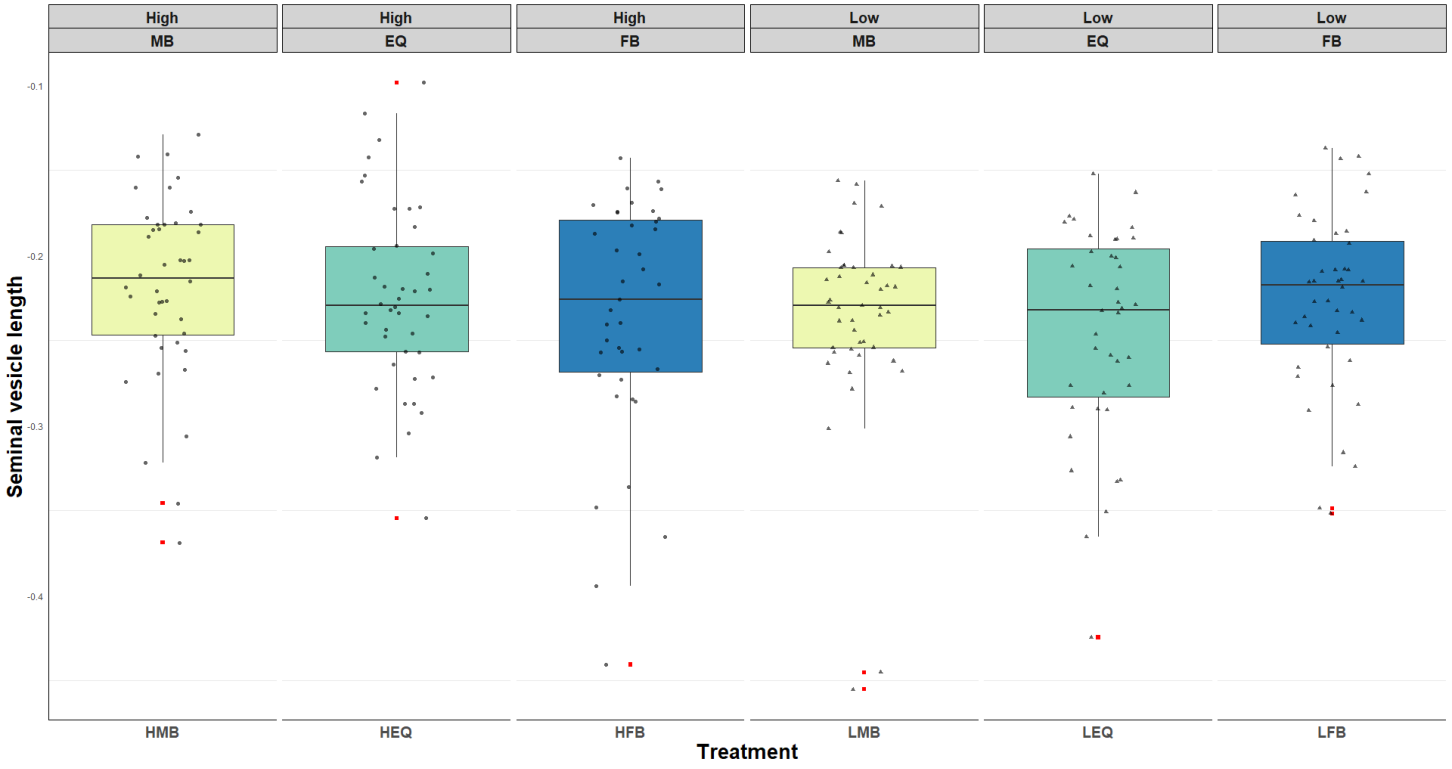

Fig S4.7. Normalised seminal vesicle length (µm) for males from the sex ratio regimes. Seminal vesicle length measurement was taken from 5-day old virgin males from female-biased (FB), Equal sex (EQ) or Male –biased (MB) sex ratios and standard protein (High) or 20% protein (Low) diet regimes. Measurements were normalised to body size using thorax length as a proxy. Boxplot showing median line, with boxes representing upper and lower 25% quartile and whiskers representing the range, and points representing individual records, outliers highlighted in red.

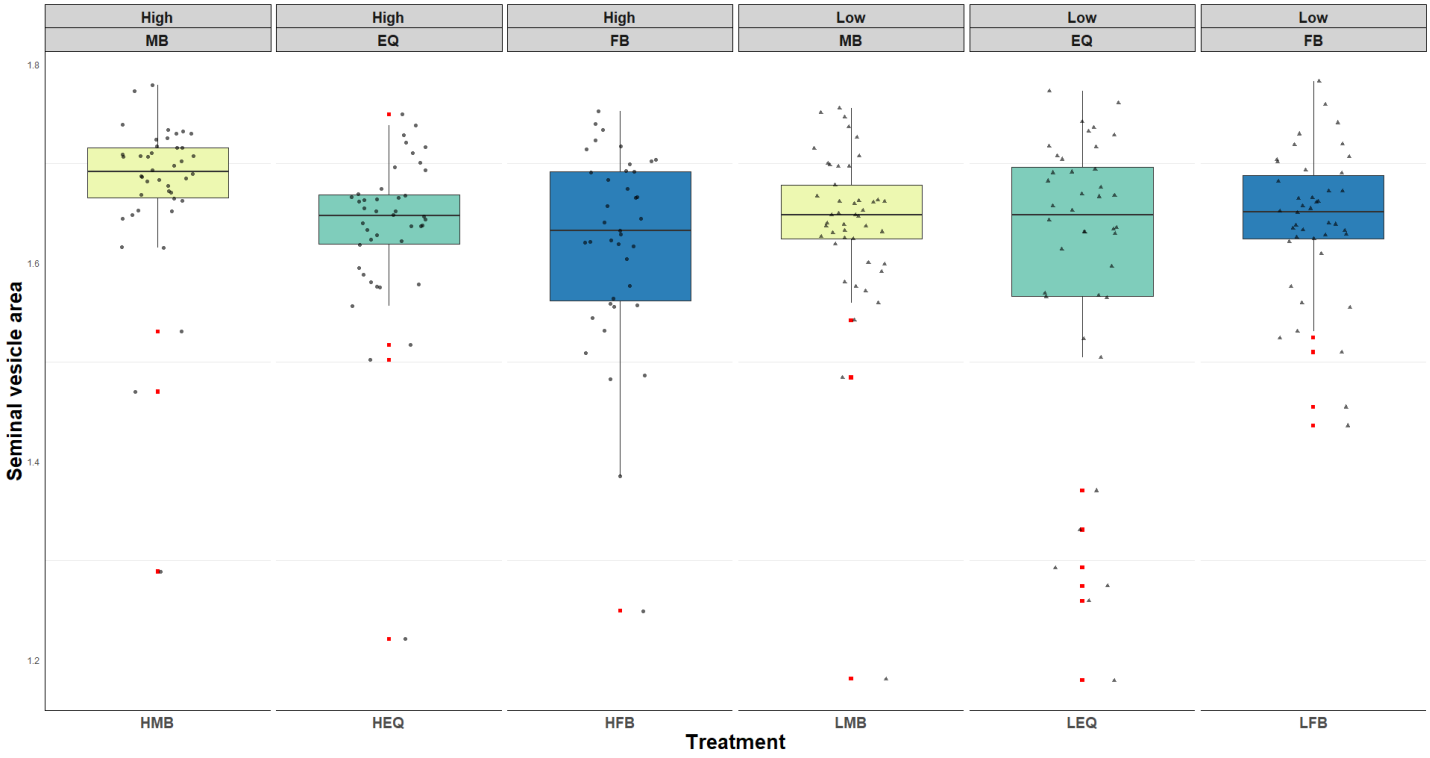

Fig S4.8. Normalised seminal vesicle area (µm²) for males from the sex ratio regimes. Seminal vesicle area measurement was taken from 5-day old virgin males from female-biased (FB), Equal sex (EQ) or Male –biased (MB) sex ratios and standard protein (High) or 20% protein (Low) diet regimes. Measurements were normalised to body size using thorax length as a proxy. Boxplot showing median line, with boxes representing upper and lower 25% quartile and whiskers representing the range, and points representing individual records, outliers highlighted in red.

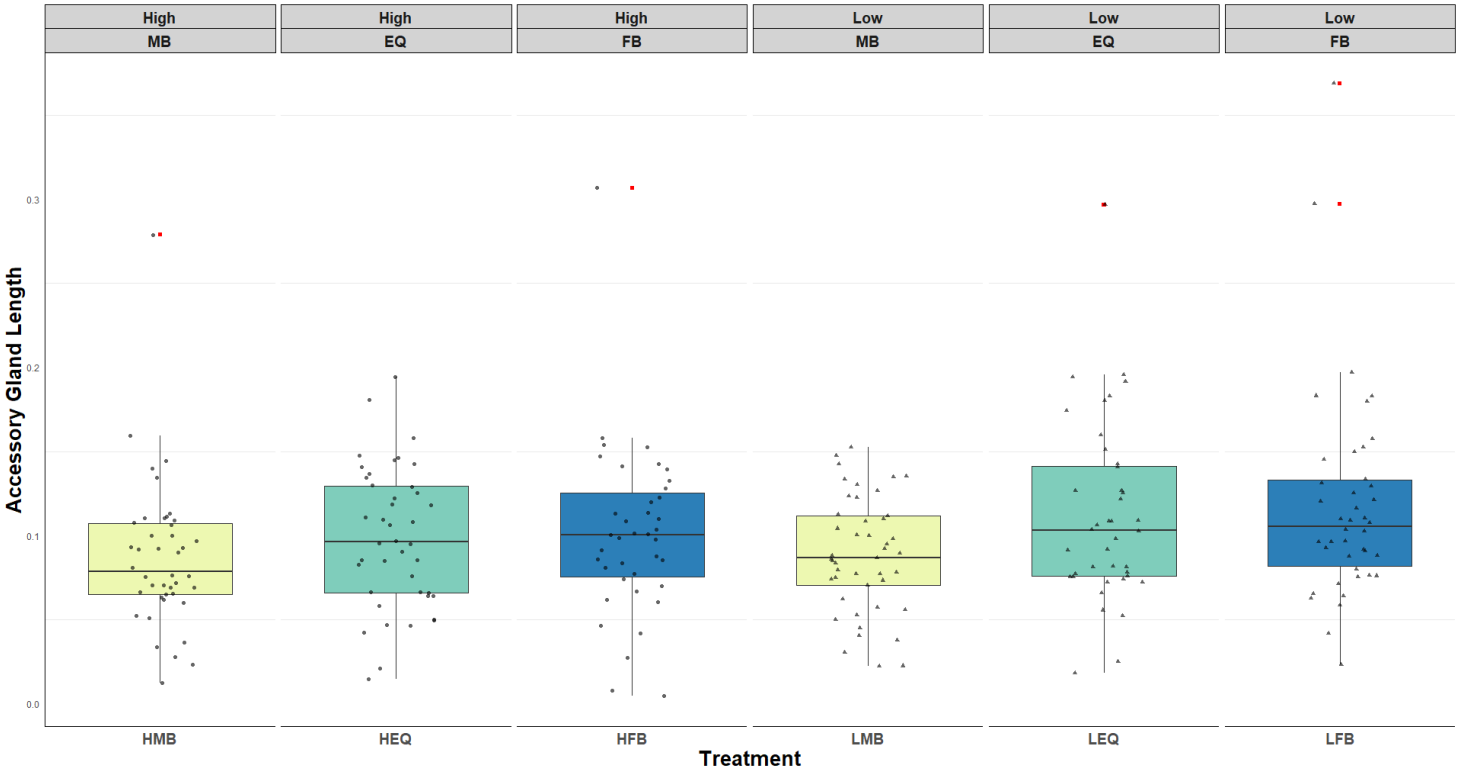

Fig 4.9a. Normalised Accessory gland length (µm) for males from the sex ratio regimes. Accessory gland length measurement was taken from 5-day old virgin males from female-biased (FB), Equal sex (EQ) or Male –biased (MB) sex ratios and standard protein (High) or 20% protein (Low) diet regimes. Measurements were normalised to body size using thorax length as a proxy. Boxplot showing median line, with boxes representing upper and lower 25% quartile and whiskers representing the range, and points representing individual records, outliers highlighted in red.

