Love thy neighbour? Social and sexual accommodation in fruit flies

Thesis submitted for PhD

By

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Abstract

Many animals plastically adjust their reproductive phenotype in response to their social and sexual environment. A common example of this type of plasticity occurs when males tailor their reproductive effort to the risk and intensity of sperm competition. In this thesis, I studied reproductive plasticity in male *Drosophila melanogaster* in response to cues of sexual competition. I found some evidence that cues signalling the likelihood of male-male competition affected the morphology of male reproductive structures and wings differently at two developmental stages, suggesting a high degree of environmental sensitivity in these traits. However, these findings were not fully consistent, highlighting the limitations of proxies when measuring complex, multi-faceted traits. I also showed that reproductively plastic behaviours can evolve in response to the prevailing social/sexual environment. Male *D. melanogaster* that evolved under a high degree of male-male competition expressed longer overall mating duration, reduced courtship delivery and altered courtship repertoire, in comparison to males evolved under less intense competition. I investigated the role of redundancy in cues signalling male-male competition and showed that occluding one sensory modality did not reduce the ability of male *D. melanogaster* to detect rivals and express behavioural responses. However, responding to rivals by extending mating duration did not confer any clear fitness benefits under the conditions tested. Finally, I tested the hypothesis that responses to redundant environmental cues can be underpinned by redundancy at the gene expression level. I found preliminary evidence that quasi-equivalent behavioural responses to rivals by male *D. melanogaster* can be reached by alternative transcriptomic pathways. Overall, this thesis demonstrates the important and varied effects that the social and sexual environment can have on individual development, behaviour, fitness, and the evolution of populations. My findings highlight the important context-dependence of many key reproductive traits and suggest several important avenues for future research.

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Declarations and author contributions

The work presented in this thesis is my own, with the following acknowledgements:

Chapter 1 – General introduction

I adapted this chapter from the following paper: Dore et al. (2018) The role of complex cues in social and reproductive plasticity. *Behavioral Ecology and Sociobiology* 72(8), 124. I conceived the original paper with Tracey Chapman, Amanda Bretman and Matthew Gage and I wrote it with input from the above, Laurin McDowall and James Rouse. Chapter 1 includes excerpts from this paper with additional content authored by me.

Chapter 2 - The effect of social cues on developmental and adult plasticity

I conceived and designed these experiments with Tracey Chapman, Amanda Bretman and Matthew Gage. Jennifer Perry advised on morphometric analysis. All other experimental work and analysis was carried out by me.

Chapter 3 - Plastic male mating behaviour evolves in response to the competitive environment

I conceived and designed these experiments with Tracey Chapman, Amanda Bretman and Matthew Gage. Tracey Chapman, Emily Fowler, Wayne Rostant, Stewart Leigh, Michael Darrington, Aaron Thomas, Nicholas West, Kris Sales, Rebecca Lewis, Jenny Donelan, Adam Constantine, Claudia Martin and Siobhan Hillman helped to monitor mating assays. Wayne Rostant and Kris Sales assisted with statistical analysis. All other experimental work and analysis was carried out by me.

Chapter 4 - Fitness consequences of redundant cues of competition in male *D. melanogaster*

I conceived and designed these experiments with Tracey Chapman, Amanda Bretman and Matthew Gage. Tracey Chapman, Emily Fowler, Wayne Rostant, Stewart Leigh, Michael Darrington and Kris Sales helped to monitor mating assays. Adam Constantine helped with counting offspring and scoring paternity. Wayne Rostant and Kris Sales assisted with statistical analyses. All other experimental work and analysis was carried out by me.

Chapter 5 - Redundant networks and alternative expression pathways: a test case using conspecific competitive responses in male fruit flies

The experimental work for this chapter was conceived and designed by Tracey Chapman, Emily Fowler, Amanda Bretman and Irina Mohorianu. Experimental work was carried out by Emily Fowler. Bioinformatics were carried out by Irina Mohorianu. I designed the analysis with Tracey Chapman, Emily Fowler, Amanda Bretman and Irina Mohorianu. Analysis was carried out by me.

1. General introduction

N.B. sections of this chapter are adapted from:

Dore et al. (2018) The role of complex cues in social and reproductive plasticity. *Behavioral Ecology and Sociobiology* 72(8), 124 (Appendix 1).

1.1 The role of reproductive traits in fitness and evolution

Traits directly involved with reproduction, such as mating behaviour, reproductive morphology, mating success and offspring production, are key contributors to the fitness of organisms, and can be shaped by many biotic processes that operate over short and long-term scales. These processes include the reproductive success and mortality of focal individuals, their mating partners and their offspring, as well as sexual competition, social behaviour, mating systems and mutation load (West-Eberhard 1989; Wigby and Chapman 2005; Amdam et al. 2006; Bretman et al. 2009; Immler et al. 2014; Lumley et al. 2015; Anholt et al. 2020). Understanding the role of reproductive traits in these processes can be facilitated by identifying and quantifying the costs and benefits of their expression in various contexts. The balance between the costs and benefits of expressing a reproductive trait in the prevailing environment can determine the strength and direction of selection on the trait, with effects on the evolutionary trajectory of individuals within populations. The applications of research in this area are broad. For example, reproduction and lifespan are closely linked (Lee et al. 2008; Travers et al. 2015). Relationships between fertility and longevity in both male and female humans have been identified (Jasienska 2009; Eisenberg et al. 2014). Furthermore, the effect of heatwaves on male fertility and sperm competitiveness has recently been studied as a driver of reduced reproductive output, with potential impacts on biodiversity declines through climate change (Sales et al. 2018). Finally, female mate recognition and the effects of male accessory gland products have important roles in determining the effectiveness of satyrization (a form of reproductive interference) as a method of controlling the population of dengue mosquitos (Bargielowski et al. 2013). Thus, increasing understanding of the expression, costs, benefits and evolution of male reproductive traits can provide a broad range of fundamental biological insights.

In this thesis, I consider how the reproductive phenotypes of males show both plastic and evolutionary adaptation to the social and sexual environment. Primarily, I focus on tailoring of male reproductive investment in response to cues signalling male-male

competition perceived during both development and adulthood. I investigate how the extent of male investment in reproductive behaviours, and the degree of flexibility in these behaviours, evolves in response to the social/sexual and nutritional conditions. Thereby, I aim to gain understanding of how the costs and benefits of reproductive investment and plasticity are mediated by the environment. Furthermore, I explore the possibility that accurately matching a phenotype to the environment is facilitated by the perception of cues made up of multiple components, which may be fully or partially redundant. Finally, I test whether plasticity informed by redundant cues may be underpinned by further redundancy within genetic networks – i.e., that equivalent phenotypes may be achieved via alternative transcriptional pathways.

Reproductive traits can be subject to strong selection, including sexual selection. A primary driver of sexual selection is proposed to be that female gametes are large and contain resources for developing offspring, making them costly to produce. Male gametes are suggested to be smaller, cheaper and more numerous, giving males a higher reproductive potential than females (Bateman 1948; Kodric-Brown and Brown 1987). This leads to the general prediction that females should primarily exhibit choosiness (intersexual selection) and males should compete with other males for fertilisations (intrasexual selection; Bateman 1948). The large variation in the reproductive success of males, dependent on their mating rate (Bateman 1948), can lead to the evolution of elaborated male traits to attract females, even to the extent that these traits have negative effects on the male's survival (Darwin 1871; Zahavi 1975). These male ornaments may be genetically linked with the female preference for that ornament, leading to a rapid runaway evolutionary process by which the trait becomes highly exaggerated (Fisher 1930). Selection for males to successfully compete with each other for fertilisations can also act on traits with roles in competitions with other males for mates, or in post-copulatory sperm competition (Darwin 1871; Parker 1970; Simmons et al. 2017). However, sexual selection has considerable and far-reaching consequences on individuals, populations and species beyond just elaborated male traits. Sexual selection can cause particularly rapid evolution, due to the high and constant selection pressure to access mates and its potential for runaway evolution (Lande 1981; West-Eberhard 1983). Furthermore, sexual conflict arising from the different reproductive optima of males and females can also be a driver of rapid evolution (Gage 2004; Anholt et al. 2020). These processes can lead to sexual dimorphism, phenotypic divergence between populations, evolution of mating systems and, ultimately, reproductive isolation with the potential to drive speciation (Bateman 1948; Lande 1981; West-Eberhard 1983; Anholt et al. 2020). Sexual selection also has important effects on

population performance: removing sexual selection can decrease male aggression and increase female resistance to male-induced post-mating effects (Holland and Rice 1999) but may also ultimately lead to higher mutation load and extinction risk (Lumley et al. 2015).

1.2 Reproductive plasticity in response to the social and sexual environment

The strength and direction of sexual selection on reproductive traits is dependent on the social and sexual environment, which encompasses factors such as the risk and intensity of sperm competition, and the availability and condition of potential mates. This can be a particularly fast-changing aspect of the environment, showing substantial variation within individual lifespans (Kasumovic et al. 2008; Bretman et al. 2011a). The optimal reproductive strategy by which an animal can maximise its fitness will depend upon these social/sexual conditions. These shifting optima are expected to select for phenotypic plasticity in reproductive traits (Parker et al. 1997; Wedell et al. 2002; Rebar et al. 2019). Phenotypic plasticity, which allows an organism with a fixed genotype to express alternative phenotypes in different environments, can confer a substantial adaptive advantage in heterogeneous environments that change with moderate to high predictability (Via et al. 1995; Botero et al. 2015).

Plasticity has important evolutionary implications, as it may increase the diversity of traits upon which selection can act (West-Eberhard 1989; Pfennig et al. 2010). Conversely, environmentally-induced variation in the phenotype has been proposed to 'shield' the genotype from selection, thus slowing the rate of adaptive evolution (Ghalambor et al. 2007). Types of plasticity including indirect genetic effects (IGEs) and learning have been proposed to have particular effects on evolution. IGEs occur when the phenotypes of two individuals interact, affecting the expression of one other. This may be observed in behaviours such as aggression and courtship, where the behaviour of one individual influences the behaviour of an interacting individual (Moore et al. 1997; Schneider et al 2017). An evolutionary implication of this is that cross-generational phenotypic evolution in one trait may occur without change to the genes directly affecting that trait. This occurs because the expression of the trait responds to a trait in another individual, which itself may be undergoing genetic evolution (Moore et al. 1997; Wolf et al. 1998). Moreover, depending on the relationship between the interacting traits, IGEs may lead to either an increase or a decrease in the rate of evolutionary change (Wolf et al. 1998). In the case of learning, behaviours such as mate choice may be

influenced by earlier experiences, which may in turn affect speciation (Servedio et al. 2009). For example, *D. melanogaster* males can learn to reduce courtship towards *D. simulans* females following a period of exposure (Dukas 2004). Learned preferences such as these may contribute to reproductive isolation. These preferences can eventually become innate through the process of reinforcement by genetic assimilation (Magurran and Ramnarine 2004; 2005). A phase of accelerated phenotypic evolution, followed by a phase of slow genetic assimilation, may be commonly associated with the emergence of plasticity as a response to sudden evolutionary change. (Lande 2009).

In addition to its role in evolution, plasticity can also have substantial implications for individual fitness, for example by allowing individuals to tailor their reproductive investment to the circumstances of each mating opportunity. This can enable organisms to invest more heavily in profitable mating opportunities while being more prudent in others, avoiding the depletion of costly reproductive resources and optimising lifetime fitness (Kaitala 1991; Jordan and Snell 2002; Wedell et al. 2002). It has long been acknowledged that female reproductive resources, such as eggs and parental care, are costly and often limiting, leading to trade-offs between current reproduction, future reproduction and survival (Bateman 1948; Stearns 1989; Queller 1997). However, it is now also accepted that male reproductive characteristics, such as sperm and other ejaculate components, may also be costly and thus limited and potentially easily depleted (Preston et al. 2001; Wedell et al. 2002; Linklater et al. 2007). Therefore, both males and females may be expected to show plasticity in their reproductive phenotypes, in order to invest their limited resources to the optimal effect. Plastic responses can be modelled as a reaction norm – a function describing the expression of an individual's phenotype across an environmental gradient (Via et al. 1995; Nussey et al. 2007). A plastic trait may be characterised by both the elevation (the degree of the expression of the response) and the slope (the extent to which the expression of the response changes across the environmental gradient, i.e. the degree of plasticity; see Dore et al. 2018). Individuals may vary in both the elevation and slope of a response due to genetics and non-genetic factors, such as experience and condition (Blumstein and Bouskila 1996; Nussey et al. 2007). Furthermore, social interactions are proposed to influence the evolution of reaction norms, affecting both between-individual and within-individual variation in the elevation and slope of a response (Dingemanse and Araya-Ajoy 2015). Plastic responses can occur on a spectrum of specialist to generalist (Gabriel et al. 2005). A more specialist strategy is characterised by a steeper gradient, resulting in a highly expressed response in some environments and a low level of expression in others, whereas a generalist strategy can be modelled by a flatter reaction norm.

Limitations to the adaptive value of plasticity, such as time lags between environmental change and response, or receiving incomplete information on the environmental conditions (DeWitt et al. 1998), are predicted to result in a trade-off between specialist and generalist strategies (Gabriel et al. 2005).

The sensitivity of plastic traits to the environment, and the fitness consequences of plasticity, often vary across life stages (Groothuis and Taborsky 2015). Plastic traits can be fixed irreversibly during development (developmental plasticity), either in anticipation of the future environment or as a response to the current conditions (Kasumovic and Brooks 2011; Kasumovic 2013; Snell-Rood 2013). Alternatively, plasticity can occur as a rapid, flexible and potentially reversible response at any life stage (Snell-Rood 2013). During specific periods of development, known as sensitive windows, phenotypes can be particularly strongly influenced by the environment (Fawcett and Frankenhuis 2015). The adaptive value of plasticity is always dependent upon receiving accurate and relevant information on the environment (DeWitt et al. 1998), and this may be particularly important during sensitive windows and early in development. Later in life, when an individual has more experience of the long-term prevailing environment and the potential for future reproduction diminishes, the fitness consequences of monitoring and responding to the current environment may decrease (Frankenhuis and Panchanathan 2011; Rebar and Greenfield 2017).

There is evidence of both males and females expressing developmental and reversible plasticity in response to the social and sexual environment. For example, female mate choice is often highly plastic. The preference of female mice (*Mus musculus*) for male songs is influenced by auditory experience during development (Asaba et al. 2014). Similarly, the brood size that female swordtail fish (*Xiphophorus multilineatus*) experience as embryos affects preference for visual male signals (Lyons et al. 2014). In both instances, these developmentally plastic female preferences are also mediated by factors in the adult environment, such as the presence of male pheromones and nutrition. This demonstrates that the expression of plastic, reproductive traits can be determined by an interaction between the developmental and adult environment. In the fruit fly *Drosophila melanogaster*, larval density significantly affects both male- and female-mediated sperm precedence via developmental plasticity of seminal receptacle and sperm length (Amitin and Pitnick 2007). Furthermore, male *D. melanogaster* express well-characterised plastic responses to encountering conspecific male rivals. When a male is exposed to a rival before mating, the subsequent mating duration is significantly longer. This response is associated with increased ejaculate investment and higher reproductive success (Bretman et al. 2009; Figure 1.1). The behavioural plasticity in mating

duration expressed by male *D. melanogaster* is highly sensitive to the social environment, and fully reversible when competitors are removed (Bretman et al. 2012).

1.3 Male plasticity in response to sperm competition

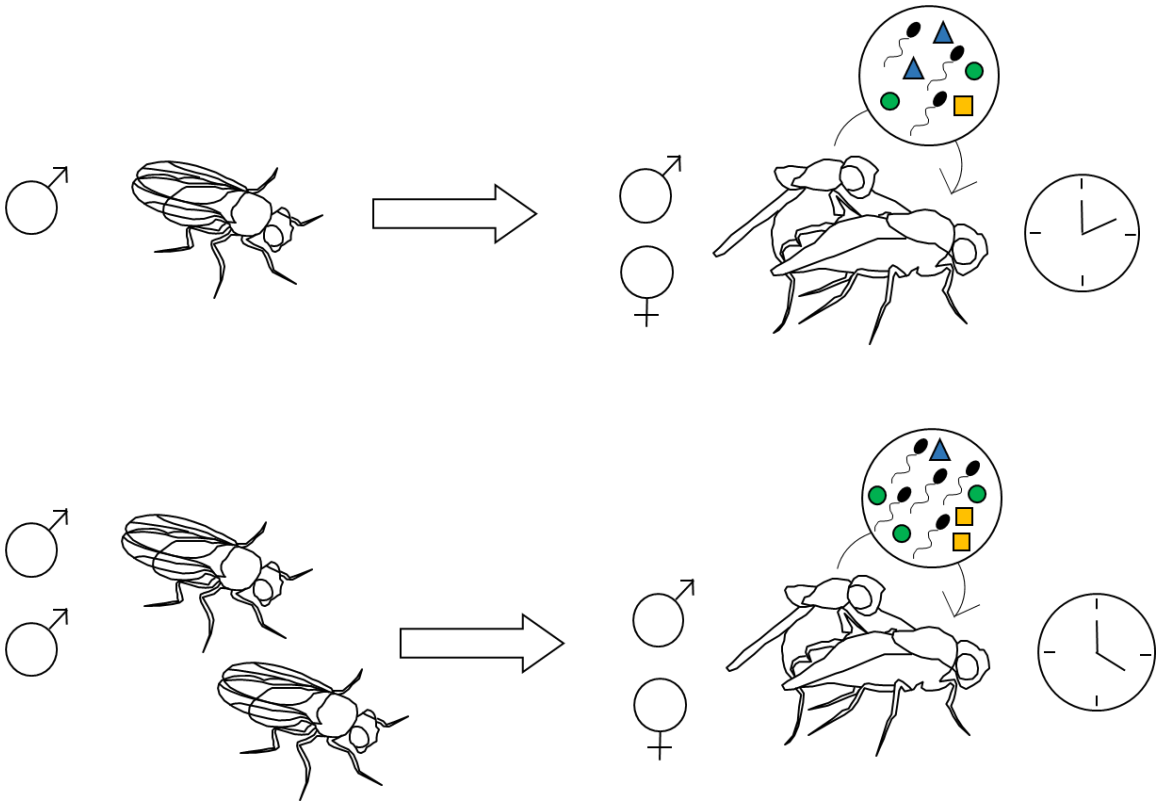
For males, sperm competition is a key element of the social and sexual environment to which plastic, reproductive traits respond (Figure 1.1). Sperm competition occurs whenever the sperm of two or more males compete within a single female to fertilise an ovum (Parker 1970) and can be a strong selective force affecting male behaviour and ejaculate investment (Wedell et al. 2002). The risk of sperm competition (i.e. the likelihood that it will occur) and the intensity (i.e. the number of competitors) can both have distinct effects on male reproductive traits (Parker et al. 1996, 1997). Across species, there is a general pattern of male reproductive investment increasing with the intensity of male-male competition: a positive correlation between relative testis size and the degree of polyandry has been established in many taxa (Smith 1984; Birkhead 1998; Wedell et al. 2002). Within species, models predict that plastic ejaculate investment will increase with the risk of sperm competition, but that investment will decrease when the number of competitors is greater than two, as the likelihood of successful fertilisation diminishes (Parker 1990; Parker et al. 1996; Bretman et al. 2011a). Males are also predicted to adjust their investment in sperm competition depending on the order of mating and their role in the mating system. Furthermore, per-mating investment is subject to trade-offs with obtaining further mates (Parker 1990). In addition to sperm competition, there is also frequently pre-copulatory competition between males to obtain mates (Bretman et al. 2011a). The balance of pre- and post-copulatory sexual selection can itself be mediated by the social/sexual environment, specifically the level of polyandry (Morimoto et al. 2019). Collectively, these factors select for male responses that can be fine-tuned to various social conditions. In addition to ejaculate allocation, these plastic male responses include traits such as courtship, mating duration and mate guarding (reviewed in Bretman et al. 2011; Wedell et al. 2002). Detailed adjustments of various male reproductive responses to the social/sexual environment has been identified across taxa including mammals, birds, fish and insects (Engqvist and Sauer 2003; Ramm and Stockley 2008; Immler et al. 2010; Bierbach et al. 2011). However, I focus primarily on *D. melanogaster*, the plastic male responses of which are well evidenced (e.g. Bretman et al. 2009; Wigby et al. 2009b; Hopkins et al. 2009).

There is ample evidence to show that the quantity of sperm and other components of the ejaculate can be increased or decreased in response to the risk and intensity of sperm

competition (Wedell et al. 2002; Wigby et al. 2009b; Sloan et al. 2018). However, recent research has showed that insight can be gained by examining the finely tuned adjustments to seminal components that can affect the composition, not just the overall quantity, of the ejaculate (Perry et al. 2013; Hopkins et al. 2019). The costs of producing each seminal fluid component (sperm, seminal fluid proteins (SFPs), salts, sugars, lipids, water, etc.) and their effects on the female differ (Perry et al. 2013). Insect SFPs have a range of female effects including decreasing female receptivity to remating, increasing egg production, changing female sleep and activity patterns, promoting sperm storage, mediating aggression and forming mating plugs (Chapman and Davies 2004; Avila et al. 2011; Abraham et al. 2016; Bath et al. 2017). Thus, the optimal transfer of each component may differ across gradients of the social and sexual environment. Indeed, the composition of SFPs in the ejaculate has been found to be adjusted according to cues of sperm competition in several species (Ramm et al. 2015; Simmons and Lovegrove 2017; Hopkins et al. 2019). In the *D. melanogaster* ejaculate, sperm and clusters of SFPs have been found to differ in their sensitivity to perceived competition and their abundance across a sperm competition gradient. This demonstrates that fine-grain adjustments to the ejaculate composition can be made in response to the social environment (Hopkins et al. 2019).

In Chapter 2, I address outstanding questions related to the expression of developmental plasticity of the accessory glands of male *D. melanogaster* in response to the social environment (Bretman et al. 2016). The developmental social environment experienced by male *D. melanogaster* has been proposed to cue the anticipated level of competition in adulthood and impacts several aspects of the adult phenotype. Male *D. melanogaster* that developed at high larval density were larger, produced two key SFPs in greater quantities, and had higher mating success with previously mated females (Wigby et al. 2016). Furthermore, males that develop at either higher larval densities or in the presence of adult males have larger accessory glands at maturity (Bretman et al. 2016). I investigated the degree of sensitivity of developmentally reproductive traits to details of the social environment, as well as whether the strength and direction of the effect of competition on reproductive traits changes at different stages of development.

Figure 1.1 – Reproductive plasticity of male *Drosophila melanogaster* in response to cues of sperm competition. Following exposure to rivals, males significantly extend mating duration (Bretman et al. 2009) and adjust the quantity and composition of the ejaculate (Wigby et al. 2009a; Hopkins et al. 2019).



1.4 Evolutionary responses to the social and sexual environment

While short-term changes in the social and sexual environment can select for individual plasticity, longer-term trends can lead to population-level evolved responses to the prevailing environment. For example, seminal fluid proteins, which are allocated plastically across matings by *D. melanogaster* males (Wigby et al. 2009b), show evidence of rapid evolution under positive selection (Haerty et al. 2007; Anholt et al. 2020). Rapid evolution of seminal fluid proteins has also been identified in mammals (Clark and Swanson 2005; Dean et al. 2011). Experimental evolution approaches offer excellent potential for directly testing the effects of the social environment on the evolution of male reproductive traits. For example, the correlation between testis size and polyandry across taxa is supported by the evolution of larger testes after only ten generations of experimentally increased sperm competition in the yellow dung fly (*Scathophaga stercoraria;* Hosken and Ward 2001). In *D. melanogaster*, there is an extensive experimental evolution literature demonstrating the strong selection that can be exerted by the social and sexual environment on both male and female reproductive traits. Reuter et al. (2008) built on evidence that testis size evolves in response to the social environment by demonstrating that *D. melanogaster* males experimentally evolved under highly female-biased sex ratio had larger testes, likely as a response to sperm depletion. Other research has showed that evolutionary sex ratio has effects on patterns of ejaculate investment across matings (Linklater et al. 2007). Furthermore, female resistance to male-induced harm increased following evolution under experimentally increased sexual conflict, with effects on female survival (Wigby and Chapman 2004). The combination of experimental evolution with genetic manipulation to test responses to elevated polyandry has shown that the mating system can mediate trade-offs between male mating frequency and both pre- and post-copulatory per-mating investment (Perry et al. 2016). Collectively, these studies demonstrate that marked changes to both fixed and plastic reproductive traits can rapidly evolve in response to the social and sexual environment.

In addition to the overall level of expression of reproductive traits evolving in response to social/sexual conditions, the degree of plasticity in such traits may also be evolutionarily labile. Plasticity is expected to be particularly beneficial, and thus strongly selected for, in environments that are highly variable with a moderate-high degree of predictability (Botero et al. 2015). Conversely, when the environment is static or unpredictable, the adaptive value of plasticity can be expected to be lower. If there are costs to the maintenance of plasticity, these will be relatively higher in such environments and may lead to selection against plasticity, favouring the evolution of fixed responses (Hedrick et al. 1976; Givnish 2002; Hall and Colegrave 2008; Murren et al.

2015). Costs of plasticity may include the energetic and cognitive expenditure required to accurately monitor the environment via receiving, processing, learning and/memorising cues, and produce a modified phenotype (DeWitt et al. 1998; Relyea 2002; Rouse et al. 2018). However, the question of whether these costs are sufficient to exert negative selection on plasticity remains disputed (Masel et al. 2007; Maughan et al. 2007; Murren et al. 2015). In Chapter 3, I investigated key questions related to the evolution of reproductive investment and plasticity by male *D. melanogaster* in response to fixed sex ratio and diet. I employed an experimental evolution approach to empirically test how a range of plastic male reproductive behaviours respond to the social, sexual and nutritional environment, and whether plasticity in these responses erodes under environmental stability.

1.5 Monitoring of the social/sexual environment via complex cues

In order for individuals to adaptively respond to their social and sexual environment, they must be able to perceive accurate and reliable environmental information (DeWitt et al. 1998; Auld et al. 2010). This may be facilitated by the perception of cues comprising multiple components or modalities, i.e. complex cues (Dore et al. 2018). Throughout, I consider a 'cue' as any indicator that can be used to perceive information about the social/sexual environment by an individual. I consider 'cues' to be information transmitted between individuals either 'intentionally' or unintentionally. For instance, body size, which can potentially signal information on aspects of morphology/general condition, versus visual/auditory displays, which give potentially more targeted information, can both be considered as cues. Use of the term 'signal' may suggest targeted transmission of information via a trait that has undergone adaptation to the purpose of communication (Lehmann et al. 2014). A 'complex' social cue comprises two or more distinct elements exchanged during the course of an encounter between individuals, which is capable of inducing or influencing a response in a receiver (Hebets and Papaj 2005). This contrasts with 'simple' cues, in which information is received in a single signalling component. Complex cues can be composed of multiple components within a single modality (unimodal), or of multiple sensory modalities (multimodal; Hebets and Papaj 2005). An example of a unimodal complex cue is two or more male sexual ornaments, all processed visually, which inform female choice (Møller and Pomiankowski 1993; Auld et al. 2016). The perception of rival males, which elicits longer mating durations in male *Drosophila*, occurs via a multimodal cue comprising song, smell and touch (Bretman et al. 2011b; Maguire et al. 2015). Individual components or modalities within complex cues may elicit a response by the receiver on their own, but interact to alter this response (i.e.

'multiple signals'), or elicit a response only if perceived together ('multicomponent signals'; Hebets and Papaj 2005).

The applications of complex cue theory are broad. Complex cues can be relevant the collation of information from multiple signallers and/or the integration of multiple cue components perceived at different times (see Dore et al. 2018). For example, social experience of relevant cues may influence later plastic responses to cues in the same or a different modality, showing intriguing potential for information perceived at different points in time to interact (Bailey and Zuk 2008; Bailey 2011). However, I focus here on the transmission of information between individual signallers and receivers of the same species during one reproductive encounter. The perception of complex cues may have important effects on the receiver, facilitating the expression of social and reproductive plasticity by increasing the quantity and quality of social information that can be perceived. Complex cues are likely to have a role in several types of reproductive encounter. The use of complex cues by males to assess the level of competition (Bretman et al. 2011b; Maguire et al. 2015), and by females to inform mate choice (Møller and Pomiankowski 1993; Auld et al. 2016) is well documented. Furthermore, complex cues may have a particular role in intersexual conflict. For example, there can be a selective advantage to males conveying deceptive cues to females regarding their individual quality, while females are selected to detect honest information (Holland and Rice 1998). As females evolve resistance to one deceptive male trait, males may evolve new cue components to manipulate female perception of quality, resulting in complex mating displays. In this way, sexual conflict has been proposed to promote the evolution of complex cues (Candolin 2003; Bro-Jørgensen 2010).

1.6 The evolution of complex cues

Individuals may plastically respond to social cues that have evolved due to selection for signaller benefits. These cues may evolve for several reasons, including 1) selection for signalling between the signaller and the focal receiver, 2) selection for signalling between the signaller and a different intended receiver, upon which the focal receiver eavesdrops, 3) selection for a non-signalling purpose, which is co-opted for communication (Lehmann et al. 2014). Plastic receiver responses to these cues may be selected for when the cue provides accurate and relevant information on the social environment, whereby the resulting phenotype is better matched to the prevailing environment and fitness benefits to the receiver occur (DeWitt et al. 1998; Auld et al. 2010). Once a cue and a receiver response become functionally related, cues, sensory systems and plastic responses may undergo coevolution (Endler 1992). In some instances, the perception of a cue and

an associated receiver response may also confer benefits to the signaller, selecting for cooperative signalling systems (Johnstone 1997). In other cases, the interests of the signaller and the receiver may be misaligned, exerting selection on the signaller to produce cues that are dishonest, more difficult for the focal receiver to detect, or manipulate receiver responses to the signaller's advantage. As these cues evolve, the focal receiver may be under ongoing selection to detect and respond to honest cue components, leading to an evolutionary arms race (Burk 1988). This can arise, for instance, under sexual conflict in which the female is under selection to detect honest cues of male quality to inform mate choice, while males are under selection to produce dishonest signals to improve their mating success (Hill 1994; van Doorn and Weissing 2006).

As described above, cues may evolve in response to selection for signalling, or by a pre-existing trait being co-opted for communication. Once a functional and evolutionary link between a cue and a receiver response is established, the characteristics of the cue itself may affect the fitness consequences of the communication to both the signaller and the receiver. Here, I focus on the fitness effects of receivers responding to complex cues over simple cues, and how complexity of cues may evolve (Table 1.1). Generally, complex cues are expected to evolve via selection for the benefits they confer to signallers and/or receivers. While the advantages of complex cues to the signaller are a key aspect of the evolution of complex cues (Table 1.1; Dore et al. 2018), here I focus on benefits to the receiver. The two predominant theories on the evolution of complex cues are the 'backup signal' and 'multiple messages' hypotheses, which emphasise the role of redundant and nonredundant cues, respectively (Johnstone 1996; Partan and Marler 2005; McElroy et al. 2007; Stynoski and Noble 2012). Redundant cue components confer partly or entirely overlapping information, such that if one or more component is compromised the overall message is preserved. Conversely, nonredundant cue components each confer distinct information. While redundancy can ensure that responses are based on robust information, nonredundancy can increase the range of environmental information available to inform optimal phenotype expression (Johnstone 1996; Partan and Marler 1999). Evidence in support of the backup signal hypothesis comes from the previously mentioned expression of reproductive plasticity by male *D. melanogaster* in response to male rivals, via the perception of auditory, olfactory and tactile cues. Any two of these cues in combination, or all three, results in equivalent extension of mating duration, implying redundancy (Bretman et al. 2011b). On the other hand, the multiple messages hypothesis is supported by the example of the detection of pheromones by *D. melanogaster* males, in which separate cue components signal female presence and mating status (Siwicki et al. 2005; Lacaille et al. 2007).

It is likely that the backup signal and multiple messages hypotheses, and their associated benefits to plasticity, are not mutually exclusive. Cues could be partially overlapping in information content or may convey different information via alternative combinations (Johnstone 1996; Ay et al. 2007). Evidence for this idea comes from ornate tree lizards (*Urosaurus ornatus*), in which male quality, which may affect plastic responses of competitors and potential mates (Kolm 2001; Swierk and Langkilde 2013), is communicated by a complex cue of multiple morphological and behavioural characteristics. Some of these characteristics are correlated, indicating a repertoire of partially overlapping cue components (McElroy et al. 2007). This may confer benefits to the receiver in terms of both the robustness of the cue and the range of information transmitted. Nevertheless, the possibility for multimodal cues to act in a redundant or compensatory way likely depends on flexibility in cue production and in the cue components that can initiate a receiver response. It is possible that this imposes substantial evolutionary constraints (Gray et al. 2014). This idea was tested in field crickets (*Teleogryllus oceanicus*), in which female choice is based on both male song and CHC composition. A flatwing male morph, unable to produce song, has recently evolved in some Hawaiian populations. However, there has been no concomitant increase in the attractiveness of cuticular hydrocarbons, suggesting that the reduced ability to attract females via acoustic cues is not compensated through other sensory modalities (Gray et al. 2014). Therefore, insights into the evolution of complex cues could be gained by considering a full spectrum from fully redundant to fully non-redundant cues, as well as by recognising that some cues may combine in different ways to convey different messages (Ay et al. 2007).

Whether the benefits of receiving complex cues fall under the multiple messages or backup signal hypothesis may also depend on the extent to which the social/sexual environment is predictable (Botero et al. 2015). In scenarios where the future conditions are closely correlated with current cues, the selective pressure to receive redundant complex cues as 'backup' for cue components with poor predictive accuracy is likely to be weaker. On the other hand, receiving multiple, highly predictive cue components may increase the amount of environmental information upon which a future phenotype can be based, as described by the multiple messages hypothesis (Johnstone 1996). When the environment is moderately predictable, redundant complex cues may allow for robust information to be received and an appropriate response to be expressed, even if one or more cue component has declined in predictive accuracy.

Table 1.1 - **Hypotheses for the evolution of complex cues in animal communication, developed in the context of social/sexual plasticity**. Evidence for the potential selective advantage of each hypothesis is given.

Hypothesis	Theory	Evidence	Possible role in social/sexual plasticity
'Backup signal' 'Redundant signal'	Multiple cues convey one message. The receiver benefits by assessing the message with increased accuracy. The signaller may benefit when the cost of signalling is reduced by spreading investment across multiple components (Møller and Pomiankowski 1993; Johnstone 1996).	Female swordtail fish distinguish hetero- and con-specific males more accurately based on both chemical and visual cues (Hankison and Morris 2003); male wolf spiders use more visual courtship displays when seismic components are inhibited (Gordon and Uetz 2011).	Improved robustness of information transmission in fluctuating social environments (Bro-Jørgensen 2010) and/or accelerated passing of a stimulus threshold (Rouse and Bretman 2016), resulting in phenotypes better suited to the current environment.
'Multiple messages'	Each cue conveys a different message to one receiver. For example, different sexual ornaments could reflect different aspects of male quality. The signaller and/or the receiver may benefit by increasing the scope of information that can be exchanged (Møller and Pomiankowski 1993).	Components of great tit (*Xiphophorus* pygmaeus) birdsong are related to different measures of male quality (Rivera-Gutierrez et al. 2010); agonistic male-male signalling in eland antelopes (*Tragelaphus oryx*) reflect separate aspects of fighting ability (Bro-Jørgensen and Dabelsteen 2008).	Plastic responses can be fine-tuned to multiple features of the environment.
'Unreliable signal'	Only one cue is a reliable indicator of quality. Any other signals are maintained because they are not costly to produce and are subject to weak Fisherian runaway selection (Fisher 1930). The signaller gains some benefit from the additional, more minor mate preference. The receiver does not gain any increase in the accuracy of the message (Møller and	Bill brightness is significantly correlated with male mating success in mallards (*Anas platyrhynchos*) and plumage only loosely correlated (Omland 1996); female red jungle fowl (*Gallus gallus*) show a primary preference for male comb colour and	No likely application to social/sexual plasticity.

	Pomiankowski 1993; Hankison and Morris 2003).	weaker preferences for other ornaments (Johnsen and Zuk 1996).	
'Emergent message'	A single message emerges through the combination of non-redundant cue components. May benefit the receiver by conveying a more general and informative message based on multiple factors (Partan and Marler 2005; Bro-Jørgensen 2010).	Multiple species of songbirds account for a trade-off between trill rate and frequency bandwidth when assessing trills (Ballentine et al. 2004; Illes et al. 2006; Bro-Jørgensen 2010).	Plastic responses can be fine-tuned to multiple features of the environment, and information transmission is more robust.
'Alerting signal'	One cue component may catch the attention of the receiver and direct it towards one or more other, informative signals. The signaller and the receiver may benefit from improved transmission of the message(s) (Hebets and Papaj 2005; Bro-Jørgensen 2010).	Bornean ranid frog (*Staurois guttatus*) calls direct the attention of conspecifics towards a visual foot-flagging display (Grafe and Wanger 2007); olfactory signals from male Gasterosteidae sticklebacks may make females alert to subsequent visual signals and increase detection (McLennan 2003).	Informative cues are made more salient, resulting in a phenotype better-matched to the social environment.
'Receiver psychology'	Complex cues may benefit the signaller and the receiver by enhancing detection, discrimination, learning and memory of the message (Guilford and Dawkins 1993; Candolin 2003; Hebets and Papaj 2005; Bro-Jørgensen 2010).	The presence of auditory signals improves the speed of colour discrimination in domestic chicks (*Gallus gallus domesticus*; Rowe 2002); audiovisual stimuli enhances song acquisition and quality in nightingales (*Luscinia megarhynchos*; Hultsch et al. 1999).	Informative cues are more salient, influential or efficiently processed; as in 'alerting signal'.

'Sensory overload'	In agonistic interactions, the signaller may benefit from the transmission of complex cues by reducing the accuracy and/or speed of message transmission (Hebets and Papaj 2005; Bro-Jørgensen 2010).	Dark-eyed juncos (*Junco hyemalis*) react more slowly when exposed to alarm calls in addition to a visual cue, compared to the visual cue alone (Randolet et al. 2014).	The response of the receiver may be rendered less advantageous or costly due to time-lags (Padilla and Adolph 1996; DeWitt et al. 1998) and phenotype-environment mismatches, to the benefit of the signaller.

1.7 The role of complex cues in facilitating reproductive plasticity

In the specific context of providing information on the social and sexual environment as a basis for the expression of reproductive plasticity, the benefits of complex cues to the receiver may be: 1) complex cues provide robust information in variable environments, 2) perception of complex cues can fine-tune plastic responses based on multiple features of the environment, 3) complex cues can reduce time lags between environmental change and response (Dore et al. 2018; Table 1.2). The first benefit is relevant to scenarios in which the efficiency of simple cues could be compromised by environmental fluctuations, for example when the content of the message to be transmitted is spatiotemporally variable, but the cue is fixed (Robinson et al. 2008; Bro-Jørgensen 2010). In such scenarios, individuals that receive simple cues would obtain incomplete or inaccurate information concerning the social and sexual environment. However, if complex cues are received, alternative cue components can be available for scrutiny if one is comprised, which could allow receivers to more accurately track a range of environments (Lyons et al. 2014; Reparaz et al. 2014; Rhebergen et al. 2015). This could both enhance the benefits of plasticity and avoid costly 'mismatches' between phenotype and environment. The second way in which complex cues may facilitate reproductive plasticity is by providing a greater volume of information about multiple environmental factors, if the cue components convey at least partially distinct messages, allowing plastic responses to be fine-tuned to multiple strands of environmental information (Griffith and Ejima 2009; Rivera-Gutierrez et al. 2010; Simmons et al. 2013). While there may not be a 'one-to-one' association between cues and messages, complex cues may increase the range of information that can be perceived via incomplete redundancy, or by combining in a degenerate manner (Ay et al. 2007). Finally, complex cues may facilitate plasticity by enabling faster responses. One scenario in which this would be pertinent is if sensory thresholds need to be exceeded in order to initiate a response (Figure 1.2). Complex cue components may additively or synergistically contribute to reaching these thresholds more rapidly than a simple cue (Page et al. 1998; Scheuber et al. 2004; Partan and Marler 2005; Brown et al. 2006; Smith and Evans 2013). Because time lags between environmental change and phenotypic adaptation can reduce or negate the benefits of plasticity (Padilla and Adolph 1996; DeWitt et al. 1998), the potential for complex cues to increase the speed of response could be an important factor in facilitating social plasticity.

Table 1.2 - Hypotheses on the use of complex cues and their underlying assumptions.

Hypothesis	Underlying assumptions
1. Complex cues can prevent information loss in variable environments	(i) Simple cues are significantly compromised in variable environments. (ii) This loss of information lead to phenotypes mismatched to the environment. The resulting deleterious effects reduce fitness and hence exert selection for processing complex rather than simple cues. (iii) Cue components can convey equivalent information and are interchangeable to the extent that the overall message is intact if one or more components are compromised.
2. Complex cues can fine-tune plastic responses based on multiple features of the environment	(i) Cue components provide at least partially different information. (ii) Perception of a greater quantity of environmental information results in a better phenotype-environment match.
3. Complex cues can reduce time lags between environmental change and response	(i) Sensory thresholds for initiating responses exist. (ii) Complex cues components additively or synergistically contribute to meeting these thresholds. (iii) The information transferred by each cue component is correlated. (iv) A fast-responding phenotype confers adaptive benefits. (v) These benefits outweigh potential costs of changing the phenotype in response to an ephemeral environmental fluctuation.

All of the above	(i) Genetic and phenotypic variation in ancestral populations existed, upon which natural selection acted to promote the processing of complex cues (ii) Organisms have the sensory and cognitive capacity to receive, process and integrate more than one cue component. (iii) The production of complex cues either directly benefits the signaller or occurs for other purposes and is co-opted by the receiver. (iv) A phenotype that is more closely matched to the social/sexual environment confers fitness benefits. (v) These benefits outweigh the potential costs of processing complex cues.

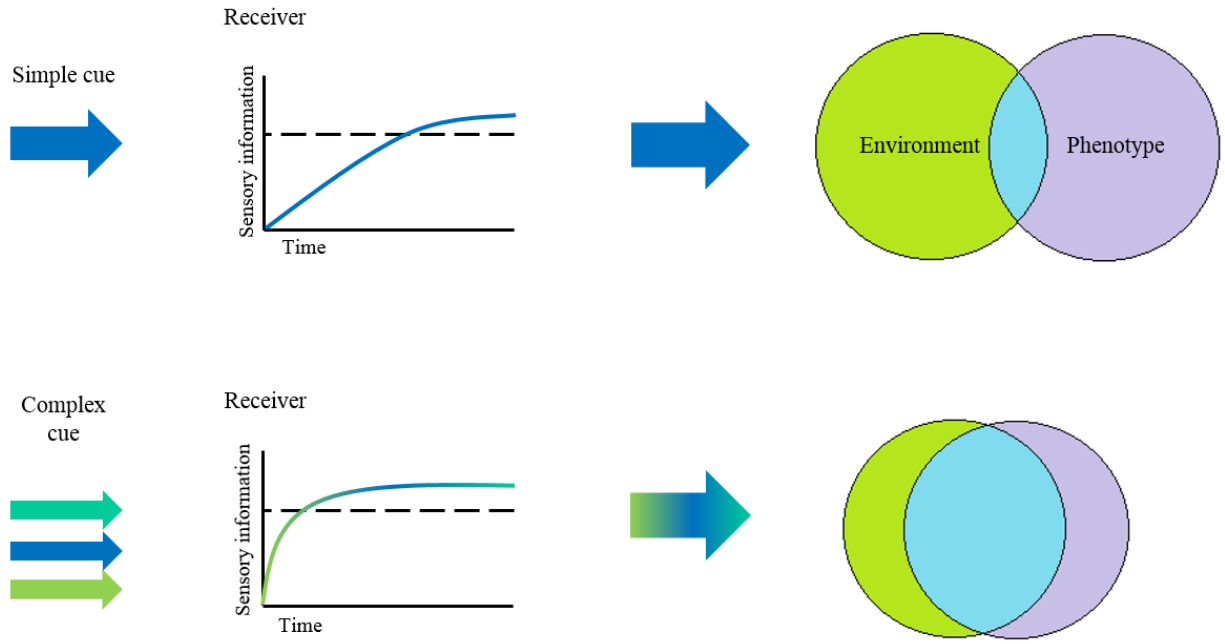


Figure 1.2 - Complex cues can reduce time lags. The perception of cues from the social and sexual environment comprising multiple distinct sensory components (complex cues in multiple colours vs simple cues in one colour) can decrease the time taken to reach sensory thresholds required to initiate a response (dotted line), resulting in a shorter time lag between environmental change and phenotypic change, and hence a better-adapted phenotype.

1.8 Costs and constraints limiting complex cues

Despite the potential benefits of receiving complex cues, their evolution may sometimes by limited by costs, or by cognitive and evolutionary constraints. Costs may be incurred by the signaller due to greater energy expenditure, higher risk of predation or disease and increased potential for eavesdropping when transmitting complex, rather than simple, cues (Hebets and Papaj 2005; Bro-Jørgensen 2010). The receiver may also pay costs associated with the increased energetic and cognitive effort of processing a greater quantity of information (DeWitt et al. 1998). Furthermore, there may be instances when the components of a complex cue are unreliable, contradictory or asynchronous, leading to disruption of the overall message and increasing environmental uncertainty (Taylor et al. 2011; Munoz and Blumstein 2012). Whether the benefits of complex cues outweigh the costs may depend upon the individual's experience, the 'missed opportunity' cost of not responding to the environmental change, and the features of the current social environment (Munoz and Blumstein 2012; Munoz 2015). The many examples of complex cues being used in the context of phenotypic plasticity suggests

that the benefits may frequently prevail, and that even relatively 'simple' organisms may not be precluded from processing complex cues by cognitive constraints (Wessnitzer and Webb 2006; Bailey 2011; Bretman et al. 2011b; Gordon and Uetz 2011; Leonard and Masek 2014; Lyons et al. 2014).