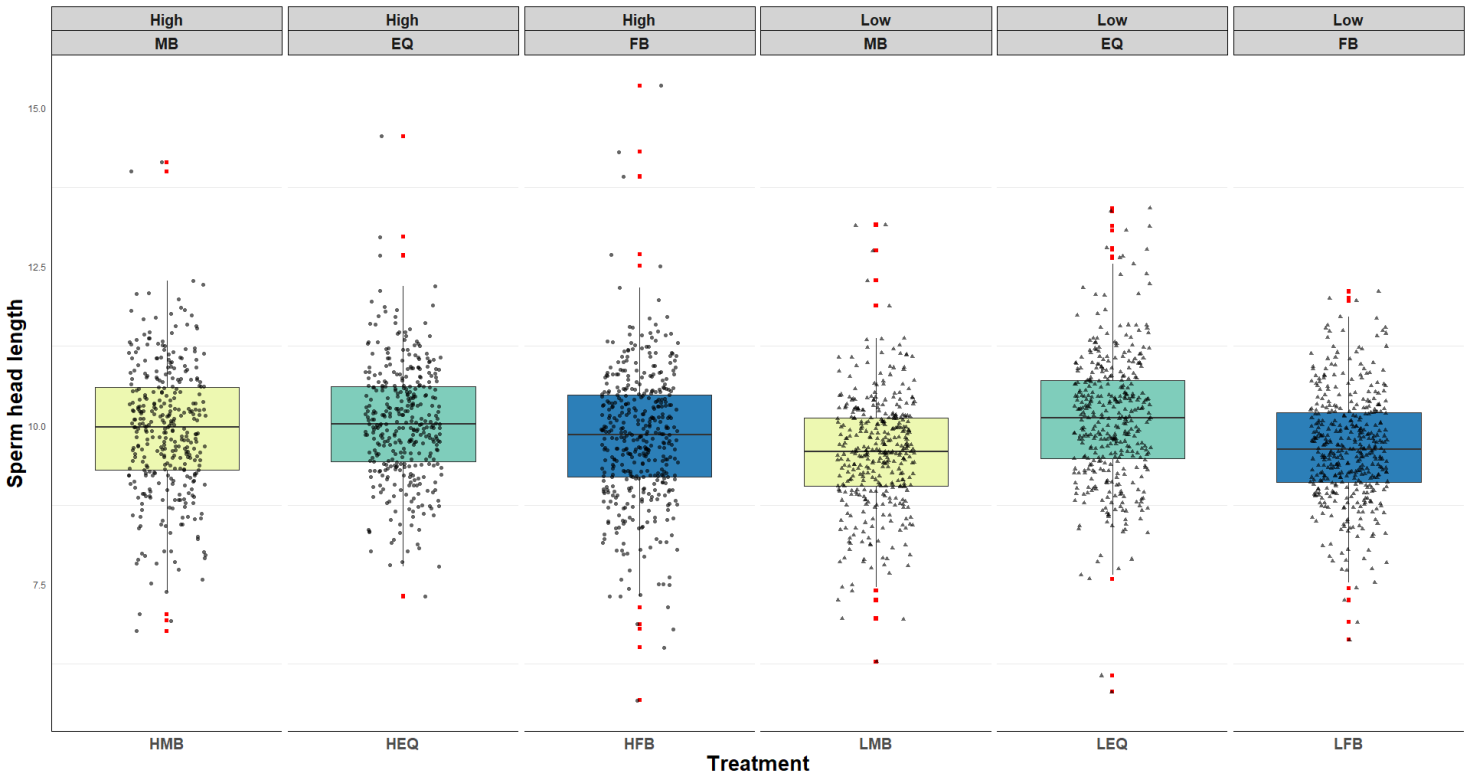

Fig S4.10. Sperm head length (µm) for virgin males from the sex ratio regimes. Sperm head length measurement was taken from 5-day old virgin males from female-biased (FB), Equal sex (EQ) or Male –biased (MB) sex ratios and standard protein (High) or 20% protein (Low) diet regimes. Boxplot showing median line, with boxes representing upper and lower 25% quartile and whiskers representing the range, and points representing individual records, outliers highlighted in red.

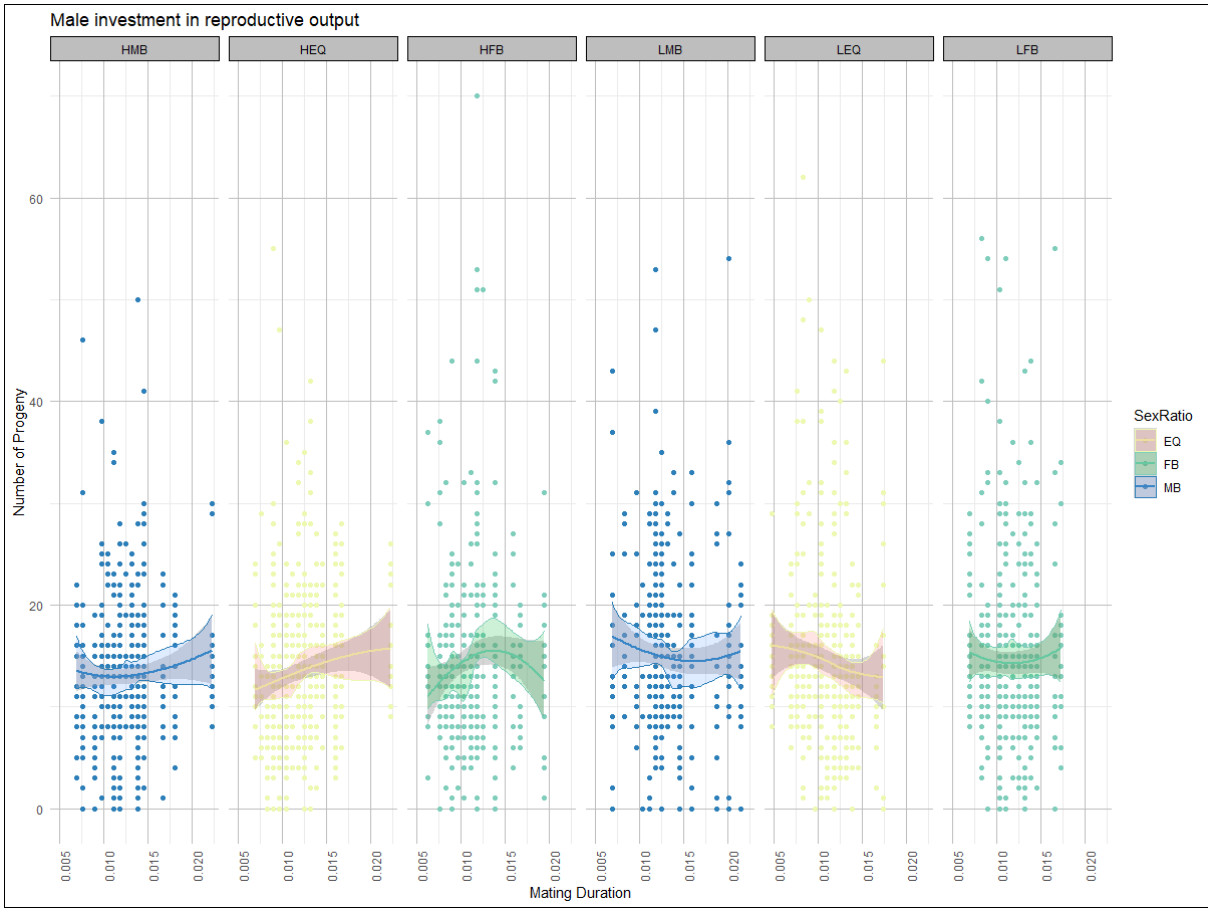

Fig S4.12b. Duration of mating for females from the sex ratio regimes mated to wildtype males. Duration of mating using 5-day old virgin wild type males mated to females from either female-biased (FB), Equal sex (EQ) or Male –biased (MB) sex ratios and standard protein (H) or 20% protein (L) diet regimes. Each data point is color-coded based on the sex ratio condition (legend on the right). Solid lines represent loess smoothed curves, showing the general trend in the data, shaded

ribbons around the curves represent 95% confidence intervals. X- axis represents an individual's mating duration, y-axis the number of eggs produced by that female.

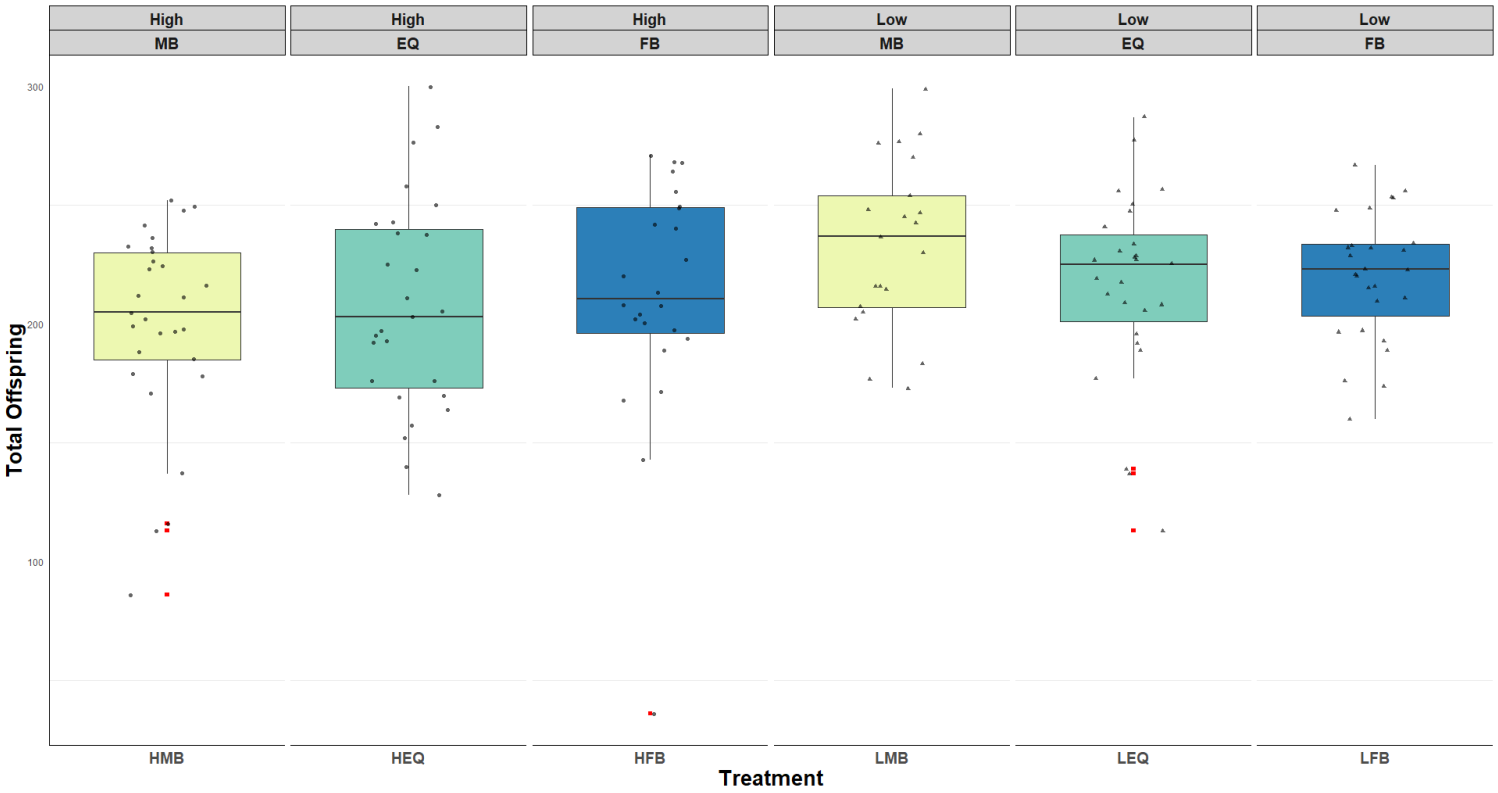

Fig S4.13. Total progeny for females from the sex ratio regimes mated to wildtype males. Total progeny of females from either female-biased (FB), Equal sex (EQ) or Male –biased (MB) sex ratios and standard protein (High) or 20% protein (Low) diet regimes mated to wild type males and allowed to lay eggs in fresh vials for 15 consecutive days. Boxplot showing median line, with boxes representing upper and lower 25% quartile and whiskers representing the range, and points representing individual records, outliers highlighted in red.

Table S4.2. Regression Coefficients for Female Thorax Length.

Female Thorax Length	Estimate	Std. Error	DF	t value	Pr(>|t|)
Intercept	685.8721	8.4515	17.3562	81.154	<2e-16 ***
SexRatioFB	-11.7172	11.9742	17.4778	-0.979	0.341
SexRatioMB	0.7299	11.9847	17.4884	0.061	0.952
FoodLow	4.3761	11.9768	17.4993	0.365	0.719
SexRatioFB:FoodLow	1.5331	17.0009	17.7482	0.090	0.929
SexRatioMB:FoodLow	-10.2050	16.9821	17.6537	-0.601	0.556

Table S4.3a. Regression Coefficients for Female Spermatheca.

Female Spermatheca size	Estimate	Std. Error	DF	t value	Pr(>|t|)
Intercept	74.5770	1.7035	17.7748	43.778	<2e-16 ***
SexRatioFB	-0.2731	2.4125	17.8693	-0.113	0.9111
SexRatioMB	5.1728	2.4131	17.8694	2.144	0.0461 *
FoodLow	-0.2607	2.4115	17.8445	-0.108	0.9151
SexRatioFB:FoodLow	2.3176	3.4206	18.0505	0.678	0.5067
SexRatioMB:FoodLow	-0.5723	3.4160	17.9488	-0.168	0.8688

Table S4.3b. Regression Coefficients for Female Spermatheca normalised to body size.

Normalised female Spermatheca	Estimate	Std. Error	DF	t value	Pr(>|t|)
Intercept	0.1088819	0.0017080	17.8008854	63.748	< 2e-16 ***
SexRatioFB	0.0013912	0.0024228	17.9982108	0.574	0.57293
SexRatioMB	0.0073039	0.0024223	17.8792146	3.015	0.00748 **
FoodLow	-0.0011825	0.0024210	17.9639463	-0.488	0.63115
SexRatioFB:FoodLow	0.0032533	0.0034464	18.3967307	0.944	0.35741
SexRatioMB:FoodLow	0.0009413	0.0034350	18.1344560	0.274	0.78715

Table S4.4a. Regression Coefficients for Female Seminal receptacle.

Female Seminal receptacle	Estimate	Std. Error	DF	t value	Pr(>|t|)
Intercept	1357.819	40.315	17.854	33.680	<2e-16
SexRatioFB	18.655	57.592	18.510	0.324	0.750
SexRatioMB	6.614	57.012	17.719	0.116	0.909
FoodLow	45.742	57.046	17.903	0.802	0.433
SexRatioFB:FoodLow	32.326	81.523	18.606	0.397	0.696
SexRatioMB:FoodLow	-73.644	80.718	17.876	-0.912	0.374

Table S4.4b. Regression Coefficients for Female Seminal receptacle normalised to body size.

Normalised female Seminal receptacle	Estimate	Std. Error	DF	t value	Pr(>|t|)
Intercept	1.98002	0.04277	17.18097	46.297	<2e-16
SexRatioFB	0.06294	0.06151	18.16137	1.023	0.320
SexRatioMB	0.01422	0.06031	16.56273	0.236	0.816
FoodLow	0.05494	0.06056	17.29780	0.907	0.377
SexRatioFB:FoodLow	0.04409	0.08718	18.39549	0.506	0.619
SexRatioMB:FoodLow	-0.08685	0.08560	17.05349	-1.015	0.324

Table S4.5. Regression Coefficients for Male Thorax Length.

Male Thorax length	Estimate	Std. Error	DF	t value	Pr(>|t|)
Intercept	944.055	12.058	17.529	78.292	<2e-16 ***
SexRatioFB	23.710	17.112	17.773	1.386	0.183
SexRatioMB	17.992	17.048	17.514	1.055	0.306
FoodLow	5.015	17.089	17.681	0.293	0.773
SexRatioFB:FoodLow	-38.070	24.181	17.720	-1.574	0.133
SexRatioMB:FoodLow	-21.137	24.098	17.483	-0.877	0.392

Table S4.6a. Regression Coefficients for Male Testes Length.

Male Testes length	Estimate	Std. Error	DF	t value	Pr(>|t|)
Intercept	3.458549	0.009999	17.996068	345.872	<2e-16 ***
SexRatioFB	-0.004117	0.014194	18.260359	-0.290	0.775
SexRatioMB	0.018359	0.014137	17.980744	1.299	0.210
FoodLow	-0.019477	0.014173	18.160721	-1.374	0.186
SexRatioFB:FoodLow	0.030738	0.020056	18.202823	1.533	0.143
SexRatioMB:FoodLow	-0.001294	0.019983	17.947230	-0.065	0.949

Table S4.6b. Regression Coefficients for Male Testes Length normalised to body size.

Normalised Male Testes length	Estimate	Std. Error	DF	t value	Pr(>|t|)
Intercept	0.388464	0.010223	18.126867	37.999	<2e-16 ***
SexRatioFB	-0.016740	0.014523	18.454615	-1.153	0.2638
SexRatioMB	0.011897	0.014453	18.110771	0.823	0.4211
FoodLow	-0.020150	0.014498	18.332668	-1.390	0.1812
SexRatioFB:FoodLow	0.052072	0.020518	18.385528	2.538	0.0204 *
SexRatioMB:FoodLow	0.007364	0.020427	18.070160	0.360	0.7227

Table S4.7a. Regression Coefficients for Male Testes Area

Male Testes Area	Estimate	Std. Error	DF	t value	Pr(>|t|)
Intercept	5.269380	0.017143	17.837523	307.376	<2e-16 ***
SexRatioFB	0.010830	0.024337	18.109092	0.445	0.6616
SexRatioMB	0.049418	0.024237	17.822151	2.039	0.0566.
FoodLow	0.003496	0.024301	18.006921	0.144	0.8872
SexRatioFB:FoodLow	0.016581	0.034387	18.050264	0.482	0.6355
SexRatioMB:FoodLow	-0.036905	0.034259	17.787837	-1.077	0.2958

Table S4.7b. Regression Coefficients for Male Testes Area normalised to body size.

Normalised Male Testes Area	Estimate	Std. Error	DF	t value	Pr(>|t|)
Intercept	5.269380	0.017143	17.837523	307.376	<2e-16 ***
SexRatioFB	0.010830	0.024337	18.109092	0.445	0.6616
SexRatioMB	0.049418	0.024237	17.822151	2.039	0.0566 .
FoodLow	0.003496	0.024301	18.006921	0.144	0.8872
SexRatioFB:FoodLow	0.016581	0.034387	18.050264	0.482	0.6355
SexRatioMB:FoodLow	-0.036905	0.034259	17.787837	-1.077	0.2958

Table S4.8a. Regression Coefficients for Male Seminal vesicle length

Male Seminal vesicle length	Estimate	Std. Error	DF	t value	Pr(>|t|)
Intercept	2.7505241	0.0141188	17.2442016	194.812	<2e-16 ***
SexRatioFB	-0.0001439	0.0201034	17.7142151	-0.007	0.994
SexRatioMB	0.0132969	0.0199587	17.2321316	0.666	0.514
FoodLow	-0.0207294	0.0200519	17.5450451	-1.034	0.315
SexRatioFB:FoodLow	0.0146749	0.0283882	17.6235484	0.517	0.612
SexRatioMB:FoodLow	-0.0039000	0.0282011	17.1775101	-0.138	0.892

Table S4.8b. Regression Coefficients for Male Seminal vesicle length normalised to body length.

Normalised Male Seminal vesicle length	Estimate	Std. Error	DF	t value	Pr(>|t|)
Intercept	-0.224253	0.011881	17.355653	-18.875	5.22e-13 ***
SexRatioFB	-0.010760	0.016973	18.059444	-0.634	0.534
SexRatioMB	0.005343	0.016795	17.361009	0.318	0.754
FoodLow	-0.022624	0.016910	17.816817	-1.338	0.198
SexRatioFB:FoodLow	0.031334	0.023954	17.941006	1.308	0.207
SexRatioMB:FoodLow	0.005089	0.023722	17.286982	0.215	0.833

Table S4.9a. Regression Coefficients for Male Seminal vesicle area.

Male Seminal vesicle Area	Estimate	Std. Error	DF	t value	Pr(>|t|)
Intercept	4.6073319	0.0270194	16.9902837	170.519	<2e-16 ***
SexRatioFB	0.0007752	0.0384243	17.3691317	0.020	0.984
SexRatioMB	0.0512409	0.0381968	16.9757168	1.341	0.197
FoodLow	-0.0330363	0.0383430	17.2303152	-0.862	0.401
SexRatioFB:FoodLow	0.0367290	0.0542726	17.2923733	0.677	0.508
SexRatioMB:FoodLow	-0.0118975	0.0539790	16.9301005	-0.220	0.828

Table S4.9b. Regression Coefficients for Male Seminal vesicle area normalised to body length.

Normalised Male Seminal vesicle Area	Estimate	Std. Error	DF	t value	Pr(>|t|)
Intercept	1.633029	0.023363	16.765486	69.899	<2e-16 ***
SexRatioFB	-0.010649	0.033288	17.268151	-0.320	0.753
SexRatioMB	0.042766	0.033026	16.755978	1.295	0.213
FoodLow	-0.035118	0.033195	17.088875	-1.058	0.305
SexRatioFB:FoodLow	0.054028	0.047000	17.173736	1.150	0.266
SexRatioMB:FoodLow	-0.002675	0.046661	16.698687	-0.057	0.955

Table S4.10a. Regression Coefficients for Accessory gland length normalised to body length.

Male Accessory gland length	Estimate	Std. Error	DF	t value	Pr(>|t|)
Intercept	0.098483	0.010748	17.769209	9.163	3.77e-08 ***
SexRatioFB	0.002401	0.015330	18.370128 SexRatioMB	0.157	0.877
SexRatioMB	-0.011299	0.015194	17.763859 FoodLow	-0.744	0.467
FoodLow	0.012570	0.015282	18.158641 SexRatioFB:	0.823	0.421
SexRatioFB:FoodLow	0.005512	0.021641	18.261747	0.255	0.802
SexRatioMB:FoodLow	-0.011232	0.021464	17.697351	-0.523	0.607

Table S4.10b. Regression Coefficients for Accessory gland length normalised to body length.

Normalised Male Accessory gland length	Estimate	Std. Error	DF	t value	Pr(>|t|)
Intercept	3.072889	0.013219	17.837274	232.453	<2e-16 ***
SexRatioFB	0.013114	0.018792	18.205516	0.698	0.494
SexRatioMB	-0.002832	0.018688	17.821618	-0.152	0.881
FoodLow	0.014815	0.018755	18.069805	0.790	0.440
SexRatioFB:FoodLow	-0.011144	0.026545	18.129764	-0.420	0.680
SexRatioMB:FoodLow	-0.020712	0.026411	17.776784	-0.784	0.443

Table S4.11a. Regression Coefficients for Accessory gland Area.

Male Accessory gland Area	Estimate	Std. Error	DF	t value	Pr(>|t|)
Intercept	2.34025	0.01288	17.78900	181.639	<2e-16 ***
SexRatioFB	0.04076	0.01834	18.23369	2.223	0.0391 *
SexRatioMB	0.03413	0.01821	17.77509	1.874	0.0775 .
FoodLow	0.02412	0.01829	18.07239	1.319	0.2037
SexRatioFB:FoodLow	-0.01505	0.02589	18.14602	-0.581	0.5684
SexRatioMB:FoodLow	-0.05162	0.02574	17.72258	-2.006	0.0604 .

Table S4.11b. Regression Coefficients for Accessory gland Area normalised to body length.

Normalised Male Accessory gland Area	Estimate	Std. Error	DF	t value	Pr(>|t|)
Intercept	5.31466	0.01465	17.88979	362.828	<2e-16 ***
SexRatioFB	0.05138	0.02081	18.22554	2.469	0.0237 *
SexRatioMB	0.04266	0.02071	17.87388	2.060	0.0543 .
FoodLow	0.02652	0.02078	18.10093	1.277	0.2179
SexRatioFB:FoodLow	-0.03200	0.02940	18.15522	-1.088	0.2907
SexRatioMB:FoodLow	-0.06132	0.02927	17.83247	-2.095	0.0507 .

Table S4.12. Regression Coefficients for sperm head length

Normalised Male sperm head length	Estimate	Std. Error	DF	t value	Pr(>|t|)
Intercept	10.04343	0.14437	18.03352	69.567	<2e-16 ***
SexRatioFB	-0.19756	0.20345	17.77515	-0.971	0.345
SexRatioMB	-0.04818	0.20482	18.24290	-0.235	0.817
FoodLow	0.04319	0.20388	17.92778	0.212	0.835
SexRatioFB:FoodLow	-0.25112	0.28751	17.72239	-0.873	0.394
SexRatioMB:FoodLow	-0.48242	0.28889	18.05817	-1.670	0.112

Table S4.13a. Regression Coefficients for Mating Latency

	Estimate	Std. Error	DF	t value	Pr(>|t|)
Intercept	-1.692366	0.025780	17.777306	-65.647	<2e-16 ***
SexRatioFB	-0.050301	0.036627	18.106502	-1.373	0.1864
SexRatioMB	-0.058494	0.036423	17.709457	-1.606	0.1260
FoodLow	-0.076762	0.036493	17.846342	-2.103	0.0499 *
SexRatioFB:FoodLow	0.004635	0.051727	18.008370	0.090	0.9296
SexRatioMB:FoodLow	0.111446	0.051615	17.854036	2.159	0.0447 *

Table S4.13b. Regression Coefficients for Mating Duration

Comparison	Estimate	Std. Error	z value	Pr(>|z|)
EQ - MB	-0.005664	0.002728	-2.076	0.0949 .
FB - MB	-0.006790	0.002766	-2.454	0.0376 *
FB - EQ	-0.001126	0.002714	-0.415	0.9095

Table S4.14. Regression Coefficients for total progeny over 15 days

Total progeny	Estimate	Std. Error	z value	Pr(>|t|)
Intercept	5.260951	0.070480	74.644	<2e-16 ***
SexRatioFB	0.003464	0.102364	0.034	0.973
SexRatioMB	-0.062532	0.099037	-0.631	0.528
FoodLow	0.079112	0.100386	0.788	0.431
SexRatioFB:FoodLow	-0.020337	0.143336	-0.142	0.887
SexRatioMB:FoodLow	-0.017535	0.142282	-0.123	0.902

Chapter 5: Effect of experimental evolutionary manipulations of adult sex ratio on the expression of a key seminal fluid protein, the Sex Peptide, in *Drosophila melanogaster* males

Abstract

Long-term generational manipulation of adult sex ratio is often utilised as a tool for investigating evolved responses to variation in sexual selection. Previous work has shown that *Drosophila melanogaster* can respond to the degree of short-term intrasexual selection by adjusting ejaculate composition, and that this confers significant reproductive advantage. However, exactly how longer-term variation in sexual competition might select for evolved differences in ejaculate gene expression is less clear. Using populations of *D. melanogaster* held at evolutionarily stable sex ratios of male bias (MB), equal sex (EQ), or female bias (FB) for 110 generations I investigated how constant exposure to increased or decreased levels of sexual selection had affected the expression of a key seminal fluid protein 'sex peptide' (SP) gene, both prior to, and immediately after, mating. SP transfer has been shown to give males a competitive advantage by reducing female receptivity to rival males and increasing female oviposition. However, recent evidence also suggests that SP is also an essential facilitator for the transfer and effects of other ejaculate components. I predicted that MB regimes would evolve to express more SP prior to mating due to their evolutionary history of elevated exposure to increased levels of competition and the competitive advantage conferred by SP when males are the first to mate with a female. FB regime males were predicted to express SP at higher rates after mating because of the evolved differences seen in accessory gland size from earlier experiments, linked to increased opportunities to mate and a pilot study which showed an increase in expression after mating in FB lines. Counter to predictions, there was no difference in relative expression of sex peptide before or after mating. However, I did detect a fold change (1.4) difference in the expression of the sex peptide gene between unmated and mated males from the MB regime. This suggested that MB males upregulated the expression of SP more markedly upon

mating than did EQ or FB males. Overall, the study suggests that the sensitivity of gene expression varies in response to male mating status can evolve.