1.9 Partial and complete redundancy

Although redundancy within complex cues may be common due to the selective advantage of having 'backup' cue components available, components that appear redundant on the basis of the immediate response they elicit may show distinctions in their processing and in the subtleties of subsequent responses. For example, although auditory, olfactory and tactile cues of rival presence appear to be interchangeable in eliciting longer mating duration by male *D. melanogaster* (Bretman et al. 2011b), removing the olfactory or auditory cue results in a longer time lag until the response is expressed (Rouse and Bretman 2016). Understanding the role of redundancy in how organisms monitor and process cues on their environment can be important for identifying plastic responses and shedding light on benefits, constraints and facilitators of plasticity. In Chapter 4, I describe investigating complete vs. partial redundancy of a complex cue signalling rival presence to male *D. melanogaster*. Previous research showed that the auditory, olfactory and tactile cue components are interchangeable for allowing focal males to express a behavioural response to rivals, with associated fitness benefits (Bretman et al. 2011b). I built on this by testing the equivalency of fitness benefits to males exposed to alternative combinations of cue components across different environments: in the presence and absence of sperm competition.

Redundancy has broader implications beyond the perception of environmental cues for providing robustness to organismal responses. Here, I use 'redundancy' as a general term to mean different components performing the same function or producing equivalent outputs, including cases also known as 'degeneracy' where the elements are structurally different (Edelman and Gally 2001). A degree of redundancy may be inherent in biological systems at many levels of organisation, including in genetics, protein functions, metabolism, cell organisation, signalling, development, immunity and neural networks (Edelman and Gally 2001). As in redundancy among cue components, redundancy in other biological systems may provide robustness against external and internal perturbations (Kitano 2004). Redundancy in antigen-recognition sites in vertebrate immune systems has also been proposed to be advantageous by increasing the range of pathogens that can be protected against.

Furthermore, redundancy may play a key role in the highly complex patterns of connectivity among neurons (Edelman and Gally 2001), allowing alternative neural connections to elicit similar motor responses (Briggman et al. 2006). Evidence in support of genetic redundancy has been identified in mice (*Mus musculus*) and yeast (*Saccharomyces cerevisiae*), in which over 30% of selected gene knockdowns had minimal phenotypic consequences, suggesting compensatory mechanisms (Melton 1994; Winzeler et al. 1999). In some cases, this genetic robustness may be underpinned by alternative transcriptional pathways that lead to the expression of functionally equivalent phenotypes (Greenspan 2012; Mohorianu et al. 2017).

As in redundancy among cue components, genetic redundancy may often involve elements with partially overlapping functions or differing efficacies, rather than identical ones. Modelling shows that the existence of two genes that perform the same function with equal effectiveness may only be evolutionarily stable if the mutation rates of the two genes are identical – although, evolutionarily unstable redundancy may still persist for a substantial time before it is removed from the population (Nowak et al. 1997). A stable equilibrium of two redundant genes may occur if the two genes perform the same function with slightly different efficacies, or if the genes are pleiotropic and are maintained by selection on their alternative functions. Pleiotropy in redundant genes can lead to complex genetic networks in which multiple genes perform one function, and multiple functions are performed by one gene (Nowak et al. 1997). Complex gene networks characterised by redundancy and pleiotropy may provide flexibility and robustness, allowing the network to be reconfigured in response to perturbations and preserving the higher-level outcomes (Greenspan 2009). In Chapter 5, I propose that genetic redundancy may be empirically tested in order to shed light on the mechanisms bridging the perception of environmental cues and the expression of a plastic phenotypic response. I describe a test case in which I investigated whether the expression of equivalent extension in mating duration by *D. melanogaster* males exposed to alternative cue components signalling rival presence may be underpinned by the expression of alternative, functionally equivalent sets of genes. I built on previous findings by Mohorianu et al. (2017) showing there may be alternative pathways of gene expression to produce the longer mating phenotype, to address outstanding questions about whether genetic redundancy may facilitate the processing of redundant, complex cues.

1.10 Summary

In summary, throughout the following chapters I address questions on the expression and evolution of reproductively plastic male behaviours, using *D. melanogaster*. I focused on how male phenotypes respond to the perceived level of male-male competition, in both the proximate and evolutionary environment. Firstly, I examined the influence of the social environment during two stages of development on plastic male morphology, in order to determine the degree of sensitivity in developmental plasticity and the point at which it is fixed (Chapter 2). Then, I investigated how male behavioural responses to competition, including mating duration, latency to mate, courtship and male-male aggression, evolve in response to the social and nutritional environment (Chapter 3). By doing this I aimed to increase understanding of how the costs and benefits of reproductive investment, and plasticity *per se,* vary under different social/sexual conditions. Furthermore, I investigated how males perceive redundant cues signalling the presence of rivals in order to adapt their behaviour and gain fitness benefits across differing social environments (Chapter 4). Finally, I tested the potential for this process to be underpinned by gene expression within a redundant genetic network (Chapter 5). My findings have ramifications for understanding how reproductive traits may be influenced by both the developmental and adult environment, and how the costs and benefits of plastic traits in various social contexts can lead to their rapid evolution. This may help to strengthen fundamental knowledge of the expression and evolution of reproductive traits, informing developing research into the various applications of theory on reproduction. In Chapter 6 I discuss how my research could be built on in the future, for example by testing reproductive responses in a wider range of social contexts, investigating the impact of complex cues on social learning, and testing how social information may be transmitted across generations.

Throughout my experiments described in this thesis I used *D. melanogaster* as a study system for testing male plastic responses to competition (Figure 1.1). This is a useful model, due to the well-characterised and repeatable reproductive plasticity expressed by males (Bretman et al. 2009; Bretman et al. 2010; Bretman et al. 2012) and the wealth of genetic resources available (Adams et al. 2000; Mackay et al. 2012; Gramates et al. 2017; Leader et al. 2018). The availability of a reference genome, for example, allows us to test the quality of transcriptomic data and improves the ability to investigate high-level questions on the expression and effects of genes. Furthermore, the experimental evolution lines I describe in Chapter 3, which have been evolving for over 60 generations under fixed sex ratio and diet, are a powerful resource which allows us to empirically test predictions on how the social/sexual environment influences the evolution of reproductive traits. In the following chapters, I

describe the use of the *D. melanogaster* study system to investigate how males perceive and process cues signalling competition, the fitness benefits associated with plastic responses to rivals, and how these responses evolve.

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2. The effect of social cues on developmental and adult plasticity

2.1 Abstract

Male *Drosophila melanogaster* show adaptive, plastic responses to the threat of sperm competition. These responses can occur via alterations to the size and physiological functioning of the accessory glands and testes. The cues to which males respond and that ultimately influence the size and development of these reproductive structures can prime individuals for the anticipated competitive environment of adulthood. The cues involved may be perceived during development and also after eclosion during the sensitive periods in which the ultimate size of adult reproductive structures, and body size itself, are determined. Here, I investigated the effect of social cues during two stages of development on reproductive morphology. I tested for developmental plasticity in accessory gland, testis and wing size of male *D. melanogaster* in response to indices of competition experienced in the larval and post-eclosion environments. The results suggest 1) plasticity in male reproductive morphology may be affected by both the presence and the sex of the adults to which males are exposed as larvae during the developmental period; 2) male accessory gland size may remain plastic in the hours immediately after eclosion; 3) the effect of cues indicating sperm competition on reproductive investment may differ across stages of development. However, overall, there was variability between replicates and between different indices of accessory gland, testis and wing size, reducing the strength of general inferences that can be made. The results highlight the importance of choosing robust measures of reproductive morphology, wing shape and size. I suggest that quantifying 3D structures on the basis of 2D images, and using univariate measures of single wing traits as proxies for body size, may introduce inconsistencies in results.

2.2 Introduction

Individuals often adaptively tailor the expression of phenotypes according to the social and sexual environment. The extent to which they can do this effectively can represent a key component of fitness. Across taxa, males can plastically alter their investment in reproduction in response to the risk and intensity of sperm competition (Wedell et al. 2002; Bretman et al. 2011). Such responses can allow a male to adjust his per-mating investment to optimise lifetime reproductive fitness (Parker 1982). For example, in *Drosophila melanogaster* and the beetle *Tenebrio molitor*, males that perceive an elevated risk of sperm competition can increase their reproductive effort, e.g. by extending mating duration, increasing sperm investment, initiating mating more quickly, and increasing post-copulatory mate guarding (Gage and Baker 1991; Bretman et al. 2009). Conversely, when the intensity of sperm competition is high and the likelihood of winning fertilisations is lower, males may reduce mating effort (Fuller 1998; Saether et al. 2001). Males of various taxa may also adapt their reproductive investment in response to the perceived quality of the female (Proshold 1996; Edward and Chapman 2011). In addition to the social/sexual environment, male reproductive effort is influenced by intrinsic factors such as the male's age, condition and mating history (Cook and Wedell 1996; Fuller 1998; Saether et al. 2001).

Plastic reproductive traits can respond to the social environment at multiple life stages. Some phenotypes are plastic during development and become fixed at maturity. In these cases, the developmental environment must be predictive of the environment the individual will experience as an adult in order for the reproductive plasticity expressed to be adaptive (Kasumovic and Brooks 2011). Developmental plasticity may be particularly important in the case of phenotypes such as such as reproductive morphology, that cannot readily be altered after sexual maturity is reached (Kasumovic and Brooks 2011; Kasumovic 2013; Snell-Rood 2013). For example, males of the moth *Plodia interpunctella* and the yellow dung fly *Scathophaga stercoraria* that experience higher larval densities develop larger testes (Gage 1995; Stockley and Seal 2001). Other phenotypes remain reversibly plastic throughout an organism's lifetime, e.g. behavioural traits. The expression of such phenotypes may be rapidly altered according to the prevailing environment at low metabolic cost (Snell-Rood 2013). For example, mating duration of male *D. melanogaster* is fully reversible and sensitive to changes in the competitive environment throughout adulthood (Bretman et al. 2012). The immediate social environment may be particularly influential early in life, when the individual has less

experience of the long-term prevailing social and sexual environment (Frankenhuis and Panchanathan 2011; Dore et al. 2018).

The size of *Drosophila melanogaster* male reproductive morphology (accessory glands and testes) can be altered plastically in response to the social environment males experience during development (Bretman et al. 2016). The accessory glands are the site of seminal fluid protein (SFP) synthesis. SFPs are transferred to the female in the ejaculate and modulate sperm storage, sperm displacement, female receptivity to remating, and egg production – collectively, these responses result in substantial effects on the extent and share of paternity (Wolfner 1997; Chapman et al. 2000; Chapman et al. 2003). The size of *D. melanogaster* accessory glands has also been found to be positively associated with mating success (Bangham et al. 2002). The testes are the site of sperm production and thus also have an important role in competitive success. Across *Drosophila* species, testis size is associated with investment in sperm competition (Pitnick and Markow 1994). Furthermore, sperm competition selects for larger testes in the yellow dung fly *Scathophaga stercoraria* (Hosken and Ward 2001). However, in *D. melanogaster*, testis size did not account for a significant portion of variation in mating success (Bangham et al. 2002), and sperm investment may not evolve in response to high-competition environments (Linklater et al. 2007). Investment in the accessory glands/SFPs and in the testes/sperm production may show independent responses to the social environment, and different investment trajectories for any given level of competition (Hopkins et al. 2019).

Bretman et al. (2016) found that the accessory glands of males that developed as larvae in the presence of adult males were significantly larger than those of males that developed with no adult males. This suggests that *D. melanogaster* are sensitive to the social environment during development and suggests that they can tailor their investment in reproductive morphology in response to the level of sperm competition predicted by this environment. However, the extent to which developing *D. melanogaster* can detect and respond to the details of the social environment is not yet known. For example, the optimal response to the presence of adult females during larval development may be different to that of adult males (Kasumovic and Andrade 2006). Furthermore, while it is known that *D. melanogaster* accessory glands continue to develop in the hours after emergence from the pupa (Ruhmann et al. 2016), it is unclear whether they remain plastic during this time or are fixed at the point of eclosion.

In these experiments, I addressed these outstanding questions and increased our understanding of the sensitivity of developing male *D. melanogaster* to the social environment. To do this, I measured the accessory glands and testes of males that developed in the presence of adult males, adult females, or no adults. I also conducted a fully factorial experiment to compare the effects of these larval environments with the influence of male population density immediately after eclosion. Measurements of the longitudinal (L)3 wing vein were made as a proxy for overall body size. Following the preliminary results, which indicated a possible effect of the post-eclosion environment on wing size, I then investigated the developmental plasticity of male wing morphology during the larval and early post-eclosion periods.

2.3 Methods

a) General methods

Fly rearing and experiments were conducted at 25°C with a 12h:12h light:dark cycle. Flies were maintained in 75x25mm^2 glass vials containing a sugar-yeast-agar (SYA) medium (100g brewer's yeast, 50g sucrose, 15g agar, 30mL Nipagin (10% solution), 3mL propionic acid, and 0.97L water per litre of medium). Flies came from wildtype Dahomey stock populations (Bretman et al. 2009). To obtain experimental flies, females were allowed to oviposit on agar-grape juice plates (50g agar, 600mL red grape juice, 42mL Nipagin (10% solution), 1.1L water) and larvae were cultured under a standard density of 100/vial, unless otherwise specified.

b) Repeatability of accessory gland and testis measurements

Prior to beginning the main experiments, a pilot study was conducted to determine the repeatability of scoring the accessory glands and testes by using measurements of perimeter and area. Thirty-five wildtype males were cultured at standard density and collected at eclosion. The males were stored 10/vial in same-sex groups after eclosion, before being frozen at -80°C. Dissections to remove the accessory glands and testes were done using sharpened forceps on a concave microscope slide in 20µl of ice-cold phosphate-buffered saline (PBS). After the accessory glands and testes were removed, they were transferred to a flat microscope slide with another 20µl of PBS for photographing. To avoid rupturing the reproductive structures, no cover slips were used (Bretman et al. 2016). Each accessory gland and testis was photographed using an AxioCamMR5 camera and a Zeiss Stereo Discovery.V12 microscope with a 1.0x objective. Each sample was then rearranged with a mounted needle

before being photographed a second time. The perimeter and area of the accessory glands and testes in each of the two images was measured using ImageJ (Schneider et al. 2012). Subsequently, the repeatability of the measurements of area and perimeter across the two images of each sample was quantified using the Pearson correlation coefficient in R (R Core Team 2016).

c) The effect of adults in larval vials on the development of male reproductive morphology and body size

Focal males were randomly assigned to one of three treatments: 1) 20 adult males present in larval vials (M), 2) 20 adult females present in larval vials (F), 3) no adults present in larval vials (N). Males in the M and F treatments had 20 conspecific, wildtype adults of the specified sex introduced to their developmental vials the point that the focal larvae were collected. The adults were removed from the vials the day before the focal flies eclosed. At eclosion, adults were separated by sex and males were stored 10/vial for three days to allow the completion of development. Subsequently, focal males were frozen at -80°C for dissection. The wings of each focal male were removed using sharpened forceps and mounted on a glass slide, adhered with approximately 5µl glycerol and a cover slip. The accessory glands and testes of each male were removed and all morphological structures were photographed as described above. The area and perimeter measurements were done by using ImageJ (Schneider et al. 2012). The length of the L3 wing vein was also measured as a proxy for body size (Gidaszewski et al. 2009). The wing vein, accessory gland and testis on the left and right side of each individual was measured and an average of the two sides was taken.

d) The effect of larval and post-eclosion environment on male reproductive morphology and wing size

To investigate a possible methodological explanation for inconsistencies between the results of the first experiment and previous findings by Bretman et al. (2016), the above experiment was repeated with an additional post-eclosion treatment. At eclosion, focal males were collected and either stored in groups of 10/vial, or individually 1/vial, for three days. This was done to determine whether male reproductive morphology and wing size/shape remain plastic during this period or are fixed at pupation. The rationale for thinking that post-eclosion modification of the size and shape of these structures might be possible is that their development is known to be completed in the hours after eclosion (Matamoro-Vidal et al. 2015; Ruhmann et al. 2016),

Focal males were randomly assigned to one of six treatments: 1) 20 adult males in larval vials, grouped post-eclosion (M-group); 2) 20 adults males in larval vials, alone post-eclosion (M-alone); 3) 20 adult females in larval vials, grouped post-eclosion (F-group); 4) 20 adult females in larval vials, alone post-eclosion (F-alone); 5) no adults in larval vials, grouped post-eclosion (N-group); 6) no adults in larval vials, alone post-eclosion (N-alone). As above, the adults were removed from the vials the day before the focal flies eclosed. Focal males were collected at eclosion and stored either in single-sex groups or alone for three days, before being frozen at -80°C. The wings, accessory glands and testes of each male were dissected, photographed and measured as described in section b). In addition to L3 wing vein length, wing perimeter and roundness of the wing (width divided by length; Menezes et al. (2013)) were also measured, again using ImageJ (Schneider et al. 2012).

e) The effect of larval and post-eclosion population density on male wing morphology

The results of the first two experiments suggested that the post-eclosion environment may have an influence on wing morphology. However, the pattern of these results was inconsistent. Therefore, a final experiment was carried out to directly test the effects of larval and post-eclosion population density on the size and shape of males' wings. Larval density has previously been found to affect wing development, such that the wings develop larger relative to thorax size at higher densities (BitnerMathé and Klaczko 1999). Here, I tested for effects of the post-eclosion environment on the wings of males that developed under standard (100/vial) or high (200/vial) larval density, to determine possible interaction effects between the two environments.

Focal males were randomly assigned to one of four treatments: 1) standard larval density, grouped post-eclosion (S-group); 2) standard larval density, alone post-eclosion (S-alone); 3) high larval density, grouped post-eclosion (H-group); high larval density, alone post-eclosion (H-alone). At eclosion, focal males were collected and stored either alone or in groups of 10 for three days, then were frozen at -80°C. The wings of each male were then removed, mounted and photographed as described above. The L3 wing vein and the perimeter, length and width of each wing were measured. Then, the position of 15 landmarks on the wing (identified in Gidaszewski et al. (2009)) were recorded using the landmark digitisation tool in tpsDig (Rohlf 2016). A Procrustes superimposition of the landmark coordinates was carried out in R using the procGPA() command in the 'shapes' package (Dryden 2019), in order to scale and align all sets of landmarks.

f) Statistical analyses

Statistical analyses were carried out in R v3.6.1 (R Core Team 2016). All data on accessory gland, testis and wing size were analysed using linear models. Throughout, stepwise model simplification was conducted to determine which terms were significant. Post-hoc pairwise tests were conducted. The length of the L3 wing vein (a proxy for body size) was included as a covariate for accessory gland and testis size in all models (Bretman et al. 2016). The Procrustes coordinates obtained from the 15 landmarks on the wings were analysed with a MANOVA model, using the adonis() function in the package 'vegan' (Oksanen et al. 2018), and a PCA was carried out in MorphpoJ (Klingenberg 2011), using the steps outlined in Swiderski et al. (2012). Within each experiment, all p-values were corrected using the Benjamini-Hochberg procedure. Figures were produced in R and MorphoJ (R Core Team 2016; Klingenberg 2001).

2.4 Results

a) Repeatability of accessory gland and testis measurements

The Pearson correlation coefficient showed high intra-individual repeatability in measurements of accessory gland area (0.97) and perimeter (0.93). The repeatability of measuring testes by perimeter was similarly high (0.90). However, measurements of testes by area showed lower repeatability between the two images of each sample (0.38).

b) The effect of adults in larval vials on the development of male reproductive morphology

To determine whether developmental plasticity of male accessory glands and testes is sensitive to the sex of adults in the environment during larval development, either 20 adult females, 20 adult males or no adults were introduced to the larval vials during focal male development. The area of the accessory glands (LM: $F=9.97$, $df=2$ & 144, $p=0.0011$; Figure 2.1a) but not their perimeter (LM: $F=2.01$, $df=2$ & 144, $p=0.21$; Figure 2.1b; Table 2.1; see Table S2.1 for full simplified models with significant terms retained) was significantly influenced by the social environment during larval development. Males that developed with adult males present in their larval vials had significantly smaller accessory glands by area than males that developed with no adults in the environment (Tukey: $t=4.46$, $df=144$, $p=0.0060$), while males that developed with females present had intermediate sized accessory glands (Figure 2.1a). Neither the area (LM: $F=2.74$, $df=2$ & 122, $p=0.12$; Figure 2.2a) nor the perimeter (LM: $F=1.52$, $df=2$ & 122, $p=0.29$; Figure 2.2b; Table 2.1) of the testes was significantly influenced by

treatment. The length of the L3 wing vein of males was also not significantly influenced by the social environment during development (LM: $F=0.37$, $df=2$ & 146; $p=0.26$).

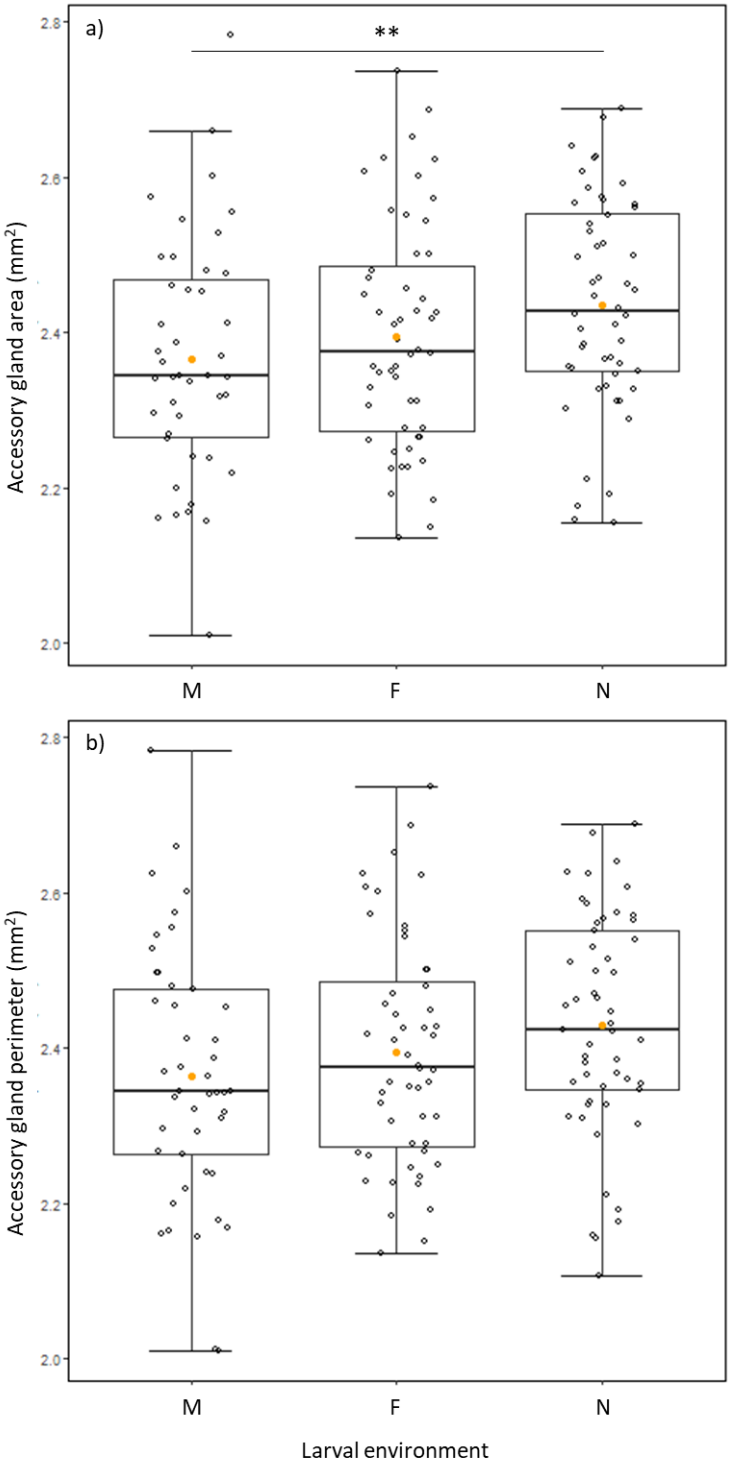

Figure 2.1 – The area (a) and perimeter (b) of the accessory glands of male *Drosophila melanogaster* in response to the larval environment. Focal males developed as larvae in vials containing either 20 adult males (M), 20 adult females (F), or no adults (N). Boxplots show interquartile range and median with raw data points also plotted. Orange dots indicate means; asterisks indicate significant pairwise differences between groups on each end of the horizontal line: *** p>0.001; ** p>0.01; * p>0.05.

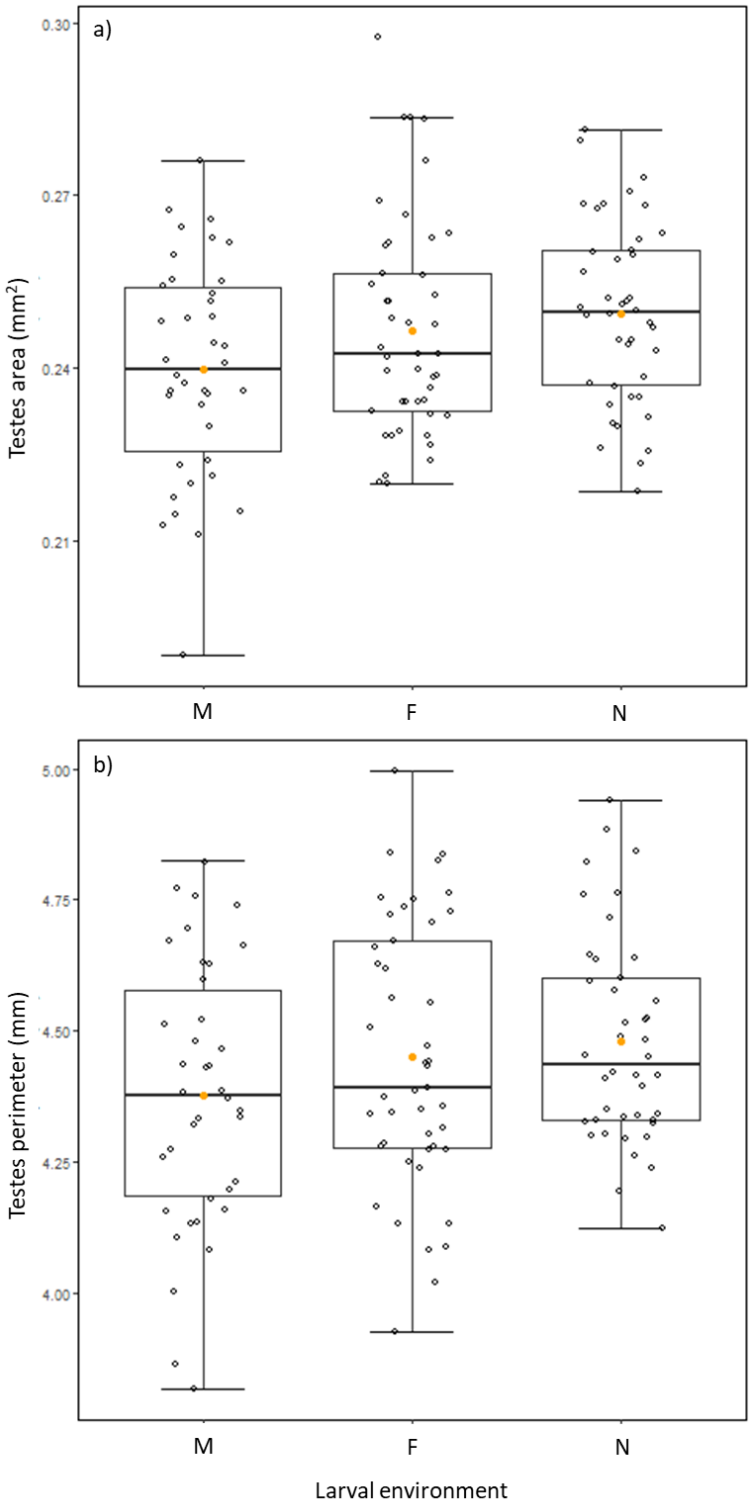

Figure 2.2 – **The area (a) and perimeter (b) of the testes of male *Drosophila melanogaster* in response to the larval environment.** Focal males developed as larvae in vials containing either 20 adult males (M), 20 adult females (F), or no adults (N). Boxplots as described in Figure 2.1.

c) The effect of larval and post-eclosion environment on male reproductive morphology and wing size

To investigate inconsistencies between the results described above and previous research by Bretman et al. (2016), the experiment was repeated with males from each developmental treatment either stored in groups or alone immediately after eclosion. In this experiment, neither the area (LM: F=2.30, df=2 & 245, p=0.23; Figure 2.3a) nor the perimeter of the accessory glands (LM: F=0.83, df=2 & 245, p=0.61; Figure 2.3b; Table 2.1) was influenced by the presence of adults during larval development. However, the post-eclosion environment significantly affected the perimeter of the accessory glands (LM: F=10.18, df=1 & 246, p=0.014; Figure 2.3b; Table 2.1). Among males that developed with no adults in the larval vials, those that were stored in groups post-eclosion had accessory glands with a significantly larger perimeter than those that were housed alone (T-test: t=2.71, df=90.37, p=0.033). The accessory gland area was independent of the post-eclosion as well as the larval environment (LM: F=2.69, df=1 & 246, p=0.23; Figure 2.3b; Table 2.1).

In contrast to the results of the first iteration of this experiment, testis area was significantly influenced by the presence of adults during larval development (LM: F=6.54, df=2 & 219, p=0.014; Figure 2.4a; Table 2.1). Among males that were stored in groups post-eclosion, males that developed with adult males in the larval vials had significantly smaller testes than males that developed with no adults present (T-test: t=3.16, df=80.29, p=0.015). The effect of the post-eclosion environment on testis area (LM: F=0.47, df=1 & 220, p=0.63; Figure 2.4a), and the effects of both the larval (LM: F=2.21, df=2 & 219, p=0.24) and the post-eclosion environment (LM: F=0.041, df=1 & 220; p=0.91; Figure 2.4b; Table 2.1) on testis perimeter were all nonsignificant.

The length of the L3 wing vein in this experiment was significantly affected by the presence of adults in the larval environment (LM: F=5.77, df=2 & 259, p=0.021) but not by the post-eclosion environment (LM: F=2.70, df=1 & 260, p=0.23; Figure 2.5; Table 2.1). Among males stored in groups post-eclosion, those that developed with females in the larval vials had longer L3 wing veins than those that developed with males (T-test: t=3.27, df=85.70, p=0.014) or with no adults present (T-test: t=3.61, df=82.86, p=0.011). Despite the overall nonsignificant effect of post-eclosion environment, males that developed with no adults in the larval vials had significantly longer L3 veins when they were stored in groups post-eclosion compared to when they were stored alone (T-test: t=2.87, df=95.74, p=0.026). This result was unexpected, as

body size, as well as wing development itself, are thought to be fixed at eclosion (French et al. 1998; McGuigan 2009).

Although the results of the two iterations of the experiment were somewhat inconsistent (Table 2.1), across both sets of results males that developed in the presence of adult males generally tended to have smaller reproductive structures, in contrast to the results reported by Bretman et al. (2016). The finding that L3 wing vein length could significantly vary with the environment after eclosion was surprising, suggesting that wing development may remain plastic for longer than previously thought and that this measure may not be a reliable proxy for body size.

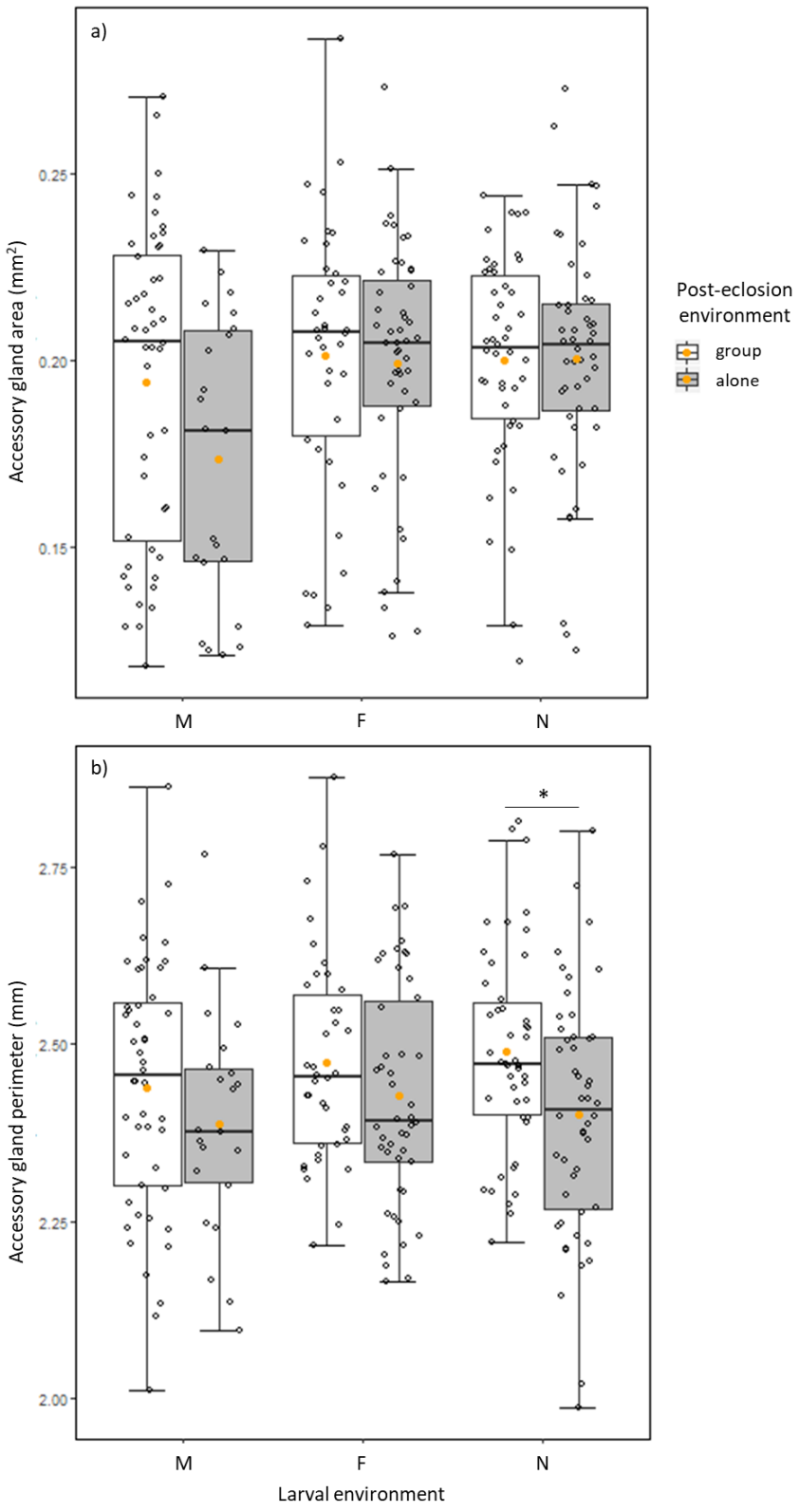

Figure 2.3 – The area (a) and perimeter (b) of the accessory glands of male *Drosophila melanogaster* in response to larval and post-eclosion environment. Focal males developed as larvae in vials with either 20 adult males (M), 20 adult females (F), or no adults present (N). Immediately after eclosion, until development was completed, males were either stored in same-sex groups of 10/vial (group) or singly 1/vial (alone). Boxplots as described in Figure 2.1.

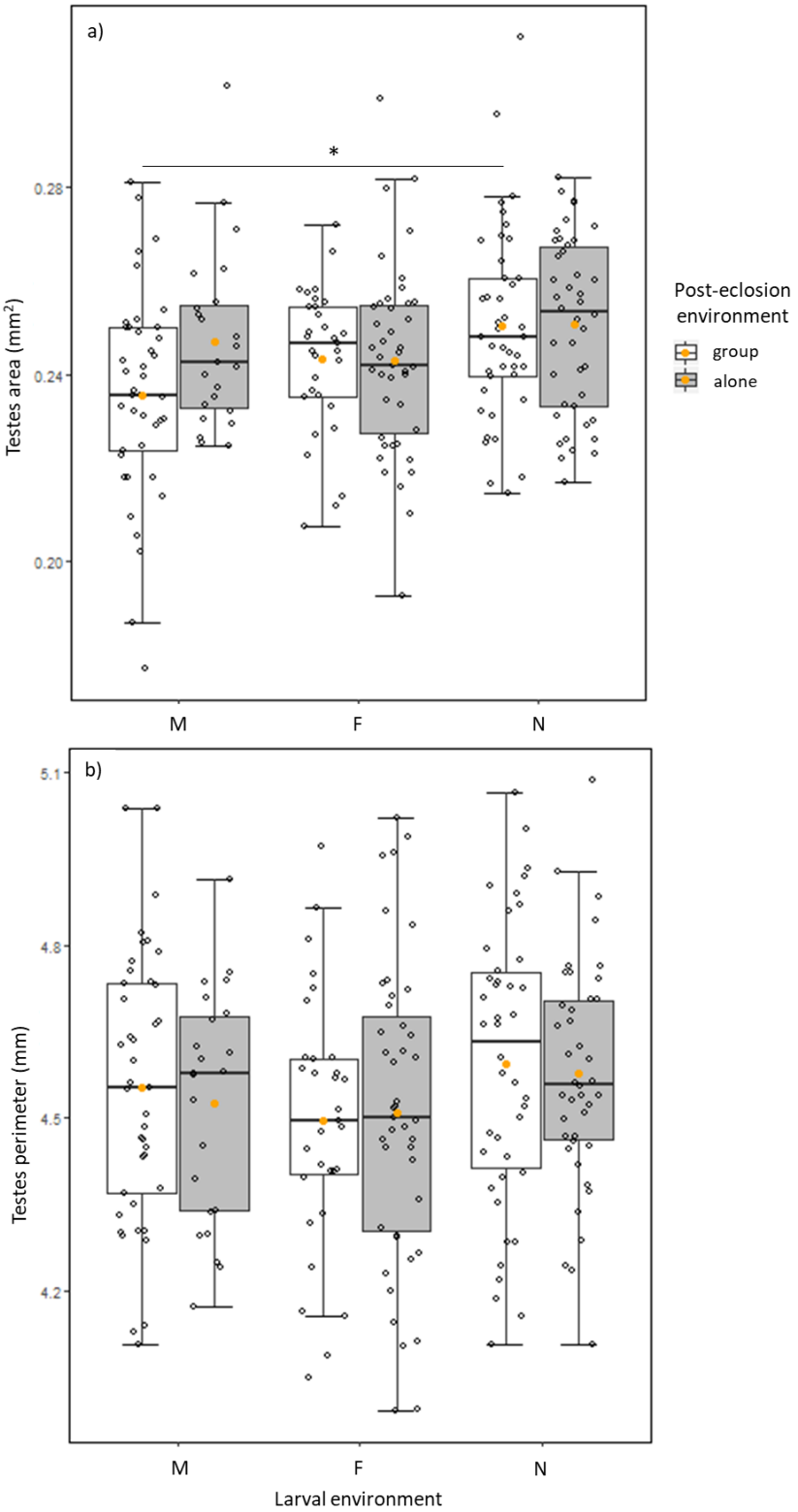

Figure 2.4 – The area (a) and perimeter (b) of the testes of male *Drosophila melanogaster* in response to larval and post-eclosion environment. Focal males developed as larvae in vials with either 20 adult males (M), 20 adult females (F), or no adults present (N). Immediately after eclosion, until development was completed, males were either stored in same-sex groups of 10/vial (group) or singly 1/vial (alone). Boxplots as described in Figure 2.1.

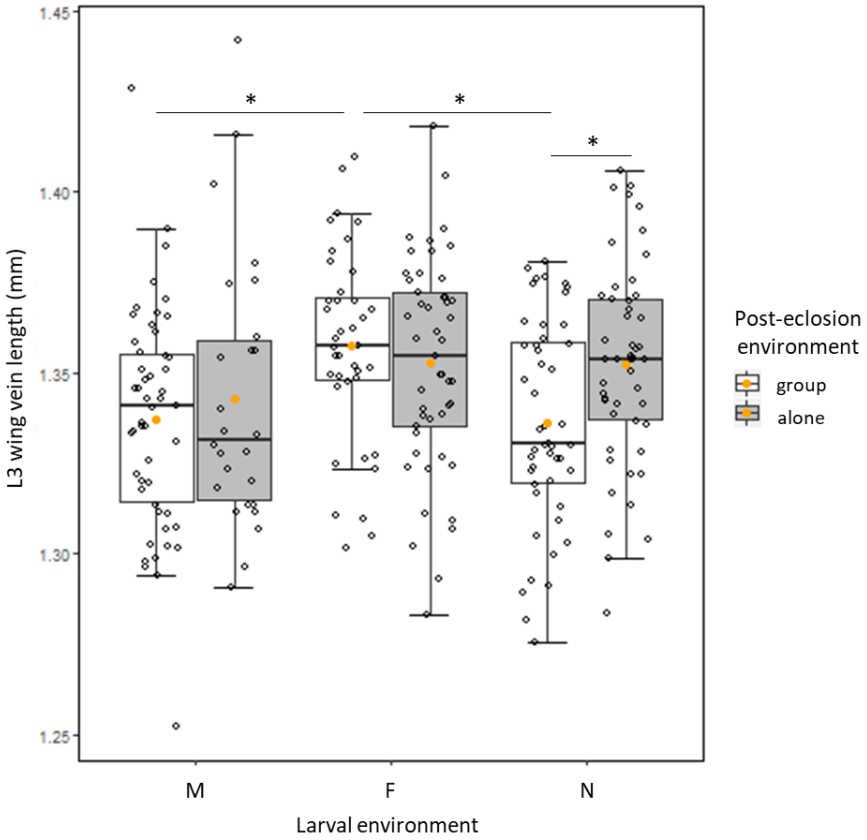

Figure 2.5 – **The length of the L3 wing vein (a proxy for body size) of male *Drosophila melanogaster* in response to larval and post-eclosion environment.** Focal males developed as larvae in vials with either 20 adult males (M), 20 adult females (F), or no adults present (N). Immediately after eclosion, until development was completed, males were either stored in same-sex groups of 10/vial (group) or singly 1/vial (alone). Boxplots as described in Figure 2.1.

d) The effect of larval and post-eclosion population density on male wing morphology

In order to further investigate the potential influence of the plasticity of male wings at different stages of development, the shape and size of the wings of male *D. melanogaster* that developed at either standard or high larval density, then were stored either in groups or alone immediately after eclosion, were measured. L3 length was not significantly influenced by larval (LM: F=1.08, df=1 & 176, p=0.51) or post-eclosion density (LM: F=2.23, df=1 & 176, p=0.28; Table 2.1). The perimeter of the wings too was neither affected by the larval (LM: F=0.92, df=1 & 16, p=0.51) nor the post-eclosion social environment (LM: F=0.71, df=1 & 176, p=0.53; Table 2.1).

The roundness of the wings, quantified by dividing the width by the length as described in Menezes et al. (2013), was significantly affected by the larval density (LM: F=7.49,

df=1 & 176, p=0.041; Figure 2.6; Table 2.1). Among males stored in groups after eclosion, those that developed at high larval density had significantly rounder wings than those that developed at standard larval density (T-test: t=3.55, df=82.26, p=0.0078). Despite the overall nonsignificant effect of the post-eclosion environment on wing roundness (F=3.45, df=1 & 176, p=0.16; Table 2.1), males that developed at high larval density had significantly rounder wings when they were stored in groups after eclosion compared to when they were stored alone (T-test: t=2.59, df=82.86, p=0.045).

A more comprehensive comparison of wing shape was conducted by analysing the superimposed coordinates of 15 landmarks on the wing. Multivariate analysis of these coordinates showed that the overall shape of the wing was not significantly influenced by either the larval (MANOVA: F=2.05, df=1 & 167, p=0.087) or post-eclosion social environment (MANOVA: F=0.50, df=1 & 167, p=0.97; Figure 2.7; Table 2.1).

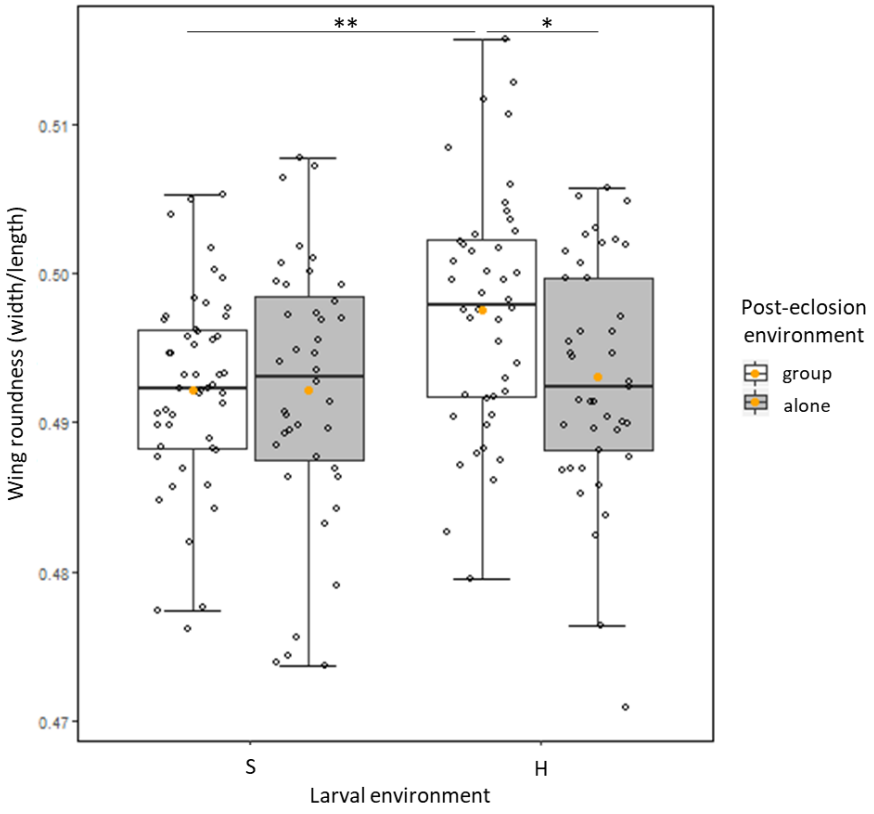

Figure 2.6 – The roundness (width divided by length) of the wings of male *Drosophila melanogaster* in response to larval and post-eclosion density. Focal males developed as either at a standard density (S) of 100/vial or high density (H) of 200/vial. Immediately after eclosion, until development was completed, males were either stored in same-sex groups of 10/vial (group) or singly 1/vial (alone). Boxplots as described in Figure 2.1.

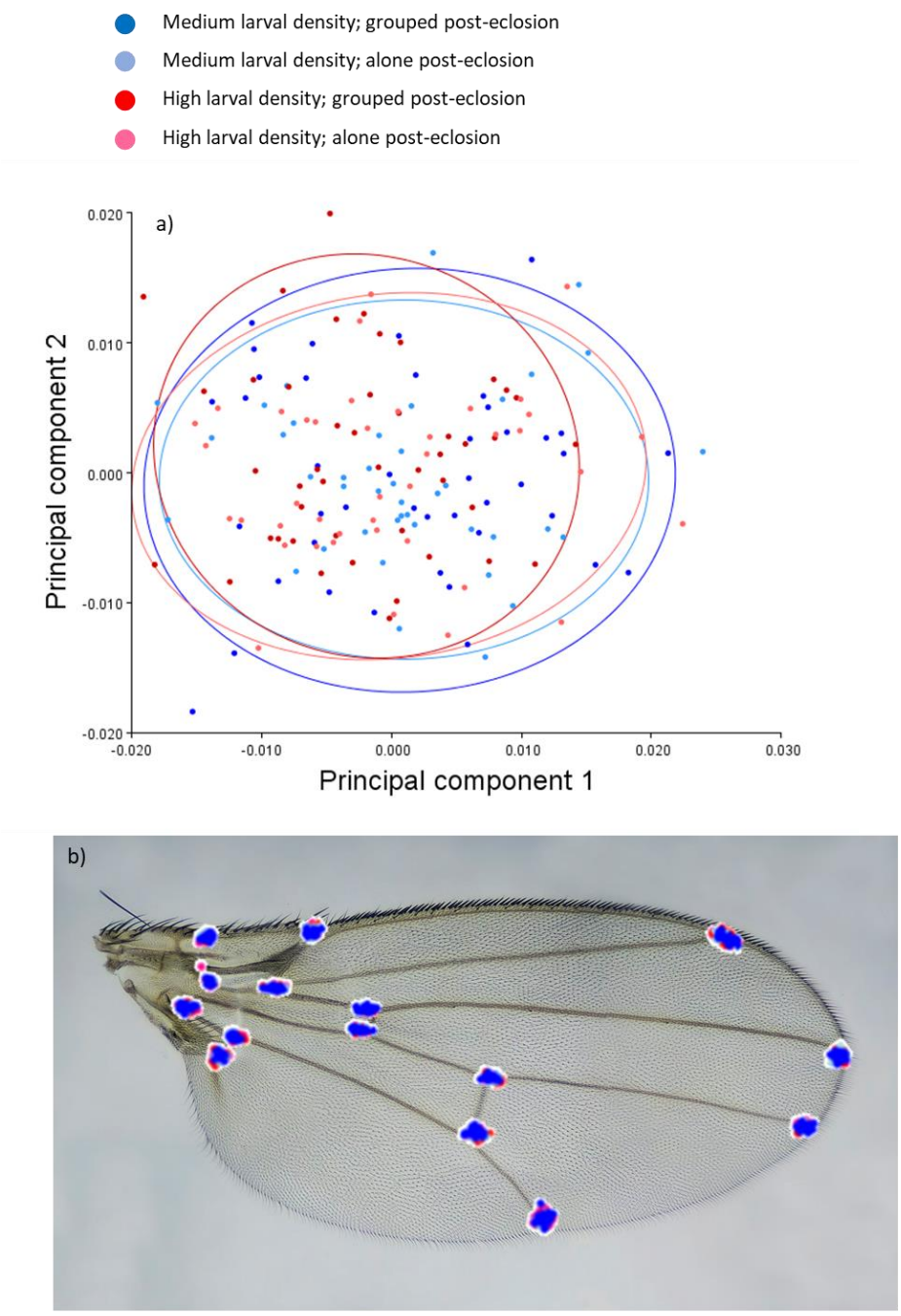

Figure 2.7 – **The Procrustes coordinates of 15 landmarks of the wings of male *Drosophila melanogaster* in response to larval and post-eclosion density. a)** Principal components analysis of the Procrustes coordinates. **b)** The superimposed coordinates of each sample. Focal males developed as either at a standard density of 100/vial (blue) or high density of 200/vial (red). Immediately after eclosion, until development was completed, males were either stored in same-sex groups of 10/vial (darker shade) or singly 1/vial (lighter shade).

Table 2.1 – **Statistical summary of the effects of the larval and post-eclosion social environment on reproductive and wing morphology of male *Drosophila melanogaster*. a)** The overall effect of the presence of adults (either 20 adult males, 20 adult females, or no adults) on accessory gland (AG) and testis (T) size, and L3 wing vein length (a proxy for overall body size). **b)** The overall effect of the presence of adults (treatments as above) and post-eclosion density (10 males/vial or 1/vial) on AG and T size, and L3 length. **c)** The overall effect of larval density (100/vial or 200/vial) and post-eclosion density (10 males/vial or 1/vial) on wing size and shape. Asterisks indicate significance: *** p>0.001; ** p>0.01; * p>0.05.

a) The effect of adult males or females in larval vials on reproductive morphology

Larval environment	**F**	**df**	**p**
AG perimeter	2.01	2 & 144	0.21
AG area	9.97	2 & 144	**0.0011 ****
T perimeter	1.52	2 & 122	0.29
T area	2.74	2 & 122	0.12
L3 length	0.37	2 & 146	0.29

b) The effect of adults in larval vials and post-eclosion density on reproductive morphology

Larval environment	**F**	**df**	**p**
AG perimeter	0.83	2 & 245	0.61
AG area	2.30	2 & 245	0.23
T perimeter	2.21	2 & 219	0.24
T area	6.54	2 & 219	**0.014 ***
L3 length	5.77	2 & 259	**0.021 ***
Post-eclosion density	**F**	**df**	**p**
AG perimeter	10.18	1 & 246	**0.014 ***
AG area	2.69	1 & 246	0.23
T perimeter	0.041	1 & 220	0.91
T area	0.47	1 & 220	0.63
L3 length	2.70	1 & 260	0.23

c) The density of larval and post-eclosion density on wing morphology

Larval environment	**F**	**df**	**p**
L3 length	1.08	1 & 176	0.51
Wing perimeter	0.92	1 & 176	0.51
Wing roundness	7.49	1 & 176	**0.041 ***
Wing shape	2.05	1 & 167	0.087

Post-eclosion density	**F**	**df**	**p**
L3 length	2.23	1 & 176	0.28
Wing perimeter	0.71	1 & 176	0.53
Wing roundness	3.45	1 & 176	0.16
Wing shape	0.50	1 & 167	0.97

2.5 Discussion

Overall, the results suggest a subtle influence of the social environment during larval development, and potentially in the period immediately eclosion, on the development of male *D. melanogaster* reproductive morphology. The presence of adult males in the environment during larval development sometimes resulted in males developing smaller accessory glands and/or testes. Conversely, housing males in groups rather than alone in the hours immediately after eclosion generally resulted in larger accessory glands. This suggests that cues of competition may exert different effects on reproductive morphology depending on the stage of development at which they are detected. However, these results were inconsistent across the two iterations of the experiment, and also differed from previous findings by Bretman et al. (2016). The size of the accessory glands and testes may show high between-individual variability and/or sensitivity to extraneous factors, which may confound the effects of the social environment. The inconsistencies in the results, particularly the differing patterns in accessory gland and testis size when measured by area vs. perimeter, also raise questions about the legitimacy of inferring the size of these 3D structures from 2D image measurements.

There was an overall trend towards males developing in the presence of adult males having smaller accessory glands and testes compared to males that developed with no adults present. Larval social environment was a significant predictor of accessory gland area in the first iteration of the experiment, and of testis area and L3 wing vein length (a proxy for body size) in the second iteration – although, this pattern was inconsistent between experiments and across the two measures of size (perimeter and area). Furthermore, as shown by the repeatability measures, area is not a robust way of quantifying testis size. *D. melanogaster* testes have a complex 3-dimensional shape, which may lead to differing measures of area depending on positioning on the surface. Generally, males that developed with adult females in the environment were found to have intermediate sized accessory glands and testes.

Although the interpretation of the results is complicated by weak repeatability, the overall pattern suggests reduced size of reproductive structures when adult males were present in the developmental environment compared to when there were no adults, with the presence of adult females eliciting intermediate size. This implies that during larval development, male *D. melanogaster* show plasticity in the development of reproductive morphology which is sensitive not only to the presence of adults in the environment, but to the sex of those adults. The presence of adult males and females may be detected by pheromonal cues, as has been demonstrated in developing redback spiders (*Latrodectus hasselti*; Kasumovic and Andrade, 2006) or alternatively by food breakdown products or metabolites.

The trend towards developing smaller accessory glands and testes in environments signalling a high likelihood of sperm competition is surprising in the context of previous findings. Earlier studies have found that the size of the accessory glands increases in response to cues of competition through developmental plasticity (Bretman et al. 2016) and is positively associated with mating success (Linklater et al. 2007). However, the relationship between testis size and sperm competition in *D. melanogaster* has been shown not to be direct: previous research found no association between testis size and either pre-copulatory or post-copulatory success (Bangham et al. 2002). Furthermore, experimental evolution has shown that testis size increases under female-biased sex ratio in response to mating frequency and sperm depletion, not in high-competition male-biased environments. Thus, the relationship between sperm competition and reproductive investment may not be simple, particularly in the case of sperm production and transfer. Moreover, the absence of the expected positive influence on accessory gland and/or testis size by cues of competition during the development may be a product of the fast-changing nature of the social environment. As SFP and sperm production remain plastic after maturity (Moatt et al. 2014; Mohorianu et al. 2017), the prevailing social environment during adulthood may be the predominant influence on plastic reproductive investment, rather than the developmental environments. Reversible plasticity that can respond to ongoing variation in the social environment may be more advantageous in this context than fixed, developmental plasticity.

Alternatively, the finding of smaller accessory glands and testes in response to cues of male-male competition may be explained by the exposure to 20 adult males throughout larval development signalling high intensity sperm competition, which may result in diminishing returns to reproductive investment by the developing males (Parker et al. 1996; Wedell et al. 2002). Furthermore, the absence of females in the developmental environment may have signalled that early mating opportunities were likely to be scarce or non-existent. The

developing males may have adapted to this social environment by reducing their early investment in reproduction in favour of later, more profitable mating opportunities. Males developing in the presence of adult females, with no adult males in the environment, may receive cues that the risk of sperm competition will be low upon eclosion, making high investment in reproduction unnecessary (Parker et al. 1997; Wedell et al. 2002). This could explain why males developing in the presence of neither adult females nor adult males expressed significantly larger reproductive structures. To better understand the adaptive value of investing in reproductive development in different social environments, it would be interesting to investigate the reproductive morphology of males developing in environments with a lower number of adult males that may signal intermediate intensity of sperm competition, or with a mix of males and females at various sex ratios.

The social environment immediately after eclosion from the pupa was found to have a significant influence on the perimeter of the accessory glands. Across all three larval environment treatments, males developed larger accessory glands by perimeter when they were housed in same-sex groups of ten after eclosion, compared to when they were housed alone – although, this difference was only significant when males developed as larvae with no adults present. In the hours after a male *D. melanogaster* emerges from the pupa, the accessory glands continue to develop and increase in size as they fill with seminal fluid proteins (Ruhmann et al. 2016). The results suggest that this stage of development may be plastic in response to the prevailing social environment, and that males that infer a high risk of sperm competition at this stage may increase their production of seminal fluid proteins. It is unclear why this effect was only significant among males that developed as larvae in an environment with no adults present. It is possible that the absence of any cues on the social environment during larval development made the cues experienced after eclosion more salient. The pattern of increased investment in the accessory glands elicited by cues of sperm competition in the post-eclosion environment is the reverse of the observed effect of cues during larval development. This may suggest that adaptive value of increasing reproductive investment in response to sperm competition differs across stages of development. However, the effect of the post-eclosion social environment on accessory gland size was only detected when measured by accessory gland perimeter, not area. The incongruence between these two measures of gland size suggests that more work is needed to develop fully robust indicative measures of accessory gland size.

The results on the influence of the social environment on reproductive development were somewhat inconsistent between the two iterations of the experiment and between the two

measures of size (perimeter and area). The results also contrasted with previous findings by Bretman et al. (2016), who found that accessory gland size increased among males who were exposed to adult males during larval development. This suggests that the effect of the developmental social environment on accessory gland and testis size, when measured as area or perimeter, is not repeatable. This may be due to high inter-individual variability or sensitivity to confounding influences in the environment leading to inconsistent results. Alternatively, slightly different ages of males in these experiments and those described by Bretman et al. (2016) may have contributed to inconsistencies, due to possible changes in responses to the social environment across male lifespan. Furthermore, measuring the reproductive structures by perimeter vs. area did not always yield the same pattern of results. Area as a measurement of testes size showed low within-individual repeatability in the pilot experiment, weakening conclusions drawn based on this measure. The extent to which these measures are robust proxies of the actual size of 3D structures may be limited. The accessory glands and testes are 3D structures which may be better quantified by volume, for example, rather than by the perimeter or area of flat images. It is also not clear whether freezing has any effect on the dimensions of the reproductive structures. Future studies could compare the size of the accessory glands and testes or males from equivalent social environments both after freezing and following immediate dissection at room temperature, to determine the optimal protocol for carrying out such dissections.

Moreover, the biological significance of the size of the accessory glands and testes soon after emergence is not entirely clear. For example, the overall size of the testis may not be an accurate proxy for sperm production capacity – other factors such as the rate of spermatogenesis, the size of the seminal vesicle, etc. may also have a role in predicting sperm investment (Schärer and Vizoso 2007). The accessory glands are known to shrink after successive matings (Hihara 1981; Linklater et al. 2007), showing that their size is dependent upon the amount of SFPs stored in them at a given time. As SFP production remains plastic throughout male adulthood (Fedorka et al. 2011; Hopkins et al. 2019), the size of the accessory glands when maturity is reached may have limited effects on reproductive potential. On the other hand, it is conceivable that variation in the size of the accessory glands shortly after eclosion demonstrates 'priming' for imminent sperm competition via increased early SFP production, and/or is reflective of an upper limit on the volume of the accessory glands and the quantity of proteins that can be stored. To better understand the influence of the developmental environment on reproductive investment, it may be interesting to track the mating effort and reproductive effort across early matings of males from different

developmental environments. This could shed light on how well any developmental plasticity in accessory gland and/or testes size correlates with reproductive effort in adulthood.

The length of the L3 wing vein, which is often used as a proxy for body size (Bretman et al. 2016) varied significantly with the larval social environment in the second iteration of the experiment, though not in the first. Although the overall effect of the post-eclosion social environment was nonsignificant, males that developed with no adults in the larval environment had significantly longer L3 veins when they were housed alone post-eclosion, compared to those stored in same-sex groups. This result was surprising, as body size, though plastic during larval development, is not expected to be influenced by the environment after eclosion, by which time the adult cuticle has hardened (French et al. 1998). *Drosophila* wings themselves show developmental plasticity and continue to develop in the hours after eclosion (Bitner-Mathe and Klaczko 1999; Matamoro-Vidal et al. 2015) but are not thought to remain plastic past the point of emergence from the pupa (McGuigan 2009). Further investigation into wing size and shape also showed that the roundness of the wing (width divided by length) significantly varied with the post-eclosion environment in some contexts. Wing shape has been found to influence mating success among male *D. melanogaster*, so plasticity in this trait in response to the developmental social environment could suggest an adaptive response to the perceived level of male-male competition (Menezes et al. 2013). Furthermore, this result could suggest that the wings remain plastic later in development than previously thought. However, a more comprehensive, multivariate analysis of wing shape based on the position of 15 landmarks showed no significant influence of either the larval or the post-eclosion environment on shape. The result of the multivariate morphometric analysis, combined with the inconsistencies in the results on L3 wing vein length, suggest that measures of single wing traits may not be reliable. The apparent potential for L3 length to vary in different post-eclosion social environments suggests this measure may not always be an appropriate proxy for overall body size.

Overall, the results suggest that male *D. melanogaster* accessory glands and testes are sensitive to multiple features of the social environment during larval development and may remain plastic in the hours immediately after eclosion from the pupa. Cues of potential sperm competition in the larval environment tended to lead to the development of smaller reproductive structures, while cues of competition in the post-eclosion environment seemed to generally lead to larger accessory glands. This suggests that reproductive morphology remains plastic until its development is completed after eclosion, and that the effect of competition on this plasticity may change at different stages of development. However, the

apparent effect of the social environment on accessory glands and testes did not show high repeatability, depended on whether measures of perimeter or area were used, and contrasted with previous findings (Bretman et al. 2016). Similarly, the length of the L3 wing vein showed an unexpectedly high degree of variability, and in one instance seemed to significantly vary with the post-eclosion environment. Measuring wing morphology based on multiple landmarks may lead to more robust conclusions (BitnerMathé and Klaczko 1999; Debat et al. 2003). Similarly, better understanding of the developmental plasticity of the accessory glands and testes may be gained by using techniques such as 3D fluorescence imaging, rather than 2D imaging (Kibanov et al. 2013; Fretaud et al. 2017).

2.6 Supplementary information

Table S2.1 - Simplified statistical models with significant terms retained - the effects of the larval and post-eclosion social environment on reproductive and wing morphology of male *Drosophila melanogaster.*

Dependent variable	**Simplified model**	**LRT**	**df**	**p**	
a) The effect of adult males or females in larval vials on reproductive morphology					
Accessory gland perimeter	Linear model on untransformed data				
	No significant terms				
Accessory gland area	Linear model on untransformed data	**F**	**df**	**p**	
	Area ~ larval environment	9.97	2	**0.0011**	**
	Pairwise comparisons	**t**	**df**	**p**	
	M / F	2.30	144	0.12	
	M / N	4.46	144	0.0060	**
	F / N	2.27	144	0.12	
Testis perimeter	Linear model on untransformed data				
	No significant terms				
Testes area	Linear model on untransformed data				
	No significant terms				
b) The effect of adults in larval vials and post-eclosion density on reproductive morphology					
Accessory gland perimeter	**Simplified model**	**F**	**df**	**p**	
	Linear model on untransformed data				
	Perimeter ~ post-eclosion environment	10.18	1	0.014	*
	Pairwise comparisons	**t**	**df**	**p**	
	M group / M alone	1.24	45.86	0.41	
	F group / F alone	1.44	80.78	0.30	
	N group / N alone	2.71	90.37	0.033	*
	M group / F group	0.99	83.85	0.53	
	M group / N group	1.48	89.46	0.29	
	F group / N group	0.46	78.42	0.76	
	M alone / F alone	0.96	41.50	0.54	
	M alone / N alone	0.32	44.35	0.86	
	F alone / N alone	0.76	91.82	0.61	

Accessory gland area	**Simplified model**	**F**	**df**	**p**	
	Linear model on untransformed data				
	Area ~ L3 vein	12.81	1	0.011	*
Testes perimeter	**Simplified model**	**F**	**df**	**p**	
	Linear model on untransformed data				
	No significant terms				
Testes area	**Simplified model**	**F**	**df**	**p**	
	Linear model on untransformed data				
	Area ~ larval environment + L3 vein	6.47	3 & 218	0.012	*
	Larval environment	6.54	2	0.014	*
	L3 vein	7.29	1	0.033	*
	Pairwise comparisons	**t**	**df**	**p**	
	M group / M alone	2.17	51.75	0.12	
	F group / F alone	0.10	71.69	0.96	
	N group / N alone	0.045	81.42	0.96	
	M group / F group	1.74	69.70	0.23	
	M group / N group	3.16	80.29	0.015	*
	F group / N group	1.67	70.95	0.23	
	M alone / F alone	0.81	47.79	0.61	
	M alone / N alone	0.74	45.31	0.62	
	F alone / N alone	1.81	82.83	0.43	
L3 wing vein length	**Simplified model**	**F**	**df**	**p**	
	Linear model on untransformed data				
	L3 length ~ larval environment	5.77	2	0.021	*
	Pairwise comparisons	**t**	**df**	**p**	
	M group / M alone	0.65	42.64	0.65	
	F group / F alone	0.79	84.21	0.61	
	N group / N alone	2.87	95.74	0.026	*
	M group / F group	3.27	85.70	0.014	*
	M group / N group	0.24	95.87	0.90	
	F group / N group	3.61	82.86	0.011	*
	M alone / F alone	1.15	41.50	0.46	
	M alone / N alone	1.13	39.82	0.46	
	F alone / N alone	0.054	96.58	0.96	

c) The effect of larval and post-eclosion population density on male wing morphology

L3 wing vein length	**Simplified model**	**F**	**df**	**p**	

	Linear model on untransformed data				
	No significant terms				
Wing perimeter	**Simplified model**				
	Linear model on untransformed data				
	No significant terms				
Wing roundness (width/length)	**Simplified model**				
	Linear model on untransformed data				
	Roundness ~ larval environment	7.49	1	0.041	*
	Pairwise comparisons	**t**	**df**	**p**	
	S group / S alone	0.032	69.43	0.97	
	H group / H alone	2.59	82.86	0.045	*
	S group / H group	3.55	82.26	0.0078	*
	S alone / S alone	0.52	77.44	0.72	
Wing shape	**Simplified model**	**F**	**df**	**p**	
	MANOVA on Procustes coordinates				
	No significant terms				

2.7 References

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3. Plastic male mating behaviour evolves in response to the competitive environment

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3.1 Abstract

Male reproductive phenotypes can evolve in response to the social and sexual environment. The expression of many such phenotypes may also be plastic within an individual's lifetime. For example, male *Drosophila melanogaster* show significantly extended mating duration following a period of exposure to conspecific male rivals. The costs and benefits of reproductive investment, and plasticity itself, can be shaped by the prevailing socio-sexual environment and by resource availability. I investigated these ideas by using experimental evolution lines of *D. melanogaster* evolving under three fixed sex ratios (high, medium and low male-male competition) on either rich or poor adult diets. I found that males evolving in high-competition environments evolved longer mating durations overall. In addition, these males expressed a novel type of plastic behavioural response following exposure to rival males: they both significantly reduced and altered courtship delivery, and exhibited significantly longer mating latencies. Plasticity in male mating duration in response to rivals was maintained in all of the lines, suggesting that the costs of plasticity were minimal. None of the evolutionary responses tested were consistently affected by dietary resource regimes. Collectively, the results show that fixed behavioural changes and new augmentations to the repertoire of reproductive behaviours can evolve rapidly.

3.2 Introduction

Male reproductive investment is shaped by the level of pre- and post-mating sexual competition in many species. Across taxa, males from species with higher levels of polyandry have been found to have larger testes and produce more sperm (Birkhead 1998; Wedell et al. 2002; Smith 2012). Furthermore, individual males can express plasticity in their reproductive investment and mating behaviour, allowing them to adapt to variation in the social environment within their lifetime. Plasticity in reproductive traits enables individuals to adjust their investment in each mating or reproductive bout in response to the environment, including social context, thus optimising lifetime fitness (Dewsbury 1982; Gage and Baker 1991; Wedell et al. 2002; Bretman et al. 2011a). There are many examples of individuals adapting their reproductive effort according to factors such as the risk of sperm competition, the mating status or quality of a potential mate, or to the developmental environment (Wedell et al. 2002; Kasumovic and Brooks 2011; Kelly and Jennions 2011). In this chapter, I investigate how male reproductive behaviours evolve in response to the competitive environment.

Investment in reproduction, particularly sperm and seminal fluid protein production, is known to be costly to males (Dewsbury 1982; Nakatsuru and Kramer 1982; Wedell et al. 2002; Perry et al. 2013). *D. melanogaster* males that were repeatedly exposed to competitors, and responded by extending mating duration, throughout their lifetime suffered significant costs later in life, indicating that reproductive resources can be limiting (Bretman et al. 2013b). Furthermore, plasticity *per se* may also carry costs. For example, maintaining the capability to accurately monitor the environment, process cues and alter phenotype expression accordingly is expected to be energetically costly (DeWitt et al. 1998; Relyea 2002; Auld et al. 2010). Producing a phenotype that is rapidly and accurately matched to a changing environment may require stringent and sophisticated receiving, processing, learning and/or memorising of multiple sensory cue components (Bretman et al. 2011b; Mohorianu et al. 2017; Rouse et al. 2018). The relative costs and benefits of expressing plasticity are also likely to be context-dependent. Thus, the adaptive value of maintaining plasticity in a trait versus expressing a fixed response may vary temporally and spatially (Givnish 2002). Plasticity is predicted to be particularly beneficial in rapidly-changing environments (Botero et al. 2015) and may become neutral or even costly if the environment is stable or constant. Therefore, both the overall level of investment in a reproductive trait and the degree to which it is plastic may be subject to trade-offs, and may be targets of selection imposed by the social environment.

If reproductive investment and plasticity are costly, they may be mediated by resource availability, as well as selection from the social environment. Diet is known to mediate trade-offs between reproduction and longevity, such that dietary restriction limits fecundity (Flatt 2009; Edward and Chapman 2011). Remating frequency, egg production and lifespan are affected by reducing the levels of protein and carbohydrate in the diet of female *D. melanogaster* (Chapman and Partridge 1996) and protein availability may also mediate male reproductive success (e.g. Fricke et al. 2008). The balance of costs and benefits of plasticity *per se* may also interact with nutrition availability, as investment in maintaining costly plasticity may itself be resource-limited (Steinger et al. 2003; Cipollini 2004). Therefore, the expression of costly, plastic reproductive traits may be affected by an interaction between the social environment and resource availability.

Experimental evolution approaches offer excellent potential for testing explicit predictions on how male reproductive behaviours evolve in response to the social environment, whether the expression of plasticity is reduced when environments are more stable, and how these responses may be mediated over evolutionary time by resource availability (Murren et al. 2015). Previous studies have utilised lines of *D. melanogaster* experimentally evolved under male- or female-biased sex ratio to study male and female responses to the level of male-male competition and sexual conflict. A strongly female-biased sex ratio can select for larger male testis size, suggesting an adaptation to mating rate and sperm depletion (Reuter et al. 2008). Male-biased adult sex ratios have been found to select for increased female resistance to male-induced harm (Wigby and Chapman 2004) and faster ejaculate depletion over serial matings (Linklater et al. 2007). Edward et al. (2010) tested plastic male responses to rivals in male-biased and female-biased lines of *D. melanogaster* and found that males from both regimes maintained responses to rivals, while males from male-biased lines expressed a nonsignificant tendency to mate for longer overall. Here, I built on these previous studies by conducting a comprehensive investigation into male plastic reproductive behaviour in male-biased, equal-sex and female-biased experimental evolution lines maintained under two dietary regimes. The inclusion of the equal-sex lines served the purpose of distinguishing between the effects of biased sex ratio *per se* from other possible influences of the evolutionary environment. New to this study were tests of the reproductive behaviour of males from these lines in response to both wildtype and own-regime rivals and females, allowing the disentangling of potential effects arising from co-evolution as well as from context-dependence. I also studied plastic male mating duration and latency among males evolved under both fixed sex ratio and either rich or poor adult diet regimes, to test the

effects of, and interactions between, the social environment and resource limitation on male reproductive investment and plasticity. Finally, I investigated the previously unanswered question of how male courtship behaviour has evolved in response to fixed sex ratio.

I used experimental evolution lines in which each generation is subjected to a fixed adult sex ratio and either a rich or poor adult diet. This allowed testing of how male reproductive behaviours evolve in response to different degrees of male-male competition and resource availability. Furthermore, the relatively stable level of male-male competition induced by controlling sex ratio allowed investigation into whether plasticity in male reproductive behaviours diminishes when environmental stability increases. I measured mating duration, which shows a highly repeatable and well-characterised response to male-male competition (Bretman et al. 2009; Bretman et al. 2010; Bretman et al. 2011b; Bretman et al. 2017; Rouse et al. 2018), latency to mate and courtship behaviour in males from these regimes. I first measured male behaviour in response to standardised wildtype rivals and with wildtype females. In subsequent experiments, I tested for context specificity by comparing the behaviour of focal males exposed to either wildtype or co-evolved rivals and females.

My first prediction was that males evolved under the fixed sex ratios, and thus divergent levels of male-male competition, would show evidence of directional selection on mating behaviour. I expected that males from the high-competition (male-biased lines) would be selected to mate for longer overall, indicating an increase in reproductive investment. The second prediction was that males from all the sex ratio regimes would show reduced plasticity overall in their reproductive behaviours. This prediction was based on the assumption that plasticity is less beneficial in the more stable social environments in which the sex ratio lines have been maintained, thus increasing the relative costs of expressing plasticity in comparison to the originating stock populations. The final prediction was that the adult dietary regime on which males were evolved would interact with sex ratio to influence plastic male mating behaviour, assuming that male investment in reproduction and/or the expression of plasticity is limited by protein restriction.

3.3 Methods

a) General methods

Experiments were conducted in a 25°C humidified room with a 12 h light: 12 h dark cycle. Unless otherwise specified, flies were maintained on a standard sugar-yeast-agar (SYA) medium (100g brewer's yeast, 50g sucrose, 15g agar, 30mL Nipagin (10% solution), 3mL

propionic acid, 0.97L water). Wildtype rivals and females were from a Dahomey stock population (Bass et al. 2007; Bretman et al. 2009) maintained in large cages with overlapping generations and in which sex ratio was allowed to vary naturally. Experimental flies were cultured by allowing females to oviposit on agar-grape juice plates (50g agar, 600mL red grape juice, 42mL Nipagin (10% solution), 1.1L water). Larvae were collected from the plates and reared under a controlled density of 100 per vial. At eclosion, adults were separated by sex to ensure virginity, and stored 10 per vial. Post-collection, rival males and females were maintained on standard SYA medium supplemented with live yeast paste. Focal treatment males were maintained on their evolutionary diet. Experiments took place when the focal males were aged 7-10 days old.

b) Experimental evolution under fixed adult sex ratios and standard and low yeast diets

Experimental evolution lines of *D. melanogaster* originated from a laboratory population of wildtype Dahomey flies and were maintained under three fixed adult sex ratios and two dietary regimes. Lines were maintained on either standard SYA medium, or a protein-restricted SYA medium containing only 20% the standard amount of yeast (Fricke et al. 2008). Within these two dietary treatments, lines were maintained under fixed sex ratios, either male-biased (MB, 70 males:30 females), equal sex (EQ, 50:50) or female-biased (FB, 25:75). The MB lines were propagated at a sex ratio of 70:30 (rather than 75:25) to ensure sufficient eggs were produced to set up each next generation. There were three replicate populations for each diet/sex ratio combination (3 sex ratio regimes x 2 diets x 3 replicates each = 18 experimental evolution lines). These lines were maintained in non-overlapping generations, each consisting of 100 individuals of the same age. This created a stable social environment relative to the originating wildtype (which was maintained in large populations in which sex ratio and age structure were allowed to fluctuate). These experimental populations had been evolving under fixed sex ratio and diet for over 66 generations at the time the experiments were conducted. Although there may be some inbreeding depression in the lines, Snook et al. (2009) calculated that the effective population sizes of equivalent regimes did not substantially differ between sex ratio treatments, thus any differential effects across lines can be expected to be minimal.

The sex ratio lines were maintained in 1 litre ventilated plastic boxes with two vials of water plugged with cotton bungs to maintain adequate humidity, and two vials of SYA (either standard SYA or 20% yeast). Food was replaced with fresh vials on a regular schedule, every 2-3 days. On the 8th day after each generation was set up, the SYA vials were replaced with agar-

grape juice plates, containing a smear of live yeast paste, for egg collection. Three-hundred larvae were collected from these plates and cultured at 100 per vial on standard SYA. After eclosion, 100 individuals in the correct sex ratio were randomly selected from these offspring. Thus, the lines were maintained in non-overlapping generations, within same age cohorts. Treatment males were offspring of individuals from the experimental evolution lines, obtained by standard density culturing of eggs laid on agar-grape juice plates.

c) Reproductive plasticity of males evolved under fixed sex ratios and two dietary resource levels

Experiment 1. Evolution of plastic male behaviour. Males cultured from experimental evolution lines were randomly assigned to either rivals (+) or no rivals (-) treatments. Males in the +rivals treatments were housed in a vial with three wildtype males for three days immediately prior to the mating assay. Rival males had their wings clipped under CO_2 anaesthesia, to differentiate the focal and rival males without affecting mating success (Ehrman 1966). Males in the no-rivals treatments were housed alone. During the ± rivals exposure treatment period, all males were maintained on the evolutionary diet of the focal male. All focal males, rival males and females used in experiments were virgins, in order to control for confounding effects of prior social experience, and for consistency with previous studies of male *D. melanogaster* reproductive behaviours (Bretman et al. 2009; Bretman et al. 2011b; Rouse and Bretman 2016). Females were transferred to individual vials of SYA with live yeast supplementation a day prior to mating. Each focal male was introduced to a female by aspiration. Latency to mate (the time from when the male was introduced to the vial with the female to when mating began) and mating duration were recorded to the nearest minute. Labels on vials were coded so that observers were blind to the treatment of each sample. Pairs that did not mate within 2.5 h were discarded. Males were removed after mating to avoid remating and females were left to oviposit for 24 h. Vials were retained until all offspring eclosed, when adult offspring were frozen and counted. Replicate populations 1 of each experimental evolution regime were tested in block one (at generation 66 of experimental evolution), replicate populations 2 of each regime tested in block two (at generation 67), and replicate populations 3 in block three (at generation 68). Data were pooled for analysis and analysed as described below.

A separate control experiment was also conducted to determine the effects on reproductive responses to rivals of maintaining wildtype males on a proximate diet of either

100% or 20% yeast diets. This was done to give further insight into the determination of evolutionary versus proximate diet effects in the main experiments with the sex ratio lines. Wildtype individuals from stocks maintained on standard SYA were cultured as described above, and males were randomly assigned to a rivals or no-rivals treatment, and to a 100% or 20% yeast diet. Males were collected as adults and housed with (+) or without (-) three conspecific, wildtype male rivals for three days on their experimental test diet. Rival males and females were collected and stored in standard SYA vials with live yeast supplementation. Females were transferred to individual vials of SYA with live yeast a day prior to mating. Mating duration and latency to mate were recorded as described above.

The results from experiment 1 revealed that males from MB lines had evolved to become significantly slower to mate following exposure to rival males. In order to investigate potential male- and female-mediated drivers of this novel plasticity in mating latency, additional experiments were then conducted to test the influence of the evolutionary history of rival males and females on focal male mating behaviour. Furthermore, a detailed analysis of male courtship behaviour was performed to examine which elements had changed (details below). These experiments 2-4 focused on MB lines, due to the plasticity in mating latency expressed specifically by these populations. The EQ lines were included as a control group against which to infer evolved patterns of male mating behaviour in the MB lines, and the FB lines were excluded from these further experiments. As no consistent effect of diet on male mating behaviour was found, these subsequent experiments were also conducted only on lines derived from the standard diet regimes.

Experiment 2. Interaction of male reproductive plasticity with rival male evolutionary history: Focal regime males were tested with wildtype rivals vs. coevolved rivals from within their own experimental evolution regime, when mating with wildtype females. Focal males were randomly assigned to treatments in which they were housed for three days with either three wildtype rivals (+WT), with three co-evolved rivals from within their own experimental evolution regime (+own regime), or alone (-). To investigate male aggressive encounters as a potential driver of evolved changes to male courtship repertoires, behavioural spot checks of the focal male were conducted during the period of exposure to rival males. On each of the three days, spot checks were made every half an hour from 8:30 (ZT0)-10:30am (ZT2.5), a period of peak activity for *D. melanogaster* (De et al. 2013). The number of times the focal male was observed in physical contact with a rival male (encompassing fencing, lunging,

boxing, tussling, etc, Chen et al. 2002) was recorded, as a proxy for the frequency of aggressive interactions. Following rival/no rival exposure treatment, focal males were introduced to a virgin female and mating latency and duration were recorded as described in experiment 1. All three replicate populations were tested simultaneously. This experiment was conducted twice, independently, at generations 85 and 89 of experimental evolution, and the data were pooled across generations for analysis.

Experiment 3. Interaction of male reproductive plasticity with female evolutionary history: To investigate potential female-mediated drivers of MB male plasticity in mating latency, the responses of focal line males to wildtype rivals, when mating to wildtype vs. coevolved, own-regime females were tested. Focal males were randomly assigned to treatments in which they were housed for three days either with three wildtype rivals (+) or alone (-), then mated to either a wildtype virgin female (xWT) or a virgin co-evolved female from within the male's own experimental evolution regime (xMB or xEQ). Assays to measure mating latency, duration and offspring production were conducted as described for experiment 1. All three replicate populations were tested simultaneously. This experiment was conducted on individuals drawn from generation 92 of the experimental evolution.

Experiment 4: Evolutionary changes in courtship behaviour: To investigate the behavioural drivers underpinning MB male plasticity in mating latency, the courtship repertoire of males from MB and EQ experimental evolution lines were analysed, with and without prior exposure to wildtype rivals. Focal males were cultured as above and either exposed to one wildtype rival for three days (+) or housed alone (-). Following this, each focal male was aspirated into a circular Perspex mating arena (diameter 22mm, depth 5mm) with a wildtype female and filmed for up to 30 min, or until copulation began. Video recordings were made using Sony Handycam HDR cameras from 9:30am (ZT0)-11:00am (ZT1.5) over six adjacent days. The first minute of footage of each pair was disregarded to allow for acclimation. The courtship videos were blinded with respect to identity and analysed using *JWatcher* (Blumstein and Bouskila 1996; Blumstein and Daniel 2007). A time log of each video was created, which recorded the occurrence, duration and sequence of the following courtship behaviours (Lasbleiz et al. 2006): stationary (male (M)), chasing (M), orientating (M), tapping (M), wing flicking (female (F)), kicking (F), singing (M), licking (M), attempted copulation (M), copulation (M), circling (M), decamping (M/F), movement (general movement around the courtship arena not directed at

the other individual; M/F). The following behaviours were removed prior to statistical analysis, because they occured in <10% of samples: decamping (M), movement (F), wing flicking (F), kicking (F). Courtship latency and copulation latency were also recorded, as before.

d) Statistical analyses

Statistical analyses were performed using R v 3.6.1 (R Core Team 2016). Mixed models were used to account for units of replication. In experiment 1, replicate population 1 of each experimental evolution treatment was tested in one block, replicate populations 2 in a second, and replicate populations 3 in a third. Thus, replicate population and experimental block were confounded, so were included in mixed models as one random effect ('block'; Table S3.1a). In experiment 2, all populations were tested simultaneously, in the two replicate assays. Thus, in this case population and experiment were included in mixed models as two random factors (Table S3.1b). In experiment 3, all replicate populations were tested once, simultaneously, thus population alone was included in mixed models as a random factor (Table S3.1c). In experiment 4, samples were tested across several days in a randomised order. Both population and the date of testing were included as random factors in models analysing these data.

Where mating duration and latency data were normally distributed or could be transformed to fit a normal distribution, Gaussian linear models were used. Where data were not normally distributed, generalised linear mixed models with gamma distributions and log links, as was determined to be the best fit for the data, were implemented in the package 'lme4' (Bates et al. 2015). Maximal models included the main effects of evolutionary sex ratio, evolutionary diet, rival exposure, rival evolutionary identity and female evolutionary identity, where relevant as well as interaction effects. Stepwise model simplification was conducted, with analysis of deviance to determine significant terms.

Multivariate data showing the time budget of male courtship (the proportions of courtship duration spent on each recorded behaviour) were analysed using a principal components analysis with the function prcomp(). The eigenvalues of each principal component were extracted, and those with a value of >1 (PCs 1 and 2) included in linear mixed models to determine the influence of sex ratio and rival exposure. To complement this analysis and determine the consistency of patterns of courtship intensity across individual behaviours, the courtship data were also analysed by using univariate testing. The numbers of times behaviours were performed were analysed with generalised linear models with Poisson distributions and log links. Some behaviours (singing, stationary, circling and general

movement) were performed for highly variable durations and could not be analysed as simple counts of occurrence. In these cases, Kruskal-Wallis tests were run on individual measures to analyse the proportion of time the individual spent performing the behaviour. Courtship duration and latency were also analysed using Kruskal-Wallis tests. The probability of successful copulation within the 30 min window was analysed using a generalised linear model with a binomial distribution and a logit link. Finally, the probability of transitions between courtship behaviours were analysed to investigate differences in the sequence of the courtship routine. Occurrences of single-order transitions between behaviours were pooled for all males within each treatment, to give a transition matrix for each. Transitions that never occurred across all treatments were considered structural zeroes and not included. A generalisation of the Fisher's Exact test was used to test for non-randomness at each transition, using the function aylmer.function() in the package 'aylmer' (West and Hankin 2008).

Throughout, planned pairwise comparisons were carried out on estimated marginal means using the emmeans() function in the package emmeans (Lenth et al. 2018). Within each set of experiments, p-values were adjusted for multiple comparisons using the Benjamini-Hochberg procedure.

3.4 Results

a) Longer overall mating duration and a novel behavioural plasticity phenotype evolved in response to strong male-male competition

The first prediction, that male mating behaviour would evolve in response to the level of male-male competition imposed by the fixed sex ratio regimes, was supported. Males evolved under male-biased (MB) sex ratio evolved longer matings overall and novel, behaviourally plastic, responses to rivals in mating latency and courtship behaviour. The evolution of this plasticity in mating latency and courtship was specific to the males from the MB sex ratio regimes and was not observed among FB, EQ or wildtype males.

Across all experiments, there was evidence that baseline mating duration had evolved in the sex ratio regimes (Table 3.1). Increased male-male competition generally led to longer overall mating duration, with males from FB lines tending to mate for the shortest duration (Figure 3.1). There was a general pattern of MB males mating for longer than EQ males in equivalent diet/rival treatments (Figure S3.1). This effect was statistically significant in some, but not all comparisons. However, the pattern was repeatable across experiments 1-3 (Figure 3.1, S1; Table S3.1a-S1c). This supported the prediction that sex ratio imposed directional

selection on overall mating duration, leading to extended mating duration among MB males in response to the consistently high level of competition exerted in the male-biased regimes.

Table 3.1 - Statistical models and summary of effects of exposure to rivals on mating behaviour of focal males. Experiment 1: responses of experimentally evolved focal males to wildtype rivals and wildtype females. Experiment 2: responses of focal males to wildtype vs. co-evolved rivals and wildtype females. Experiment 3: responses of focal males to wildtype rivals and wildtype vs. co-evolved females. Experiment 4: courtship behaviour of focal males in response to wildtype rivals and wildtype females. Asterisks indicate significant pairwise differences:; *** p>0.001; ** p>0.01; * p>0.05. See Table S3.1 for full reporting of models and pairwise comparisons.