Attribution statement

I would like to thank Suzanne Bennett Keki for conducting the RNA extractions and qPCR and Dr Emily Fowler for teaching me the techniques.

Introduction

Darwin first introduced the concepts of sexual selection in the late 19th century (Darwin, 1871). Since then, our understanding of the complex interaction that sexual selection can have on phenotypic variation, and how it can drive evolutionary change has greatly expanded. A phenotype that conveys an advantage in securing an individual's opportunity to attract and secure a mate is a pre-copulatory sexually selected trait (Darwin, 1871). A phenotype that maximises fertilization opportunity is a post-copulatory sexually selected trait. Examples include traits involved in securing success in sperm competition (Trivers, 1972) and in cryptic female choice. Many pre-copulatory traits increase an organism's fitness via mechanisms involving combat, colouration, or song (Harrison et al., 2021, Nowicki and Searcy, 2004, McComb, 1991). Post-copulatory traits can mediate inhibitory contests between males as each attempt to maximise fertilisations (Emlen, 1997, Radwan and Siva-Jothy, 1996, Chapman et al., 2003). Pre and post copulatory traits can also be bound by negative trade-offs (Emlen, 1997, Simmons and García-González, 2008, Wedell et al., 2002, Chapman et al., 2003). An increase in polyandry, for example, has been shown to weaken rather than strengthen pre-copulatory sexual selection, and promote alternative post-copulatory strategies (Morimoto et al., 2019). A significant pre-copulatory strategy is mate choice and is often influenced most strongly by the actions of females (Andersson, 1994). It is frequently based on a selection of metrics or indicators that are predicted to convey fitness. For example, female stalk-eyed flies show a preference for males with the widest eye-span (Wilkinson and Reillo, 1994) and eye span is generally seen as an honest indicator of fitness (Cotton et al., 2004b). Hence mating with the widest-eyed male should confer fitness benefits. However, in situations by which females mate multiply, or are less choosy, males do not need to be the first male to mate if they can secure fertilization through post copulatory tactics such as sperm competition (Firman and Simmons, 2008).

Post-copulatory mechanisms are generally less well understood than pre-copulatory characteristics because techniques required to clearly document paternity "winners" often require molecular analyses. Sperm competition is one notable way in which males may improve the odds that they achieve fertilizations. Whilst the number of sperm that males transfer to a female can be stored is not unlimited, it typically

outnumbers significantly the number of eggs that can be fertilised (Parker and Pizzari, 2010). However, in response to an increase in competition, male *Drosophila melanogaster* transfer even more sperm and increase their fitness because there is a corelation between ejaculate size and fertilisation success (Garbaczewska et al., 2013a) even despite the supernumerary sperm numbers already present. Sperm morphology can vary greatly across taxa, even between closely related species (Pitnick et al., 2009) with many studies indicating that at least part of this diversity is driven by the effects of sperm competition (Godwin et al., 2017a, Calhim et al., 2007). For example, longer sperm may swim faster or be better able to dislodge rival sperm (Lüpold et al., 2012). Whilst sperm are considered relatively cheap to produce (Trivers, 1972) increasing sperm numbers might not be the most economical use of energy expenditure if it comes at a cost of reducing other competitive components, such as sperm morphology (Degueldre and Aron, 2023) or other strategies such as mate guarding (Yokoi et al., 2016).

An emerging picture is that the non-sperm components of male ejaculates are also crucial to a male's success in post-copulatory competition. The ejaculate transferred by males is not solely comprised of sperm, a large assortment of seminal fluid proteins (SFPs) are transferred to the female during copulation (Chapman and Davies, 2004, Rowe et al., 2020, Mrinalini et al., 2021). The diversity of SFPs transferred across sexually reproducing organisms is extensive, and whilst the complete range of these proteins and their interactions is not yet fully known (Sepil et al., 2019, Chapman, 2001), researchers are continuing to uncover the various ways in which SFPs contribute to a male's reproductive success (Mueller et al., 2007). Many investigations into SFP components have used *D. melanogaster* as a model (Sepil et al., 2019) (Chapman and Davies, 2004). For example, seminal proteins such as Ovulin (Acp26A) when transferred to the female stimulates the release of oocytes (Heifetz et al., 2000a). The protein Acp36DE is associated with sperm storage (Neubaum and Wolfner, 1999b) and experiments have shown that males without this protein obtain fewer fertilizations when they are mating with already mated females, suggesting that this protein is a key component of success in sperm competition (Chapman et al., 2000). Additionally, the protein PEB-me forms part of the post-copulatory mating plug, and when knocked down fertility is drastically decreased (Avila et al., 2015). There are almost three hundred seminal fluid proteins

identified in *D. melanogaster* (Wigby et al., 2020), however, we lack a complete understanding of the function for the vast majority of these. In addition, we know that SFPs can provide males with a post-copulatory advantage (Wigby and Chapman, 2005) and that gene expression can evolve in response to sex biases (Veltsos et al., 2017), including specifically those that regulate seminal fluid proteins (Hollis et al., 2019a). Yet it is not fully understood how levels of sexual selection may impact the expression of sex peptide due to its unpredictable nature (Veltsos et al., 2017) or whether it is influenced by other accessory gland proteins (Smith et al., 2012).

Here, I used populations of *D. melanogaster* maintained for the long-term under evolutionarily fixed sex ratios of male bias, female bias, and equal sex to determine the effect of sexual selection on the relative base line expression of a key seminal fluid protein 'Sex Peptide' (SP). SP is synthesised in male *D. melanogaster* reproductive accessory glands (Monsma et al., 1990) and is transferred to the female during copulation where it binds to the sex peptide receptor (SPR) (Yapici et al., 2008). At least some SP is bound to the sperm and is gradually released within the female over an extended period, which prolongs its effects (Peng et al., 2005). SP elicits a striking range of responses in the mated female, which impact significantly upon a male's competitive ability. For example, SP increases egg production and decreases a female's sexual receptivity to other males (Chen et al., 1988) (Liu and Kubli, 2003, Chapman et al., 2003). Other effects of SP within the female include a heightened immune response (Domanitskaya et al., 2007), the stimulation of food intake (Carvalho et al., 2006), the type of food eaten by females (Ribeiro et al., 2010) and female circadian rhythm (Delbare et al., 2023). Whilst other SFPs may also influence some of these important post mating phenotypes (Chapman et al., 2001, Saudan et al., 2002) it is striking that SP on its own has strong effects on a range of traits. This makes SP a good target for the investigations described in this chapter, which aimed to test whether the level of expression of the SP gene could itself evolve in response to sexual selection.

In the short-term and in response to the immediate level of competition in the environment, *D. melanogaster* males have been shown to plastically increase sperm numbers transferred to the female following exposure to rivals (Garbaczewska et al., 2013a). Further investigations have also shown that the transfer of seminal fluid components including SP is also sensitive to a male's competitive environment

Wigby et al., 2011 (Current Biology) (Hopkins et al., 2019a). Hopkins et al. (2019) found that sperm transfer was highest under conditions of low competition, but that SFP investment was generally elevated by high competition. In addition, it has been shown that the accessory glands of males evolved under high competition deplete their accessory glands more quickly over serial mating's than do those held under low competition (Linklater et al., 2007). These findings reveal significant interactions between levels of sexual competition and the evolution of ejaculate allocation.

Significant variation in accessory gland size (the structures that produce SFPs) within the lines used for this experiment has already been demonstrated (Chapter 4). These data showed that accessory glands for males held over the long-term under low competitive environments (female biased) were significantly larger by area than their high competition counterparts from the male-biased lines. This demonstrates that evolutionary manipulation of adult sex ratio has selected on accessory gland size, predicting that larger accessory glands may potentially confer on males the ability to transfer more SFPs to females during mating, or to successfully engage in more mating's. What is not clear, and which is tested here, is whether this selection also includes changes to the base line level of expression of SFPs genes such as SP. A pilot study was conducted at several timepoints before and after mating (see Appendix). These indicated the potential for differences in the expression of SP in the MB, EQ and FB lines immediately after mating. However, the samples sizes for the early post mating timepoint were low, prompting the main experiment described here that was completed using larger sample sizes.

The specific hypotheses I tested were as follows:

Table 5.1. Predicted responses of SP gene expression in unmated (virgin) and mated males subject to long-term variation in sexual selection (male biased (MB); female biased (FB)).

Treatment	Expectation	Rationale
Unmated	MB males will show higher base line expression (in virgins) of the SP gene	Males have been shown to increase SFP gene expression and SFP transfer in response to higher short term sexual competition (Hopkins et al., 2019a, Mohorianu et al., 2017).
Mated	MB males will show higher expression of the SP gene after mating.	Males from MB lines will be expected to have transferred more SP (Ramm, 2020) and so expression of SP should be higher to achieve SP replenishment.

Methods

Experimental evolution line maintenance

Experimental evolution lines comprised nine Sex Ratio lines consisting of three independent replicates of each of Equal sex (EQ (50M: 50F)), Female-Biased (FB (25M: 75F)) and Male-Biased (MB (70M: 30F)) lines, maintained as adults on standard SYA diets (see below). Populations were maintained within plastic boxes (12cmW x 18cmL x 8.5cmD, with gauze lid) at 25°C in a humidified room with a 12 h light: 12 h dark cycle. High protein lines were given access as adults to two fresh, standard SYA medium vials every two or three days (100g brewer's yeast, 50g sucrose, 15g agar, 30mL Nipagin (10% solution), 3mL propionic acid, 0.97L water). All regimes were raised at standardised densities (one hundred larvae per vial) and on standard SYA medium during development. Nine days after setting up the adults in the boxes, each line was supplied with an initial agar-grape juice egg collection plate (50g agar, 600mL red grape juice, 42mL Nipagin (10% solution), 1.1L water), this was repeated on the 10th day. Egg collection plates were maintained at 25°C following removal from the boxes and kept in cotton bags for two days to allow egg hatching. Four hundred larvae were then picked from each of these egg collection plates and placed in densities of 100 per vial for each line (Four replicates vials of

100 larvae each), on standard SYA for all regimes. After eclosion, flies were anaesthetised using CO_2 and counted into their specified sex ratios and placed in the plastic boxes as described above to initiate the next generation. The lines had been maintained under these conditions since 2013 until COVID-19 restrictions began in March 2020. Maintenance for these lines was then relaxed whereupon cultures were maintained in bottles on SYA medium (two bottles per replicate) and maintained at 18°C. This reduced the number of generations of relaxed selection. All flies used in this experiment had experienced 110 generations of selection under the sex ratio and dietary regimes as described above. Flies used in experiment one were from generation 143, flies from experiment two were from generation 146. The lines had spent a total of 33 and 36 generations under subsequent relaxed selection imposed by COVID-19 restrictions in these experiments, respectively.

The effect of evolutionary manipulation of adult sex ratio and nutrition on the expression of sex peptide.

The rearing environment of the experimental flies was standardised. To do this, equal sexes (50 males and 50 females) from all populations were isolated using CO_2 and transferred to the standard rearing plastic boxes (12cmW x 18cmL x 8.5cmD, gauze lid) and supplied with two agar-grape juice egg collection plates for 24h. 100 larvae from each population were collected and transferred to fresh SYA medium vials (50 larvae per vial; SYA: 100g brewer's yeast, 50g sucrose, 15g agar, 30mL Nipagin (10% solution), 3mL propionic acid, 0.97L water). Twenty males from each population were collected as virgins as they eclosed and kept in vials of 10 for five days to ensure sexual maturity. At five days old flies males were assigned a treatment: Treatment A = five-day old virgin males from each treatment were transferred to 1.5ml microtubes and flash frozen in liquid nitrogen, then transferred to storage at -80°C. Treatment B = five-day old males from each population were mated with a virgin wild-type female and after mating finished, males were immediately flash frozen in liquid nitrogen and transferred to storage at -80°C.

Sample preparation.

Extract total RNA using the miRvana miRNA Isolation kit (Invitrogen, available from ThermoFisher). The workspace was initially cleaned using RNase Zap (ThermoFisher) to ensure sterile working conditions. Each sample was designated its own pestle, which was first washed with warm soapy water, and then wiped clean using RNase Zap. Samples were taken from the -80°C freezer and kept on dry ice until used. Samples were transferred into new sterile 2ml microtubes and held in liquid nitrogen for five seconds, the pestle was simultaneously submerged in liquid nitrogen to standardise temperatures before crushing the sample until a fine powder, 300µl of lysis buffer and kept on ice. 60 µl of homogenate additive was added to the sample and the pestle was removed, before resting on ice for 10 minutes. Under a fume hood 600µl of Acid Phenol: Chloroform was added to the sample, vortexed for 30sex and centrifuged for 5min. The RNA was carefully pipetted from the top layer into a fresh microtube and 625µl of 100% ethanol was added, mixing by gentle inversion of the tube. The sample was transferred into the miRVana cartridge and collection tube before being centrifuged at 10,000 rpm for 15 seconds and the flow through was discarded. This step was repeated three times, adding wash solution at the start and discarding flow through. A final 1minute spin ensured the removal of all remaining solutions and the cartridge was transferred to a fresh collection tube. Adding 30µl of RNA storage solution (pre-heated to 95°C) the sample was centrifuged at 13000 rpm for 1min. Samples were checked using before storage at -80°C.