Model	**LRT**	**df**	**p**
Experiment 1. Experimentally evolved focal males with wildtype rivals and wildtype females.			
Mating duration ~ rival + SR + diet + SR:diet + (1|block)	151	13	<0.001 ***
Mating latency ~ rival + SR + diet + rival:SR + rival:diet + (1|block)	93.53	11	<0.001 ***
Number of offspring ~ rival + (1|block)	23.67	11	<0.001 ***
Experiment 2. Experimentally evolved focal males with wildtype vs. co-evolved rivals and wildtype females.			
Mating duration ~ SR + rival.presence + (1|experiment) + (1|population)	49.98	2	<0.001 ***
Mating latency ~ rival.presence + (1|experiment) + (1|population)	28.01	1	<0.001 ***
Frequency of contact with rival ~ SR + (1|experiment) + (1|population)	5.34	1	0.047 *
Experiment 3. Experimentally evolved focal males with wildtype rivals and wildtype vs. co-evolved females.			
Mating duration ~ rival + (1|population)	23.08	7	<0.001 ***
Experiment 4. Courtship behaviour of experimentally evolved focal males with wildtype rivals and wildtype females.			
Courtship behaviour PC1 ~ rival + (1|date) + (1|population)	6.85	1	0.026 *

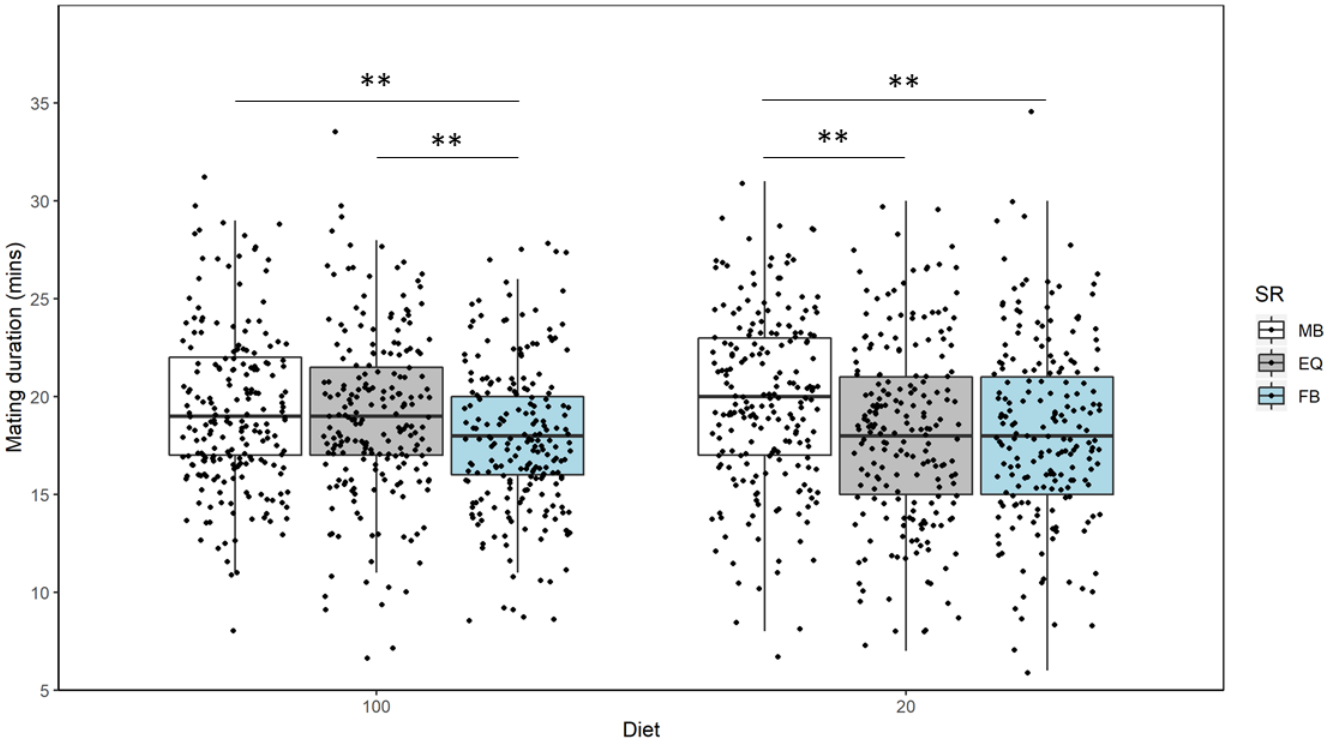


Figure 3.1 – **Mating duration of experimentally evolved focal male *Drosophila melanogaster* in response to wildtype rivals and wildtype females.** The mating duration of males evolved under male-biased (MB; white boxes), equal (EQ; grey boxes) or female-biased (FB; blue boxes) sex ratio and standard (100% yeast) or protein-restricted (20% yeast) diet regimes. Rival exposure treatments within each sex ratio/diet treatments are pooled to show differences in overall mating duration. Boxplots showing interquartile range and median. Asterisks indicate significant pairwise differences in planned comparisons of estimated marginal means between groups on each end of the horizontal line: *** p>0.001; ** p>0.01; * p>0.05. For boxplots split by replicate populations, see Figure S8. Each end

Males from MB sex ratio regimes showed longer mating latencies following exposure to rivals (Table 3.1): in experiments 1 and 2 MB males significantly extended mating latency in response to both wildtype and own-regime rivals (Table S3.1a-b). The tendency to extend mating latency in response to rivals was not generally apparent among wildtype males or those evolved under equal (EQ) or female-biased (FB) sex ratio (Table S3.1a-b). In experiment 1, in which the mating behaviour of males from all experimental evolution regimes was tested in response to wildtype rivals and wildtype females, mating latency was influenced by a significant interaction between evolutionary sex ratio and rival exposure (GLMM: X^2=12.16, df=2, p=0.0088). MB males evolved on both the 100% yeast (Z-test: z=2.90, p=0.029) and 20% yeast diets (Z-test: z=2.51, p=0.032) expressed significantly longer mating latencies following exposure to rivals (Figure 3.2; Table S3.1a). In experiment 2, in which the evolutionary history

of male rivals was varied, rival exposure (GLMM: X^2=28.01, df=1, p<0.001), but not sex ratio, significantly influenced mating latency. Pairwise comparisons showed that MB males exposed to both wildtype (Z-test: z=4.37, p<0.001) and co-evolved rivals (Z-test: z=3.51, p=0.0014) significantly extended mating latency in comparison to males kept alone (Figure S3.2a; Table S3.1b). In experiment 3, in which the influence of female evolutionary history on focal male responses to competition was tested, there were no significant effects of sex ratio or rival exposure on male mating latency (Table S3.1c). Nevertheless, there was a nonsignificant pattern of MB males extending mating latency following exposure to rivals (Figure S3.2b). Previous studies have not found a consistent effect of rival exposure on mating latency, suggesting that the behaviour I observed here in MB males is an evolved response (Bretman et al. 2009; Bretman et al. 2013a; Bretman et al. 2013b).

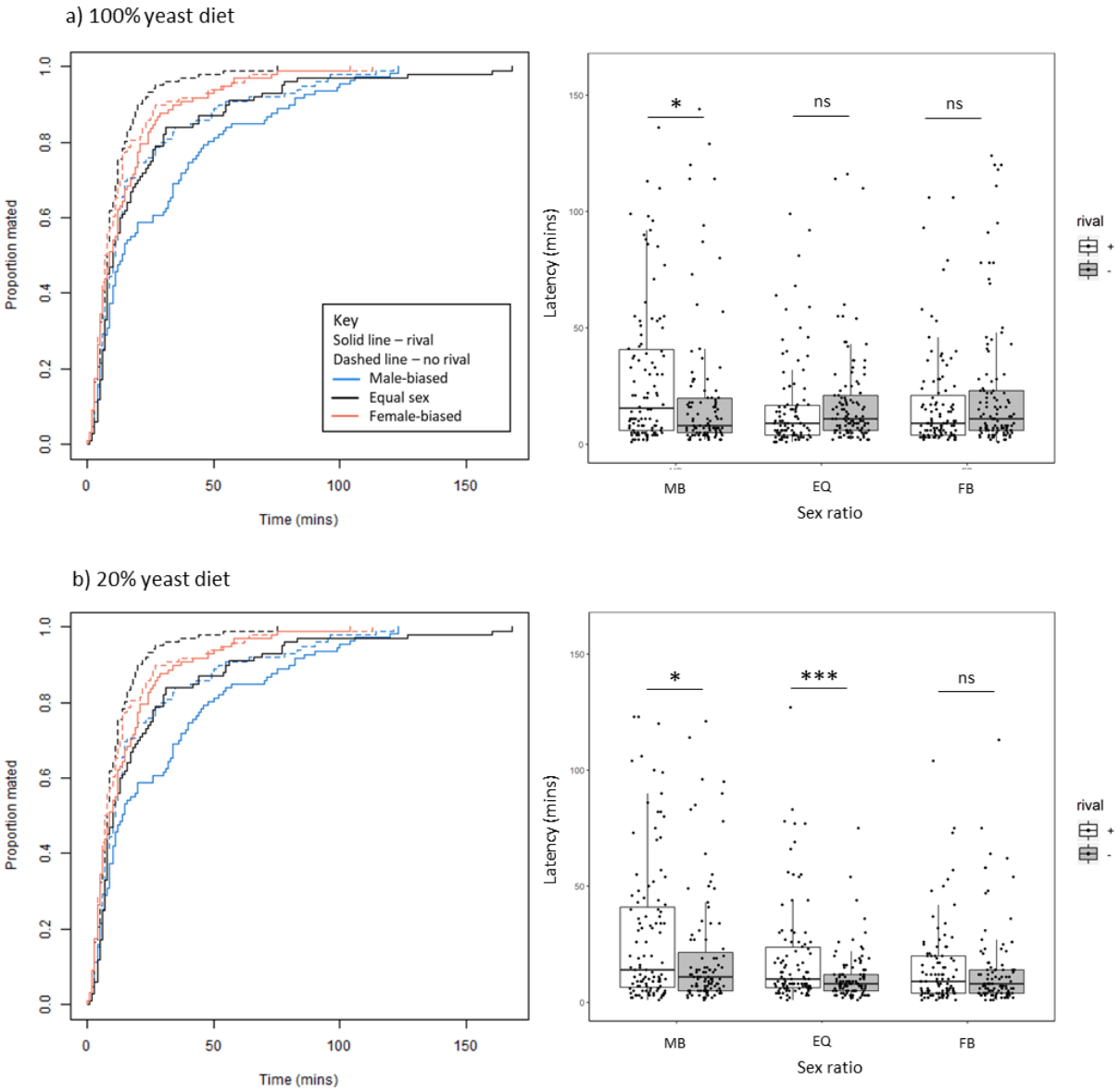


Figure 3.2 – **Mating latency of experimentally evolved focal male *Drosophila melanogaster* in response to wildtype rivals and wildtype females.** Left hand panel plots: the latency to mate of males (shown as the proportion of males that mated over time) evolved under male-biased (MB; blue), equal (EQ; black) or female-biased (FB; orange) sex ratio and standard (100% yeast) or protein-restricted (20% yeast) diet regimes. Focal males were either exposed to three conspecific male rivals ('rivals', solid line) or housed alone ('no rival'; dashed line) prior to mating. Right hand column: the same data visualised as boxplots (as described in Figure 3.1). For boxplots split by replicate populations, see Figure S3.9.

To investigate the mechanistic basis of the long latency expressed by MB males following exposure to rivals, the detailed courtship sequences of males from MB and EQ lines, with and without prior rival exposure, were analysed (experiment 4). MB males responded to

rival exposure by exhibiting a marked reduction in the expression of all courtship behaviours, evident as significantly extended courtship latency (Kruskal-Wallis: X^2=17.64, df=3, p=0.019; Table S3.1e) and a significantly altered courtship routine. The principal components with eigenvalues >1 were PC1 (explaining 41.59% of variation in courtship behaviour) and PC2 (explaining 13.14% of the variation). The first principal component was significantly affected by rival exposure (LMM: X^2=6.85, df=1, p=0.026; Table 3.1) with a borderline nonsignificant interaction between evolutionary sex ratio and rival exposure (LMM: X^2=3.77, df=1, p=0.052; Table S3.1d). The second principal component was not significantly predicted by sex ratio or rival exposure. The time the male spent tapping the female had the highest loading on PC1 (0.46), followed by time spent chasing the female (0.40) and time spent licking the female (0.39). Time spent circling the female had the highest loading on PC2 (0.58), followed by time spent chasing (0.43) and time spent orientating (0.40; Figure S3.3). Additional univariate tests showed that across 6 of the 7 male courtship behaviours tested MB males responded to rivals by performing the behaviour significantly less frequently, or for a significantly shorter proportion of time (Table S3.1e).

This effect of rivals on courtship behaviour was seen only in MB, not EQ, males (Figure 3.3; Figure S3.4; Table S3.1e). MB males also responded to rivals by spending a significantly higher proportion of their courtship time stationary and thus less time performing courtship behaviours (Exact Wilcoxon rank sum: w=672.5, p=0.013; Figure S3.4; Table S3.1d). However, the MB males did not spend less time engaged in general movement (i.e. moving around the courtship arena without interacting with the female; Exact Wilcoxon rank sum: w=440, p=0.80; Figure S3.4h; Table S3.1d). This suggested that the decrease in courtship behaviour was not driven by lower activity levels overall among MB males exposed to a rival. Furthermore, the number of times the female decamped (i.e. abruptly jumped or flew away from the male, which can be interpreted as a signal that the female is not receptive to mating) was not elevated in the MB rival treatment, suggesting that the reduced courtship intensity observed in the MB rival treatment group was not a response to reduced female receptivity (Table S3.1d). Extended courtship latency and reduced courtship intensity are likely to be the drivers of longer latency to mate among MB males following rival exposure. MB males retained the ability to express normal courtship behaviour, as demonstrated in the no rivals treatments (Figure 3.3, S3.4) and these males had comparable copulation success to that of EQ males in an equivalent rival treatment (Figure S3.4c).

Courtship was less stereotypical in MB males that had been exposed to rivals. This was indicated by an overall lower incidence of statistically significant transitions between

behaviours, and followed from their lower overall courtship activity. There were few cases where the likelihood of transitions between behaviours showed a significant response to sex ratio or rival exposure. However, the MB rivals treatment was the only group in which males were significantly likely to be stationary following female decamping, and not to follow decamping with chasing (Table S3.1f). This shows that MB males exposed to rivals appear more likely to respond to female rejection behaviour by ceasing courtship delivery.

Among males exposed to rivals, the identity of the rival males did not significantly predict the frequency of aggressive interactions between focal and rival males, though MB males generally showed less contact with rivals overall (Figure S3.5 Table S3.1b).

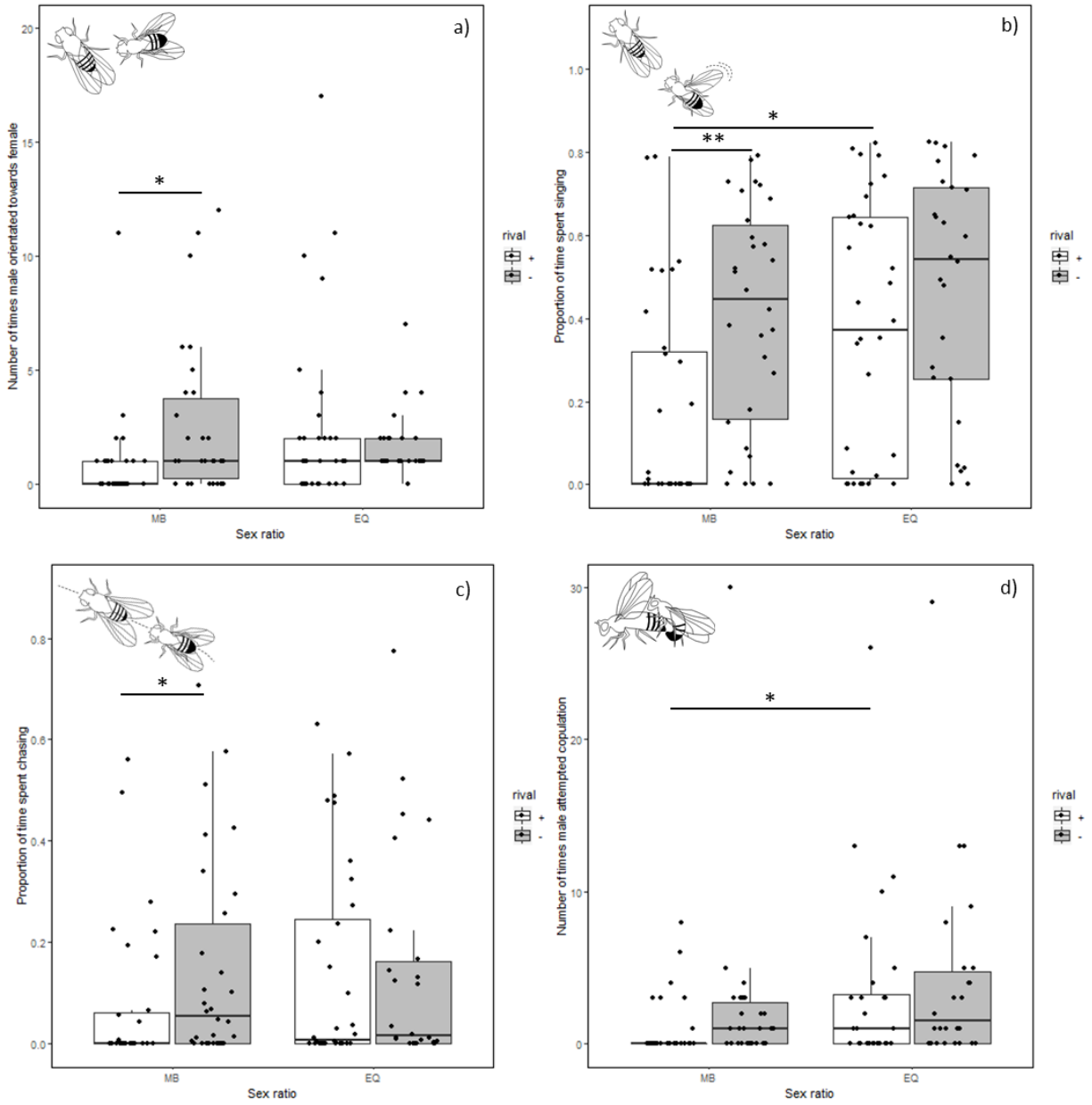


Figure 3.3 – **Courtship behaviour of experimentally evolved focal male *Drosophila melanogaster* in response to wildtype rivals and wildtype females.** The courtship intensity of males experimentally evolved under male-biased (MB) or equal (EQ) sex ratio. Focal males were either exposed to a conspecific male rival (+; white boxes) or housed alone (-; grey boxes) prior to introduction to the female (boxplots defined as described in Figure 3.1). **(a)** The number of times the male **orientated** towards the female. **(b)** The proportion of time (of the total duration spent in the courtship arena; 30 min or until courtship occurred) the male spent **singing**. **(c)** The proportion of time the male spent **chasing** the female. **(d)** The number of times the male **attempted copulation** with the female. For boxplots split by replicate populations, see Figure S3.10.

b) Plasticity was maintained in the fixed sex ratio and diet regimes

Counter to the second prediction, males evolving under the different fixed sex ratio regimes maintained plasticity in mating duration in response to rivals (Table 3.1). The presence of rivals remained a significant predictor of mating duration of focal males in response to both wildtype rivals and to wildtype females (experiment 1; LMM: X^2=93.87, df=1, p<0.001), to co-evolved rivals (experiment 2; LMM: X^2=44.24, df=1, p<0.001) and to co-evolved females (experiment 3; LM: X^2=23.08, df=1, p<0.001; Table S3.1a-c; Figure S3.1). Thus, plasticity in mating duration was not reduced by evolution in a relatively stable social environment. Males from the experimental evolution lines did not express significantly different responses to wildtype rivals compared to coevolved rivals. Among focal males exposed to rivals, the evolutionary identity of the rival did not predict latency to mate, mating duration or the frequency of contact with rival males (Figure S3.1, S3.2, S3.5; Table S3.1b). Although behavioural plasticity was maintained among experimentally evolved males, mating duration did not show a consistent relationship with the number of offspring fathered (Figure S3.6; Table S3.1a, S3.1c). In some instances, males that were exposed to rivals had lower reproductive success than those that experienced no competition. This was inconsistent with earlier studies showing that the extended mating phenotype expressed in response to rivals is associated with increased ejaculate investment and greater offspring production (e.g. Bretman et al. 2009). However, recent research with wildtype male *D. melanogaster* has also failed to find fitness benefits of extended mating and suggested that there may not be a direct relationship between rival exposure, behavioural response, ejaculate transfer and reproductive fitness (Dore et al. 2020).

Although the pattern of extended mating duration in response to rivals was consistent across treatments and across experiments, it was less pronounced among MB males mating with co-evolved females (experiment 3). Unlike experiments 1-2, in experiment 3 there were no significant pairwise differences in mating duration between treatments exposed to competitors and those that were not. Nevertheless, the size of the effect of rival exposure on mating duration was markedly lower in the case of MB x MB matings (T-test: t =1.13, df=297, p=0.42; Table S3.1c) than in other comparisons. This suggests that the expression of plasticity can be context-dependent, and that plasticity was diminished among MB males in their selective context with MB females.

c) Nutritional restriction had no consistent effect on male reproductive investment or plasticity

In the tests using wildtype rivals and wildtype females (experiment 1), there was a significant interaction between evolutionary sex ratio and adult diet on mating duration (LMM: p=0.035, Table S3.1a). However, this did not appear to be driven by reduced mating duration among males evolved on the poor diet (20% yeast) medium (Figure 3.1, Table S3.1a). This was counter to the prediction that a protein-restricted evolutionary diet would impose resource limitations leading to reduced investment in reproduction. Similarly, the limited protein dietary regime did not result in a reduction in mating duration or limit the expression of reproductive plasticity in wildtype males, again giving no evidence that resource limitation affected the ability of males to invest in reproduction (Figure S3.7; Table S3.1g).

3.5 Discussion

a) Directional selection on mating duration imposed by fixed sex ratio

The results supported the prediction that the evolutionary manipulation of adult sex ratio would impose directional selection on overall mating duration. There was a general trend for overall mating duration to be longer in males from the MB lines that experienced higher male-male competition, with mating duration in FB males tending to be the shortest. Additionally, in comparisons between MB and EQ males held under equivalent conditions, MB males generally mated for longer. Males are predicted to increase their reproductive investment when there is a high risk of sperm competition and when future mating opportunities are low (Linklater et al. 2007). Support for this prediction is observed across populations and species (Birkhead 1998; Hosken et al. 2001; Wedell et al. 2002; Smith 2012). In *D. melanogaster,* for example, males evolved in a polygamous mating system are more successful in sperm competition and elicit stronger post-mating responses from females compared to monogamous males, likely driven by higher investment in seminal fluid proteins (Hollis et al. 2019). *Drosophila spp.* males from populations with higher sperm competition have also been found variously to have larger testes, higher investment in spermatogenesis, larger accessory glands and higher offspring production (Pitnick et al. 2001; Crudgington et al. 2009). In the environment of the MB experimental evolution lines, each female may mate up to three times as often as each male (Wigby and Chapman 2004; Rostant et al. 2020). Thus, in order to contribute to the next generation of the MB lines, males must achieve reproductive success under consistently high sperm competition. The results of this study are consistent with previous findings that male *D. melanogaster* evolving in MB regimes invest more heavily in early mating opportunities, as evidenced by more rapid declines in productivity and accessory gland sizes than males from FB

lines (Linklater et al. 2007). Despite expressing longer overall mating, males from MB lines did not father a higher number of offspring than males from other lines, hence it is unclear if this extension of mating duration is adaptive. Extended mating may result in other reproductive benefits not measured, such as delaying female remating or promoting sperm defence (Bretman et al. 2009; Dore et al. 2020) and these would be interesting to explore further. Moreover, the evolution of longer mating duration could be a correlated response to another trait targeted by selection. A contribution of maternal effects towards the differences in male mating duration and plastic courtship behaviour observed between the sex ratio lines cannot be ruled out, as the focal males were the offspring of parents maintained in the regimes. Nevertheless, the results suggest a directional, potentially adaptive, response of male reproductive plasticity to the social environment.

b) Evolution of delayed and reduced courtship in response to rivals among MB males

Males evolved under the MB sex ratio expressed novel, plastic responses to rivals in mating latency and courtship behaviour, which were not observed in control (wildtype or EQ) males. Males from MB lines frequently responded to exposure to rivals by shutting down their courtship delivery and becoming significantly slower to initiate mating. This was driven by longer courtship latency and reduced courtship intensity. Reducing courtship intensity, and thus extending latency, in response to encountering rivals was not evidenced among EQ, FB or wildtype males, and to my knowledge has not been previously reported. Previous research has suggested that elements of courtship behaviour can evolve rapidly in response to the mating system (Holland and Rice 1999) and reduced latency to the initiation of courtship song is reported in promiscuous populations of *Drosophila pseudoobscura* (Snook et al. 2005). The results show that plasticity in courtship behaviour can evolve rapidly in response to the social environment.

In the evolutionary environment of the MB lines, it is likely that courtship is frequently interrupted or interfered with by the immediate presence of other males. The presence of rival males in the mating arena can reduce mating duration, suggesting that interference from rivals can interrupt and terminate copulation (Bretman et al. 2009). A similar effect is likely to occur during courtship - the structure of courtship song may often be masked by overlapping songs of other males, and it may be rare for males to complete a courtship sequence without interruption. These factors are proposed to drive a lower rate of courtship song delivery and shorter song duration by male *D. melanogaster* in the presence of competition (Tauber and

Eberl 2002) as well as shorter courtship bouts in more male-biased groups (Ewing and Ewing 1984). Similarly, interruption by rival males has been found to reduce the amount of time male guppies (*Poecilia reticulata*) and Pacific blue-eye fish (*Pseudomugil signifer*) spend courting in competitive environments (Jirotkul 1999; Wong 2004). Ubiquitous interruption of courtship by competitor males in the MB lines may have selected for plasticity whereby shorter and less intensive bouts of courtship behaviour are expressed by males when cues of rival presence are received prior to mating. This could explain the lower courtship intensity following exposure to rivals that was observed in males from MB, but not EQ lines, despite the fact that there were no competitors present in the mating arena to directly interrupt courtship in this experiment. Overall, the results show that novel elements of plasticity in courtship behaviour can rapidly evolve in response to evolution under high male-male competition.

In contrast to the generally longer mating duration expressed by MB males, implying increased reproductive investment, the lower courtship intensity elicited by rival exposure among MB males implies reduced mating effort. Together, these results may represent a re-focusing of reproductive effort that has evolved in response to the high level of male-male competition. Previously, polyandry has been shown to weaken pre-copulatory sexual selection and increase the relative strength of post-copulatory selection, demonstrating that the social environment can influence the balance of these two selective forces (Morimoto et al. 2019). The mating rate of females in the male-biased lines is high (Rostant et al. 2020), which may increase the relative importance of post-copulatory selection. In combination with the high likelihood of courtship being interrupted in this environment, this may select for a shift in reproductive effort from long, high-energy courtship sequences towards investment in post-mating competition.

Though possible, it seems unlikely that the evolved changes to mating behaviour expressed by MB males were strongly influenced by genetic drift and/or inbreeding. The effective population size of these regimes differ only slightly (Snook et al. 2009) minimising the potential for differential effects due to differential genetic drift. The extension of mating latency and reduction of courtship intensity in response to rivals also showed high consistency across the replicate MB populations (Figure S8; S9). Furthermore, MB males did not show evidence of inbreeding depression in that they retained the ability to express all the standard elements of the male courtship repertoire (Figure 3.3). I posit that this context-dependent courtship behaviour is more consistent with selection under high male-male competition than with the influence of inbreeding or drift.

c) Maintenance of reproductive plasticity in a fixed social and sexual environment

When environments become more stable the benefits of maintaining plasticity are expected to decrease. If there are net costs to maintaining plasticity it may then be selected against, leading to the evolution of more fixed phenotypes (Hedrick et al. 1976; Givnish 2002; Hall and Colegrave 2008; Murren et al. 2015). Overall, the results did not support the prediction that plasticity in mating duration would be reduced within a relatively stable selective environment. Males evolving under fixed adult sex ratio regimes that were female-biased, equal or male-biased all retained the ability to fully express extended mating duration as a response to rival males. Assuming that plasticity is heritable to some degree, this suggested that benefits of plasticity remained in the sex ratio regimes, or that costs were insufficient for any substantial negative selection. While some studies have supported the existence of costs of plasticity (Agrawal et al. 2002; Merila et al. 2004; Aubret and Shine 2010), which may select for fixed genotypes in stable environments, others have failed to find evidence for it (Scheiner and Berrigan 1998; Maughan et al. 2007; van Buskirk and Steiner 2009). It has been suggested that costs of maintaining plasticity *per se*, independent of any cost of the phenotype, may be negligible (Murren et al. 2015). Hence the accumulation of mutational effects, rather than costs of plasticity, may be the primary driver of erosion of plasticity under stability (Masel et al. 2007; Maughan et al. 2007; Murren et al. 2015).

Alternatively, the maintenance of plasticity in mating duration could be driven by remaining variation in the competitive environment of the sex ratio lines, to which males may continue to adaptively respond. The result that MB males extended mating duration in response to rivals more markedly when mating with wildtype females, compared to coevolved females, suggests that while the capacity for plastic responses was maintained in these lines, it may not actually be expressed in the environment in which they have been evolving. The reason why this was not observed in males from other lines could be due to differences in selection pressures across regimes. The data do not support the existence of plasticity costs, as MB males were still capable of expressing plasticity in mating duration when mating with wildtype females, and suggests instead that fixed reproductive behaviours may become more beneficial than plasticity when the social environment increases in stability.

d) Adult resource levels did not affect the expression or evolution of plastic mating behaviour

Overall, the results showed that the dietary resource level regimes did not affect the ability of males to invest in reproduction or express plasticity. When the responses of focal males to

wildtype rivals and wildtype females were tested, there was a significant interaction between sex ratio and diet for predicting mating duration. However, this appeared to be driven by particularly short mating duration among the 20% yeast no rivals EQ treatment. There was no general pattern of males evolved on the protein-restricted diet mating for shorter durations, or fathering fewer offspring. This does not support the prediction that nutritional limitation within the evolutionary regimes affected the allocation of reproductive resources. The dietary protein restriction imposed by the evolutionary 20% yeast diet does not appear to have selected for more prudent reproductive strategy in the lines maintained on this diet. Furthermore, maintaining wildtype flies on poor or rich yeast diets in the three days prior to mating also had no effect on mating duration. Taken together, these results suggest that this dietary restriction did not limit the level or flexibility of male *D. melanogaster* mating duration. Previous findings suggested that protein restriction resulted in males fathering few offspring and securing fewer rematings (Fricke et al. 2008) and affected courtship intensity and testis mass (Droney 1998). However, the effects of protein restriction were not consistent across male reproductive traits in the current study, and it may be that other dietary components have a stronger impact on male reproductive investment. For example, carbohydrate may be the primary requirement for energetically-demanding male mating behaviour, while protein may be more important for female egg production (Maklakov et al. 2008). Previous research has similarly found that a low yeast dietary regime did not limit the expression of plastic mating duration by male *D. melanogaster*, but suggested that imbalance in dietary components can cause loss of the extended mating response (Mason et al. 2016). Overall, there does not seem to be a simple relationship between dietary restriction and reproductive investment in male *D. melanogaster.* However, the finding that males retained the ability to express plasticity in mating duration under protein restriction offers further support for the idea that the costs of this plasticity may be small, or even negligible.

e) Conclusions

I found that fixed and plastic reproductive behaviours of male *D. melanogaster* can rapidly evolve in response to the competitive environment. The level of sexual competition exerted directional selection on overall mating duration, resulting in MB males generally mating for longer than EQ or FB males. This is consistent with the idea that MB males are strongly selected for 'per-mating' rather than 'repeated-mating' investment. MB males also expressed novel responses to rival exposure, whereby they were slower to begin mating and showed reduced courtship intensity across a range of behaviours. Interruption of courtship by rival males is likely to be ubiquitous in the MB regimes, and may have selected for the expression of

alternative or truncated courtship sequences when cues of competition are detected. Plasticity in male mating duration was not found to be reduced following evolution in a relatively stable competitive environment. Taken with the finding that protein restriction had no consistent effect on the expression of reproductive plasticity, this suggests that the maintenance of plasticity itself may carry low costs.

3.6 Supplementary information

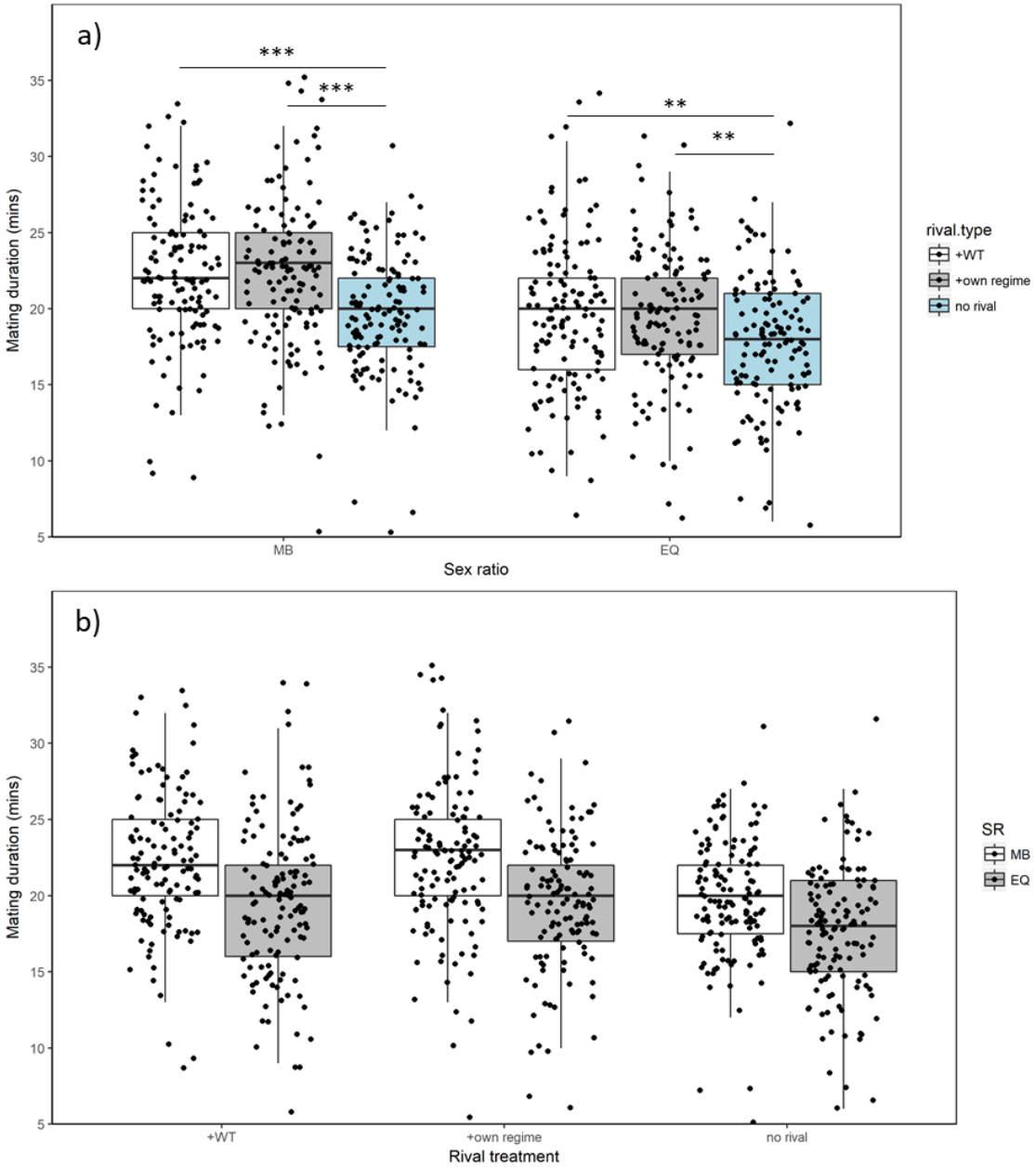


Figure S3.1 – The mating duration of experimentally evolved male *Drosophila melanogaster* in response to wildtype vs. co-evolved rivals and wildtype females. Focal males were evolved under male-biased (MB) or equal (EQ) sex ratio, then housed alone (no rival), exposed to three conspecific wildtype rivals (+WT), or exposed to rivals from within their own experimental evolution line (+own regime) prior to mating. All females were wildtype. (a) Comparisons of rival treatments within sex ratio lines; (b) data re-visualised to show comparisons of sex ratio lines within rival treatments. Boxplots as described in Figure 3.1.

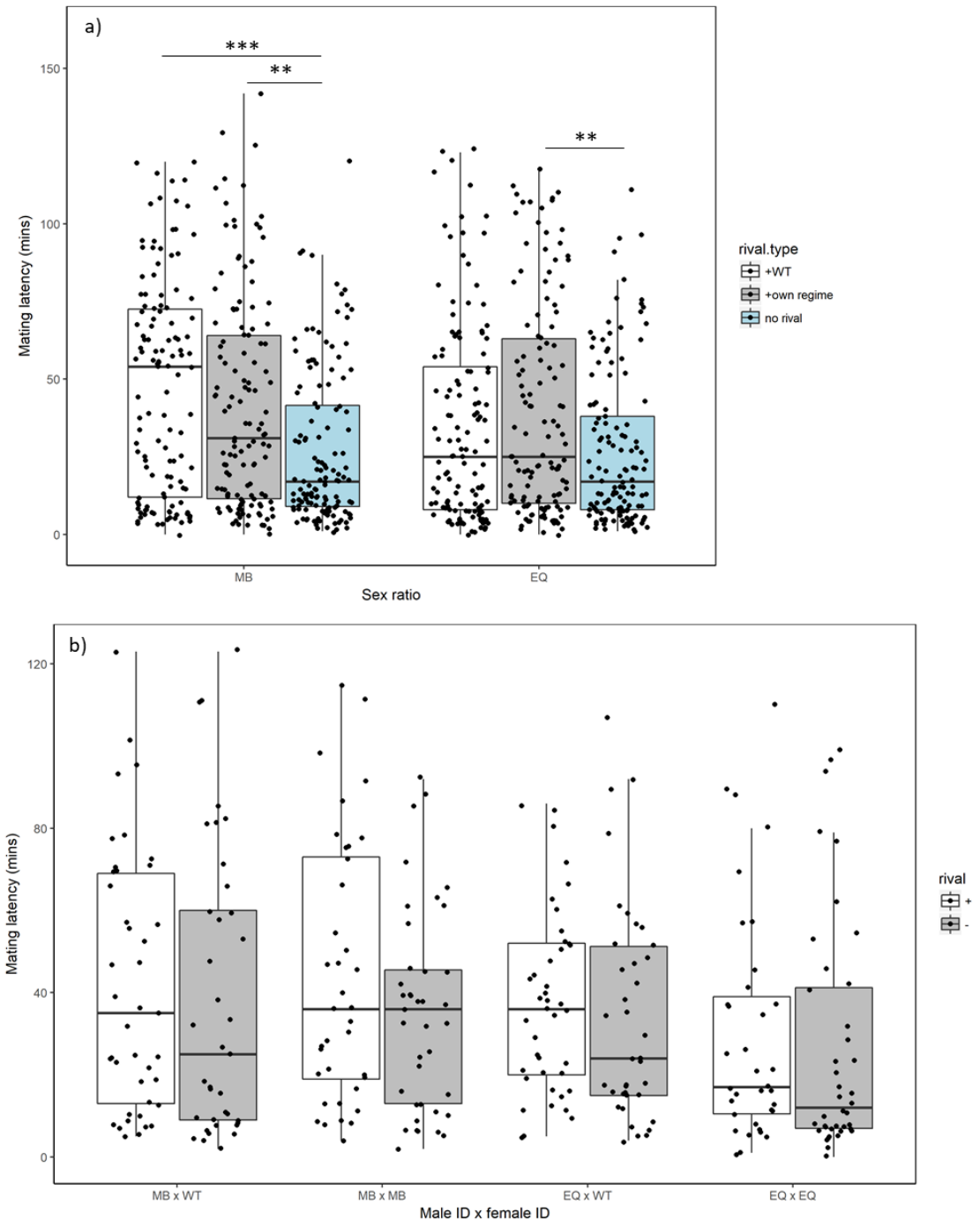


Figure S3.2 – **The latency to mate of experimentally evolved male *Drosophila melanogaster* in response to wildtype rivals and wildtype vs. co-evolved females**. Focal males were evolved under male-biased (MB) or equal (EQ) sex ratio, then housed alone (-), exposed to three conspecific male rivals (+) prior to mating. Asterisks indicate significant pairwise differences. (a) Rival males were either wildtype (+WT) or came from the same experimental evolution lines as the focal male (+own regime). (b) females were either wildtype (xWT) or came from the same experimental evolution lines as the focal male (xMB or xEQ). Boxplots as described in Figure 3.1.

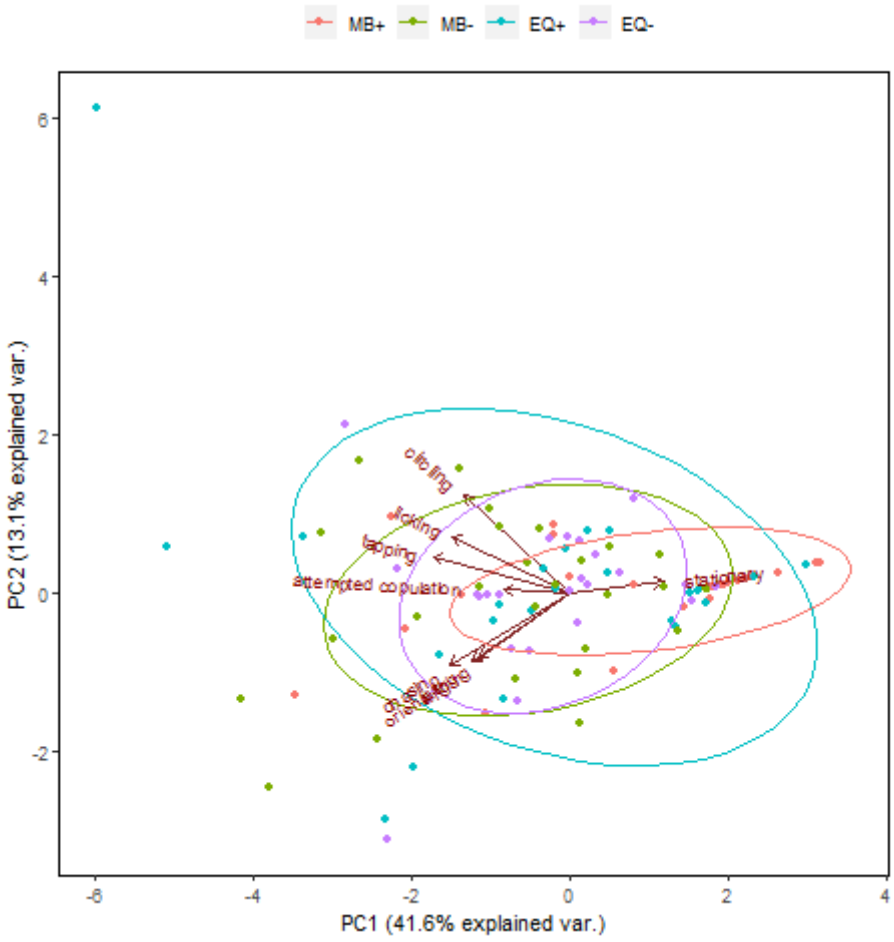
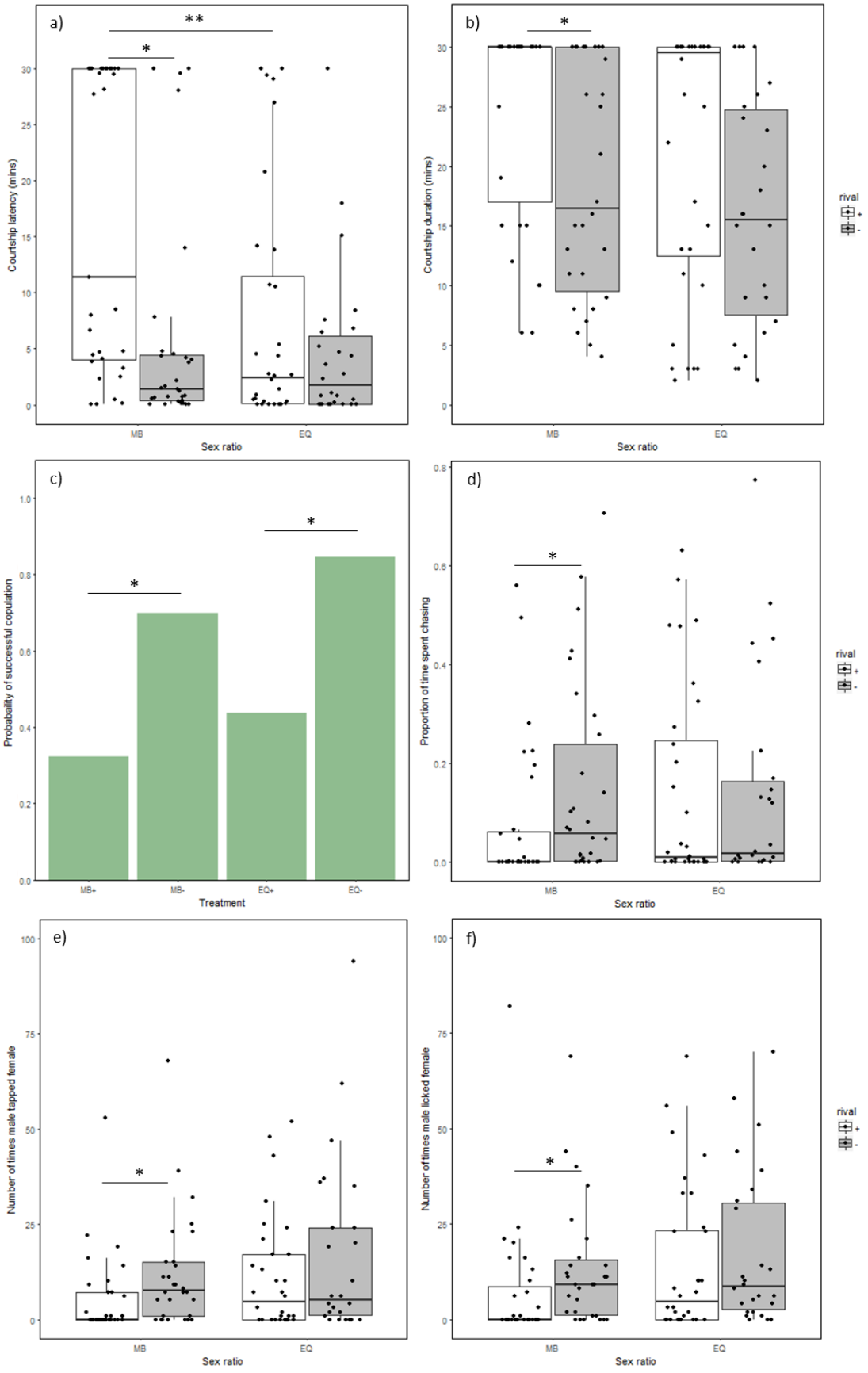


Figure S3.3 – Proportion of courtship duration spent performing each courtship behaviour by experimentally evolved male *Drosophila melanogaster*, represented by a principal components analysis (PCA). Focal males were evolved under male-biased (MB) or equal (EQ) sex ratio, then were housed alone (-) or exposed to a conspecific male rival (+) prior to meeting the female. PCA biplot shows the percentage of variation in courtship time budget explained by PC1 and PC2, and loading of behaviours on the first two PCs.


a)
**
*
Courtship latency (mins)
0
5
10
15
20
25
30
MB
EQ
Sex ratio
b)
*
Courtship duration (mins)
0
5
10
15
20
25
30
MB
EQ
Sex ratio
rival
+
-
c)
*
*
Probability of successful copulation
0.0
0.2
0.4
0.6
0.8
1.0
MB+
MB-
EQ+
EQ-
Treatment
d)
*
Proportion of time spent chasing
0.0
0.2
0.4
0.6
0.8
MB
EQ
Sex ratio
rival
+
-
e)
*
Number of times male tapped female
0
25
50
75
100
MB
EQ
Sex ratio
f)
*
Number of times male licked female
0
25
50
75
100
MB
EQ
Sex ratio
rival
+
-

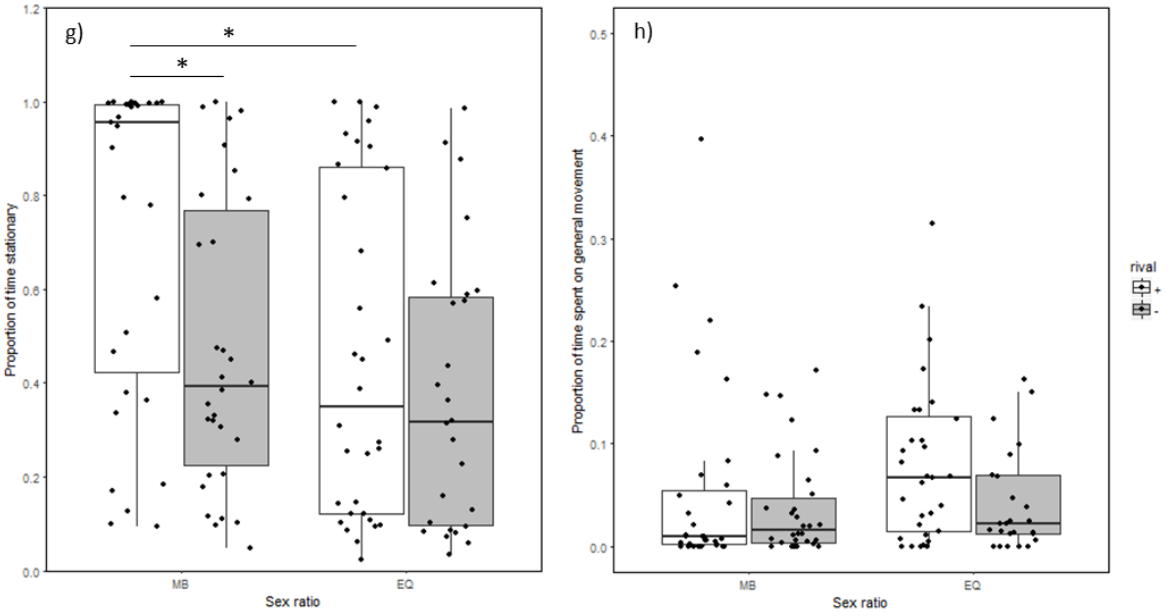


Figure S3.4 – The courtship intensity of experimentally evolved male *Drosophila melanogaster* in response to wildtype rivals and wildtype females. Focal males were evolved under male-biased (MB) or equal (EQ) sex ratio, then either housed alone (-) or exposed to a conspecific male rival (+) prior to being introduced to the female. All rival males and females were wildtype. Asterisks indicate significant pairwise differences. **(a)** The courtship latency (time from when the male and female were introduced to when the first courtship behaviour occurred). **(b)** The courtship duration (from when the first courtship behaviour occurred to when either copulation occurred or the 30 min window ended). **(c)** The average likelihood of successful copulation occurring within the 30 min window. **(d)** The proportion of time (of the total duration spent in the courtship arena; 30 min or until courtship occurred) the male spent chasing the female. **(e)** The number of times the male tapped the female. **(f)** The number of times the male licked the female **(g)** The proportion of time the male spent stationary. **(h)** The proportion of time the male spent on general movement around the courtship arena not directed at the female. Boxplots as described in Figure 3.1.

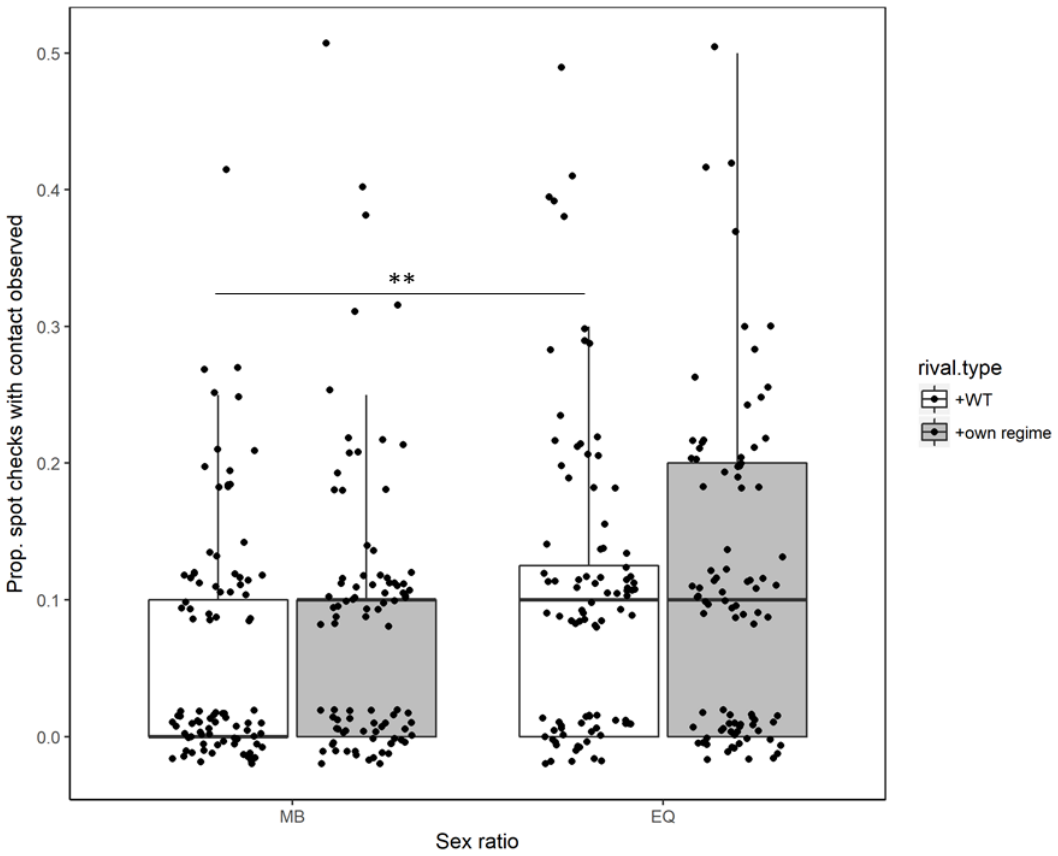


Figure S3.5 – The frequency of aggressive encounters between experimentally evolved male *Drosophila melanogaster* and wildtype vs. co-evolved rivals. The proportion of behavioural spot checks in which focal males experimentally evolved male-biased (MB) or equal (EQ) sex ratio were observed in contact with any one of three rival males (taken as a proxy for male-male aggression). Rival males were either wildtype (+WT) or came from the same experimental evolution lines as the focal male (+own regime). Boxplots as described in Figure 3.1.

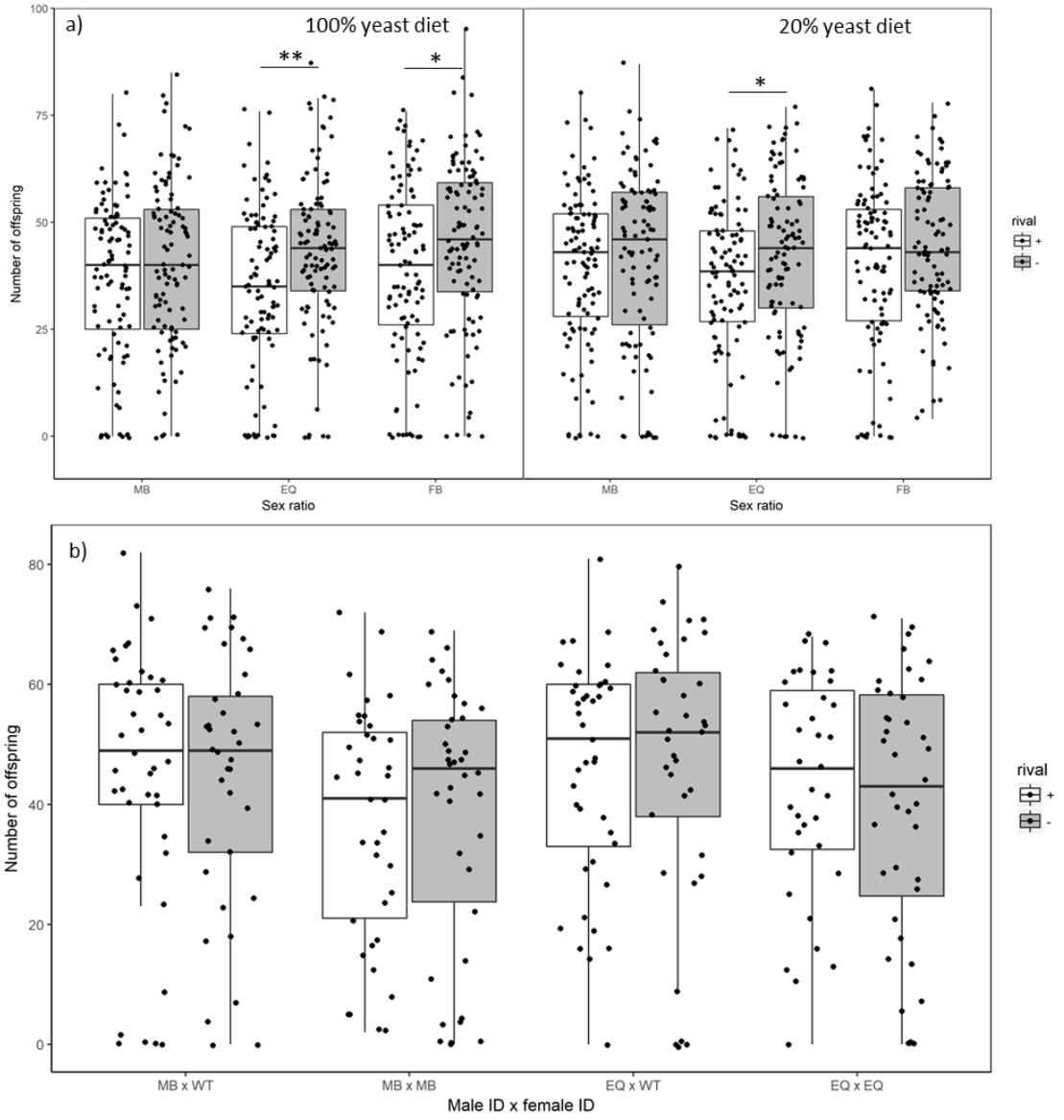


Figure S3.6 – The number of offspring fathered by experimentally evolved male *Drosophila melanogaster,* in response to wildtype rivals and wildtype vs. co-evolved females. Focal males were evolved under male-biased (MB), equal (EQ) or female-biased (FB) sex ratio, then housed alone (-), exposed to three conspecific male rivals (+) prior to mating. (a) All rival males and females were wildtype. During experimental evolution, focal males were maintained on either a standard (100% yeast) or protein-restricted (20% yeast) diet. (b) All rival males were wildtype. Females were either wildtype (xWT) or from the same experimental evolution line as the focal male (xMB or xEQ). Standard diet lines only tested. Boxplots as described in Figure 3.1.

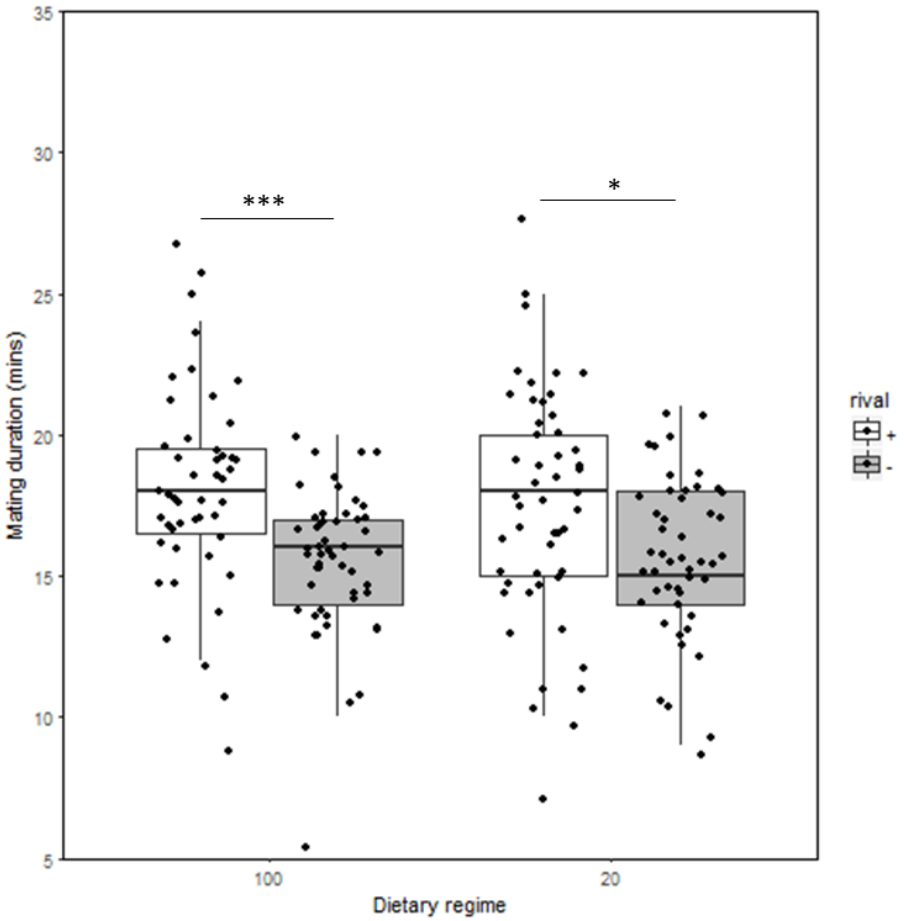


Figure S3.7 – **The mating duration of wildtype male *Drosophila melanogaster* in response to diet and rival exposure.** Focal males were housed alone (-) or exposed to three conspecific wildtype rivals (+) prior to mating. Males were maintained on a standard (100% yeast) or protein-restricted (20% yeast) diet. Boxplots as described in Figure 3.1.

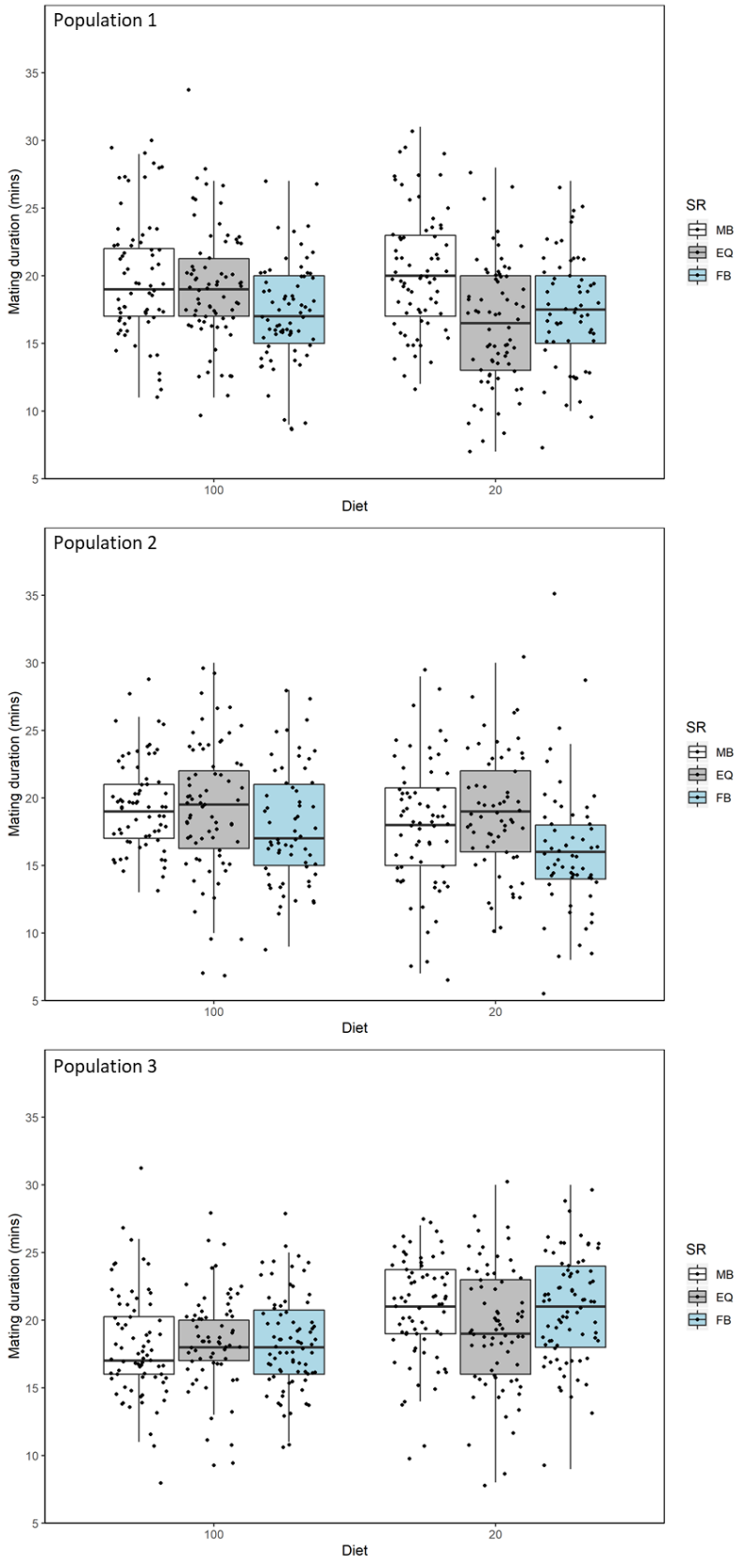


Figure S3.8 – The mating duration of experimentally evolved male *Drosophila melanogaster* in response to wildtype rivals and wildtype females. Focal males were evolved under male-biased (MB), equal (EQ) or female-biased (FB) sex ratio, on standard (100) or protein-restricted (20) diet. Plots by replicate population; pooled data displayed in Figure 3.1.

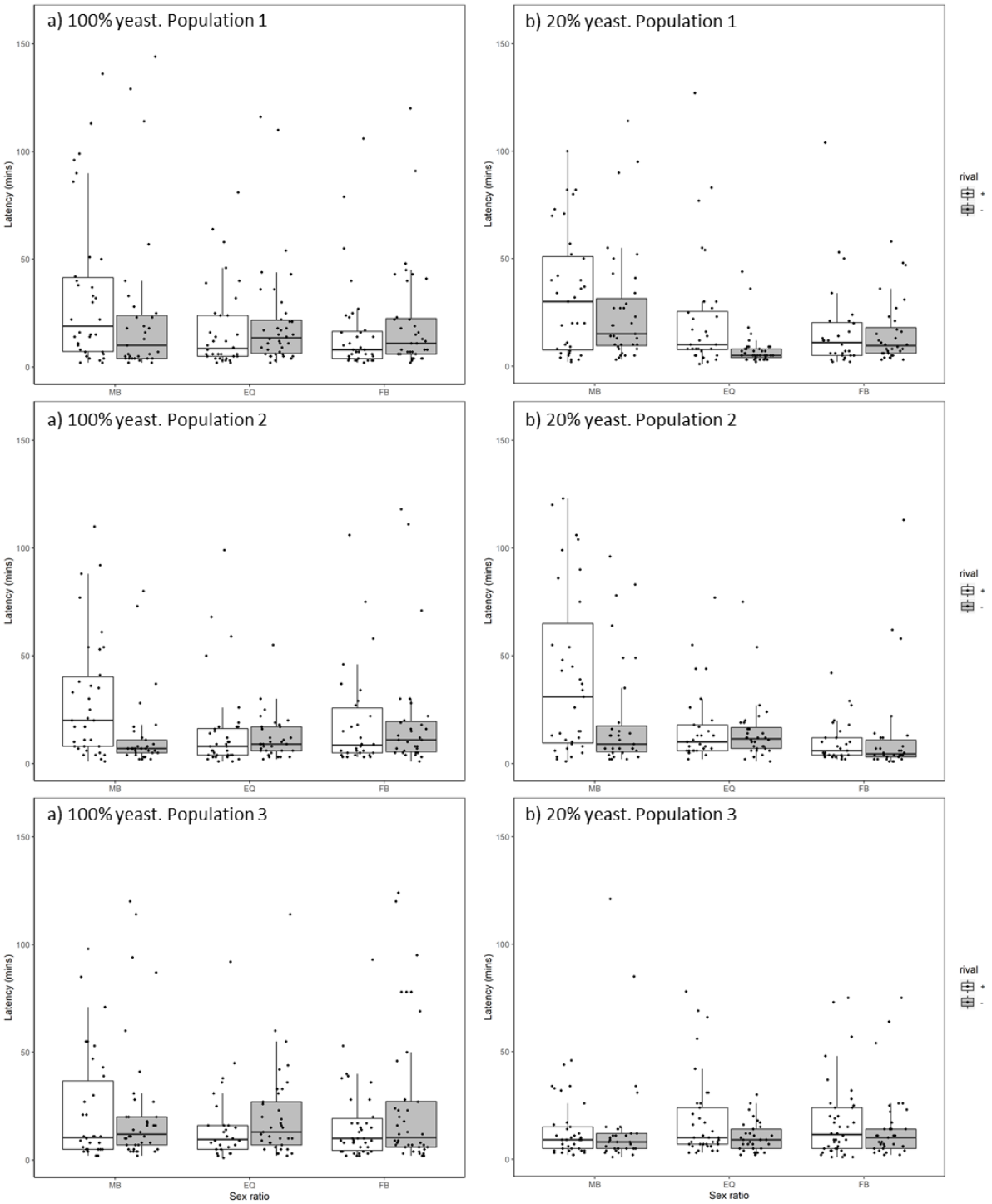
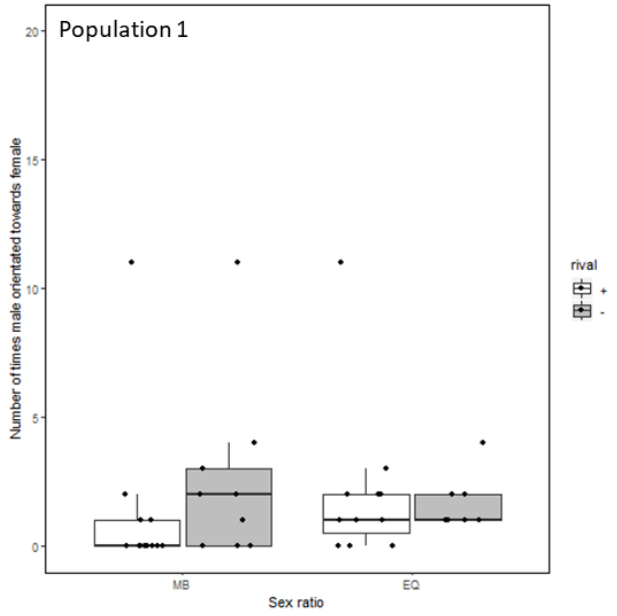
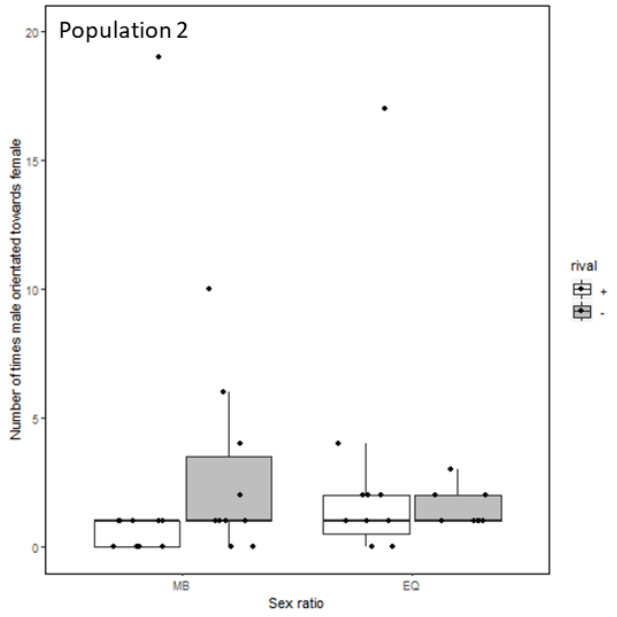


Figure S3.9 – The latency to mate of experimentally evolved male *Drosophila melanogaster* in response to wildtype rivals and wildtype females. Focal males were evolved under male-biased (MB), equal (EQ) or female-biased (FB) sex ratio, on standard (100% yeast) or protein-restricted (20% yeast) diet, then either exposed to a conspecific rival male (+) or housed alone (-). Plots by replicate population; pooled data displayed in Figure 3.2.


Population 1
Number of times male orientated towards female
20
15
10
5
0
MB
EQ
Sex ratio
rival
+
-


Population 2
Number of times male orientated towards female
20
15
10
5
0
MB
EQ
Sex ratio
rival
+
-

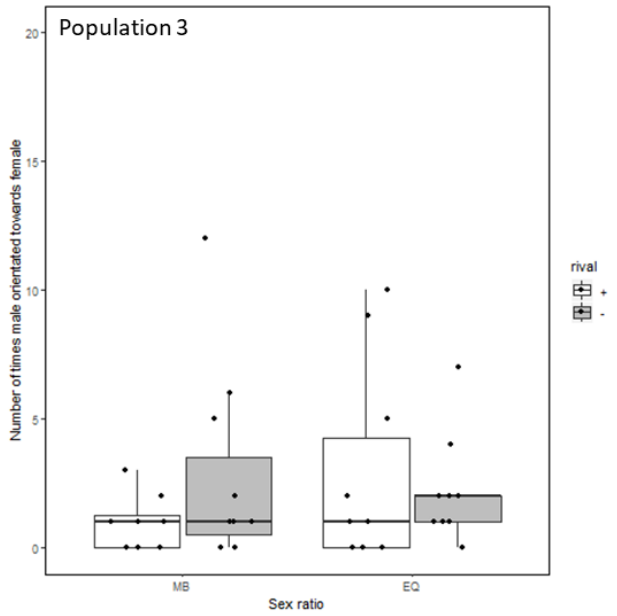
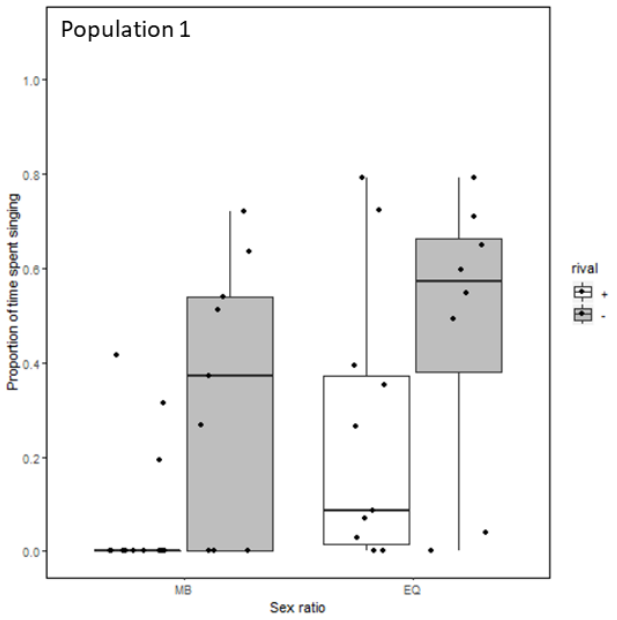
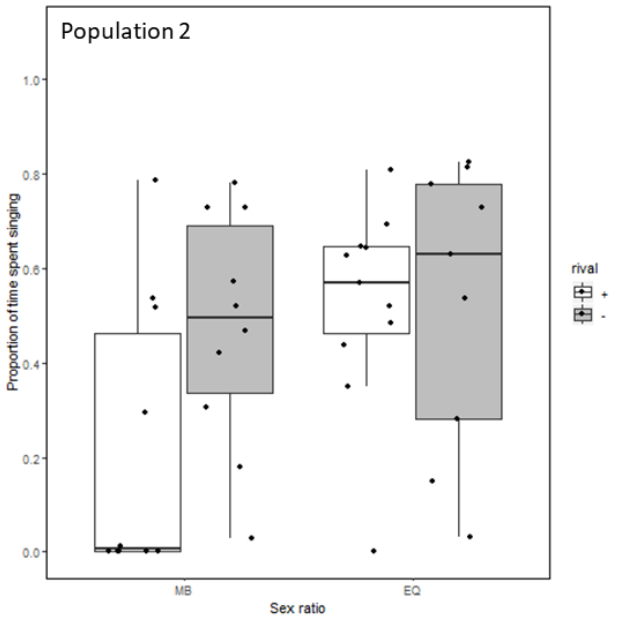

Population 3
Number of times male orientated towards female
20
15
10
5
0
MB
EQ
Sex ratio
rival
+
-

Figure 3.10a


Population 1
Proportion of time spent singing
1.0
0.8
0.6
0.4
0.2
0.0
MB
EQ
Sex ratio
rival
+
-


Population 2
Proportion of time spent singing
1.0
0.8
0.6
0.4
0.2
0.0
MB
EQ
Sex ratio
rival
+
-

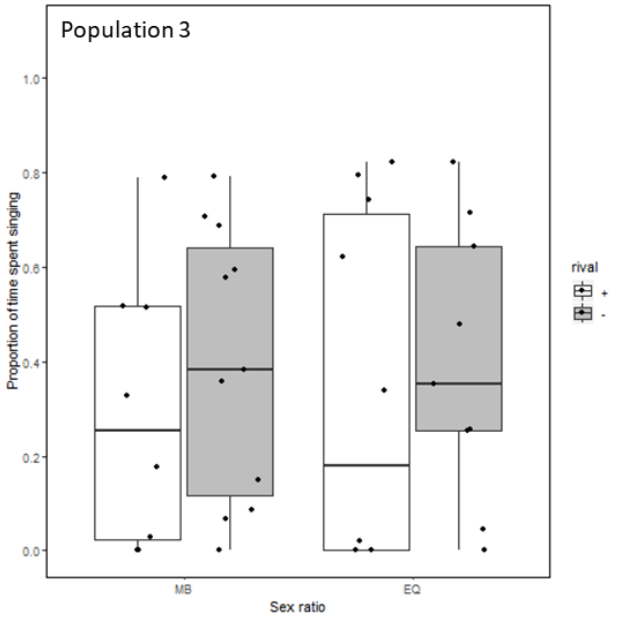
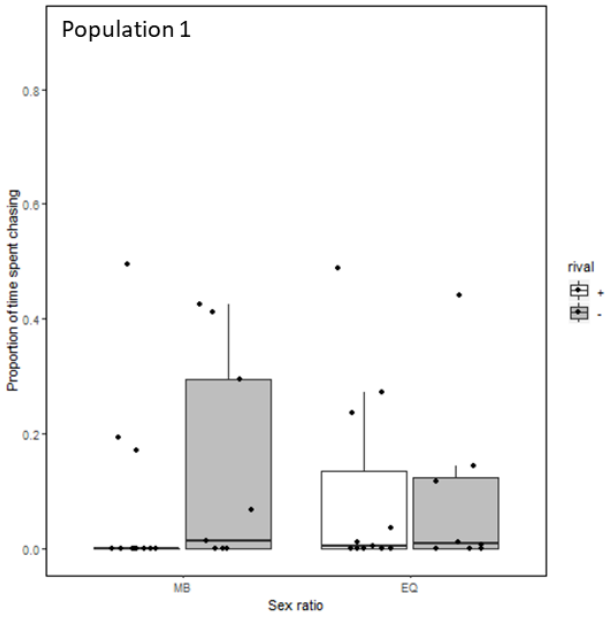
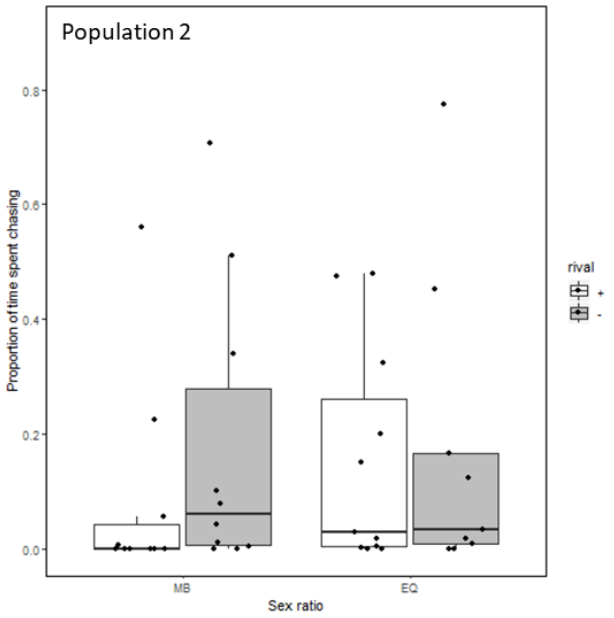

Population 3
Proportion of time spent singing
1.0
0.8
0.6
0.4
0.2
0.0
MB
EQ
Sex ratio
rival
+
-

Figure 3.10b


Population 1
Proportion of time spent chasing
0.0
0.2
0.4
0.6
0.8
MB
EQ
Sex ratio
rival
+
-


Population 2
Proportion of time spent chasing
0.0
0.2
0.4
0.6
0.8
MB
EQ
Sex ratio
rival
+
-

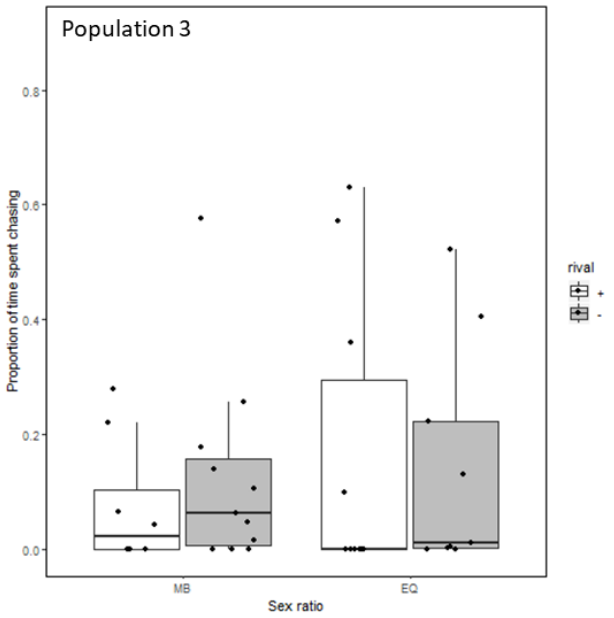

Population 3
Proportion of time spent chasing
0.0
0.2
0.4
0.6
0.8
MB
EQ
Sex ratio
rival
+
-

Figure 3.10c

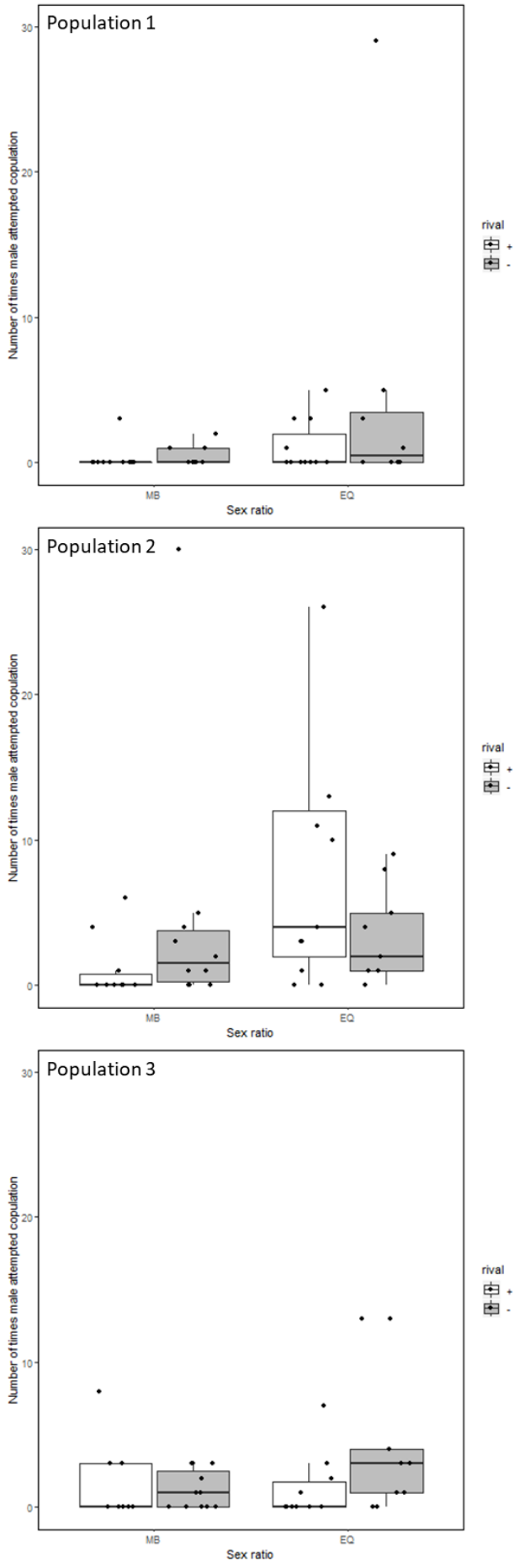

Population 1
Number of times male attempted copulation
30
20
10
0
MB
EQ
Sex ratio
rival
+
-
Population 2
Number of times male attempted copulation
30
20
10
0
MB
EQ
Sex ratio
rival
+
-
Population 3
Number of times male attempted copulation
30
20
10
0
MB
EQ
Sex ratio
rival
+
-

Figure 3.10d

Figure S3.10 – **Courtship intensity of experimentally evolved male *Drosophila melanogaster* in response to wildtype rivals and wildtype females.** Focal males were evolved under male-biased (MB) or equal (EQ) sex ratio, exposed to a conspecific rival male (+) or housed alone (-) prior to introduction to a female. (a) The number of times the male orientated towards the female. (b) The proportion of time (of the total duration spent in the courtship arena; 30 min or until courtship occurred) the male spent singing. (c) The proportion of time the male spent chasing the female. (d) The number of times the male attempted copulation with the female. Plots by replicate population; pooled data displayed in Figure 3.3.

Table S3.1a - Experiment 1. Responses of experimentally evolved male *Drosophila melanogaster* to wildtype rivals and wildtype females. Statistical analyses of mating duration, latency to mate and number of offspring produced by male *D. melanogaster* evolved under male-biased (MB), equal (EQ) or female-biased (FB) sex ratio and standard (100) or protein-restricted (20) diet, compared to a control group of wildtype males (CT). Focal males were exposed to rivals (+) or housed alone (-) prior to mating . All rival males and females were wildtype. 'Block' refers to the replicate population from which focal males were taken, and the date of testing (all replicates 1 were tested on one day, all of replicates 2 on another, etc).

Dependent variable	**Simplified model**	**LRT**	**df**	**p**	
Mating duration	Linear mixed model on untransformed data				
	duration ~ rival + SR + diet + SR:diet + (1|block)	151	13	<0.001	***
	rival	93.87	1	<0.001	***
	SR	23.66	2	<0.001	***
	diet	0.060	1	0.85	
	SR:diet	8.56	2	0.035	*
	Pairwise comparisons	**t.ratio**	**df**	**p**	
	CT+ / CT-	2.94	1338	0.011	*
	100MB+ / 100MB-	3.99	1338	<0.001	***
	100EQ+ / 100EQ-	4.60	1338	<0.001	***
	100FB+ / 100FB-	5.30	1338	<0.001	***
	20MB+ / 20MB-	1.25	1338	0.32	
	20EQ+ / 20EQ-	4.90	1338	<0.001	***
	20FB+ / 20FB-	3.81	1338	<0.001	***
	100MB+ / CT+	4	1340	0.023	*
	100EQ+ / CT+	2.82	1340	0.015	*
	100FB+ / CT+	1.15	1340	0.35	
	100MB- / CT-	2.46	1340	0.035	*
	100EQ- / CT-	2.16	1339	0.070	
	100FB- / CT-	-0.18	1340	0.88	
	100MB+ / 100EQ+	-0.23	1338	0.85	

	100MB+ / 100FB+	1.74	1338	0.15	
	100EQ+ / 100FB+	1.95	1338	0.10	
	100MB- / 100EQ-	0.37	1338	0.81	
	100MB- / 100FB-	3.02	1338	0.0091	**
	100EQ- / 100FB-	2.70	1338	0.020	*
	20MB+ / CT+	2.40	1340	0.039	*
	20EQ+ / CT+	1.61	1340	0.19	
	20FB+ / CT+	1.30	1340	0.31	
	20MB- / CT-	4.56	1339	<0.001	***
	20EQ- / CT-	0.57	1339	0.66	
	20FB- / CT-	1.17	1340	0.35	
	20MB+ / 20EQ+	0.90	1338	0.49	
	20MB+ / 20FB+	1.24	1338	0.33	
	20EQ+ / 20FB+	0.35	1338	0.82	
	20MB- / 20EQ-	4.53	1338	<0.001	***
	20MB- / 20FB-	3.81	1338	0.0055	**
	20EQ- / 20FB-	-0.68	1338	0.59	
	100MB / 100EQ	0.27	1345	0.85	
	100MB / 100FB	3.30	1345	0.0041	**
	100EQ / 100FB	3.04	1345	0.0090	**
	20MB / 20EQ	3.78	1345	0.001	**
	20MB / 20FB	3.48	1345	0.0022	**
	20EQ / 20FB	-0.26	1345	0.85	
Mating latency	**Simplified model**	**LRT**	**df**	**p**	
	Generalised linear mixed model with gamma distribution and log link				
	latency ~ rival + SR + diet + rival:SR + rival:diet + (1|block)	93.53	11	<0.001	***
	rival	7.26	1	0.020	*
	SR	47.16	2	<0.001	***
	diet	8.34	1	0.012	*
	rival:SR	12.16	2	0.0088	**
	rival:diet	9.21	1	0.0090	**
	Pairwise comparisons	z.ratio		p	
	CT+ / CT-	-1.23		0.33	
	100MB+ / 100MB-	2.54		0.029	*
	100EQ+ / 100EQ-	-0.85		0.51	
	100FB+ / 100FB-	-2.08		0.081	
	20MB+ / 20MB-	2.50		0.032	*
	20EQ+ / 20EQ-	4.12		<0.001	***
	20FB+ / 2-FB-	0.82		0.51	
	100MB+ / CT+	3.97		<0.001	***
	100EQ+ / CT+	0.30		0.84	
	100FB+ / CT+	0.80		0.52	

	100MB- / CT-	0.48		0.73	
	100EQ- / CT-	-0.31		0.84	
	100FB- / CT-	1.28		0.31	
	100MB+ / 100EQ+	4.25		<0.001	***
	100MB+ / 100FB+	3.72		0.001	**
	100EQ+ / 100FB+	-0.57		0.66	
	100MB- / 100EQ-	0.91		0.49	
	100MB- / 100FB-	-0.90		0.49	
	100EQ- / 100FB-	-1.83		0.13	
	20MB+ / CT+	4.07		<0.001	***
	20EQ+ / CT+	1.40		0.27	
	20FB+ / CT+	0.049		0.96	
	20MB- / CT-	0.58		0.66	
	20EQ- / CT-	-0.55		0.0014	**
	20FB- / CT-	-2.03		0.088	
	20MB+ / 20EQ+	3.06		0.0087	**
	20MB+ / 20FB+	4.65		<0.001	***
	20EQ+ / 20FB+	1.56		0.20	
	20MB- / 20EQ-	4.68		<0.001	***
	20MB- / 20FB-	2.94		0.011	*
	20EQ- / 20FB-	-1.73		0.15	
Number of offspring	**Simplified model**	**LRT**	**df**	**p**	
	Linear mixed model on untransformed data				
	offspring ~ rival + (1|block)	23.67	11	<0.001	***
	Pairwise comparisons	t.ratio	df	p	
	CT+ / CT-	1.22	1320	0.33	
	100MB+ / 100MB-	-1.28	1320	0.31	
	100EQ+ / 100EQ-	-3.50	1320	0.0022	**
	100FB+ / 100FB-	-2.40	1320	0.039	*
	20MB+ / 20MB-	-0.88	1320	0.50	
	20EQ+ / 20EQ-	-2.41	1320	0.039	*
	20FB+ / 2-FB-	-1.47	1320	0.23	
	100MB+ / CT+	-0.85	1322	0.51	
	100EQ+ / CT+	-1.73	1322	0.15	
	100FB+ / CT+	-0.13	1322	0.91	
	100MB- / CT-	1.62	1322	0.19	
	100EQ- / CT-	2.68	1322	0.021	*
	100FB- / CT-	3.35	1322	0.0034	**
	100MB+ / 100EQ+	1.03	1320	0.42	
	100MB+ / 100FB+	-0.83	1320	0.51	
	100EQ+ / 100FB+	-1.85	1320	0.12	
	100MB- / 100EQ-	-1.18	1320	0.34	
	100MB- / 100FB-	-1.93	1320	0.11	

	100EQ- / 100FB-	-0.77	1320	0.54	
	20MB+ / CT+	-0.070	1322	0.95	
	20EQ+ / CT+	-1.20	1322	0.33	
	20FB+ / CT+	0.20	1322	0.88	
	20MB- / CT-	2.05	1322	0.086	
	20EQ- / CT-	2.26	1322	0.055	
	20FB- / CT-	2.86	1322	0.013	*
	20MB+ / 20EQ+	1.32	1320	0.30	
	20MB+ / 20FB+	-0.31	1320	0.84	
	20EQ+ / 20FB+	-1.61	1320	0.19	
	20MB- / 20EQ-	-0.24	1320	0.85	
	20MB- / 20FB-	-0.92	1320	0.49	
	20EQ- / 20FB-	-0.69	1320	0.59	

Table S3.1b - **Experiment 2. Responses of experimentally evolved male *Drosophila melanogaster* to wildtype vs. co-evolved rivals and wildtype females.** Statistical analyses of mating duration, latency to mate and frequency of contact with rivals of male *D. melanogaster* evolved under male-biased (MB) or equal (EQ) sex ratio (standard diet lines). Focal males were exposed to wildtype rivals (+WT), exposed to rivals from within their own experimental evolution line (+MB or +EQ), or housed alone (-) prior to mating. All females were wildtype flies. 'Experiment' refers to when samples were tested (two independent replicate experiments were conducted and the data pooled); 'population' refers to the replicate population from which focal flies were taken.