DNA was removed from the samples following Invitrogen Turbo DNA-free kit protocol (ThermoFisher, cat# AM1907). Diluting the samples to at least 200ng/µl using nanodrop data, a reaction containing 30µl of the RNA sample, 3µl of DNase buffer and 0.6µl DNase was set up and incubated at 37°C for 30min. 5.5µl of inactivation reagent was added and incubated at room temperature for 2min, mixing the sample regularly to re-disperse the inactivation reagent. Centrifuging the sample at 10000rpm for 1.5min allowed for the collection of RNA, which was transferred to a new sterile tube. Samples were verified using the nanodrop.

The conversion of RNA to cDNA was performed using Qiagen's QuantiTect reverse transcriptase kit (Qiagen cat# 205311) and protocol. 1µg of DNased RNA was calculated using nanodrop data were gDNA wipeout buffer was added along with 14µl of RNase free water and incubate a 42°C for 2min and placed immediately on ice to stop the reaction. A mastermix was then added and incubated for a further 15 minutes at 42°C, inactivation of this reaction was instigated by incubating the sample for a further 3min at 95°C. The samples were then stored at -20°C until use.

Gene expression

Gene of interest "Sex Peptide" and the two reference genes "alpha Tubulin" and "eIF-1A" primer sequences was selected using FlyRNAi (https://www.flyrnai.org/). Primer efficiencies were checked using a 5-fold standard curve of cDNA with at 50 ng total cDNA. I used primer concentrations that yielded efficiencies of between 90 and 110%. The two reference genes (αTub84B and eIF1A) were selected because of their stable expression in *Drosophila melanogaster*. αTub84B is highly conserved across eukaryotic life (Raff, 1984) and greatly expressed at all life stages by both sexes (Brown et al., 2014). The gene for eIF1A is also highly expressed at all life stages in each sex (Brown et al., 2014) and is highly conserved across taxa (Liang and Biggin, 1998). Samples were run in triplicate and each plate contained an intra-plate calibrator, to account for any plate variation, and a non-template control (molecular grade H_2O).

Quantitative RT-PCR was performed using a Bio-Rad CFX Connect Thermal Cycler (software CFX maestro) and iTaq universal SYBR green supermix (Bio-Rad no. 1725121). Primers were manufactured salt-free by Eurofins.

Table 5.2. Gene of interest and the qPCR primers used for experiment 1 and 2.

Target Gene	Description	Forward primer	Reverse primer	FBgn	Primer efficiency 5pg/mol
Sex Peptide	Encodes a peptide produced by the male accessory glands that is transferred to females in the ejaculate	GGAATGGCCGTGGAATAGGA	TAACATCTTCCACCCCAGGC	FBgn0003034	102.42%
Alpha Tubulin	Major constituent of microtubules	CACACCACCCTGGAGCATTC	CCAATCAGACGGTTCAGGTTG	FBgn0087040	100.5%
eIF1A	Encodes a protein required for mRNA translation	AAGAATCGTCGTCGTGGTAAGA	CTGCGCGTACTCCTGTTGG	FBgn0026250	93.84%

Statistical analysis

All statistical analysis were performed using R-4.0.2 (R Core Team, 2020). The three replicates of each sex ratio were analysed simultaneously, with the replicates (indicated by Population) designated as a random factor. Where possible individual data were nested within Population as an additional random factor. The Shapiro-Wilk test was used to check data were normally distributed and the Levene's test to check the homogeneity of variances across treatments. Cycle threshold (CT) values were converted using the delta CT and delta-delta CT method (2^ΔΔCt) (Rao et al., 2013). The 2^-ΔΔCt steps are as follows:

1) Set a control group and average the ΔCt

For example, when comparing EQ virgin vs EQ mated, the control is the average ΔCt of the EQ virgin males.

2) Then subtract average ΔCt from the ΔCt of each sample in your treatment. For example, EQ mated sample 1 – average virgin EQ ΔCt

3) Then transform the data 2-fold, giving the 2^-ΔΔCt.

each plate additionally had an intraplate calibrator used to normalise expression across each plate.

Analysis was performed using mixed effects models using the lme4 package (Bates et al. 2015). The Akaike's Information Criterion (AIC) was used to check for model fit. Post-hoc analysis was performed using the Tukey's HSD Test.

Results

Sex Peptide expression of virgin males from adult sex ratio regimes

Counter to the predictions, the relative expression of sex peptide was not significantly different between the sex ratios prior to mating (Fig. 5.1.) (*FB: t value = 0.146, residual DF = 9.2335, p = 0.887, MB: t value = -1.105 residual DF = 8.9875, p = 0.298*).

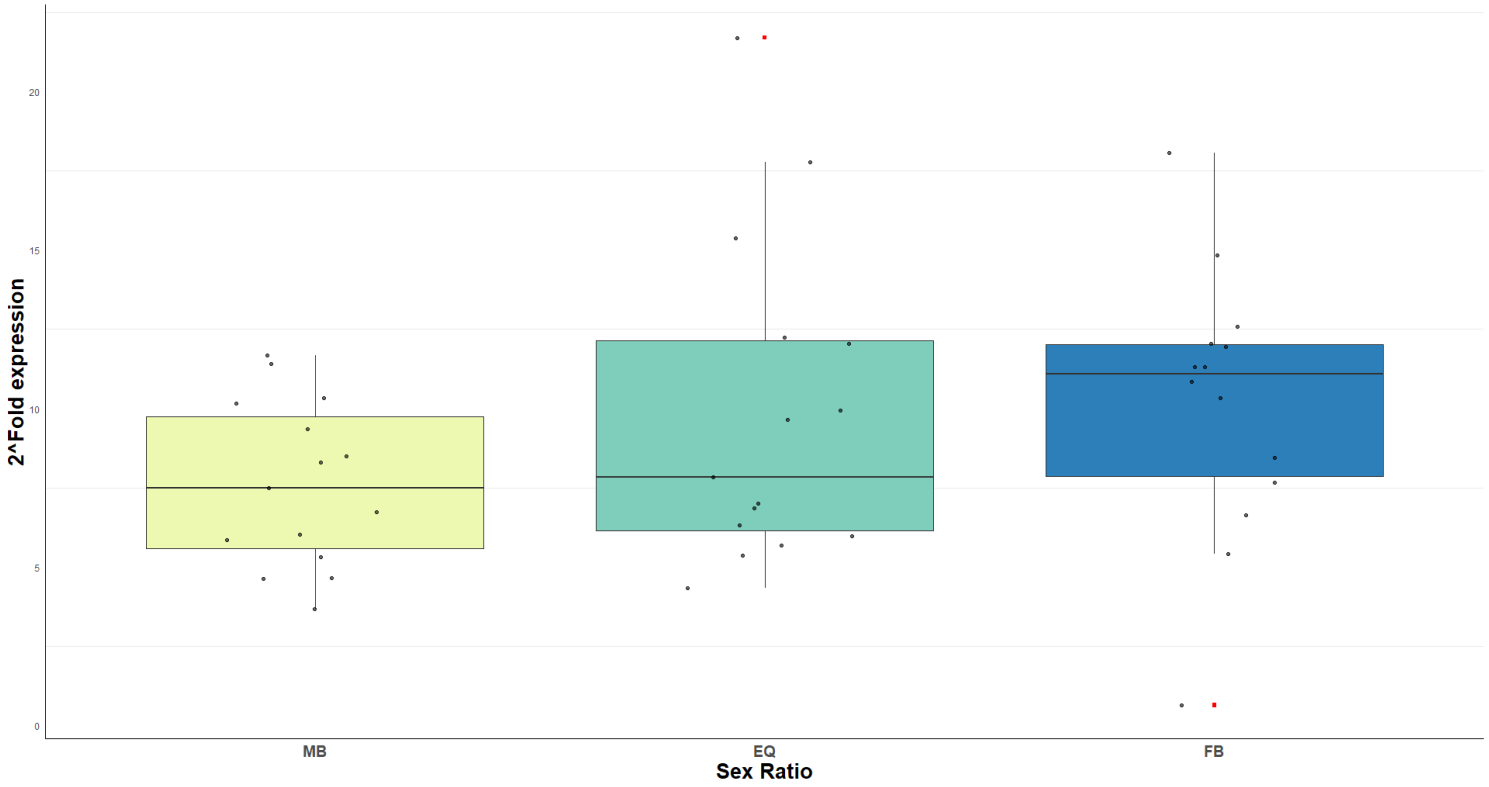

Fig 5.1. Normalised expression of the Sex Peptide (SP) gene for virgin males from the sex ratio regimes. 2^ gene expression for Sex Peptide compared to the house keeping gene eIF1a from 5-day and old virgin males from female-biased (FB), Equal sex (EQ) or male –biased (MB) sex ratios. Boxplot showing median line, with

boxes representing upper and lower 25% quartile and whiskers representing the range, and points representing individual records, outliers highlighted in red.

Sex Peptide gene expression of immediately mated males from adult sex ratio regimes.

As with the virgin males, the expression of sex peptide also did not differ significantly between the regimes after mating (Fig 5.2.) (*FB: t value = 1.190, residual DF = 45, p = 0.902, MB: t value = 0.123, residual DF = 45, p = 0.240*).

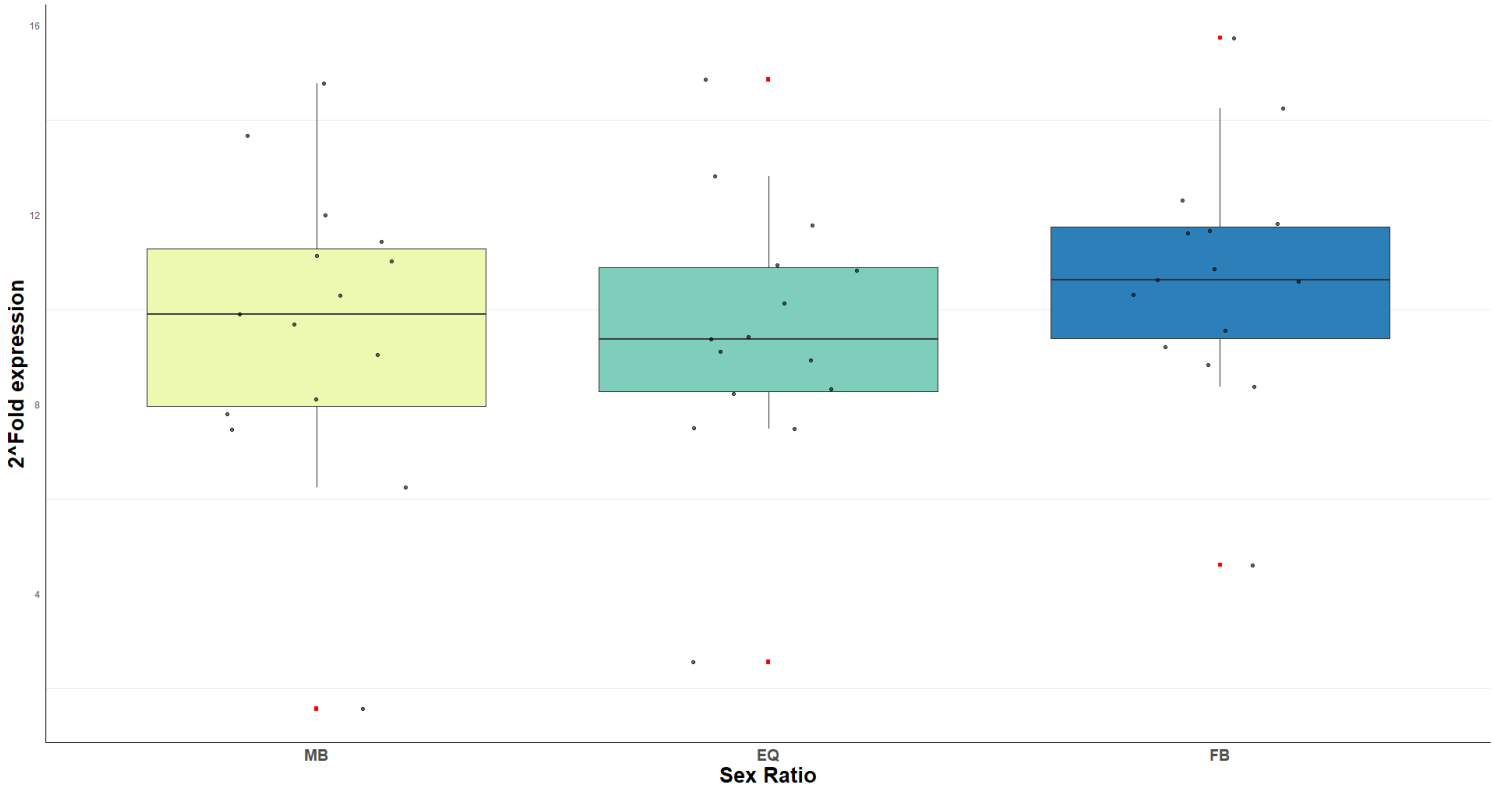

Fig 5.2. Normalised expression of Sex Peptide (SP) for mated males from the s ex ratio regimes. 2^ gene expression for Sex Peptide compared to the house keepi ng gene eIF1a from 5-day and old mated males from female-biased (FB), Equal sex (EQ) or male –biased (MB) sex ratios. Boxplot showing median line, with boxes repr esenting upper and lower 25% quartile and whiskers representing the range, and poi nts representing individual records, outliers highlighted in red.

Sex Peptide expression of males from adult sex ratio regimes, immediately after mating.