Dependent variable	**Simplified model**	**LRT**	**df**	**p**	
Mating duration	Linear mixed model on untransformed data				
	mating duration ~ SR + rival.presence + (1|experiment) + (1|population)	49.48	2	<0.001	***
	SR	5.12	1	0.049	*
	rival.presence	44.24	1	<0.001	***
	Pairwise comparisons	**t.ratio**	**df**	**p**	
	MB+WT / MB+MB	0.11	754	0.96	
	MB+WT / MB-	4.65	753	<0.001	***
	MB+MB / MB-	4.79	753	<0.001	***
	EQ+WT / EQ+EQ	0.00	753	1.00	
	EQ+WT / EQ-	3.53	753	0.0014	**
	EQ+EQ / EQ-	3.50	753	0.0015	**
	MB+WT / EQ+WT	2.31	5.63	0.11	
	MB+MB / EQ+EQ	2.36	5.62	0.11	

	MB- / EQ-	1.76	5.54	0.18	
Mating latency	**Simplified model**	**LRT**	**df**	**p**	
	Generalised linear mixed model with gamma distribution and log link				
	latency ~ rival.presence + (1|experiment) + (1|population)	28.01	1	<0.001	***
	Pairwise comparisons	**z.ratio**		**p**	
	MB+WT / MB+MB	0.94		0.43	
	MB+WT / MB-	4.34		<0.001	***
	MB+MB / MB-	3.52		0.0014	**
	EQ+WT / EQ+EQ	1.03		0.39	
	EQ+WT / EQ-	2.22		0.052	
	EQ+EQ / EQ-	3.17		0.0041	**
	MB+WT / EQ+WT	1.62		0.15	
	MB+MB / EQ+EQ	0.35		0.85	
	MB- / EQ-	0.09		0.96	
Freq. contact with rival	**Simplified model**	**LRT**	**df**	**p**	
	Generalised linear mixed model with binomial distribution and logit link				
	contact ~ SR + (1|experiment) + (1|population)	5.34	1	0.047	*
	Pairwise comparisons	**z.ratio**		**p**	
	MB+WT / MB+MB	1.67		0.15	
	EQ+WT / EQ+EQ	0.24		0.92	
	MB+WT / EQ+WT	2.97		0.0074	**
	MB+MB / EQ+EQ	1.66		0.15	

Table S3.1c - Experiment 3. Responses of experimentally evolved male *Drosophila melanogaster* to wildtype rivals and wildtype vs. coevolved females. Statistical analyses of mating duration, latency to mate and number of offspring produced by male *D. melanogaster* evolved under male-biased (MB) or equal (EQ) sex ratio (standard diet lines). Focal males were housed alone (-) or exposed to wildtype rivals (+) prior to mating with either wildtype females (xWT) or females from within their own experimental evolution lines (xMB or xEQ). 'Population' refers to the replicate population from which focal males were taken.

Dependent variable	**Simplified model**	**LRT**	**df**	**p**	
Mating duration	Linear model on untransformed data				

	duration ~ rival + (1|population)	23.08	7	<0.001	***
	Pairwise comparisons	**t.ratio**	**df**	**p**	
	MB(+)xWT / MB(-)xWT	2.61	296	0.058	
	MB(+)xMB / MB(-)xMB	1.13	297	0.42	
	MB(+)xWT / MB(+)xMB	1.63	296	0.24	
	MB(-)xWT / MB(-)xMB	0.17	297	0.95	
	EQ(+)xWT / EQ(-)xWT	2.05	296	0.12	
	EQ(+)xEQ / EQ(-)xEQ	2.65	296	0.06	
	EQ(+)xWT / EQ(+)xEQ	0.017	296	0.99	
	EQ(-)xWT / EQ(-)xEQ	0.69	296	0.57	
	MB(+)xWT / EQ(+)xWT	1.39	16.20	0.36	
	MB(+)xMB / EQ(+)xEQ	0.016	19.30	0.99	
	MB(-)xWT / EQ(-)xWT	0.88	18.20	0.55	
	MB(-)xMB / EQ(-)xEQ	1.32	17	0.36	
	MBxMB / EQxEQ	0.88	7.78	0.55	
	MBxWT / EQxWT	1.41	7.45	0.36	
Mating latency	**Simplified model**	**LRT**	**df**	**p**	
	Generalised linear model with gamma distribution and log link				
	No significant terms				
Number of offspring	**Simplified model**	**LRT**	**df**	**p**	
	Generalised mixed model with gamma distribution and log link				
	No significant terms				

Table S3.1d - **Experiment 4. Analysis of courtship behaviours of experimentally evolved male *Drosophila melanogaster* (multivariate stats).** Statistical multivariate analysis of the composition of time spent performing courtship behaviours by male *D. melanogaster* evolved under male-biased (MB) or equal (EQ) sex ratio (standard diet lines). Focal males were housed alone (-) or exposed to a wildtype rival (+) prior to courting wildtype females. 'Population' refers to the replicate population from which focal males were taken.

Principal components analysis on proportions of courtship duration spent performing each behaviour				
Linear mixed models	**LRT**	**df**	**p**	
PC1				
PC1~ rival + (1|date) + (1|population)	6.85	1	0.026	*
SR:rival	3.77	2	0.052	
PC2				

No significant terms

Table S3.1e - Experiment 4. Analysis of courtship behaviours of experimentally evolved male *Drosophila melanogaster* (univariate stats). Statistical univariate analyses of the frequency of or proportion of time spent performing courtship behaviours by male *D. melanogaster* evolved under male-biased (MB) or equal (EQ) sex ratio (standard diet lines). Focal males were housed alone (-) or exposed to a wildtype rival (+) prior to courting wildtype females.

Univariate analysis					
Courtship latency	**Model**	**LRT**	**df**	**p**	
	Kruskal-Wallis test latency ~ treatment	17.64	3	0.0044	**
	Pairwise comparisons	**w**		**p**	
	MB+ / MB-	702		0.019	*
	EQ+ / EQ-	432.5		0.84	
	MB+ / EQ+	731.5		0.0071	**
	MB - / EQ-	417		0.74	
Courtship duration	**Model**	**LRT**	**df**	**p**	
	Kruskal-Wallis test duration ~ treatment	13.94	3	0.015	*
	Pairwise comparisons	**w**		**p**	
	MB+ / MB-	639		0.022	*
	EQ+ / EQ-	543.5		0.088	
	MB+ / EQ+	594		0.23	
	MB - / EQ-	458.5		0.38	
Freq. attempted copulation	**Model**	**LRT**	**df**	**p**	
	Kruskal-Wallis test attempted.copulation ~ treatment	85.34	3	<0.001	***
	Pairwise comparisons	**w**		**p**	
	MB+ / MB-			0.052	
	EQ+ / EQ-			0.48	
	MB+ / EQ+			0.032	*
	MB - / EQ-			0.27	
Copulation success	**Model**	**LRT**	**df**	**p**	
	Kruskal-Wallis test copulation(y/n) ~ treatment	21.25	3	0.0016	**
	Pairwise comparisons	**LRT**	**df**	**p**	
	MB+ / MB-	7.24	1	0.022	*

	EQ+ / EQ-	8.51	1	0.02	*
	MB+ / EQ+	0.46	1	0.60	
	MB - / EQ-	0.95	1	0.43	
Freq. licking	**Model**	**LRT**	**df**	**p**	
	Kruskal-Wallis test				
	licking ~ treatment	160.16	3	<0.001	***
	Pairwise comparisons	**w**		**p**	
	MB+ / MB-	282		0.022	*
	EQ+ / EQ-	334.5		0.30	
	MB+ / EQ+	358		0.094	
	MB - / EQ-	370.5		0.80	
Freq. orientating	**Model**	**LRT**	**df**	**p**	
	Kruskal-Wallis test				
	orientating ~ treatment	12.61	3	0.022	*
	Pairwise comparisons	**w**		**p**	
	MB+ / MB-	307		0.039	*
	EQ+ / EQ-	356		0.46	
	MB+ / EQ+	351		0.078	
	MB - / EQ-	356		0.67	
Freq. tapping	**Model**	**LRT**	**df**	**p**	
	Kruskal-Wallis test				
	tapping ~ treatment	211.61	3	<0.001	***
	Pairwise comparisons	**w**		**p**	
	MB+ / MB-	284		0.022	*
	EQ+ / EQ-	358.5		0.46	
	MB+ / EQ+	365.5		0.12	
	MB - / EQ-	393		0.96	
Freq. decamping (F)	**Model**	**LRT**	**df**	**p**	
	Kruskal-Wallis test				
	decamping ~ treatment	11.8	3	0.024	*
	Pairwise comparisons	**w**		**p**	
	MB+ / MB-	384.5		0.30	
	EQ+ / EQ-	354		0.38	
	MB+ / EQ+	493		0.96	
	MB - / EQ-	395.5		0.96	
Prop. time singing	**Model**	**LRT**	**df**	**p**	
	Kruskal-Wallis test				
	singing ~ treatment	19.15	3	0.0034	**
	Pairwise comparisons	**w**		**p**	
	MB+ / MB-	233		0.0044	**

	EQ+ / EQ-	333		0.30	
	MB+ / EQ+	282		0.013	*
	MB - / EQ-	331		0.43	
Prop. time general movement (M)	**Model**	**LRT**	**df**	**p**	
	Kruskal-Wallis test movement ~ treatment	7.96	3	0.094	
	Pairwise comparisons	**w**		**p**	
	MB+ / MB-	440		0.80	
	EQ+ / EQ-	515		0.22	
	MB+ / EQ+	330		0.051	
	MB - / EQ-	338		0.48	
Prop. time stationary	**Model**	**LRT**	**df**	**p**	
	Kruskal-Wallis test stationary ~ treatment	18.74	3	0.0035	**
	Pairwise comparisons	**w**		**p**	
	MB+ / MB-	672.5		0.013	*
	EQ+ / EQ-	478		0.44	
	MB+ / EQ+	723		0.010	*
	MB - / EQ-	478		0.25	
Prop. time chasing	**Model**	**LRT**	**df**	**p**	
	Kruskal-Wallis test chasing ~ treatment	6.95	3	0.14	
	Pairwise comparisons	**w**		**p**	
	MB+ / MB-	298		0.032	*
	EQ+ / EQ-	387.5		0.74	
	MB+ / EQ+	382		0.18	
	MB - / EQ-	410.5		0.80	
Prop. time circling	**Model**	**LRT**	**df**	**p**	
	Kruskal-Wallis test circling ~ treatment	11.16	3	0.030	*
	Pairwise comparisons	**w**		**p**	
	MB+ / MB-	264.5		0.013	*
	EQ+ / EQ-	355		0.43	
	MB+ / EQ+	374.5		0.14	
	MB - / EQ-	359		0.71	

Table S3.1f - Experiment 4. Analysis of courtship behaviours of experimentally evolved male *Drosophila melanogaster* (transition probabilities). Statistical analyses of significance of transition probabilities between courtship behaviours by male *D. melanogaster* evolved under

male-biased (MB) or equal (EQ) sex ratio (standard diet lines). Focal males were housed alone (-) or exposed to a wildtype rival (+) prior to courting wildtype females.

	MB(+)	MB(-)	EQ(+)	EQ(-)
stationary - stationary	ns	ns	ns	ns
stationary - chasing	ns	ns	ns	ns
stationary - attempted copulation	ns	ns	ns	ns
stationary - general movement (M)	s	s	s	s
statonary - decamping (F)	ns	ns	ns	ns
stationary - circling	ns	ns	ns	ns
stationary - licking	ns	ns	ns	ns
stationary - orientating	s	s	s	s
stationary - singing	s	s	s	s
stationary - tapping	ns	ns	ns	ns
chasing - stationary	s	s	s	s
chasing - chasing	ns	ns	ns	ns
chasing - attempted copulation	ns	ns	ns	ns
chasing - copulation	ns	ns	ns	ns
chasing - general movement (M)	ns	ns	ns	ns
chasing - decamping (F)	ns	ns	ns	s
chasing - circling	ns	ns	ns	ns
chasing - licking	ns	ns	ns	ns
chasing - orientating	ns	ns	ns	ns
chasing - singing	s	s	s	s
chasing - tapping	ns	ns	ns	ns
attempted copulation - stationary	s	s	s	s
attempted copulation - chasing	ns	ns	ns	ns
attempted copulation - attempted copulation	ns	ns	ns	ns
attempted copulation - copulation	ns	ns	ns	ns
attempted copulation - decamping (F)	ns	ns	ns	ns
attempted copulation - circling	ns	ns	ns	ns
attempted copulation - licking	ns	ns	ns	ns
attempted copulation - orientating	ns	ns	ns	ns
attempted copulation - singing	ns	ns	ns	ns
attemped copulation - tapping	ns	ns	ns	ns
general movement (M) - stationary	s	s	s	s
general movement (M) - chasing	ns	ns	ns	ns
general movement (M) - general movement (M)	ns	ns	ns	ns
general movement (M) - decamping (F)	ns	ns	ns	ns
general movement (M) - orientating	s	s	s	s
general movement (M) - singing	ns	ns	ns	ns
general movement (M) - tapping	ns	ns	ns	ns
decamping (F) - stationary	s	ns	ns	ns

decamping (F) - chasing	ns	s	s	s
decamping (F) - attempted copulation	ns	ns	ns	ns
decamping (F) - general movement (M)	ns	s	ns	ns
decamping (F) - decamping (F)	ns	ns	s	ns
decamping (F) - circling	ns	ns	ns	ns
decamping (F) - orientating	ns	s	s	ns
decamping (F) - singing	ns	ns	ns	ns
circling - stationary	ns	s	s	s
circling - chasing	ns	ns	ns	ns
circling - attempted copulation	ns	ns	ns	ns
circling - copulation	ns	ns	s	s
circling - decamping (F)	ns	ns	ns	ns
circling - circling	ns	ns	ns	ns
circling - licking	s	ns	ns	ns
circling - singing	s	ns	ns	ns
circling - tapping	ns	ns	ns	s
licking - stationary	ns	ns	s	s
licking - chasing	ns	ns	ns	ns
licking - attempted copulation	s	s	s	s
licking - copulation	s	s	ns	ns
licking - decamping (F)	s	ns	ns	ns
licking - circling	ns	ns	ns	ns
licking - licking	ns	ns	ns	ns
licking - singing	ns	ns	ns	ns
licking - tapping	ns	s	s	ns
orientating - stationary	ns	ns	ns	ns
orientating - chasing	s	s	s	ns
orientating - general movement (M)	ns	ns	ns	ns
orientating - decamping (F)	ns	ns	ns	s
orientating - circling	ns	ns	ns	ns
orientating - singing	s	s	s	s
orientating - tapping	ns	ns	ns	ns
singing - stationary	ns	ns	ns	ns
singing - chasing	ns	s	ns	ns
singing - attempted copulation	ns	ns	ns	ns
singing - copulation	ns	ns	ns	ns
singing - general movement (M)	ns	ns	ns	ns
singing - decamping (F)	ns	ns	ns	ns
singing - circling	s	s	s	s
singing - licking	s	s	s	s
singing - orientating	ns	ns	ns	ns
singing - singing	ns	ns	ns	ns
singing - tapping	s	s	s	s
tapping - stationary	ns	ns	s	s
tapping - chasing	s	ns	ns	s

tapping - attempted copulation	ns	s	s	ns
tapping - copulation	s	ns	ns	ns
tapping - decamping (F)	ns	ns	ns	ns
tapping - circling	s	ns	ns	s
tapping - licking	ns	ns	s	s
tapping - orientating	ns	ns	ns	ns
tapping - singing	ns	ns	ns	ns
tapping - tapping	ns	ns	ns	ns

Table S3.1g - The effect of proximate diet on wildtype male *Drosophila melanogaster*. Statistical analysis of mating behaviour of wildtype male *D. melanogaster* either exposed to wildtype rivals (+) or housed alone (-) while being maintained on a diet of either standard 100% yeast or 20% yeast.

Dependent variable	**Simplified model**	**LRT**	**df**	**p**	
Mating duration	Linear model on untransformed data				
	duration ~ rival	21.57	1 & 196	<0.001	** *
	Pairwise comparisons	**t**		**p**	
	100+ / 100-	3.96		<0.001	** *
	20+ / 20-	2.616		0.047	*
	100+ / 20+	1.08		0.70	
	100- / 20-	0.28		0.99	
Mating latency	**Simplified model**	**LRT**	**df**	**p**	
	Linear model on log10 transformed data				
	No significant terms				

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4. Fitness consequences of redundant cues of competition in male *D. melanogaster*

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4.1 Abstract

Phenotypic plasticity can allow animals to adapt their behaviour, such as their mating effort, to their social and sexual environment. However, this relies on the individual receiving accurate and reliable cues of the environmental conditions. This can be achieved via the receipt of multimodal cues, which may provide redundancy and robustness. Male *Drosophila melanogaster* detect presence of rivals via combinations of any two or more redundant cue components (sound, smell and touch) and respond by extending their subsequent mating duration, which is associated with higher reproductive success. Although alternative combinations of cues of rival presence have previously been found to elicit equivalent increases in mating duration and offspring production, their redundancy in securing success under sperm competition has not previously been tested. Here, I explicitly tested this by exposing male *D. melanogaster* to alternative combinations of rival cues and examining reproductive success in both the presence and absence of sperm competition. The results supported previous findings of redundancy of cues in terms of behavioural responses. However, there was no evidence of reproductive benefits accrued by extending mating duration in response to rivals. The lack of identifiable fitness benefits of longer mating under these conditions, both in the presence and absence of sperm competition, contrasted with some previous results, but could be explained by: 1) damage sustained from aggressive interactions with rivals leading to reduced ability to increase ejaculate investment, 2) presence of features of the social environment, such as male and female mating status, that obscured the fitness benefits of longer mating, 3) decoupling of behavioural investment with fitness benefits.

4.2 Introduction

Many animals exhibit plasticity in their reproductive behaviour and/or reproductive investment in response to the other organisms around them, allowing them to allocate resources across mating opportunities in order to maximise lifetime reproductive success (Dewsbury 1982; Parker 1982; Gage 1995; Wedell et al. 2002; Kokko and Rankin 2006; Rodriguez et al. 2013). However, in order for plasticity to be adaptive, cues that confer accurate, reliable and robust information on the current conditions must be received (DeWitt et al. 1998; Auld et al. 2010). One way in which the information conferred by environmental cues may be made more robust is through the receipt of multicomponent or multimodal (complex) cues. Cues can be categorised as multicomponent if they are received via one sensory modality or as multimodal if the components are received through multiple sensory modalities (Candolin 2003; Hebets and Papaj 2005). Here, I use 'complex cue' as a general term for multicomponent and multimodal cues, and 'cue component' as an umbrella term for the separate modalities or components that comprise the complex cue. Redundancy among cue components can mean that even if one component is lost or compromised, the overall information within any message can remain intact (Johnstone 1996; Bro-Jørgensen 2010). This suggests that receiving alternative combinations of cue components should elicit equivalent phenotypic changes and equivalent associated fitness benefits. However, redundancy may also be incomplete, whereby separate components relay partially overlapping, but not identical, information about the environment (Bretman et al. 2011b; Dore et al. 2018). In this scenario, altering the combination of cue components to which an individual is exposed may result in subtle effects on subsequent phenotypes, with associated fitness consequences.

Both multimodal and multicomponent cues are abundant in mating systems and may often be subject to sexual selection via their effects on both the signaller and the receiver (Bro-Jørgensen 2010). The separate elements of a cue may be entirely or partially redundant, convey distinct information, or interact – for example, one cue component may grab the attention of the receiver while the others convey information (Bro-Jørgensen 2010). In field crickets (*Teleogryllus oceanicus*), female responses to male acoustic performances are mediated by the presence of CHCs, suggesting that mate choice is dependent on a multimodal signal encompassing auditory and olfactory components (Bailey 2011). In this instance, the two modalities are found to interact and may increase the amount of information that can be perceived. Male wolf spiders (*Schizocosa ocreata*) also perform multimodal courtship displays which consist of visual and seismic components, but in this case the cue components appear to

be redundant. On substrates that weakened the effectiveness of seismic components of courtships males were found to increase their use of visual signals, suggesting that the two cue components can act as 'backups' to ensure the signal can be received across varying environments (Gordon and Uetz 2011). An example of complex mating signals being contained within one sensory modality can be found in swordtail fish (*Xiphophorus nigrensis*), in which females respond to multicomponent male visual displays. Male size and courtship vigour did not have an additive effect on female preference, but females responded more quickly to males when both components were increased (Reding and Cummings 2017). This offers further evidence that cue components can interact to influence the mating decisions of the receiver. Overall, these examples demonstrate that identifying complex cues and understanding how the components overlap or interact can shed light on complex mating behaviours and how these behaviours can vary across environments.

A well-studied example of redundant, multimodal signalling comes from the reproductive behaviour of male *Drosophila melanogaster*, which offers excellent potential for studying how redundancy in cue components can affect plastic behaviour. Male *D. melanogaster* express behavioural plasticity, whereby individuals exposed to rival males will subsequently mate for longer and increase their transfer of some seminal fluid proteins, in comparison to males housed alone (Bretman et al. 2009; Wigby et al. 2009a). Extended matings following exposure to rivals are reported to be associated with increased paternity share (Bretman et al. 2009). However, exposure to rivals over a male's whole lifetime results in the expression of reproductive costs later in life (Bretman et al. 2009; Bretman et al. 2013b). The behavioural response of male *D. melanogaster* to rival males is highly sensitive to the level of competition and can rapidly be reversed upon the removal of competition (Bretman et al. 2012). Male *D. melanogaster* can detect rival males via three sensory cue components: tactile, olfactory and auditory (Bretman et al. 2011b). Males exposed to any two of these components in combination, or all three, responded with equivalent extensions to subsequent mating duration. The finding that removing any one cue of rival presence does not prevent the male from responding suggests that there is redundancy in how these cues are processed. This redundancy may confer robustness in responses to the social environment, which can be complex and rapidly variable (Kasumovic et al. 2008; Bretman et al. 2011a; Greenspan 2012; Dore et al. 2018). Although male *D. melanogaster* with one sensory cue removed were able to respond to rivals, a longer period of exposure was required to elicit the longer mating response, compared to males with all cues intact (Rouse and Bretman 2016). Furthermore, the combination of cues a male is exposed to has a role in species recognition of rivals (Bretman et

al. 2017). This suggests that there may be incomplete redundancy in how the cues of rival presence are processed in order to produce the behavioural response.

In addition to eliciting equivalent behavioural responses, perceiving any two of the three rival cues appears to result in comparable increases in the number of offspring fathered (Bretman et al. 2011b). However, thus far this has only been tested in the absence of realised sperm competition; hence, an important facet of the fitness consequences of responding to rival males is not yet known. This is the omission I tackle in this chapter. Determining whether males that have any one sensory cue systematically removed achieve equivalent success in sperm competition is important as it is expected to increase our understanding of the fitness benefits and potential costs of redundancy in general.

I explicitly tested the fitness equivalence of receiving alternative cues of rival presence under sperm competition, to investigate further whether these cues show complete redundancy. Male *D. melanogaster* were exposed to intact rivals or those subjected to a physical manipulation that removed the auditory cue of rival presence. I focused on testing auditory cue removal as this could be fully controlled, (removal of tactile and olfactory cues produced off-target effects on male behaviour; Supplementary information 1, 2; Figure S4.1, S4.2). The rationale for focusing on just this single cue removal as the exemplar was that previous tests reported that all cues were equivalent with respect to the subsequent behavioural and fitness outcomes (Bretman et al. 2011b). Thus, the effects of removing the auditory cue can inform understanding of the redundancy of all three key cues.

Males exposed to the full repertoire of cues (auditory + tactile + olfactory) and those with one cue removed (tactile + olfactory) were both predicted to show equivalent extension in mating duration and increase in non-competitive paternity compared to males that had no rival exposure, as identified by Bretman et al. (2011b). In addition, I predicted that males exposed to either of the above combinations of rival cues would achieve an equivalent increase in competitive paternity when the female subsequently remated, relative to males kept without rivals. This would support the idea that the cues of rival presence perceived by male *D. melanogaster* are redundant.

4.3 Methods

a) General methods

Experiments were conducted in a 25°C humidified room held under a 12 h light: 12 h dark cycle. Flies were maintained in 75 x 25 mm glass vials containing 7 ml sugar-yeast-agar (SYA) medium (100g brewer's yeast, 50g sucrose, 15g agar, 30mL Nipagin (10% solution), 3mL propionic acid, and 0.97L water per litre of medium). Wildtype flies were sampled from the Dahomey population (Bretman et al. 2009). Females were allowed to oviposit on agar-grape juice plates (50g agar, 600mL red grape juice, 42mL Nipagin (10% solution), 1.1L water). All larvae were reared under a controlled density of 100 per vial to minimise variation in adult body size (Miller and Thomas 1958). Adults were collected and separated by sex within 8 h of eclosion to ensure virginity and stored 10 per vial. All male and female flies were age-matched, between sexes and across treatments.

b) Sensory cues removal

Each male was randomly assigned to one of three treatments: housed with a rival male with all sensory cues intact (+ all), housed with a rival with the auditory cue removed (+ no sound), or housed alone (- all). The experiment was repeated in two independent replicates, which were pooled for analysis. The auditory cue of rival presence was removed by using a physical manipulation in which the wings of the rival males were removed under CO_2 anaesthesia, preventing them from producing the song that signals their presence to competitors. To control for handling, the rival males in the +all treatment were also subjected to CO_2 anaesthesia and the tips of their wings were clipped, allowing identification of the focal male but not affecting the capacity of rival males to produce song (Ehrman 1966). The focal and rival males in the +no sound and +all treatments were housed together in a single SYA vial. The males in the -all treatment were housed alone in a vial. Focal males were maintained in their respective treatments for three days.

c) Effect of cue removal on responses to rivals and reproductive success and sperm competitive ability

Virgin wildtype females were transferred to individual vials of SYA one day prior to mating. Each treatment male was introduced to a female directly from their rival treatments by using aspiration. Latency to mate (the time from when the male was introduced to when mating began) and mating duration were recorded to the nearest minute. Pairs that did not mate within 3 h were discarded. Males were removed from the vials by aspiration shortly after mating finished to prevent any rematings. Females were allowed to oviposit in a first set of vials for 24 h, following which each female was transferred to a fresh vial. The first set of vials were then incubated, and offspring that emerged from them were counted.

Approximately 24 h after the first mating, females were given the opportunity to mate a second time, to males with a 'stubble' (*Sb*) mutation. *Sb* mutant individuals are identifiable by the shorter, thicker bristles on the back of the thorax (Overton 1967), allowing for offspring paternity to be determined by eye. *Sb* males came from a *Sb* stock which had been backcrossed into the Dahomey wild type background at least 4 times. The proportion of females that remated was recorded as in the first mating assay, the latency and duration of the rematings were recorded to the nearest minute. Pairs that did not mate within 3 h were discarded. Males were removed shortly after mating. Females were allowed to oviposit in the vials for 24 h, after which they were discarded. The vials were retained and incubated. Offspring that developed from eggs laid following the second mating had mixed paternity, some being fathered by the first (treatment) male and some by the second *Sb* male. Paternity was thus determined by the presence of the *Sb* phenotype, allowing the calculation of the proportion of the offspring fathered by the first (treatment) male (P1) and by the second (*Sb* competitor) male (P2).

d) Statistical analyses

Statistical analyses were carried out in R v 3.4.2 (R Core Team 2016). The data from the two replicates were pooled, then analysed and plotted as one dataset with replicate as a fixed factor. Mating latency data from the first and second mating were analysed using cox proportional hazards models. Shapiro-Wilk and Levene's tests were used to assess whether mating duration and offspring count data were normally distributed and whether variances were equal across treatments, respectively. Where the data were normally distributed or could be transformed to fit a normal distribution they were analysed using Gaussian linear models.

Offspring counts from the first mating in both blocks were zero-inflated, so were analysed using hurdle models. The number of zero offspring counts in each treatment and the non-zero counts were manually separated. The number of zeroes was analysed with a binomial generalised linear mixed model. Where the non-zero offspring counts were normally distributed or could be transformed to fit a normal distribution, they were analysed with a linear mixed model. Otherwise, non-zero counts were analysed using a generalised linear mixed model with a Poisson distribution and a log link. In order to infer the effect of treatment on overall offspring counts, including zeroes and non-zeroes, Kruskal-Wallis tests were used. The proportion of offspring produced following the second mating that were fathered by the

treatment male (P1) was analysed as a dual response variable using a binomial generalised linear mixed model with a logit link.

As a significant effect of treatment was found on first mating duration, pairwise differences between groups were determined using post-hoc Tukey tests with the 'multcomp' package (Hothorn et al. 2008). Pairwise comparisons between treatment groups of the number of offspring (non-zero counts) produced after the first mating were made using Wilcoxon test. The proportion of paternity achieved by the first male after the second mating was compared between treatments using two-sample proportion z-tests. Across all analyses, p-values were adjusted using the Benjamini-Hochberg procedure.

4.4 Results

The mating duration of the treatment males was significantly affected by the cues of rival presence to which males were exposed (LM: $F=7.62$, $df=2$ & 329, $p<0.001$; Figure 4.1a). Males exposed to the full repertoire of rival cues (+ all; Tukey: $t=3.14$, $df=328$, $p=0.019$) and those that had been exposed to rivals with the auditory cue removed (+ no sound; Tukey: $t=3.633$, $df=328$, $p=0.0049$) both significantly extended mating duration relative to males that had not encountered rivals. This is consistent with previous research showing that removing one cue signalling the presence of competitors does not impede a male's ability to respond by significantly increasing mating duration.

The effect of the cues of rival presence that the male was exposed to did not significantly affect mating latency (Cox: $X^2=5.56$, $df=2$, $p=0.16$; Figure 4.1b). The influence of the experimental block in which the male was tested was borderline non-significant (Cox: $X^2=5.63$, $df=1$, $p=0.051$) which, taken with previous findings, suggests that mating latency in response to rivals does not show high repeatability (Bretman et al. 2009; Bretman et al. 2013a; Bretman et al. 2013b).

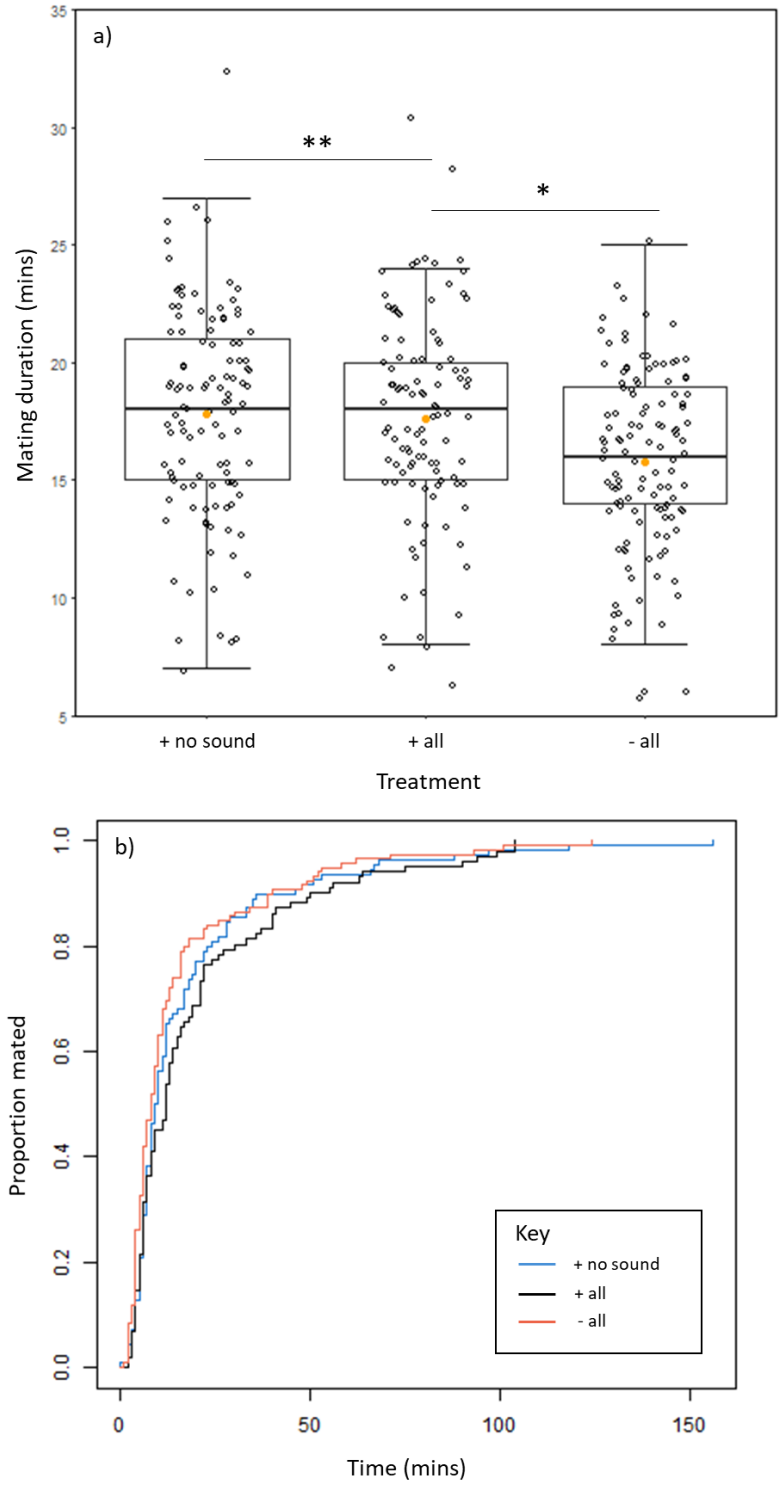

Figure 4.1 – The a) mating duration and b) mating latency of male *Drosophila melanogaster* in response to rival cues. Focal males were either exposed to a rival male with the auditory cue removed (+no sound), all cues intact (+all), or housed alone without rival exposure (-all). **a)** Boxplot shows interquartile range and median with raw data points also plotted. Orange dots indicate means; asterisks indicate significant pairwise differences between groups on each end of the horizontal line: *** p>0.001; ** p>0.01; * p>0.05. **b)** The proportion of males in each treatment (blue = + no sound; black = + all; orange = - all) that had mated over time.

Following mating with the treatment males, females were allowed to oviposit for 24 h before remating. This allowed quantification of the reproductive success of the treatment males in the absence of sperm competition before remating. A Kruskal-Wallis test showed that the number of offspring produced was significantly affected by rival cues to which males were exposed (Kruskal-Wallis: X^2=11.00, df=2, p=0.018; Figure 4.2a). As the offspring count data were zero-inflated, a hurdle model was then used in which zeroes and non-zeroes were separated and modelled. The number of zeros in offspring counts were not significantly predicted by the rival cues to which the male was exposed (GLM: X^2=4.50, df=2, p=0.23). Non-zero offspring counts were significantly influenced by an interaction between male treatment and replicate (GLM: X^2=8.52, df=2, p=0.046; Figure 4.2b), suggesting that while treatment significantly affected the number of offspring produced, this effect varied across replicates and may not show high repeatability. Contrary to predictions, pairwise testing showed that males that were not exposed to any rival cues (- all) fathered significantly more offspring than those exposed to rivals with the auditory cue removed (+ no sound; Wilcoxon rank sum: w=6168, p=0.0049). Males exposed to the full repertoire of rival cues (+ all) fathered an intermediate number of offspring.

Females were given the opportunity to remate to an *Sb* male 24 h after their first mating, in order to assess the reproductive success of the first-mating treatment males under sperm competition. The proportion of females that remated was low across treatments (+ no sound: 38%; + all: 28%; - all: 35%), and was not significantly affected by the rival cues to which the focal males were exposed (GLM: X^2=2.38, df=2, p=0.48). Neither latency to remate (Cox: X^2=2.60, df=2, p=0.27; Figure S4.3) nor remating duration (LM: F=1.12, df=2 & 110, p=0.48; Figure S4.4) were predicted by the rival cues to which the first males were exposed.

The proportion of offspring produced in the 24 h following the second mating that were fathered by the first (focal) male was significantly affected by an interaction between the rival cues the male was exposed to and experimental replicate (GLM: X^2=63.24, df=2, p<0.001; Figure 4.3). This suggested an effect of the rival cues detected by the first male on his success in sperm competition, but that this effect may vary significantly across replicates. Pairwise comparisons did not reveal any significant differences between treatment groups. This is contrary to the expectation that males exposed to rivals, either with all cues intact or with the auditory cue removed, would show equivalent increases in sperm competitiveness, compared to males that had not encountered rivals. Finally, the total number of offspring produced by the female following the second mating was not significantly affected by the treatment of the first male (LM: F=0.299, df=2 & 55, p=0.80).

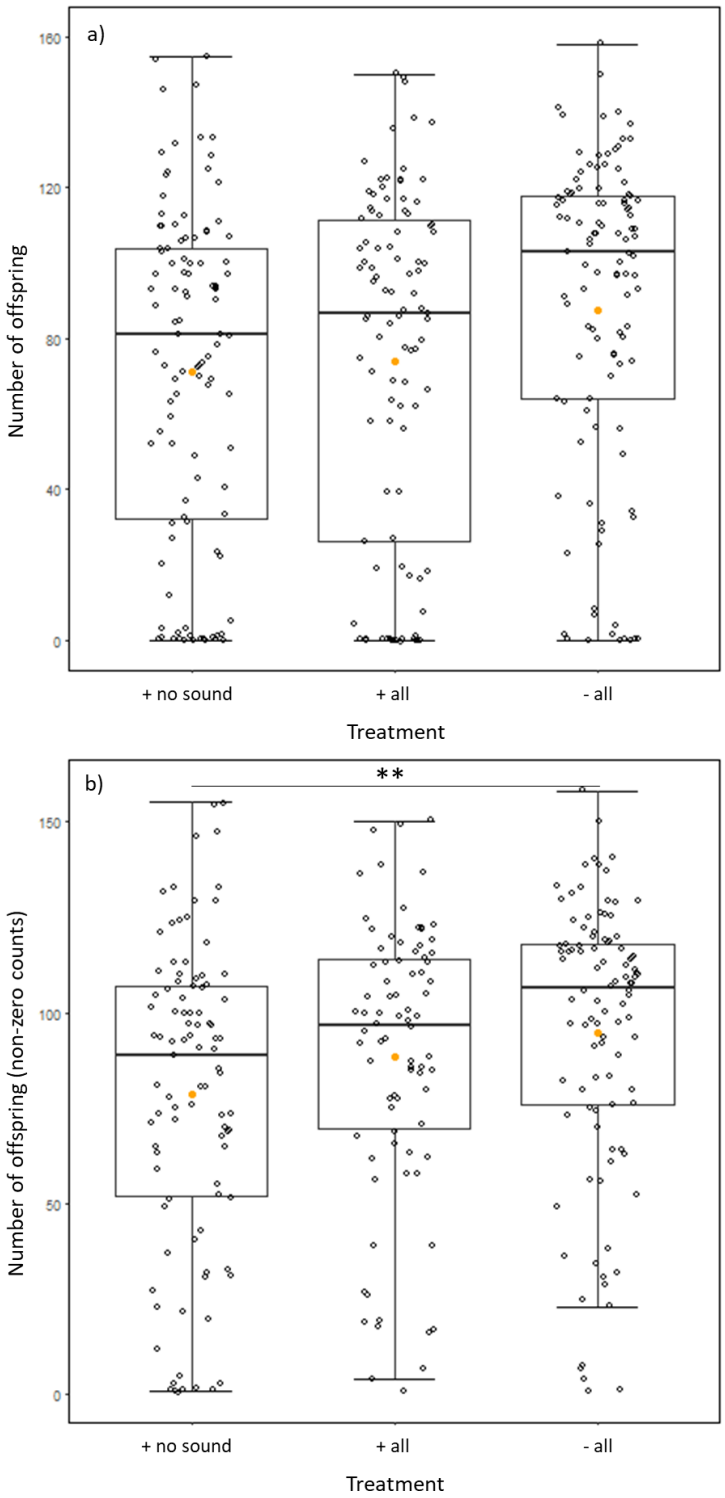

Figure 4.2 – The reproductive success of male *Drosophila melanogaster* in response to rival cues, with no sperm competition. The number of offspring fathered in 24 h following a single mating, **a)** with all data included and **b)** with zero counts removed. Focal males were either exposed to a rival male with the auditory cue removed (+no sound), all cues intact (+all), or housed alone without rival exposure (-all). Boxplots as in Figure 4.1.