The change in expression of SP between virgin and mated males from each sex ratio was significantly different for MB males (*t value = 2.090, residual DF 45, p = 0.0423*), (Fig 5.3). However, post hoc analysis suggests this is an overall effect of sex ratio as pairwise comparisons are not statistically different. (*MB - EQ: z value = 2.090, p = 0.0917, FB - EQ: z value = 1.446, p = 0.3175, FB - MB: z value = -0.645, p = 0.7954*)

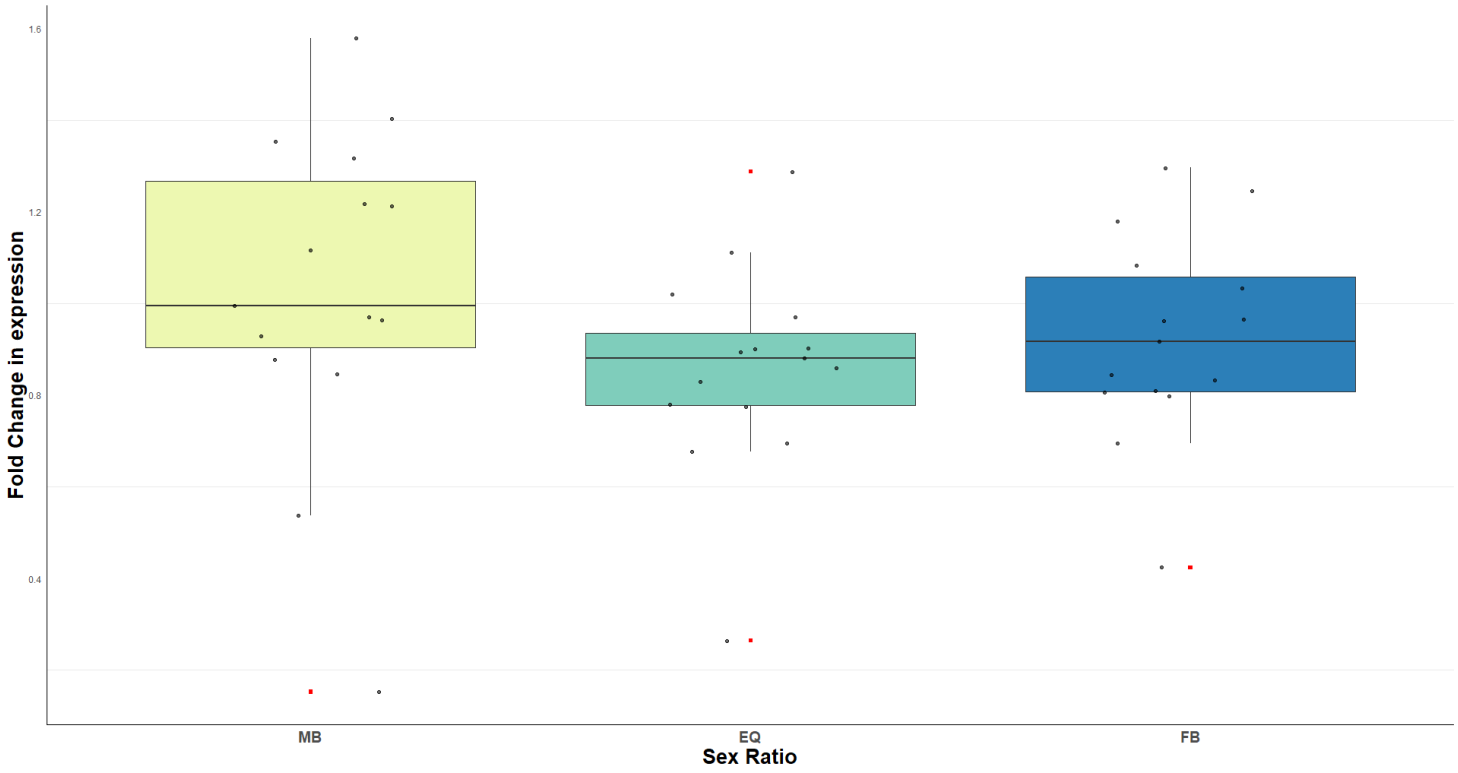

Fig 5.3. Comparative fold change in expression of Sex Peptide (SP) for mated males from the sex ratio regimes. 2^ΔΔCt expression change of SP expression between virgin and mated males from female-biased (FB), Equal sex (EQ) or male – biased (MB) sex ratios. Boxplot showing median line, with boxes representing upper and lower 25% quartile and whiskers representing the range, and points representing individual records, outliers highlighted in red.

Discussion

The overarching hypothesis tested was that SP expression would evolve in males exposed to differing adult sex ratios over 110 generations. This hypothesis is not supported in terms of base line levels of gene expression across the lines before and after mating. However, I did observe a significant difference in the change of expression between virgin and mated males before and after mating, with the expression of SP in MB increasing the most following mating. My results also highlighted a common challenge to studies of gene expression in which virgin versus mated individuals are compared. Of the two reference genes selected, only one was stable across the virgin-mated transition. The reference gene *α-Tubulin* had to be removed from the analysis as its expression increased significantly in the mated samples.

The relative expression of SP did not differ significantly by sex ratio in unmated males, counter to the prediction (table 5.1). Expression of SP appeared a little higher in FB lines post mating in comparison to the other regimes. However, this effect was not significant. When comparing expression differences between mated and virgin males, I found a significant difference in SP gene upregulation between virgin and mated MB males. The comparison of treatments show that SP is upregulated to a greater extent in MB males after mating compared EQ or FB regimes. Hence, though SP expression was generally higher in FB males, as found in a previous experiment (Supplementary material), it was the extent to which SP expression was induced that had evolved in these lines.

Expression of SP in virgin males

In contrast with our prediction that MB line males would express SP higher as virgins. The highly competitive environment these males have been subjected to over many tens of generations may have allowed the evolution of alternative strategies that could provide benefits in sperm competition, such as extended mating duration (Dore et al., 2021). Additionally, it has been shown in other studies that *D. melanogaster* divergently allocate ejaculate in response to the intensity of competition they perceive (Hopkins et al., 2019a). It could therefore be expected that

expression of SP would be higher in MB males. However, for this experiment males were housed together as virgins in vials of ten individuals for 5 days prior to the treatment and it could be that any evolved adaptation to the competitive environment might have been diminished by this exposure by the fifth day. As a result, each sex ratio male may have been able to able to produce similar quantities of SP resulting in similar SP gene expression across treatments. Whilst evidence shows that transferring more sex peptide to a female gives males a competitive advantage in a single mating (Ramm, 2020), it may in fact be a waste of resource when competition is consistently high, particularly for second males to mate (Fricke et al., 2009). Alternatively, SP not only has utility in eliciting female responses when binding to the SPR within the female, conveying fertilisation advantages, but it also facilitates the functions of other ejaculate components (Avila et al., 2010).

Expression of Sex Peptide post mating

The expression of SP immediately post mating was higher, but not significantly so, in FB lines compared to EQ and MB regimes. It has been shown that males with larger accessory glands mate more frequently (Bangham et al., 2002). Whilst results from chapter 4 show that FB males have larger accessory glands, this did not translate into a higher relative gene expression of the sex peptide gene. Male *D. melanogaster* allocate approximately a third of their accessory gland contents to a female (Monsma and Wolfner, 1988), and it is the transfer of these products that triggers the synthesis of more SFPs (Herndon et al., 1997). The lack of relative SP expression could be because all males will have transferred some SP and so the expression is turned on irrespective of the amount delivered. It could be useful to conduct similar experiments on multiply mated males to identify if expression is linked to SP transfer.

Comparison of Sex Peptide regulation before and after mating

Sex peptide provides a fitness benefit to the first male, securing a higher paternity (Fricke et al., 2009), this would suggest that MB males would benefit more from higher SP investment. Whilst I did not see a higher overall expression in relation to the experimental manipulation of sex ratio, the fold change of expression of SP after mating was higher in MB lines.

SP's effects on females are only found when SP is transferred with sperm (Chapman et al., 2003, Liu and Kubli, 2003) and the post mating effects of SP are not as strong for males that are the second males to mate (Fricke et al., 2009). It is possible that the significant upregulation of SP post mating occurs because the MB males may be transferring more ejaculate and therefore more SP. The likelihood of securing the first mating in a MB environment is low, and it has been demonstrated that males can detect whether a female is mated (Thomas, 2011). Hence, the transfer of more SP when mating first may help secure a higher paternity share. Previous studies reported MB males invest more in ejaculate than FB line males, as evidenced by a depletion of accessory glands over successive mating's (Linklater et al., 2007). It has been shown that males with larger accessory glands are more competitive – hence I would predict MB males should have larger glands (Wigby et al., 2009b). However, this was not observed (Chapter 4). I was unable to quantify SP transfer within this study, and it is not yet known whether MB individuals are able to transfer more SP to the females than FB males. Males adjust SFP components in their ejaculate in response to levels of sexual selection (Hopkins et al., 2019a). However, in this study, all males prior to mating were exposed to nine other rivals over a 5-day period. Males recognise rivals using multisensory cues (Bretman et al., 2011b) and so the differential expression of SP may only be initiated when rival males are detected in the immediate vicinity (Garbaczewska et al., 2013a).

Conclusion

This study shows that the impact long-term manipulation of the adult sex ratio can affect the way a male regulates gene expression of a key seminal fluid protein. Additionally, it shows the importance of having many stable housekeeping genes that are not differentially expressed post mating. Adult expression of accessory gland proteins is first initiated in the third larval instar (Monsma and Wolfner, 1988) and the divergent expression of sex peptide pre and post mating provides a foundation for future investigations of ejaculate composition and dosage. However, it is surprising that the larger accessory glands in FB lines was not related to evolved changes in SP gene expression. Larger accessory glands are associated with increased mating opportunities (Bangham et al., 2002) and so replenishment of the accessory gland components could be beneficial. Linklater et al. (2007), reported strong evidence of accessory gland depletion after consecutive mating's in MB lines comparative to FB males. It is possible that any evolutionary advantage large accessory glands convey in the FB lines with regards to SP gene expression may only be detected after multiple mating's. An important question to answer could be investigating whether SP gene expression is associated with SP dosage transferred to the female and whether this is altered in the presence of a rival differentially. FB males may not immediately recognise another male as a rival due to the excess of mating opportunities afforded to them in the evolutionary fixed sex ratio regimes. Additionally, previous studies have shown that exposure to SP has a potential fitness cost (Wigby and Chapman, 2005), and multiply mated females suffer lifetime fitness costs when exposed to more male attention in general (Koliada et al., 2020, Kuijper et al., 2006). Females in some instances have been shown to evolve resistance to this male harm (Rostant et al., 2020a), and it would be of interest to investigate whether females from the sex ratio lines have evolved to mediate exposure to sex peptide or other harmful components. Additionally, SFPs have been shown to evolve rapidly (Swanson et al., 2001) and I have provided evidence of sex ratio effects on only one of many seminal fluid proteins. Future, additional investigations into the evolution of whole ejaculate composition by sex ratio would be beneficial. Other SFPs that may be more utilitarian in a MB environment produced in other organs such as the ejaculatory bulb (Cohen and Wolfner, 2018) may convey an alternative competitive advantage.

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Supplementary material

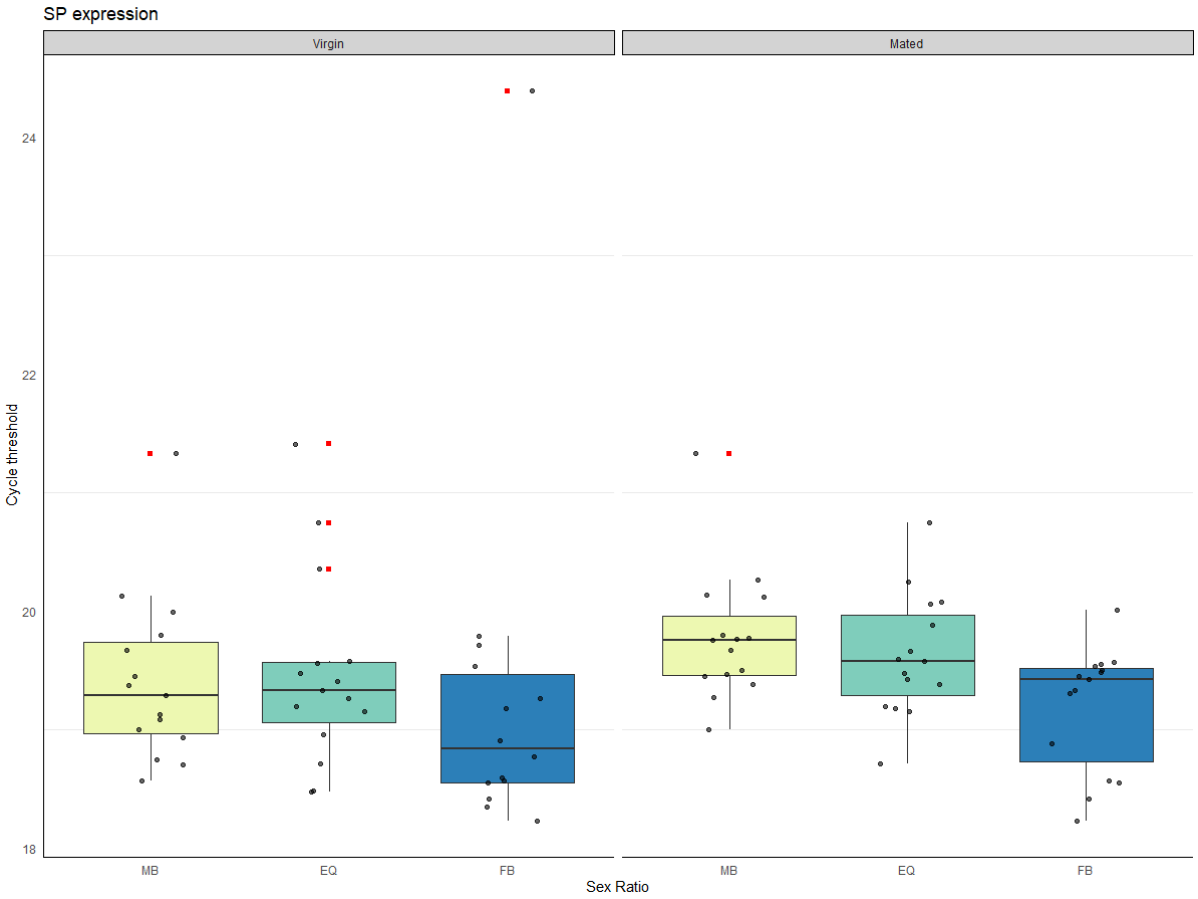

Fig S5.1a. Expression of Sex Peptide (SP) for males from the sex ratio regimes. Cycle threshold values representing gene expression from 5-day and old virgin males and 5-day-old mated males from female-biased (FB), Equal sex (EQ) or male –biased (MB) sex ratios. Boxplot showing median line, with boxes representing upper and lower 25% quartile and whiskers representing the range, and points representing individual records, outliers highlighted in red.