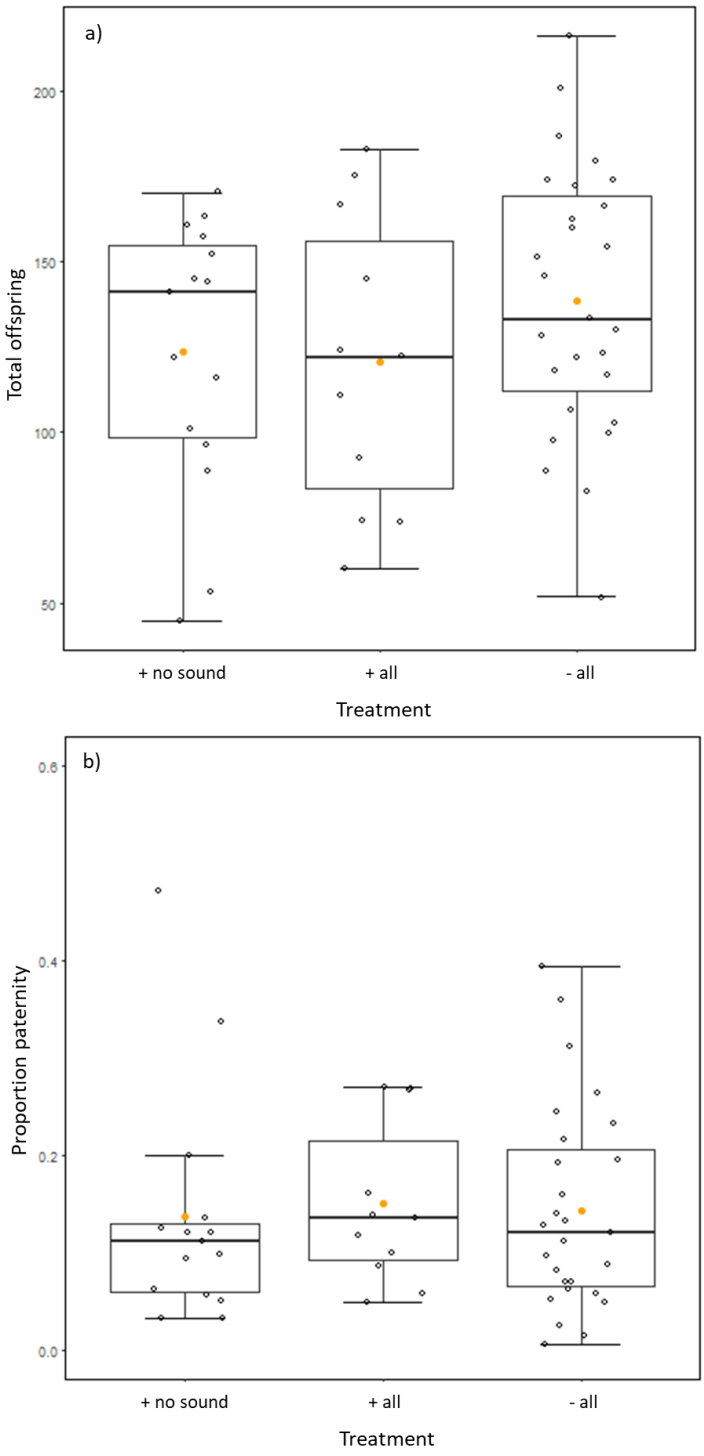

Figure 4.3 – **The reproductive success of male *Drosophila melanogaster* in response to rival cues, with sperm competition. a)** The total number of offspring that were produced following a second mating with a *Sb* mutant male, and **b)** the proportion of these offspring that were fathered by the first, focal male (P1). Focal males were either exposed to a rival male with the auditory cue removed (+no sound), all cues intact (+all), or housed alone without rival exposure (-all). Boxplots as in Figure 4.1.

4.5 Discussion

Overall, the results supported the previous finding of redundancy of cues of *D. melanogaster* male rival presence. However, this redundancy may be incomplete due to differences in the ways these cue components are perceived and processed. Unexpectedly, no fitness benefits of extending mating duration in response to rivals were observed. This suggests that there may not be a simple, direct relationship between behavioural investment in mating and fitness consequences.

a) Redundancy of cues of *D. melanogaster* rival presence

The results supported the previous finding that removing one cue of rival presence does not affect the ability of male *D. melanogaster* to detect rivals and respond to them by extending their subsequent mating duration (Bretman et al. 2011b). Males exposed to a rival with the auditory cue removed showed equivalent mating duration to males housed with rivals with all cues intact, and both groups of males mated for significantly longer than males that had not encountered a competitor. This supports the conclusion that alternative cue combinations elicit equivalent behavioural responses. Although the auditory, olfactory and tactile cues of rival presence appear to be interchangeable in terms of the behavioural response they elicit (Bretman et al. 2011b), subsequent research suggests that the processing of these cues is not fully redundant (Rouse and Bretman 2016) and may be underpinned by alternative pathways of gene expression (Mohorianu et al. 2017; Dore et al. *in review*). The results supported the idea that there is at least partial redundancy in how cues indicating the presence of rivals are processed by the receiving male (Bretman et al. 2011b).

The way in which multiple cues are perceived and processed is likely to be related to social learning, whether these cues are redundant or confer information about different components of the social environment. Learning relies on cues being perceived, stored and compared to new environmental information (Dukas 2008; Bailey and Zuk 2009). Understanding which cues are important for influencing social behaviour, and how they lead to a behavioural outcome, may in turn increase understanding of the processes underlying social learning. A form of long-term memory has been found to be necessary for male *D. melanogaster* males to respond to rivals by adjusting mating duration (Rouse et al. 2018). It has been suggested that the timing of this response is important, on the basis that a minimum period of exposure to rivals of 24 h is required to elicit a response (Bretman et al. 2010), which then persists for 12 h (Rouse and Bretman 2016). Males may be required to remember their recent social environment in order to determine whether the cues of competition have

persisted for long enough to be representative of the general level of sperm competition (Rouse et al. 2018). The mechanisms by which long-term memory facilitates responses to rivals by male *D. melanogaster* are localised to the mushroom bodies, highlighting the importance of olfactory cues (Rouse et al. 2018). Olfactory stimuli have also been found to be of particular importance for learning in the nematode *Caenorhabditis elegans*, in which learned associations with a number of aversive odours are formed at varying speeds (Choi et al. 2018). Therefore, although the multiple cues of *D. melanogaster* rival presence appear to be redundant in eliciting a longer mating response by males, there may be underlying differences related to how these cues are processed, the associative memories they form and the speed with which this happens. Indeed, the removal of auditory or olfactory cues of rivals have been found to extend the time taken for a male *D. melanogaster* to respond (Rouse and Bretman 2016). Increasing understanding of the role of social cues in learning may shed further light on the mechanisms by which reproductive plasticity is achieved (Rouse et al. 2018).

b) Fitness effects of the extension of male *D. melanogaster* mating duration following detection of rivals

There was no evidence that the extension in mating duration following detection of competitors led to any immediate reproductive fitness benefits, either in the absence or presence of sperm competition. Neither males exposed to all rival cues, nor those for which auditory cues were removed, showed an increase in the number of offspring they fathered when the female was singly mated, compared to males experiencing no cues of competition. Males exposed to rivals, with all cues intact or with the auditory cue removed, also did not increase the proportion of paternity they achieved under sperm competition. In fact, in the absence of sperm competition, there was a trend for males not exposed to a rival to father more offspring than males previously exposed to rivals. In the first set of experiments, males in the - all treatment also achieved greater success in sperm competition than those in the + all treatment. Furthermore, the longer mating demonstrated by males exposed to rivals did not reduce female receptivity to remating.

The finding that exposure to rival males (either with all cues intact or with one cue removed) and the associated longer-mating phenotype did not result in any apparent increases in reproductive fitness for *D. melanogaster* males was unexpected. Males exposed to cues of competition did not father higher numbers of offspring or reduce female receptivity to remating. This is inconsistent with previous findings (Bretman et al. 2010; Bretman et al.

2011b). There is evidence that the increase in the number of offspring fathered following longer mating occurs via increased transfer of two key seminal fluid proteins, sex peptide and ovulin, which increase female egg production and decrease receptivity to remating (Chapman et al. 2003; Chapman and Davies 2004; Wigby et al. 2009b). Neither of these effects were observed in the current study. Bretman et al. (2012) did find evidence that the behavioural response of longer mating duration can become decoupled from offspring production; however, this only occurred when males experienced a period without rival exposure prior to mating. Furthermore, Bretman et al. (2012) found evidence of males continuing to increase offspring production after mating duration was decreased, rather than of longer mating duration that did not correspond to fitness benefits. Nevertheless, Hopkins et al. (2019) found that sperm transfer and seminal fluid protein (SFP) transfer peak at different intensities of male-male competition, with the amount of SFPs transferred generally increasing with the level of competition. Additionally, the composition of SFPs in the ejaculate can change with the intensity of competition. These studies demonstrate that there may not be a simple relationship between level of competition, behavioural response and reproductive success.

One possible explanation for the absence of an increase in reproductive success among males exposed to competitors is that aggressive interactions with rivals led to the treatment males sustaining harm, reducing their condition and thus their ability to increase their ejaculate investment in response to competition. Nandy et al. (2016) found the expression of male-male aggression to be a key component of the cost of reproduction and a driver of decreased longevity under starvation in *D. melanogaster*. It has been proposed that aggression between males can impose costs via injury and energy expenditure (Bretman et al. 2013b), ultimately reducing life span (Gaskin et al. 2002; Costa et al. 2010). Males who suffer these costs from aggressive interactions during rival exposure may be less able to subsequently increase their investment in their ejaculate, negating the usual fitness benefits of extending mating duration. However, it has been argued that male-male aggression is a minor contributor to costs of reproduction (Bretman et al. 2013b; Leech et al. 2017). This is based on the findings that males housed with a rival sustained no more wing damage than males housed alone (Bretman et al. 2013b). Although social contact between male *D. melanogaster* does reduce lifespan, this could not be explained in any signature of behavioural differences between males (Leech et al. 2017). Moreover, male *Drosophila* aggression has been found to decline with prolonged exposure to the male-specific pheromone 11-*cis*-vaccenyl acetate (cVA), suggesting that continuous exposure to rivals may reduce aggressive behaviour. Thus, males housed with rivals may not be engaged in high frequencies of aggressive encounters,

reducing the likelihood that they would sustain harm during treatment that would decrease their reproductive success.

Male competitive success can respond to various features of the social environment in addition to the presence of competitors, including female condition and female mating status (Lewis and Iannini 1995; Bonduriansky 2001; Friberg 2006). The ejaculate investment of male *D. melanogaster* in this study may respond to these other variables, masking responses to the presence of competitors. This may explain why there was no elevation in offspring production from longer matings following exposure to rivals. For example, all females in this experiment were virgins prior to mating with the treatment males. Friberg (2006) found that males increased their investment in reproduction, leading to reduced female remating, when they perceived females to have previously mated. The virgin status of females in the current study may have cued to males the low probability of sperm competition, confounding the effects of the prior exposure to rivals. Furthermore, the virgin females in this study may have detected CHC components of previously encountered rivals on males in the + all and + no sound treatments, signalling the presence of other potential mates, while the females that mated with - all males may have inferred that this was the only likely mating opportunity. Therefore, females mating with the - all treatment males may have increased their per-mating investment, thereby counterbalancing any increase in offspring production elicited by the males responding to rival cues. An alternative explanation for the uniformity in reproductive success across treatment groups is that all males were also virgins prior to the experimental mating. In polyandrous butterfly species, the first ejaculate produced by a male is larger and contains more protein than subsequent ones (Bissoondath and Wiklund 1996; Hughes et al. 2000). The male *D. melanogaster* tested here had not encountered a female since reaching reproductive maturity. Due to the high variation in the reproductive success of males (Bateman 1948) and the very high potential fitness cost of never mating at all, it may be beneficial for a male to invest heavily in the first reproductive encounter, whether competition is detected or not. This too may have obscured the differences between treatment groups in reproductive success.

The apparent lack of fitness benefits of extending mating duration in response to rivals could occur because longer matings conferred benefits to males in the form of increased sperm displacement, which was not measured in this study. Reproductive success under sperm competition was only measured in terms of sperm defensiveness. However, previous research has found extended mating and increased reproductive success to follow exposure to rivals whether the focal male was the first or second to mate with a female. Another possible

'hidden' fitness benefit of extending mating duration is the delaying of female remating up to 24 h. Females were isolated for 24 h following the first mating, thus their receptivity to remating during this window was not measured. Reduced receptivity during the first 24 h after mating could contribute to the adaptive value of increasing mating duration following rival exposure, despite the apparent lack of increase in offspring production. Nevertheless, it has been shown that behavioural responses to rivals can be decoupled from fitness benefits (Bretman et al. 2012; Hopkins et al. 2019). Furthermore, a recent study on *D. melanogaster* similarly found that longer matings by males exposed to competitors did not correspond to increased paternity share (Marie-Orleach et al. 2020).

Gilchrist and Partridge (2000) proposed that sperm transfer is completed within the first few minutes of *D. melanogaster* matings and therefore that longer matings do not correspond to increased sperm allocation, higher offspring numbers, or improved sperm displacement ability. However, interrupting matings past the point where sperm transfer would occur completed impeded the male's ability to delay female remating, suggesting that some SFPs may continue to be transferred later in copulation. Furthermore, the sperm and SFP components of the ejaculate have been found to be under independent control. While sperm transfer peaks at a low level of sperm competition intensity, SFP transfer generally peaks under more intense competition, although the composition of proteins in the ejaculate may vary along the sperm competition gradient. (Hopkins et al. 2019). Mating duration therefore seems not to correspond to sperm transfer and may not be an appropriate proxy for overall ejaculate investment.

Together with the result that extended mating duration did not have any observed fitness benefits, these findings suggest that the relationship between cues of competition, behaviour and reproductive success may not be as simple or direct as previously thought, or that fitness benefits are expressed in an environment not tested here. This opens further questions on how sensory cues are processed to infer the intensity, as well as risk, of sperm competition, and whether redundancy among cues persists at varying degrees of competition.

c) Conclusions

The results supported the previous finding that removing one cue of rival presence does not prevent male *D. melanogaster* from detecting rivals and responding to them by extending mating duration (Bretman et al. 2011b). This suggests that the cues signalling rival presence are at least partially redundant. The redundancy of cue components may confer benefits to the receiving male by preventing information from being compromised if one component is

inaccurate or lost, thereby facilitating adaptive reproductive plasticity. It cannot be concluded whether alternative combinations of cue components signalling rival presence are equivalent in terms of the fitness benefits achieved by responding to them, as no increase in reproductive success among males exposed to a rival were detected. Males exposed to all rival cues or the restricted set of cues did not increase their paternity, either in the absence or presence of sperm competition, despite extending mating duration. The receptivity of females to remating was also not affected by male exposure to rival cues. The absence of any apparent fitness benefits of longer mating is inconsistent with previous studies (Bretman et al. 2010; Bretman et al. 2011b) but highlights that caution should be taken when indirectly extrapolating fitness benefits from behavioural responses alone. It is possible that the lack of increased offspring production following longer mating was caused by damage sustained from aggressive interactions with rivals impairing the male's ability to increase ejaculate investment. Or, the fitness benefits of longer mating may have been obscured by homogenising effects other features of the social environment, such as male and female mating status. Alternatively, longer mating following rival exposure could have conferred 'hidden' fitness benefits not measured in this study, for example sperm displacement or delaying of female remating up to 24 h. However, it is also possible that behaviour can become decoupled from increases in the transfer of sperm and SFPs, and that this may be mediated the degree of male-male competition (Hopkins et al. 2019).

4.6 Supplementary information

4.6.1 The effect of systematic removal of auditory, olfactory and tactile cues of *D. melanogaster* rival presence

Methods

a) General methods

As described in main text.

b) Removal of cues of rival presence

Each male was randomly assigned to one of the following treatments: 1) housed with a wildtype rival male with the tactile cue removed (+ no touch), 2) housed with a rival male with the olfactory cue removed (+no smell), 3) housed with a rival male with the auditory cue removed (+no sound), 4) housed with a rival male with all sensory cues intact (+all), 5) housed alone (-all). The auditory cue of rival presence was inhibited via the removal of the wings of rival males, as described in the main text. To control for handling and allow identification of the focal male, the rival males in the +no touch, +no smell and +all treatments were also subjected to CO_2 anaesthesia and their wings were clipped. The focal and rival males in the + no sound and + all treatments were housed together in a single SYA vial. To remove the tactile cue in the + no touch treatments, the focal and rival male were placed in separate vials of SYA which were joined together at their open ends, with porous nylon netting between the two. Through the netting, the two males were expected to be able to smell and hear each other, but not touch. The males in the - all treatment were housed alone in a vial. To inhibit the olfactory cue of rival presence, the third antennal segments (A3) of the focal males in the +no smell treatment were removed under CO_2 anaesthesia. Focal males in all other treatments were also briefly anaesthetised with CO_2 to control for experience. Focal males were maintained in their respective treatments for three days. Two independent replicate experiments were carried out and the data were pooled for analysis with replicate as a fixed factor.

c) Effect of cue removal on responses to rivals

A mating assay was carried out as described in the main text to measure latency to mate and mating duration.

d) Statistical analyses

As described in the main text, mating duration and latency were modelled in R v 3.4.2 (R Core Team 2016) using mixed models. Mating duration was modelled with a G aussian linear model. Mating latency data were analysed using a cox proportional hazards model. Post-hoc pairwise comparisons were conducted using the package 'multcomp' (Hothorn et al. 2008).

Results & discussion

The combination of sensory cues of rival presence to which the focal male was exposed significantly affected mating duration (LM: $F=9.62$, df=4 & 566, $p<0.001$; Figure S4.1). Latency to mate varied significantly between replicates (Cox: $X^2=22.72$, df=1, $p<0.001$), but was not significantly affected by the rival cues the focal male encountered (Cox: $X^2=2.29$, df=4, $p=0.68$).

When all cues were intact (+ all) males did not mate for significantly longer than males with no rival exposure (- all; Tukey: $t=2.67$, df=566, $p=0.059$). The significant extension of mating duration by male *D. melanogaster* in response to rivals has previously been found to be a robust and repeatable result (Bretman et al. 2009; Bretman et al. 2011a; Bretman et al. 2011b). Therefore, the nonsignificant difference in mating duration between + all and – all treatments in this study suggested confounding factors or imprecision in the data, which complicated the interpretation of the results. Nevertheless, males with no rival exposure expressed significantly shorter mating than males exposed to rivals with either the olfactory (Tukey: $t=5.23$, df=566, $p<0.001$) or the auditory (Tukey: $t=3.57$, df=566, $p=0.0036$) cue removed. Males exposed to rivals with the tactile cue removed also mated for significantly shorter duration than those with the olfactory (Tukey: $t=4.84$, df=566, $p<0.001$) or auditory (Tukey: $t=2.21$, df=566, $p=0.012$) cue occluded. This suggested that the removal of the tactile cue prevented males from responding to rivals by extending mating duration, instead showing similar mating duration to males that had not encountered rivals. To prevent focal and rival males from touching, the two were housed in separate vials with porous netting between them. This treatment differed from the other protocols to remove a cue, in which males were housed together in a single vial. This may have had resulted in confounding influences on the perception of the rival, for example by diluting the olfactory and auditory cues. Therefore, this treatment was not included in subsequent experiments, to avoid off-target effects.

4.6.2 The effect of inhibiting the olfactory cue of *D. melanogaster* rival presence by removal of the A3 antennal segment

Methods

a) General methods

As described in main text.

b) Effect of antennal segment removal on responses to rivals

Each focal male was randomly assigned to one of the following treatments: 1) A3 removed with subsequent rival exposure (A3 removed +), 2) A3 removed with no rival exposure (A3 removed -), 3) A3 intact with subsequent rival exposure (A3 intact +), 4) A3 intact with no rival exposure (A3 intact -). Focal males in the two A3 removed treatments were anaesthetised with CO_2 and the third antennal segment was removed with sharpened forceps. To control for handling, focal males in the A3 intact treatments were also anaesthetised with CO_2. To distinguish rival males from focal males, the wings of rival males were clipped under CO_2 anaesthesia. Only the tips of the wings were removed, so as not to prevent the rival males from producing the song which functions as the auditory cue of their presence (Bretman et al. 2011b). Each focal male in the A3 removed + and A3 intact + treatments was housed with a conspecific wildtype rival male in a vial of SYA for three days. Males in the A3 removed – and A3 intact – treatments were housed alone in a vial of SYA during this time.

A mating assay was carried out as described in the main text to measure latency to mate and mating duration.

c) Statistical analyses

Statistical analyses were carried out in R v 3.4.2 (R Core Team 2016). Mating duration data were analysed using a linear model. Mating latency data were analysed using a cox proportional hazards model. Post-hoc Tukey tests pairwise tests were carried out using the 'multcomp' package (Hothorn et al. 2008).

Results & discussion

The removal of A3 significantly influenced both mating duration (LM: $F=44.74$, $df=1$ & 190, $p<0.001$; Figure S4.2) and latency to mate (Cox: $X^2=9.92$, $df=1$, $p=0.0016$). No significant

response to rivals in mating latency was identified within either the A3 removed or the A3 intact treatments. Despite the overall significant effect of A3 removal on mating latency, there were no significant pairwise differences between males with A3 removed or A3 intact in equivalent rival treatments. Both males with A3 removed (Tukey: $t=2.63$, $df=190$, $p=0.045$) and males with A3 intact (Tukey: $t=4.24$, $df=190$, $p<0.001$) significantly extended mating duration following exposure to rivals, compared to males housed alone. Males in the A3 removed + treatment mated for significantly longer than the A3 intact + treatment (Tukey: $t=3.89$, $df=190$, $p<0.001$). Males in the A3 removed - treatment also significantly extended mating duration, relative to the A3 intact - treatment (Tukey: $t=5.56$, $df=190$, $p<0.001$). This showed that surgical A3 removal did affect the behaviour of male *D. melanogaster* - therefore this manipulation not only affected olfactory cues of rival presence, but also appeared to induce off target effects on mating duration.

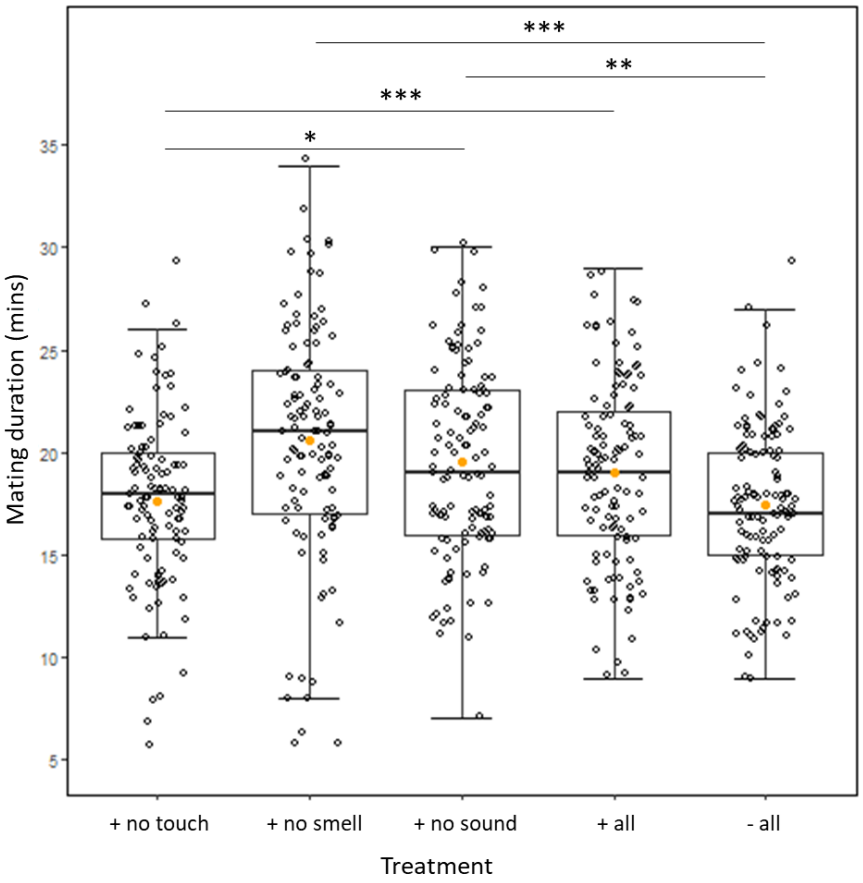

Figure S4.1 - The mating duration of male *Drosophila melanogaster* exposed to alternative combinations of rival cues. Focal males were either exposed to a rival male with the tactile cue removed (+ no touch), the olfactory cue removed (+ no smell) the auditory cue removed (+ no sound), all cues intact (+ all), or housed alone without rival exposure (- all). Boxplots show interquartile range and median with raw data points also plotted. Orange dots indicate means; asterisks indicate significant pairwise differences between groups at each end of the horizontal line: *** p>0.001; ** p>0.01; * p>0.05.

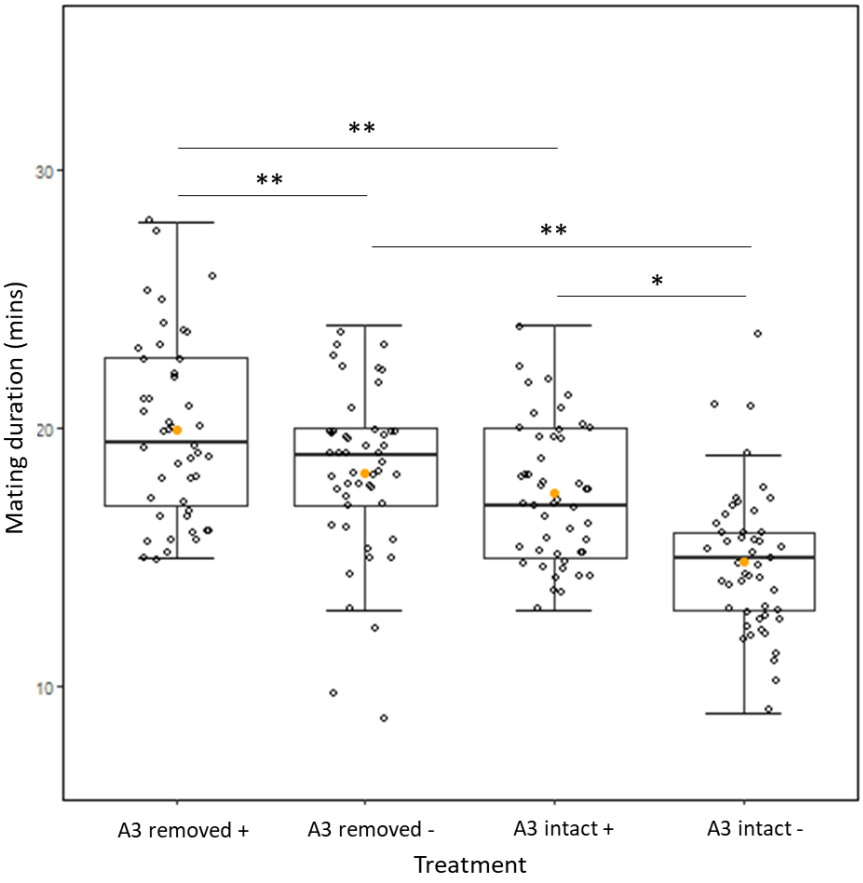

Figure S4.2 – The effect of removing the third antennal segment (A3) on male *Drosophila melanogaster* mating duration. Focal males with A3 removed or A3 intact were either exposed to rivals (+) or housing alone (-). Boxplots as described in Figure S4.1.

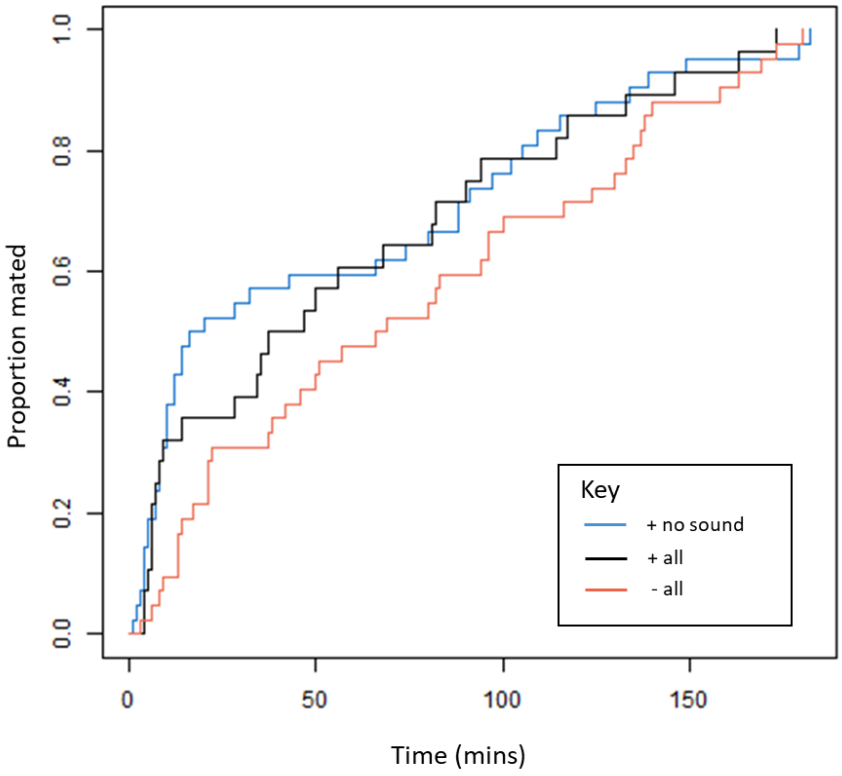

Figure S4.3 – The latency to remate of female *Drosophila melanogaster* after first mating to males exposed to alternative combinations of rival cues. Focal (first) males were either exposed to a rival male with the auditory cue removed (+no sound) or all cues intact (+all), or housed alone without rival exposure (-all). Each focal male was then introduced to a virgin female and given three hours within which to mate.

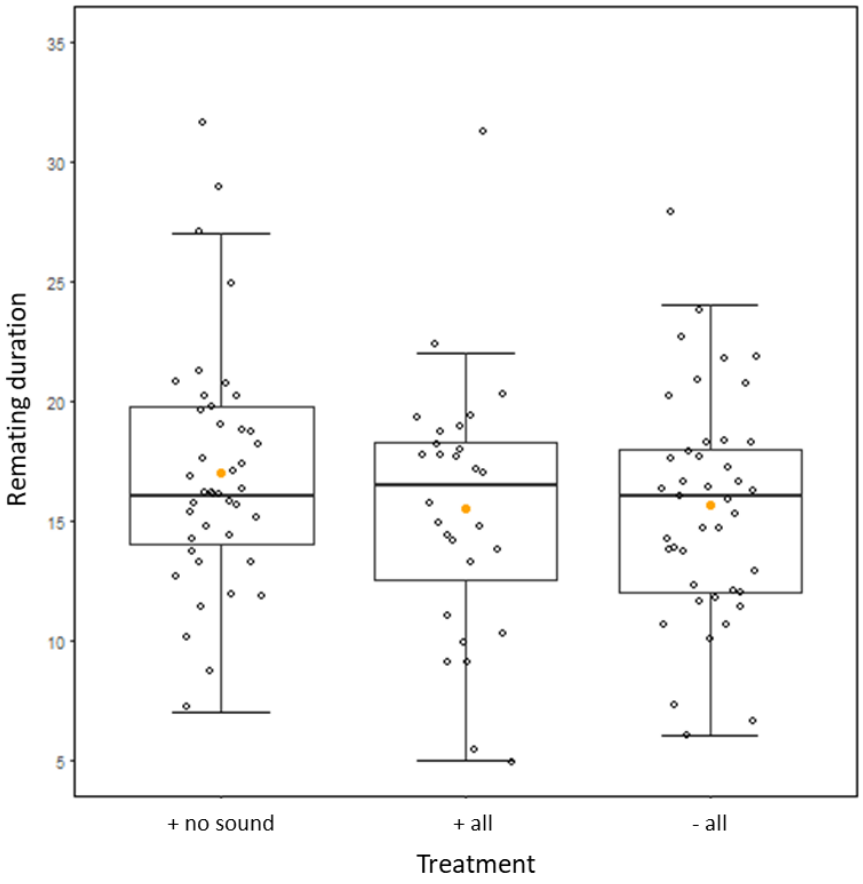

Figure S4.4 - The remating duration of female *Drosophila melanogaster* after first mating to males exposed to alternative combinations of rival cues. Focal (first) males were either exposed to a rival male with the auditory cue removed (+no sound) or all cues intact (+all), or housed alone without rival exposure (-all). Boxplots as in Figure S4.1.

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5. Redundant networks and alternative expression pathways: a test case using conspecific competitive responses in male fruit flies

5.1 Abstract

The use of alternative biological pathways to achieve quasi-equivalent phenotypes may be a common mechanism that allows organisms to robustly and flexibly respond to variable environments. However, empirical tests to identify and characterize such alternative pathways are lacking. In this chapter I present a preliminary test of the hypothesis that alternative expression pathways within redundant regulatory networks are used by male *Drosophila melanogaster* to respond to the presence of conspecific males. To do this, I evaluated and compared the body-part specific gene expression levels by using RNA-sequencing data. I compared the identity and abundance of expressed genes of focal male *D. melanogaster* exposed to a rival male, either with the full repertoire of sensory cues from rivals, or with an auditory cue removed. Males exposed to these two sets of rival cues have previously been shown to produce comparable competitive behavioural responses. I found that the two treatments led to differential expression in reproductive and sperm competition genes. This may suggest that males can produce quasi-equivalent competitive responses to rivals via the expression of alternative, but functionally equivalent, pathways. However, further testing is required to robustly support this conclusion. Overall, this study illustrates how alternative expression paths within redundant regulatory networks could contribute to the expression of adaptive phenotypic plasticity and illustrates how this concept can be tested experimentally.

5.2 Introduction

Changes in the social environment can lead to rapid and complex genomic responses, which can underpin important phenotypic changes (Cummings et al. 2007; Oliveira 2012). Such genomic responses to social stimuli have been identified in several taxa including *D. melanogaster,* Australian black field crickets (*Teleogryllus commodus)*, European starlings (*Sturnus vulgaris*) and several fish species (Sockman et al. 2002; Immonen and Ritchie 2011; Simões et al. 2015; Kasumovic et al. 2016; Oliveira et al. 2016). Distinct patterns of gene expression have been observed in response to social interactions depending on the sex, species, dominance and attractiveness of the other individual (Cummings et al. 2007; Ellis and Carney 2011; Immonen and Ritchie 2011; Simões et al. 2015). In *D. melanogaster* females, exposure to male courtship song has been shown to affect immune response genes as well as those related to signalling, demonstrating that pre-mating social interactions can be important in initiating reproductive responses (Immonen and Ritchie 2011). This shows that transcriptional changes may be key in underpinning rapid and fine-tuned phenotypic responses to the social environment. As well as initiating rapid plastic responses, research suggests that changes in gene expression in response to the social environment can have longer-term impacts by influencing life history trajectories and adaptive evolution (Kasumovic et al. 2016; Pascoal et al. 2018). The changes in gene expression underpinning social plasticity are likely to occur within a broader genomic network (Oliveira, 2012) which may often be characterised by redundancy, allowing different patterns of gene expression to elicit analogous phenotypes (Ay et al. 2007; Greenspan 2012; Mohorianu et al. 2017b).

It is increasingly clear that some organisms may have the capacity to employ different transcriptional pathways to achieve functionally analogous phenotypes, allowing them to respond flexibly when experiencing variable environments (Ay et al. 2007; Greenspan 2012; Mohorianu et al. 2017b). However, it is not yet apparent how frequently such alternative pathways are used in response to environmental variation, or which phenotypes are generally influenced by them. Alternative pathways leading to quasi-equivalent responses are likely to be key to maintaining important fitness-related responses in the face of extreme environmental variation (Mellen 2010; Joshi et al. 2013; Duthie et al. 2018). Here, 'quasi-equivalent' refers to responses that are equivalent in terms of the phenotypic trait of interest, but may have been elicited by the expression of different genes and may be associated with other phenotypic differences not measured. This type of redundancy has been described in neuroscience, where alternative neural connections can lead to similar motor responses

(Briggman et al. 2006) and in genetics, where alternative configurations of gene networks can produce quasi-equivalent effects (Greenspan 2009). A gene network that can produce similar outputs via different paths may considerably increase the robustness of a phenotype's expression, for example by buffering against the effects of deleterious mutations (Wagner 2000; Greenspan 2009). Redundancy may be a common underlying feature of phenotypic plasticity, allowing an organism to produce a behaviour or characteristic in response to multi-component signals (Hankison and Morris 2003; Bretman et al. 2011b; Gordon and Uetz 2011). Considering the role of redundancy in the context of phenotypic plasticity may offer insight into the mechanisms underpinning phenotypic responses to environmental, which are not yet well understood (Gilbert 2005; Dangles et al. 2009; Ellers and Stuefer 2010; Mohorianu et al. 2017b). Previous studies have explored modelling perception in animals using networks in order to shed light on the intermediary processes between input and output (Gurney 2007; Phelps 2007; Tosh and Ruxton 2007). The potential benefits of robustness in networks may promote redundancy within network components (Ay et al. 2007). Some empirical studies have suggested that redundant networks for modelling regulatory interactions have value in predicting and explaining the responses of animals to stimuli (Bain et al. 2007; Dalziel et al. 2008; Fuller 2009; Lewis et al. 2010). However, due to a lack of tractable experimental study systems, empirical research has been limited and evidence to support the existence of alternative components in networks and a role for redundancy is scant. In this chapter, I address this omission and present an empirical test case of alternative transcriptional pathways operating to produce quasi-equivalent phenotypes.

I used the responses of *Drosophila melanogaster* males to sexual competition as a model system for this study. The responses of male *D. melanogaster* to conspecific rivals present a valuable and tractable system for evaluating the hypothesis of redundant paths in regulatory networks for several reasons. The phenotypic responses of male *D. melanogaster* following the detection of conspecific rivals are well characterised (Bretman et al. 2009; Wigby et al. 2009b; Bretman et al. 2011b, 2012; Kim et al. 2012) and a wealth of genomic tools and resources are available (Adams et al. 2000; Gramates et al. 2017; Leader et al. 2018). Furthermore, the genes coding for reproductive responses in *D. melanogaster* have been shown to exhibit coordinated expression, indicating the presence of a tightly regulated underlying gene network (Mohorianu et al. 2018). Finally, redundant stimuli and pathways are predicted to be particularly beneficial, and therefore frequent, in systems such as this one that show plastic responses to the social environment (Dore et al. 2018). This is due to the complex and rapidly variable nature of the social environment (Kasumovic et al. 2008; Bretman et al.

2011a) which selects for the flexibility in processing and responses that can be conferred by redundant systems (Greenspan 2012). Redundancy underlying reproductive plasticity of male *D. melanogaster* may be the result of selection for flexible and robust responses in the face of rapidly-changing socio-sexual environments.

I empirically tested the concept that a redundant network built on variation in gene expression is the mechanism by which male *D. melanogaster* can adjust their mating effort in response to alternative combinations of sensory cues that indicate the presence of conspecific males (Bretman et al. 2011b; Mohorianu et al. 2017b). Male *D. melanogaster* significantly extend mating following exposure to conspecific rival males (Bretman et al. 2009). This is correlated with the increased transfer of some seminal fluid peptides to females, leading to increased offspring production (Bretman et al. 2009; Wigby et al. 2009a). The basis for testing for alternative transcriptomic pathways to this phenotype is the previous finding that the longer matings in response to potential competition can be initiated by multiple, redundant cues of rival male presence (Bretman et al. 2011b; Maguire et al. 2015). Specifically, auditory, olfactory and tactile cues are used and any two of these cues in combination, or all three, result in a significant extension of mating duration and increase in reproductive output (Bretman et al. 2011b). Some evidence suggests that the pathways used in the detection of rivals in *D. melanogaster* may be only partially redundant, as removing one cue of rival presence can slow response speeds (Rouse and Bretman 2016). Furthermore, manipulation of sensory cues can lead to variable responses to heterospecific rivals, suggesting that some cues have non-redundant roles in avoiding off-target competitive responses (Bretman et al. 2017). The quasi-equivalent response initiated by different combinations of cues raises the possibility that alternative transcriptional pathways may lead to the same behavioural response of extended mating duration (Mohorianu et al. 2017b). Alternatively, the phenotype may be produced by one expression pathway which is initiated by alternative combinations of rival cues. Previous mRNA sequencing of male *D. melanogaster* recently exposed to rivals was characterised by replicate-to-replicate variation in gene expression related to reproductive responses as opposed to a random selection of genes without any functional enrichment, supporting the possibility of alternative pathways revealed by functional convergence of gene expression (Mohorianu et al. 2017b).

Here, I tested the alternative hypothesis that different combinations of the redundant cues indicating rival presence lead to the quasi-equivalent response of extended mating duration and adjusted ejaculate transfer via alternative transcriptional pathways in male *D. melanogaster*. The null hypothesis was that alternative combinations of rival cues elicit this

behavioural response via transcription of the same genes, the expression of which is conserved when different cues are received. I assessed the existence of alternative expression pathways for achieving extended mating duration by using pre-existing mRNA-seq data collected by E. Fowler and I. Mohorianu. The transcriptomic responses of male *D. melanogaster* exposed either the full repertoire of cues signalling rival presence, or the full set minus an auditory cue were analysed. Transcriptomic data from males exposed to rival cues minus the tactile cue, and minus the olfactory cue, were removed from analysis, as the manipulations to remove these cues have subsequently been found to produce off-target effects on male behaviour. Occlusion of the auditory cue is here used as an exemplar, as previous tests have reported that the removal of any one cue was equivalent with respect to the behavioural response elicited and the associated fitness consequences (Bretman et al. 2011b). If alternative transcriptional paths to the same phenotypic response exist, initiated by different combinations of input cues (Figure 5.1), I predicted that males exposed to restricted cues of rival presence will show differential expression in mating behaviour and reproductive genes, compared to those expressed in males in which sensory input cues are unrestricted. For these transcriptional differences to represent alternative pathways initiated by alternative combinations of rival cues, relevant genes should be differentially expressed between the two treatments to a greater extent than between the biological replicates within each treatment group. Alternatively, if the extended mating phenotype is elicited by one transcriptional pathway which is initiated by alternative combinations of rival cues, I predicted that there will be no greater differential expression of mating behaviour and reproductive genes between treatments than can be explained by variation between biological replicates.

The interpretation of the data is limited by the existence of only two biological replicates per treatment, restricting the potential and power of statistical testing. Furthermore, transcriptomic data from males who were not exposed to rivals, which could have acted as a negative control group, was not obtained. Nevertheless, in this chapter I present preliminary data which demonstrate the potential value of an mRNA sequencing approach for studying redundancy in transcriptomic pathways for eliciting quasi-equivalent phenotypes. A further consideration is that a straightforward chain of causality between social cues, gene expression and phenotype cannot be assumed. Differential expression of genes following from exposure to alternative combinations of rival may not be a direct result of the sensory input. Differential expression of some genes may arise from knock-on or feedback effects of other transcriptomic changes or differential development of the phenotype. However, I propose that reconfigurations of networks of gene expression in individuals

exposed to different combinations of rival cues, but expressing the same phenotype, nevertheless suggests some2 genetic redundancy. I argue that these initial findings justify further, larger-scale studies to test the robustness and repeatability of these conclusions and further explore the concept.

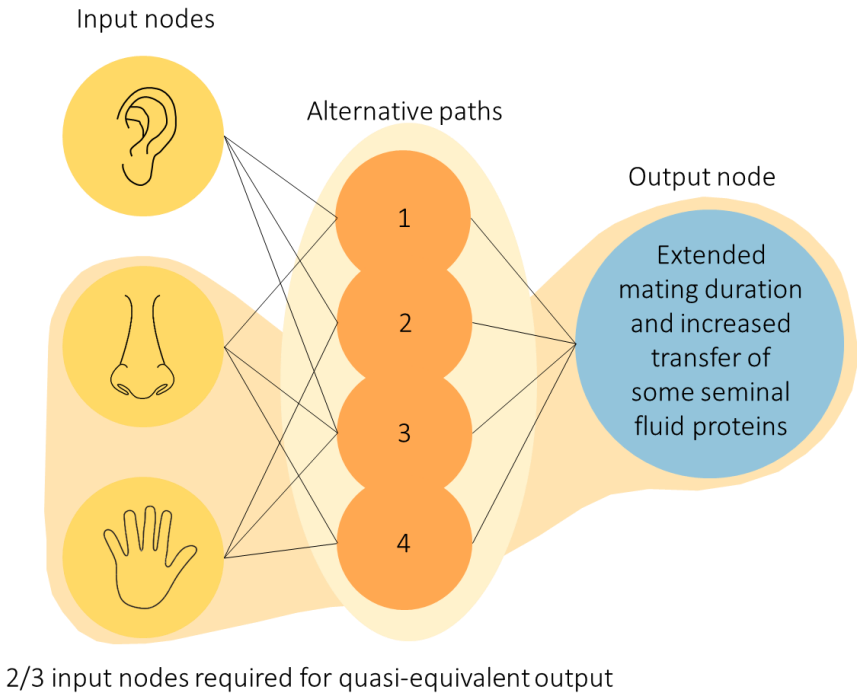


Figure 5.1 – Possible alternative pathways to quasi-equivalent phenotypes in male *Drosophila melanogaster*. Different combinations of sensory cues signalling rival presence may activate alternative expression pathways, leading to the quasi-equivalent response of extended mating duration and increased transfer of some seminal fluid proteins to the female. Highlighted in the shaded region is the subset of the network predicted to be activated when the auditory cue is removed. Note that the number of different paths is arbitrary and they may be independent or have single or multi-point interactions.