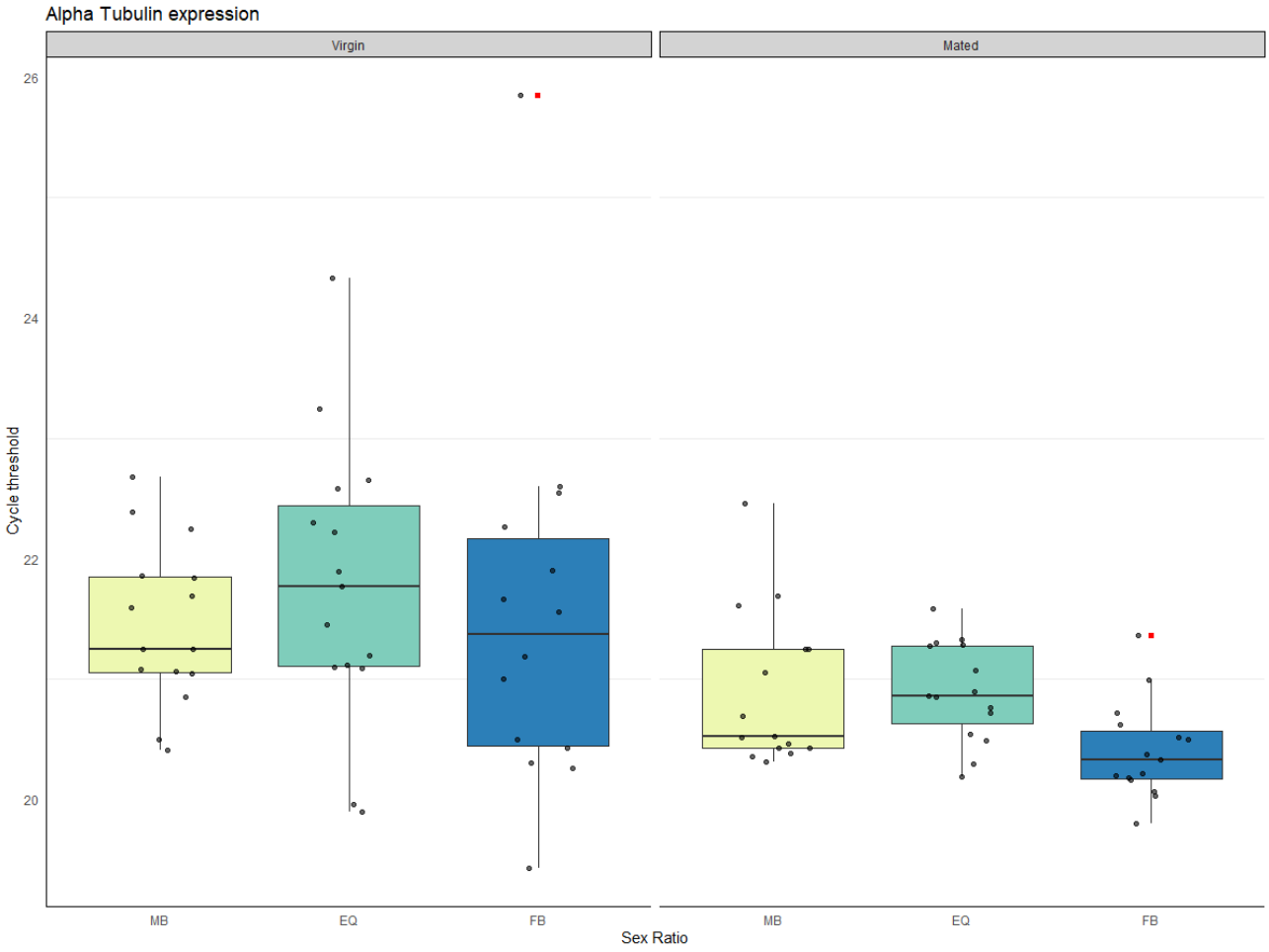

Fig S5.1b. Relative expression of alpha Tubulin for males from the sex ratio regimes. Cycle threshold values representing gene expression from 5-day and old virgin males and 5-day-old mated males from female-biased (FB), Equal sex (EQ) or male –biased (MB) sex ratios. Boxplot showing median line, with boxes representing upper and lower 25% quartile and whiskers representing the range, and points representing individual records, outliers highlighted in red.

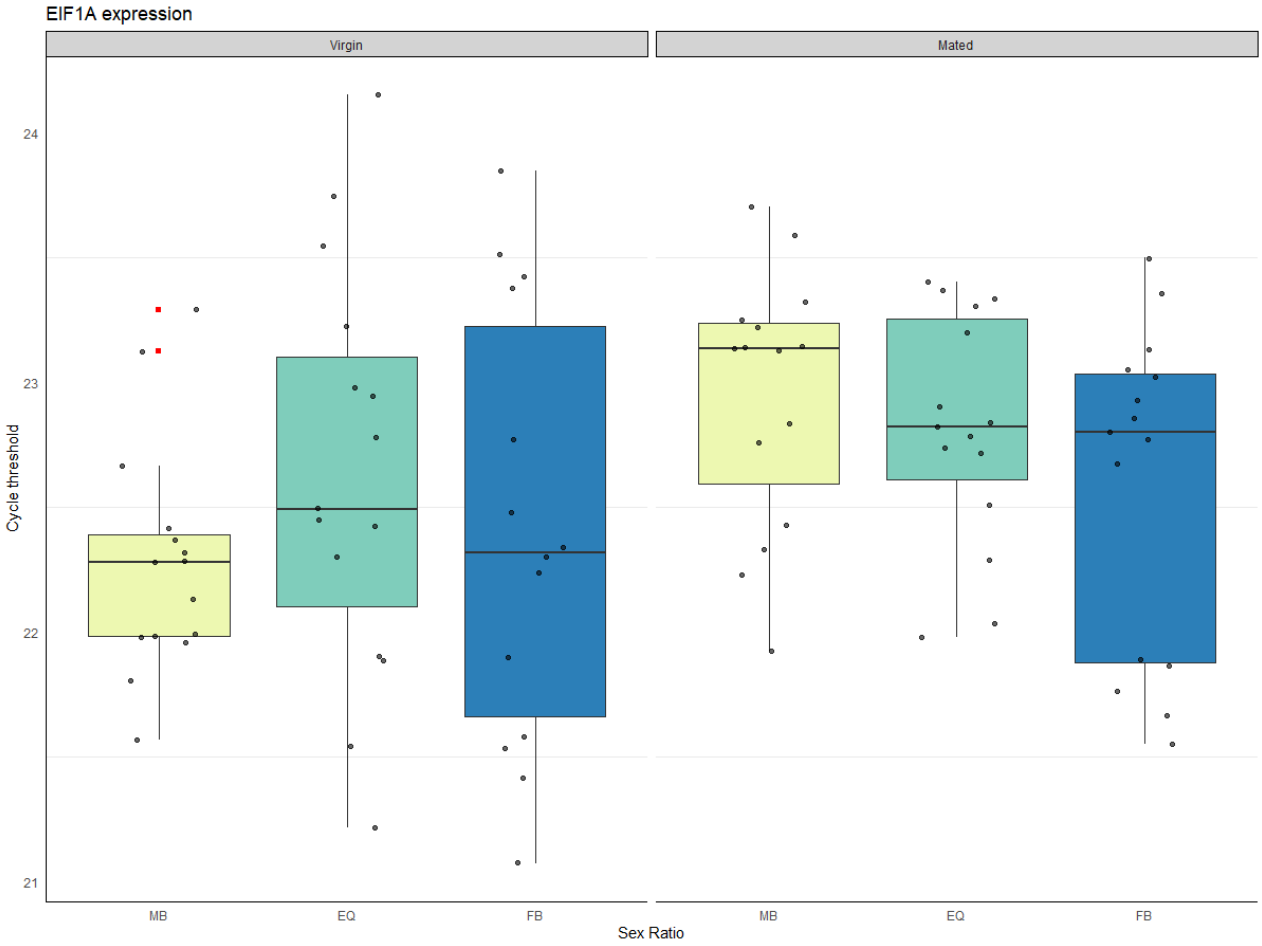

Fig S5.1c. Relative expression of eLF1A for males from the sex ratio regimes. Cycle threshold values representing gene expression from 5-day and old virgin males and 5-day-old mated males from female-biased (FB), Equal sex (EQ) or male –biased (MB) sex ratios. Boxplot showing median line, with boxes representing upper and lower 25% quartile and whiskers representing the range, and points representing individual records, outliers highlighted in red.

Pilot study Methods

Treatments used in the pilot study match those used in the main experiment with the addition of two extra treatments. At 5 days old flies males were assigned a treatment: **Treatment A** = 5-day old virgin males from each treatment were transferred to 1.5ml microtubes and flash frozen in liquid nitrogen, then transferred to storage at -80°C. **Treatment B** = 5-day old males from each population were mated with a virgin wild-type female and after mating finished, they were immediately flash frozen in liquid nitrogen and transferred to storage at -80°C. **Treatment C** = 5-day old males from each population were mated with a virgin wild-type female and 6h after mating finished, they were flash frozen in liquid nitrogen and transferred to storage at -80°C, **Treatment D** = 5-day old males from each population were mated with a virgin wild-type female and 24h after mating finished, they were flash frozen in liquid nitrogen and transferred to storage at -80°C.

Pilot results

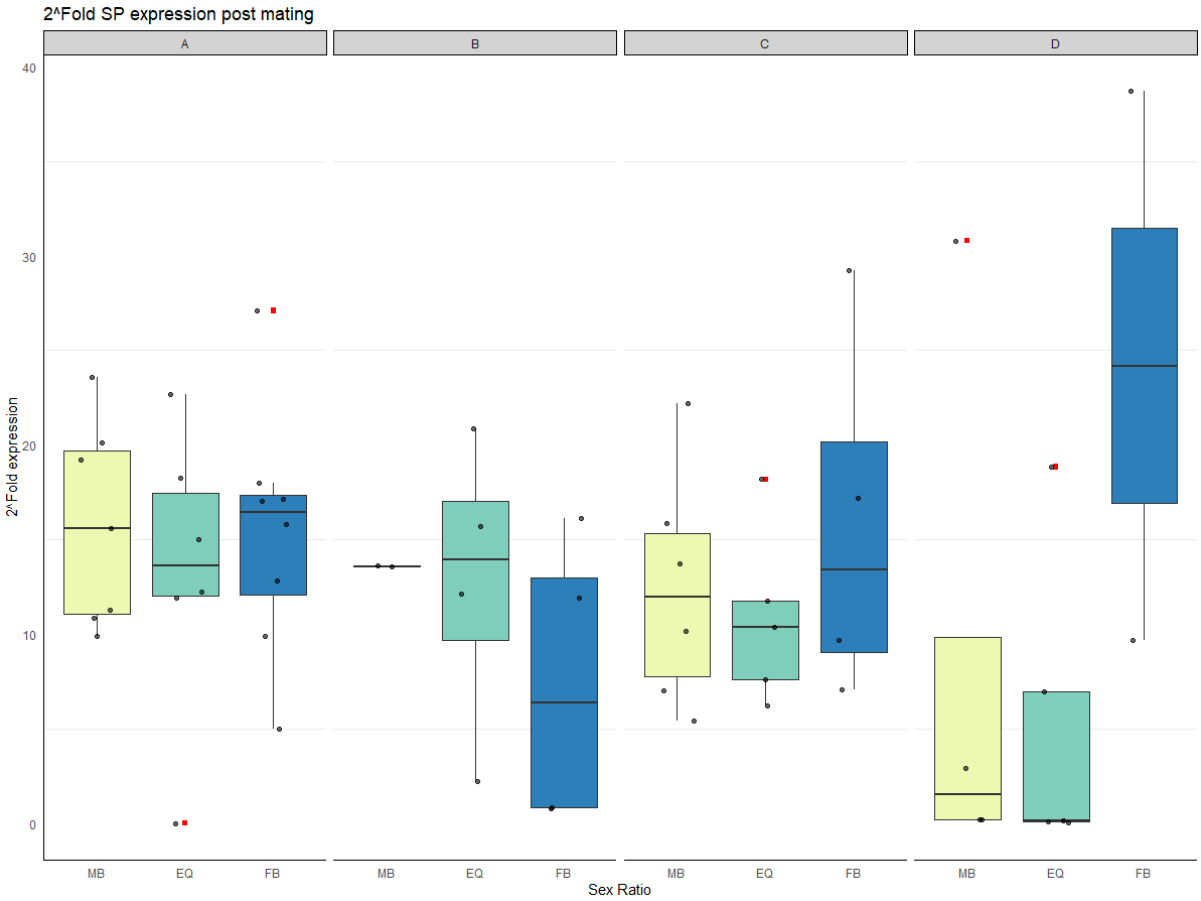

Fig S5.2. Expression of Sex Peptide (SP) for males from the sex ratio regimes. Cycle threshold values representing gene expression from 5-day males from female-biased (FB), Equal sex (EQ) or male –biased (MB) sex ratios. Treatment A = virgin males, Treatment B = Immediately after mating, Treatment C = 6hr post mating, Treatment D 24hr post mating. Boxplot showing median line, with boxes representing upper and lower 25% quartile and whiskers representing the range, and points representing individual records, outliers highlighted in red.

Chapter 6: General Discussion

Using experimental manipulation of adult sex ratios, my thesis research has provided new insights into how plastic and reproductive traits are shaped by the intensity of sexual selection experienced by individuals over generations. I first outline below the major findings of my research, before drawing out the broader themes, placing them in the wider context and finally proposing avenues for additional follow up research.

Socially plastic responses in females are robust to evolutionary manipulations of adult sex ratio and adult nutrition (Chapter 2).

I investigated here the evolution of socially plastic behaviours and their impacts on fitness. I drew from research that had shown that female fecundity is socially plastic. Specifically, wild type females held in isolation before mating produce significantly more eggs than females similarly held in same-sex environments (Churchill et al., 2021, Fowler et al., 2022b). I used lines subjected to experimental evolution to determine if these plastic responses also evolve in predictable ways, following long-term manipulation of adult sex ratio and adult nutrient availability. I predicted that evolutionary exposure to variation in adult sex ratio and nutrition would impact female responses to their pre-copulatory social environment. Specifically, I predicted that a history of increased competition amongst females (in Female-Biased, FB, regimes) would select for increasingly sensitive plastic fecundity responses to the presence of conspecifics. Additionally, I predicted that this response would be magnified in regimes subjected to long-term nutritional restriction in adulthood. In contrast to the predictions, I found that the socially plastic responses previously reported in wild type females was highly conserved and did not alter in response to long-term variation in the level of sexual competition or adult nutrient availability. The lack of response was not explained by insufficient selection as I did detect a response in virgin egg laying according to nutritional regime, and a response in virgin egg retention in response to the sex ratio regime. Overall, these results showed that females adjust their post-mating fecundity according to the social environment. This plasticity was unexpectedly robust to long-term evolutionary manipulations of sexual selection and resource levels. The results show how such plasticity can be hard wired to evolutionary perturbations.

Effect of experimental evolutionary manipulation of adult sex ratio and adult nutrition on development and body size-related fitness traits (Chapter 3)

I next investigated the evolved developmental effects of long-term-manipulation of adult sex ratio and adult nutrient restriction. Previous research using these same experimentally evolved lines showed that evolutionary manipulations of sexual selection resulted in both males and females expressing divergent courtship and mating behaviours (Dore et al., 2021), female aggression (Bath et al., 2021) and resistance to male-induced harm (Wigby and Chapman, 2004). The reported effects of adult sex ratio and nutritional restriction on the evolutionary trajectories of these key phenotypes provided foundational insights here in establishing and targeting my investigations into the developmental and size phenotypes of males and females from these lines. I examined egg size, developmental time, developmental survival, dry body weight and wing morphometry form both sexes. I found the egg sizes of High adult food (H) and Male-Biased (MB) (i.e. HMB) lines were significantly larger than their HFB (Female-Biased) counterparts. Interestingly, egg size was not significantly impacted by nutritional regime, suggesting that Low (L) nutrition females are able to compensate for the protein restriction. The development time was more difficult to tease apart, as time to pupariation was significantly slower in LMB flies, with the largest difference being between HMB and LMB, suggesting an interaction with long-term exposure to MB regimes. When investigating time to eclosion and separating by sex, the delay in development was only significant in females. Dry adult body weight was not significantly different in males. However, for females, there was an effect of sex ratio but only in the H food, suggesting that the responses were condition dependent. Perhaps the most striking result was evident in wing morphology, with both male and female flies from MB regimes having significantly larger wings than FB flies. This was found in both H and L regimes, but the nutrient restricted L lines had the largest wings overall, counter to the predictions. Overall, these results show that sex ratio and the adult nutritional environment can select for changes in development and size that are likely to underpin fitness-related phenotypes.

Effect of experimental evolutionary manipulations of adult sex ratio and adult nutrition on reproductive morphology and reproductive success (Chapter 4)

In order to identify further morphological correlates resulting from the experimental manipulation of adult sex ratio and adult nutrition, I tested how these factors select for divergent reproductive morphologies. Previous studies in these lines have revealed that long-term changes in adult sex ratio by modifying courtship behaviour and mating duration (Dore et al., 2021). Additionally, I showed in the work presented in Chapter 3 the evolution of morphological changes in these lines. I predicted that modifying the strength of selection and adult nutrition would similarly select on reproductive morphology. Specifically, the females from MB regimes will have been exposed to continually higher levels of mating and thus male ejaculates in comparison to FB regimes (Wigby and Chapman, 2004a). Therefore, I predicted that selection would favour larger sperm storage organs (spermatheca & seminal receptacle) in females from the MB lines. Conversely, males from MB lines will have been subject to higher levels of sperm competition and are predicted to respond by evolving longer sperm and larger testes. It was expected that long-term nutritional restriction in adulthood would also impact the extent of responses to selection in these phenotypes. The results showed that, in line with the prediction, females from MB lines had significantly larger spermathecae, but not seminal receptacles. Surprisingly, no differences were seen between adult dietary regimes. I tested for fitness correlates of these evolved morphological differences and interestingly the larger spermathecae of MB females did not result in higher offspring production in comparisons between once mated females allowed to lay into fresh vials each day for 15 days. However, there was a divergence in progeny production according to nutritional regime in the MB lines in the temporal production of offspring, whereby females attained a higher number of offspring 8-10 days post mating compared to other regimes. Males from FB lines also had significantly larger accessory glands, suggesting the increase in access to more mating opportunities selected for larger non-sperm ejaculates. The potential consequences of that morphological divergence in accessory gland size were subsequently tested in Chapter 5, by investigating the expression of the key seminal fluid protein sex peptide. The lack of response of reproductive morphology to adult diet regimes supports the idea that larval not adult

nutritional regimes are more important in ultimately determining the relative size of these key reproductive characteristics. The importance of these results shows that whilst evolutionary pressures are capable of driving phenotypic changes in male and female reproductive morphology, the benefit of these divergent traits may be more difficult to discern.

Effect of experimental evolutionary manipulations of adult sex ratio on the expression of a key seminal fluid protein in males (Chapter 5).

Following the evolved differences in reproductive morphology described in Chapter 4, I tested here for potential consequences of this divergence for the expression of a key ejaculate peptide gene in males. I tested whether the seminal fluid protein “Sex Peptide” (SP) gene was differentially expressed in response to the manipulation of adult sex ratio. Previous work on these lines revealed that males can respond by adjusting their ejaculate allocation patterns across mating’s (Linklater et al., 2007). The observed differences in male reproductive morphology (Chapter 4) and reported divergence in ejaculate allocation (Linklater et al., 2007) predicted that the constant exposure to increased or decreased levels of sexual selection in these lines would be likely to affect the expression of seminal fluid protein SP genes such as SP, both before, and immediately after mating. This expectation was also supported by the results of an initial pilot study (appendix Chapter 5). I predicted that MB regimes would evolve to express more SP prior to mating due to their evolutionary history of elevated exposure to increased levels of competition and the competitive advantage conferred by SP when males are the first to mate with a female. FB regime males were predicted to express SP at higher rates after mating because of the evolved differences seen in male accessory gland size from Chapter 4 and the pilot study. Counter to the predictions SP was not differentially expressed in males from the sex ratio regimes prior to or after mating. However, MB males were found to significantly upregulate the expression of the SP gene more rapidly after mating than was found for the Equal sex (EQ) or FB males. The results show overall expression of a key seminal fluid protein ‘sex peptide’ is relatively stable despite the varying levels of sperm competition and divergent reproductive morphology (Chapter 4). However, the difference in upregulation for MB males, suggest that the transfer of sex peptide may

be divergent and provides foundational work for the investigation of ejaculate composition and transfer in the future.

Conclusion and Future Directions

This thesis has provided evidence for evolutionary adaptations to the strength of sexual selection. I have explored the phenotypic plasticity of social responses, which proved highly conserved. In addition, I have shown that key morphological and reproductive traits, respond to levels of sexual selection in sometimes unpredictable and surprising ways. Furthermore, I have shown that sexual selection can also shape the differential expression of ejaculate genes.

Whilst this body of work demonstrates the impact of experimental manipulations of adult sex ratio and adult nutrition on the evolution of fitness-related traits in general, each chapter presents opportunities for future directions for clarifying and understanding the ultimate significance of the phenotypes that have been characterised.

Socially plastic responses in females are robust to evolutionary manipulations of adult sex ratio and adult nutrition. (Chapter 2)

Plasticity in differential fecundity in response to the pre-copulatory social environment is not well understood, and so additional investigations into this response are valuable. For example, we now know that these plastic responses are robust and do not require the presence of females, only their non-egg deposits, in order to be triggered (Fowler et al., 2022b). What we do not know is how long this effect lasts for, and whether it requires continual exposure to signs of female presence.

Another unknown is highlighted by the differential production of virgin eggs. Little research has been conducted on this widespread trait in *Drosophila*. Parthenogenesis was theorised as a possible function. However, in *Drosophila*

melanogaster virgin eggs are produced in large quantities and parthenogenesis has not been observed (Markow, 2013). Experiments exploring the mechanism behind the fitness significance of virgin egg production and the content of these eggs would fill knowledge gaps. Additionally, the interaction I recorded with nutrient restricted MB lines suggests that there is a condition dependency to this trait but only under a MB social environment. Further exploration of this phenotype could help understand the function (if any) of virgin egg production.

Effect of experimental evolutionary manipulation of adult sex ratio and adult nutrition on development and body size-related fitness traits in Drosophila melanogaster. (Chapter 3)

The phenotypes produced in this study have opened the door to future experiments related to male competition and nutritional restriction. For example, what advantage do larger wings in MB regimes convey for both male and female *D. melanogaster*? It is expected that the wing size variation I recorded is driven by the intensity of male competition (Menezes et al., 2013). Previous experiments demonstrating courtship divergence (Dore et al., 2021) suggests a difference in courtship song and the evolution of distinct mating regime phenotypes. Experiments involving courtship song, its competitiveness and attractiveness to opposing regimes would explore these ideas and provide more quantitative evidence of divergence in reproductive patterns.

The reported phenotype of nutrient restricted regimes possessing larger wings was unexpected. Wing size has typically been associated with fitness (Partridge et al., 1987b) and so would seem counter to the effects of restricting their adult diet, though it should be noted that wing size is primarily determined by larval nutrition which was not manipulated in these lines. Additionally, wing size is often used as a proxy for body size (Reiss, 1989). However, my work showed that adult body weight and wing size dimensions did not covary strongly. Understanding fitness effects by competing the restricted diet flies with rivals from high fitness regimes could be useful. It would also be interesting to see if individuals with larger wings are more adept at flying or singing.

Effect of experimental evolutionary manipulations of adult sex ratio and adult nutrition on reproductive morphology and reproductive success in Drosophila melanogaster. (Chapter 4)

This chapter explored the relatively understudied dynamics of sperm storage in the female reproductive tract. Many studies focus on sperm competitive traits in the male, but not the response in females. Here I showed that exposure to increased sperm competition drove the evolution of female spermatheca but not seminal receptacle size. What was interesting is that the enlarged spermathecae did not seem to correlate with any fitness effects measured. One avenue for future exploration should be assessing the secreted proteins produced by the spermatheca in response to male ejaculates. We know that females from MB lines are more resistant to male harm (Wigby and Chapman, 2004a), and additionally in other species it has been shown that increased exposure to males increases lifetime fecundity but reduces longevity (Jehan et al., 2020, Booksmythe et al., 2014). One mitigating factor for resistance may be produced in the female spermathecae in response to ejaculatory components (Barnes et al., 2008).

Effect of experimental evolutionary manipulations of adult sex ratio on the expression of a key seminal fluid protein, the Sex Peptide, in Drosophila melanogaster males. (Chapter 5)

The difference in speed of ejaculate gene expression upregulation between MB males was interesting. However, it is important to note that gene expression does not determine the investment of sex peptide actually translated and transferred into females. Future experiments should attempt to quantify the amount of sex peptide transferred and also compare the expression after mating multiply, as the depletion of accessory glands was more pronounced in MB males after multiple mating's (Linklater et al., 2007). It could also be very useful to investigate the expression some other important seminal fluid proteins such as Acp26a (Ovulin) (Herndon and Wolfner, 1995), Acp36DE (Neubaum and Wolfner, 1999a) and PEB-me (Lung and Wolfner, 2001).

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