5.3 Methods

a) General methods (conducted by E. Fowler)

All fly rearing and experiments were conducted in a 25°C humidified room with a 12 h:12 h light:dark cycle. Flies were maintained in 75x25mm glass vials containing 7 ml standard sugar-yeast-agar medium (Bass et al. 2007). Flies were sampled from a laboratory population of the

Dahomey wild type, as used in related studies (Bretman et al. 2009; Bretman et al. 2010a; Bretman et al. 2011b; Mohorianu et al. 2017b). Larvae were raised at a standard density of 100 per vial. Upon eclosion, adult males and females were separated using ice anaesthesia and males were stored at 10 per vial. Males were randomly assigned as either rivals or focal males to one of two treatments: 'All cues' (unmanipulated sensory input) or 'No sound' (auditory sensory input removed).

b) Exposure to rivals (conducted by E. Fowler)

When 1-2 days old, the wings of males assigned to be rivals in the 'No sound' treatment were surgically removed to prevent them from producing the song that functions as the auditory cue of rival presence to other males. Removal of a rival's wings has been previously shown to have an equivalent effect on focal male responses as using hearing defective mutants as the focal male, suggesting that this manipulation cleanly removes the auditory cue of rival presence (Bretman et al. 2011b). To control for the experimental manipulation, the wings of rival males in the 'All cues' treatments were clipped at the ends, such that they experienced similar anaesthesia and handling to the 'No sound' rival flies, but could still produce courtship song (Ehrman 1966; Powell 1978; Vandenberg et al. 1984). The manipulated rival males were then stored at a density of 10/vial for one further day. Each focal male was placed in an individual vial at 3 days old. The following day, males were placed in exposure treatments by adding one rival male to each focal male's vial for a period of 2 h. This exposure time was chosen as the expression of genes related to perception of and responses to rivals increases following 2 h of exposure to rivals, and in some cases shows maximal expression at this time (Mohorianu et al. 2017b). Following the 2 h exposure period, focal males were flash frozen in liquid nitrogen and stored at -80°C. Hence the focal males, upon which RNA-seq was subsequently conducted, were fully controlled for experimental handling. The entire experiment was repeated 7 days later using the same methodology with an independent cohort of males, to create a second biological replicate. There were 40 males per treatment for each biological replicate.

c) RNA extraction and sequencing (conducted by E. Fowler)

The focal males were split into abdomens (A) and head-thoraxes (HT) over dry ice and 40 body parts were pooled to form one replicate. Total RNA (Ambion mirVana miRNA Isolation Kit, Life Technologies, AM1561) was then extracted from the pooled body parts. The quantity and quality of RNA extracted was assessed using a NanoDrop 8000 Spectrophotometer (Thermo Fisher Scientific) and by running samples on 1% agarose gel. RNA was stored at -80°C until the

sequencing analysis. Single-end, stranded mRNA-seq was conducted on the Illumina HiSeq2000 platform (Baseclear provider) to generate reads of 50bp.

d) Bioinformatics analysis

Quality check, normalisation and identification of differential expression (conducted by I. Mohorianu)

Standard quality check (QC) was first conducted (Mohorianu et al. 2017b), followed by the mapping of reads to the *D melanogaster* genome and transcriptome, full length, no mismatches allowed, using PatMaN (Prufer et al. 2008; Mohorianu et al. 2017b, a). Reads incident to annotated ncRNAs (e.g. t/rRNAs) were excluded from all samples (multimaps on protein coding genes, with a t/rRNA incidence were also excluded). Sub-sampling normalization done on a total of 25M reads (Mohorianu et al. 2017b; Fowler et al. 2018); the gene expression was calculated as the algebraic sum of abundances of incident reads (against coding genes); a quantile normalization was applied to reduce any remaining low-level variation between samples. Differential expression (DE) of gene abundances within and between treatments in the HT and A was quantified. This was done by multiplying expression levels by 20, an empirically determined offset, before calculating the fold change in expression. The offset was used to avoid inflating the differential expression level of low abundance gene expression (Mohorianu et al. 2017a). Differential expression of genes within replicates was defined as an absolute offset fold-change (OFC) greater than a threshold of >1 $\log_2$ OFC (Mohorianu et al. 2017a). Differential expression between treatments was calculated using a hierarchical DE approach (body part and then treatment DE as the first and second levels in the hierarchy, as described in (Mohorianu et al. 2017b). The confidence intervals created on the minimum and maximum values for each set of replicates were compared and differences between the treatment and controls higher than 0.5 log2 OFC, identified differentially expressed genes.

Transcriptomic analysis and gene expression profiling (conducted by A. Dore)

Lists of genes that were called DE in the A and HT, both between biological replicates within a treatment and between different sensory manipulation treatments, were generated (Mohorianu et al. 2017b, a). Functional analysis of differentially expressed genes was performed through enrichment tests on the three GO ontologies (biological function, molecular process and cellular compartment) using GOrilla (Eden et al. 2009), with the full set of expressed genes from the relevant body part as the background list. The role of specific

genes was explored by cross referencing to FlyBase FB2018_05 records (Gramates et al. 2017). Genes called DE between 'All cues' and 'No sound' treatments, in the A and HT respectively, were further characterized using the BioGRID 3.5 database for known protein-protein interactions (Chatr-aryamontri et al. 2017). The known interactions for each DE gene were used to construct networks of gene interactions in Cytoscape 3.4.0 (Shannon et al. 2003). Evidence for alternative splicing was not evaluated in this analysis.

5.4 Results

a) Differential expression of genes within and between treatments

To assess the hypothesis that different subsets of differentially expressed genes driving functionally quasi-equivalent outputs (extended mating) in males in the 'All cues' and 'No sound' treatments, genes that were DE between treatments and between biological replicates were identified. I performed enrichment analyses of their GO annotations of these DE genes against the ontologies.

In the HT, 118 genes showed DE between replicates of the 'All cues' treatment, whereas 287 showed DE between replicates of the 'No sound' treatment. Thirty-seven genes were differentially expressed between the two treatments. Twenty-two were down-regulated in the HT of the 'No sound' treatment relative to the 'All cues' treatment, while 15 were up-regulated (Figure 5.2). The genes differentially expressed between treatments in the HT showed enrichment for reproductive processes including post-mating behaviour and mating plug formation (Table 5.1; e.g. *Ejaculatory bulb protein* (*Ebp*), *Accessory gland peptide 36DE* (*Acp36DE*), *Male-specific ma 57Db* (*Mst57Db*), *Male-specific RNA 84Dc (Mst84Dc)* and *CG31872*). Of these, *Mst57Db* also showed DE between replicates in the 'No sound' treatment. The other above genes were not DE between replicates of either treatment. Though differentially expressed in the HT, several of these genes (*Acp36DE, CG31872, Mst57Db* and *Mst84Dc*) have reproductive roles previously described in the A (Leader et al. 2018).

In the A, 143 genes showed DE between 'All cues' replicates, while slightly fewer (111) showed DE between 'No sound' replicates. Twenty-one genes showed DE between the 'All cues' and 'No sound' sensory manipulation treatments. Of these, 12 were down-regulated in 'No sound' males and 9 were up-regulated. These genes (Table 5.1) showed process enrichment for the metabolism of hormones, e.g. *Acp70A* (aka *Sex Peptide. SP*), which is transferred to the female during mating to promote egg-laying and a female refractory period

(Chapman et al. 2003) and was up-regulated in the abdomen of 'No sound' males. *Found in neurons (fne),* which plays a role in male courtship behaviour (Gramates et al. 2017) was also up-regulated in the 'No sound' males. The activity of genes that showed DE between treatments in the A, including *SP*, was significantly localised to the extracellular region. Neither *SP* nor *fne* showed DE between replicates of either treatment. Two genes consistently down-regulated in 'No sound' males across both the HT and the A, namely *CG31612*, associated with lateral inhibition of neurons, and *CG11775*, linked to the detection of chemical stimuli (Gramates et al. 2017), appear to reflect differences in sensory perception *per se*.

Table 5.1 – **The ontology of differentially expressed genes in male *Drosophila melanogaster* exposed to alternative combinations of rival cues.** The gene ontology terms for head-thorax (HT) and Abdomen (A) genes significantly differentially expressed (regardless of direction of DE) within replicates for 'All cues' (males exposed to all cues of rival presence) and 'No sound' (males exposed to rivals with the auditory cue removed) treatments and between the 'All cues' and 'No sound' treatments. Summarised as GO terms for which $p<0.001$ against a background of the full list of genes expressed in the HT or A, respectively.

	All cues/All cues (replicate DE)	**No sound/No sound (replicate DE)**	**All cues/No sound (treatment DE)**
HT DE			
Process	• Metabolism of chitin (8), aminoglycan (8) and sphingolipid (5) • Fatty acid metabolism (8) and chain elongation (5)	• Immune responses (15) • Responses to external biotic stimuli (19) • Protein localisation to the microtubule cytoskeleton (3)	• Reproduction (5), post-mating behaviour (3), mating plug formation (2) • Microtubule cytoskeleton organisation (5)
Function	• Activity of transferase (8), fatty acid synthase (6) and elongase (5), peptidase (5) and aminoacylase (2) • Chitin binding (7)	• Activity of metallopeptidase (9) and exopeptidase (12) • Manganese ion binding (7)	
Component	• Extracellular region (11) • Endoplasmic reticulum membrane (5)	• Extracellular region (33) • Sperm flagellum (4)	• Extracellular region (9)
A DE			
Process	• Metabolism of carbohydrate (8) and sphingomyelin (2)	• Immune response (16): humoral immune response (14); defence response to bacterium (16) • Response to hyperoxia (5) • Carbohydrate metabolism (13) • Protein deglycosylation (3)	• Hormone metabolic process (2) • Calcium-dependent cell-cell adhesion (2)

		• Proteolysis (18) • Metabolism of sphingolipid (4)	
Function	• Hydrolase activity acting on glycosol bonds (6): maltose alpha-glucosidase activity (4) • Structural constituent of the larval chitin cuticle (6) • Sphingomyelin phosphodiesterase activity (2)	• Hydrolase activity acting on glycosol bonds (13): maltose alpha-glucosidase activity (7) • Serine-type peptidase activity (13)	• Extracellular matrix structural constituent (2)
Component	• Extracellular region (17) • Nucleosome (3)	• Extracellular region (27)	• Extracellular region (6)

To test whether there was any consistency between the genes showing DE in response to cue removal with that of exposure to rivals per se (i.e. rivals present versus absent) I compared genes showing DE between treatments with those identified in a previous study (Mohorianu et al. 2017b) as showing DE following 2h of exposure to rival males. Two genes, *mitochondrial NADH-ubiquinone oxioreductase chain 4L* (*mt:ND4L*) and *mitochondrial NADH-ubiquinone oxioreductase chain 2* (*mt:ND2*) that showed the largest degree of down-regulation in both body parts in 'No sound' males were also both down-regulated in the HT following exposure to rivals (Mohorianu et al. 2017b). This implies a role for these genes in producing responses to rivals, and points to possible differences in how 'No sound' and 'All cues' males produce this response. Two further genes, *SP* and *Attacin-C* (*AttC*), that were up-regulated in the A in 'No sound' males were also found in (Mohorianu et al. 2017b) to be up-regulated in the A in response to rivals. *Lectin-37Da,* which has a role in calcium-dependent cell-cell adhesion (Wagner 2000), was up-regulated in the A of 'No sound' males here and down-regulated in the A in response to rivals (Mohorianu et al. 2017b). Finally, *Ionotropic receptor 75b* (*Ir75b*), down-regulated in the HT of 'No sound' males, was up-regulated in the HT of males in (Mohorianu et al. 2017b). The identification of some overlapping genes suggests that male responses to sensory cues overlap with the known transcriptomic changes that precede responses to rivals.

Figure 5.2 – Up- and down-regulation of genes in the head-thorax and abdomen of male *Drosophila melanogaster* in response to alternative combinations of rival cues. The number of genes called differentially expressed between the 'All cues' and 'No sound' treatments in the abdomen and in the head-thorax that were up-regulated and down-regulated.

b) Networks built on differentially expressed genes

The networks of protein-protein interactions of differentially expressed genes between 'All cues' and 'No sound' treatments in the A and HT (Figure S5.1) were constructed to investigate whether these genes formed networks, complexes or functional cascades. In the abdomen, seven genes that were differentially expressed between the 'All cues' and the 'No sound' had known protein-protein interactions with other genes (Figure S5.1). One of these was *Cadherin 88C* (*Cad88C*), which is involved with calcium-dependent cell-cell adhesion. *Acp70a* or *SP* was differentially expressed in the A between the 'All cues' and 'No sound' treatments and has known interactions with two other genes, perhaps reflecting its role in ensuring male success in sperm competition (Fricke et al. 2009). Other DE genes in the network were *Ionotropic receptor 85a* (*Ir85a*), *Found in neurons* (*fne*), *CG31612*, *AttC* and *Mucin 12Ea* (*Muc12Ea*). *Ir85a* is involved with the detection of chemical stimuli. *Fne* is implicated in the processing of RNAs, male courtship behaviour and sensory perception of pain (Gramates et al. 2017). *AttC* has functions in antibacterial immune responses (Gramates et al. 2017) and is up-regulated in response to rival exposure (Mohorianu et al. 2017b). *Muc12Ea* is involved with neurogenesis, manifests in the nervous system and neuroblast (Bretman et al. 2012), and has a potential role

in Drosophila eggshell production (Tootle et al. 2011). Of these genes, *AttC* showed DE between replicates of the 'No sound' treatment, while the rest showed DE only between treatments and not between biological replicates.

Among genes that showed DE in the head-thorax between 'All cues' and 'No sound' treatments, those with the highest numbers of protein-protein interactions (edges), in descending order, were *Syntaxin 1A* (*Syx1A*), *Male-specific RNA 84Dc* (*Mst84Dc*), *Muscle-specific protein 300 kDa* (*Msp300*), *Futsch*, *Ankyrin 2* (*Ank2*), *CG31140*, *Mutagen-sensitive 205* (*mus205*), *Lodestar* (*lds*), *Met75Cb* and *Met75Ca* (Figure S5.1). These may represent genes with greatest downstream or interacting effects (Mohorianu et al. 2018). *Syx1A,* which has 58 edges in the gene network (30 more than the second most highly-connected gene), is expressed in the nervous system of *Drosophila spp.*, localised to axons and synapses. The protein encoded by *Syx1A* is involved with the regulation of neurotransmitters, as well as nonneural secretion (Schulze et al. 1995; Wu et al. 1999), and binds many other neuronal proteins. The role of *Futsch* and *Ank2* are also manifest in axons and synapses (Gramates et al. 2017). Several of the most highly-connected genes have roles in reproductive responses: *Mst84Dc* and *Lodestar* in spermatogenesis, *Syx1A* in egg production while *Met75Ca* and *Met75Cb* have as yet unspecified roles in reproduction (Gramates et al. 2017). The finding of networks of reproductive genes showing DE between treatments suggests integrated effects and the plausibility of alternative transcriptomic pathways. *Met75Cb, Met75Ca* and *Mst84Dc* showed DE between replicates of the 'No sound' treatment; the other genes noted above did not show replicate-replicate DE within either treatment.

5.5 Discussion

This study represents a test case for the hypothesis that redundant networks can underlie the responses of organisms to multi-component stimuli. The results presented here demonstrated how this can be empirically tested by using mRNA-seq to examine and infer the structure of gene networks, using variation in gene expression as a proxy.

a) Differential expression in reproductive response genes

I tested the alternative hypothesis that the quasi-equivalent phenotypic responses elicited by exposure of males to alternative combinations of cues of rival presence are achieved via alternative transcriptional pathways, versus the null hypothesis that the phenotype is elicited by alternative cue combinations via a single pathway. I predicted that males exposed to

restricted cues of rival presence would show differential expression of subsets of genes related to mating behaviour and reproduction, in comparison to males exposed to all sensory cues. This prediction was generally supported, offering preliminary support for the alternative hypothesis. Genes that were differentially expressed between the 'All cues' and 'No sound' treatments were frequently involved in reproductive processes rather than in sensory cue processing *per se*. The majority of these DE genes with known roles in reproduction showed DE only between treatments and not between biological replicates (4/5 in the HT; 7/10 in the A), suggesting that the differential expression of these genes is not within the range of random variation. Several DE genes with reproductive functions were embedded in a network of gene interactions, suggesting integrated biological effects. Genes that showed differential expression between the 'All cues' and 'No sound' treatments in the head-thorax were enriched for processes involved with post-mating behaviour, including mating plug formation (Lung and Wolfner 2001; Chapman and Davies 2004; Bretman et al. 2010b), sperm storage and sperm competition (Chapman et al. 2000). There was also a significant enrichment for genes with extracellular roles (Table 5.1). None of these functions appear related to the perception of sound. Thus, their differential expression in the comparison of 'All cues' and 'No sound' treatments may suggest that the different cues of rival presence in these treatments initiate differing pathways of responses to rival males. The reason why some genes identified here as DE in the HT (*Acp36DE, CG31872, Mst57Db* and *Mst84Dc*) are reported from studies focused on the abdomen (Gramates et al. 2017; Leader et al. 2018) is not yet clear. *Acp36DE, CG31872, Mst57Db* and *Mst84Dc* were expressed in the HT in our study at very low levels, with much higher expression in the abdomen, in accordance with the FlyAtlas 2 database (Leader et al. 2018). Thus, it remains possible that these have different reproductive roles in the HT.

Among genes showing DE in the HT between treatments, there was also cellular component enrichment for the presynaptic membrane and functional terms related to neurotransmitter receptors (*Syx1A* and *Ionotropic receptor 68a* and *85a*). Ionotropic receptor genes are implicated in chemosensory processes in invertebrates, including the processing of olfactory signals (Leal et al. 2013; Liu et al. 2014). The differential expression of these genes between treatments is likely to be driven by the different sets of sensory cues of rival presence received by males in the 'All cues' and 'No sound' treatments.

The networks reconstructed on differentially expressed genes and the genes with which they interact showed that genes related to the functioning of neurotransmitters and reproductive responses were among those with the highest number of known protein-protein interactions. This suggests that these DE genes may be part of wider networks, and hence

changes in their transcription may have integrated effects. This offers some support for the hypothesis that males receiving different sets of sensory cues of rival presence express comparable responses via different sets of functionally equivalent genes, representing a reconfiguration of an underlying, redundant network (Mohorianu et al. 2017b).

Among genes differentially expressed between treatments in the abdomen, process enrichment for hormone metabolism was partly attributable to *Acp70a*, which induces a range of post mating responses in females, ultimately boosting a male's competitive reproductive success (Chen et al. 1988; Chapman et al. 2003; Liu and Kubli 2003; Chapman and Davies 2004; Fricke et al. 2009). *Muc12Ea* and *Muc68Ca* were identified among the extracellular enriched genes. In general, mucins are gel-like proteins that can line the reproductive tract of mammals (Lagow et al. 1999) and *Muc12Ea* is associated with the production of the *Drosophila* eggshell (Tootle et al. 2011). The protein-protein interactions of *Sex peptide* and *Mucin 12Ea* could indicate that changes in the transcription of these genes have effects across gene networks. In the abdomen, there was additional evidence of DE between treatments of other genes underlying sensory perception and/or reproductive behaviour, including *fne* and *Ionotropic receptors 68a* and *58a*. This suggests the existence of DE in genes additional to those directly attributable to differing sensory perception and supports the idea that the different combinations of rival cues initiate the expression of different sets of reproductive genes, resulting in equivalent responses.

Although differential expression of reproductive genes between the 'No sound' and 'All cues' treatments was identified, it is possible that the expression of these genes is not related to male responses to rivals. The common phenotypic response of males exposed to alternative cue combinations, i.e. extended mating duration associated with increased offspring production (Bretman et al. 2011b), could be driven by those genes that are not differentially expressed between the two treatments. The differential expression identified here could lead to other phenotypic differences between males exposed to the full repertoire of rival cues and those with a cue removed, such as the different thresholds of rival exposure time required to elicit a response (Rouse and Bretman 2016), variation in species recognition (Bretman et al. 2017), or an alternative phenotype not yet characterised. Nevertheless, several of the genes that showed DE between the 'No sound' and 'All cues' treatments (and did not show replicate-replicate DE) are known to affect offspring production, which has been shown to be equivalent when males are exposed to all cues and when the auditory cue is removed (Bretman et al. 2011b). *Sex peptide* induces egg production – females that mated with *SP* knockdown males showed almost no subsequent increase in oviposition (Liu and Kubli 2003).

Furthermore, *Acp36DE* is required for effective sperm storage by the female and affects paternity (Avila and Wolfner 2009). *Acp36DE* mutant males have been found to produce only 10% as much progeny as wildtype males (Neubaum and Wolfner 1999). There is also evidence that *Mst84Dc* is associated with variation in male reproductive success, including offspring production (Fiumera et al. 2007). The fact that 'No sound' and 'All cues' males have been found to show equivalent offspring production (Bretman et al. 2011b) despite the differential expression of these genes suggests the existence of alternative transcriptional pathways to this response. Variation in the expression of genes such as *SP* (to a lesser extent than knockdown) may be compensated for via an alternative expression pathway as part of a redundant network.

In both the A and the HT, the majority of the genes that showed DE between treatments were down-regulated in the 'No sound' males relative to the 'All cues' treatment. This could indicate a possible loss of function related to the lack of processing of the auditory rival cue. However, genes that showed DE between treatments with known roles in reproduction or mating behaviour almost all showed up-regulation in the 'No sound' treatment. This suggests that there may be an increase in some reproductive processes induced by the lack of the auditory rival cue and is consistent with the idea that males in this treatment achieved equivalent competitive responses by using alternative pathways (Mohorianu et al. 2017b). Comparison with a previous study that described gene expression profiles in male *D. melanogaster* following exposure to rivals (Mohorianu et al. 2017b) was done to determine whether the genes differentially expressed between 'No sound' and 'All cues' males were part of the overall set of genes whose expression responds to cues of competition. The two genes that showed the highest levels of differential expression in both body parts between 'No sound' and 'All cues' males were also down-regulated in the HT in response to rival exposure (Mohorianu et al. 2017b). Furthermore, *SP*, which has a known role in mediating male competitive success, and *AttC*, which was found to be a component of a genetic network of DE genes, were up-regulated in the A of 'No sound' males, and also up-regulated in the A following rival exposure (Mohorianu et al. 2017b). This shows that several genes that were differentially expressed between treatments intersected with the overall transcriptomic response of males to rival exposure. This may indicate that the 'All cues' and 'No sound' males use alternative transcriptomic pathways to reach equivalent responses, within the overall network of rival responses available.

Although the limited number of biological replicates in this study did not enable these results to be robustly supported by statistical modelling, I argue that these findings represent

preliminary evidence that exposure to alternative combinations of cues of rival presence may elicit quasi-equivalent phenotypic responses in males via expression of alternative sets of reproductive genes. This demonstrates the potential of mRNA sequencing for empirically testing this concept and warrants further investigation in a larger-scale study.

b) Replicate-replicate variation

In the head-thorax, considerably more genes were differentially expressed between biological replicates in the 'No sound' versus the 'All cues' treatment, whereas in the abdomen a similar number of genes showed DE for both. Interestingly, among the genes that showed DE between treatments and were found to have roles in reproduction, none showed replicate-replicate DE within 'All cues' but 4 showed DE between replicates in the 'No sound' treatment. Furthermore, of the five genes that showed treatment-treatment DE and were also found to be up-regulated in response to rivals by Mohorianu et al. (Mohorianu et al. 2017b), three showed replicate-replicate DE within the 'No sound' treatment. Collectively, these results suggest a higher level of variation in reproductive genes within the 'No sound' treatment than the 'All cues' treatment. It may be that the removal of a cue increases uncertainty about the environment, which increases variation between individuals in how rival responses are initiated. The perception of multi-component signals has been proposed to increase the accuracy of phenotype-environment matches (Dore et al. 2018) and allow consistent, targeted responses to cues (Bretman et al. 2011b). This theory suggests that restricting the number of cues available may reduce the accuracy and consistency of responses. Bretman et al. (2017) found that interfering with the olfactory or auditory cues of rival presence increased 'off-target' competitive responses by male *D. melanogaster* to heterospecific rivals, demonstrating that responses can become more generalised in response to increased environmental uncertainty. Removing one cue in a repertoire may inhibit an organism's ability to produce a targeted response, thus increasing variability in gene expression.

c) Conclusions

This study demonstrates that the concept of redundant networks as a mechanism for bridging the gap between environmental stimuli and phenotypic responses can be empirically tested. I examined patterns of gene expression in male *D. melanogaster* exposed to different combinations of cues of conspecific males. I found preliminary evidence that could support an underlying redundant network of gene expression, allowing males to produce comparable responses via alternative, functionally equivalent genetic pathways. This demonstrates the potential for empirically testing the redundant networks concept as a model for understanding

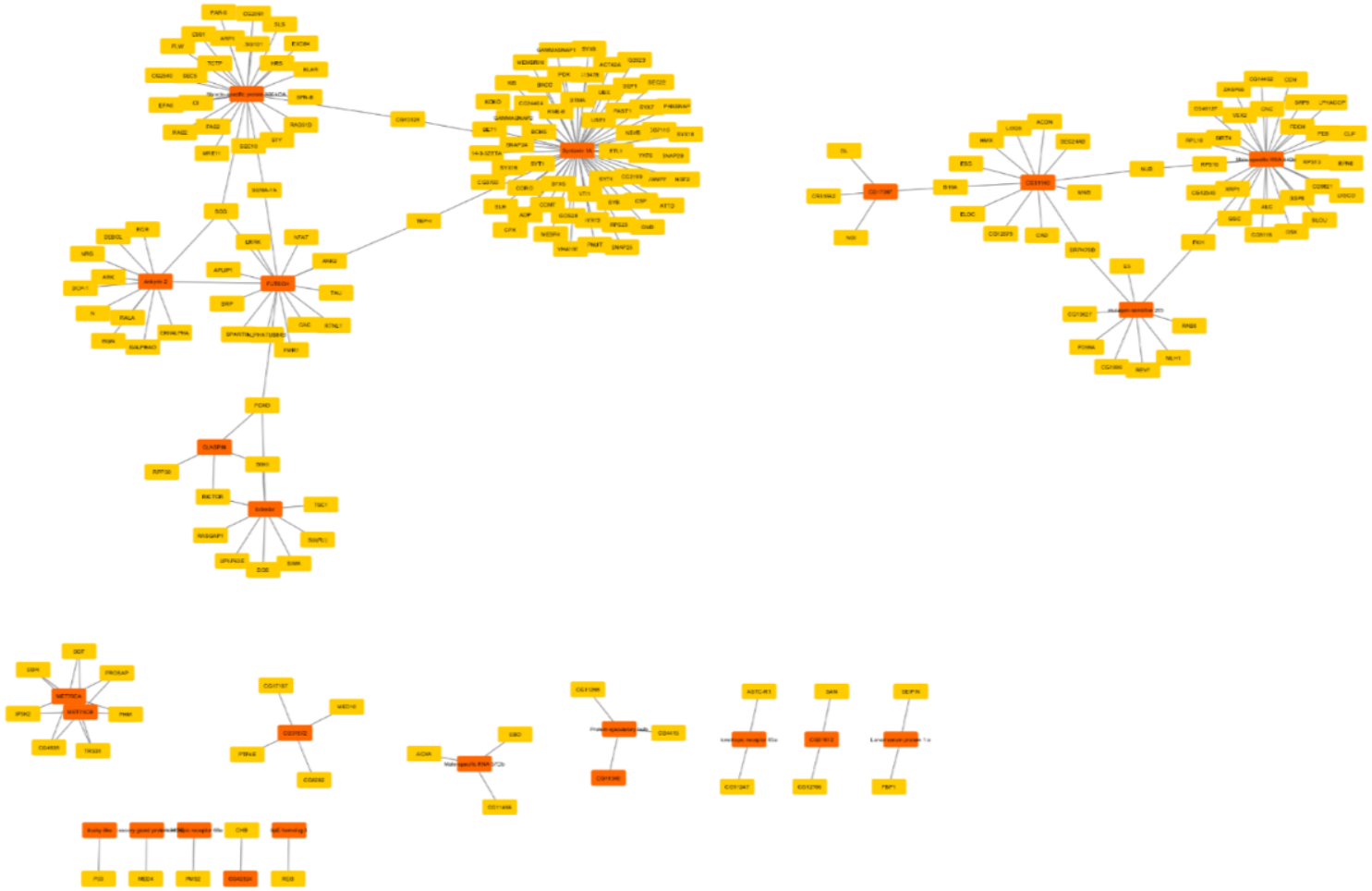
the genetic mechanisms underlying phenotypic plasticity. The findings suggest that it would be valuable to further investigate this concept with a larger number of biological replicates and more treatment groups, including a negative control, in order to determine whether the existence of alternative transcriptional pathways can be robustly supported.

Previous research has shown that changes in gene expression in response to the social environment, including courtship and competitive interactions, can underpin phenotypic plasticity, impact fitness, and influence life-history trajectory (Carney 2007; Ellis and Carney 2011; Kasumovic 2016). It has been proposed that social plasticity can be achieved by transcriptional changes within a neural network (Oliveira 2012; Simões et al. 2015). Here, I build upon this by presenting preliminary evidence that these genomic networks can be characterised by redundancy, meaning that different pathways of gene expression elicit analogous phenotypic responses. Additional research is needed to continue to shed light on the structure of such genomic networks and provide further empirical testing of these concepts.

The role of redundancy and robustness in the production of plastic responses has interesting evolutionary implications. It has been suggested that selection for robustness may result in correlations between signals that are initially independent of each other, leading to the evolution of multimodal, redundant signals. The processing of such signals may evolve by 'piggybacking' on existing signalling systems, and may be beneficial to the receiving animal by balancing the amount of information perceived with the cost of losing meaning if one cue component is knocked out (Ay et al. 2007). However, fully redundant systems may confer high robustness but low evolvability. Partial redundancy can also increase robustness and may extend the variability in phenotypes produced in response to a given set of cues, therefore boosting the evolvability of the system (Whitacre and Bender 2010; Whitacre 2010). Hence, understanding the way that redundant signals are processed may shed light on how such redundancy has evolved, and the evolutionary lability of the plastic responses that are influenced by these signals.

5.6 Supplementary information

a)



b)

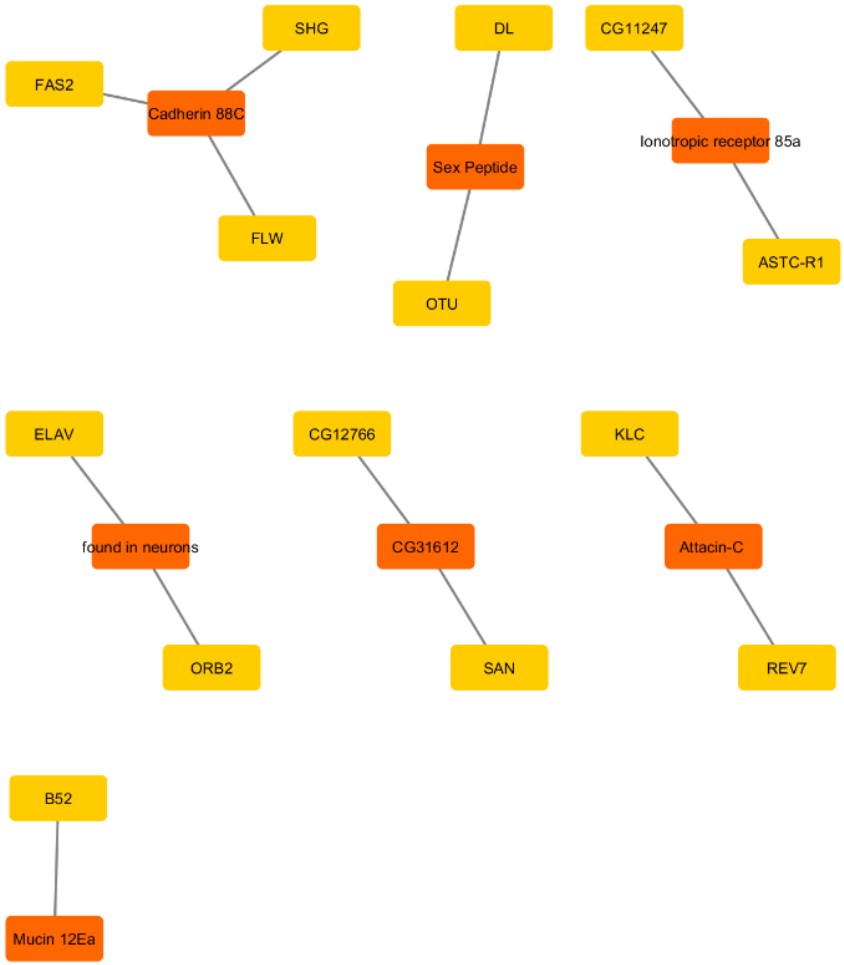

SHG
FAS2
Cadherin 88C
FLW
DL
Sex Peptide
OTU
CG11247
Ionotropic receptor 85a
ASTC-R1
ELAV
found in neurons
ORB2
CG12766
CG31612
SAN
KLC
Attacin-C
REV7
B52
Mucin 12Ea

Figure S5.1 – **Genetic networks of differentially expressed genes in male *Drosophila melanogaster* exposed to alternative combinations of rival cues.** Networks of protein-protein interactions created using genes differentially expressed in (a) the abdomen and (b) head-thorax between males exposed to all cues of rival presence ('All cues') and those exposed to rivals with the auditory cue removed ('No sound'). Genes in red are DE between treatments, while genes in yellow have known interactions with those DE genes. Note that networks with larger numbers of interactions may be more strongly supported and are less likely to represent false positive. Follow links for larger images and to zoom in: abdomen https://photos.app.goo.gl/9XTUySBmPEJ4BE237; head-thorax https://photos.app.goo.gl/yaHteBXUBvTvEqTu6.

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6. General discussion

6.1 Chapter 1: General introduction. Summary

In this thesis, I aimed to investigate the reproductive plasticity of male *D. melanogaster* in response to the social and sexual environment. In Chapter 1, I described the strong selective pressure that can be exerted by the social/sexual environment on male reproductive behaviour and morphology. In variable environments, plasticity that allows individuals to tailor their phenotype to the current conditions often confers an adaptive advantage. Such plasticity can allow males to strategically allocate reproductive effort across mating opportunities (Parker et al. 1996; Parker et al. 1997; Wedell et al. 2002). In my research, I tested the sensitivity of plastic traits to the social environment at different stages of development, measured the evolutionary responses of both mating investment and reproductive plasticity *per se* in fixed environments, and investigated the perception and processing of redundant social cues that inform plastic responses.

6.2 Chapter 2: The effect of social cues on developmental and adult plasticity. Summary of main findings and implications

In Chapter 2, I showed that the accessory glands and testes of male *D. melanogaster* show plasticity in response to the social environment during development. I built on previous research by Bretman et al. (2016) by examining the degree of sensitivity in developmental plasticity of reproductive structures. I investigated whether the presence of adult males in the larval environment elicits a different plastic response compared to the presence of adult females. Furthermore, I investigated the potential for the accessory glands and testes to remain responsive to the social environment during their final stages of development, in the hours immediately after eclosion. My findings suggested that the size of the reproductive structures respond to both the presence and the sex of adults in the larval environment, indicating a high degree of environmental sensitivity. Furthermore, there was some suggestion that both the larval and post-eclosion environment can influence the size of the accessory glands. Contrary to predictions, the presence of adult males in larval vials generally resulted in smaller accessory glands and testes. Conversely, higher male density in the post-eclosion environment appeared to result in males developing larger accessory glands. This suggested that the direction of the effect of cues indicating male-male competition may vary at different

stages of development. However, within the three experiments that were conducted in Chapter 2, the results were not fully consistent. Furthermore, the finding that accessory gland size decreased in response to the presence of adult males during development was contrary to previous findings by Bretman et al. (2016). This raised questions about the robustness of perimeter and area as measurements of accessory gland and testis size. Moreover, I proposed that to gain a more comprehensive understanding of developmental plasticity of the reproductive structures, their size should be measured at different life stages and in different social contexts. The length of the third longitudinal (L3) wing vein, which I used in these experiments as a proxy for body size, also showed an unexpectedly high degree of variability. Therefore, I discussed the plasticity of wing morphology and the potential limitations of L3 length as a proxy.

Further research could investigate alternative methods of quantifying investment in reproductive structures during development, in order to produce more robust conclusions to questions on the sensitivity of reproductive morphology to the environment at various developmental stages. The procedure I used for measuring the size of the accessory glands and testes via 2D imaging may have introduced inaccuracies. The use of 2D images to study 3D structures is common due to its simplicity and low cost. However, 2D data represent a proxy, and inevitably cannot capture all the information inherent within 3D morphology (Cardini 2014). Although it has been suggested that the error generated by transferring 3D structures to 2D data is often minimal (Cardini 2014), in the case of the complex and malleable reproductive structures such of *D. melanogaster* this process may still introduce important confounding influences. In particular, *D. melanogaster* testes are tightly coiled and must be flattened out to obtain a 2D image. This process may have distorted the dimensions of the testes, accounting for the low repeatability of measuring a testis by area (Chapter 2). Three-dimensional imaging may allow for better understanding of the architecture of the reproductive structures. For example, multicolour fluorescence imaging has been employed to image *Drosophila* testes (Kibanov et al. 2013). Alternatively, simply weighing the mass of reproductive structures (Droney 1998) may avoid some of the issues of 2D imaging. Further research could complement the experiments described in Chapter 2 by determining whether the same relationship between the developmental environment and the size of the reproductive structures is identified when the structures are measured using 3D data and/or by mass.

Furthermore, Chapter 2 could be built upon by further investigations into the biological significance of the size of the accessory glands and testes prior to a male's first

mating. In Chapter 2, I discussed the possibility that the size of these structures at a single point in time soon after emergence confers limited information on a male's potential for ejaculate production and investment. Testis size alone may not be an accurate proxy for sperm production (Schärer and Vizoso 2007). Furthermore, the size of the accessory glands is dependent upon the quantity of seminal fluid proteins (SFPs) they hold, which varies throughout adult lifespan (Hihara 1981; Linklater et al. 2007; Fedorka et al. 2011; Hopkins et al. 2019). However, it is conceivable that the size of these structures at eclosion reflects an increased capacity for sperm and SFP production. Future research could seek to clarify this relationship. Focal males could be exposed to alternative social environments during development, then a subset could be dissected for measurement of the accessory glands and testes. Mating assays could be conducted on the remaining focal males to measure mating duration, ejaculate investment, number of offspring and/or sperm competitiveness from the males' first mating. This may shed light on environmentally mediated correlations between the size of the reproductive structures and capacity for early mating investment. Further studies into the significance of the size of the reproductive structures at eclosion could be elucidated by tracking their size, and the reproductive fitness of males from different developmental environments, after varying numbers of matings and at different ages. This could illuminate whether developmental plasticity in the accessory glands and testes affects reproductive investment and fitness of early matings only, or has an influence throughout the adult lifespan. Furthermore, this could uncover possible context-dependence in the relationship between the developmental social environment and the size of the male reproductive structures, which could help to explain the inconsistencies observed between experiments.

6.3 Chapter 3: Plastic male mating behaviour evolves in response to the competitive environment. Summary of main findings and implications

In Chapter 3 I investigated how the extent of reproductive investment by male *D. melanogaster*, and the degree of plasticity in reproductive responses, can vary with the social environment. To do this, I tested the plastic reproductive behaviours of males experimentally evolved under fixed sex ratios that were either male-biased (MB), equal (EQ) or female-biased (FB), to examine how the level of male-male competition may exert selection on the extent and flexibility of male reproductive investment. Each of these sex ratio treatments was replicated on both a standard SYA diet (100g yeast per litre) and a 20% yeast diet (20g yeast per litre, with the quantities of all other components unchanged), in order to determine the effect of potential resource limitation on the expression of plastic mating investment. I found

that, in general, males that evolved under MB sex ratio expressed longer mating durations. This indicated a fixed, increased level of reproductive investment in response to the consistently high degree of sperm competition predicted to be prevalent in the MB regimes (Pitnick et al. 2001; Wigby and Chapman 2004; Rostant et al. 2020). These males also expressed novel, plastic responses to rivals not observed in wildtype, EQ or FB males: courtship intensity was significantly reduced following exposure to competitors, resulting in longer mating latencies. This was possibly an evolutionary response to frequent interruption of courtship by other males in the MB environment (Ewing and Ewing 1984; Tauber and Eberl 2002). I did not find any evidence to support my predictions that nutritional restriction would limit the expression of reproductive investment or plasticity, or that plasticity would erode under stable sex ratio. Together, these results supported the view that the costs of maintaining behavioural plasticity are small or negligible, such that the capacity for plasticity persists even when the benefits of expressing it are low (Scheiner and Berrigan 1998; Maughan et al. 2007; van Buskirk and Steiner 2009; Murren et al. 2015).

There is conflicting research on whether costs to maintaining plasticity exist, and if they do occur, whether they are substantial enough to select for the erosion of plasticity over evolutionary time. The degree of variability in an environment is predicted to influence the optimal degree of plasticity in a response – i.e. whether a more generalist (plastic) or more specialist strategy is favoured (Chapter 1; Gabriel et al. 2005; Botero et al. 2015). However, even in heterogeneous environments organisms often do not express a highly generalist phenotype, but rather show a limited range of plasticity (Murren et al. 2015). This suggests that there are limitations to the evolution of plasticity, and/or costs to its maintenance and expression (DeWitt et al. 1998). For example, maintenance costs may arise if sensory and regulatory mechanisms must be formed during development in order for plasticity to be expressed, which would not be required for the expression of a fixed response (Moran 1992; DeWitt et al. 1998). It is important to distinguish these costs of plasticity *per se* from the costs of expressing the phenotype itself (Murren et al. 2015). In Chapter 3, I discussed the potential that plasticity in the mating duration of male *D. melanogaster* is maintained under stable levels of male-male competition either because there are only small or negligible costs of the maintenance of this plasticity, or because there are significant benefits accrued from continuing to respond to even microvariations in the social environment.

In many instances, the absence or limited range of plasticity may be explained by alternative mechanisms rather than by the existence of maintenance costs. The fact that plasticity is not ubiquitous in heterogeneous environments may be largely due to limitations to

the benefits of plasticity and/or constraints on its evolution, rather than direct costs of its maintenance. For example, the benefits of plasticity may be negated by time lags between the change in environment and the matching of the phenotype (DeWitt et al. 1998). Furthermore, when the environment favours plasticity its evolution may be constrained by gene flow, genetic correlations or a lack of genetic variation (Murren et al. 2015). Where the loss of plasticity in stable environments does occur, it may be due to mutation accumulation, rather than selection (Masel et al. 2007; Maughan et al. 2007). Alternatively, the maintenance of plasticity under stable environments may relate to redundancy and pleiotropy in the genetic networks controlling plastic responses. In Chapters 1 and 5 I discussed the potential for phenotypic responses to be underpinned by complex genetic networks characterised by robustness and redundancy. It has been suggested that pleiotropic interactions may help to maintain phenotypic plasticity that is no longer under positive selection (Kingma et al. 2020).

Although there is evidence to suggest that the evolution of plasticity is not substantially influenced by maintenance costs, this may not be a universal rule for all plastic responses. Plastic responses, the sensory/regulatory mechanisms they require, and the strength of selection on them, are likely to diverse. For instance, the mechanisms necessary to monitor the presence of rival males and mount a corresponding change in mating behaviour may have relatively low costs, or may be maintained by selection due to their roles in other processes. This could account for the maintenance of plasticity in mating duration following evolution under fixed sex ratio, as described in Chapter 3. On the other hand, theoretically, a sensory system that monitors the environment for the sole purpose of informing a plastic response might be expected to be lost over evolution in a stable environment, if there is a developmental cost of producing or maintaining it (Hall and Colegrave 2008). Some plastic responses are determined via developmental selection, which involves the production of a range of phenotypes, some of which are reinforced or continually expressed on the basis of environmental feedback. Two examples of this are the diverse range of antibodies produced by vertebrate immune systems, some of which are amplified following interactions with antigens, and 'trial-and-error' behavioural learning (Snell-Rood 2012). Compared to switch-like or determinate mechanisms, plasticity produced by developmental selection may be costly to maintain due to the time and energy expenditure of sampling multiple phenotypes. It has also been suggested that the costs of maintaining plasticity are generally higher for organisms with more sophisticated brains or immune systems (Murren et al. 2015). Therefore, it is likely that the relative importance of costs of maintaining plasticity, evolutionary constraints and mutation accumulation on the evolutionary trajectory of a plastic trait will be dependent on

several factors. These factors may include the mechanisms of producing the plastic response, the role of these mechanisms in other processes, and the characteristics of the organism.

Although the results in Chapter 3 show that plasticity in mating duration by male *D. melanogaster* was maintained following evolution under fixed sex ratios, there was some evidence to suggest that the expression of this plasticity was context dependent. The extended mating phenotype elicited by exposure to rivals was diminished in matings between MB males and co-evolved MB females. This could suggest that while the capacity for plasticity in mating duration was maintained, this plasticity may not be expressed in the environment of the MB lines. Unpublished data (A. Łukasiewicz and T. Chapman) support the idea that the reproductive traits of males and females from these lines can be dependent on whether they are mating with wildtype or co-evolved individuals. These data support the finding that MB males have evolved longer mating duration, and furthermore show that following rival exposure, MB males mate for significantly longer to wildtype females than MB females. Extended mating latency expressed by MB males following rival exposure was also found by Łukasiewicz & Chapman to be more marked in matings with MB females. This could imply further adaptive plasticity in mating behaviours, whereby males are able distinguish females from different populations and tailor their mating latency and duration accordingly. Further research could seek to quantify fitness benefits from these behaviours in various social contexts, to determine whether this is an adaptive response.

As well as investigating context-dependence in the expression of reproductive plasticity, it would be interesting to conduct further studies into the evolution of novel plastic responses among males from MB regimes. The results presented in Chapter 3 showed a marked decrease in courtship intensity delivered by MB males following exposure to rivals, resulting in longer mating latency. However, these experiments only tested reproductive behaviours in scenarios with one virgin male and one virgin female in the mating arena. In the environment of the MB regimes, it is highly likely that other males will be in the immediate vicinity during courtship and copulation. Males are also likely to frequently encounter females that have previously mated (Wigby and Chapman 2004; Rostant et al. 2020). Future studies could examine courtship and mating behaviour in scenarios more closely resembling the evolutionary environment. For example, the courtship behaviour of males and their mating success could be tested with multiple males in the courtship arena, where the female is able to exercise mate choice. Furthermore, similar experiments could be conducted to compare the courtship and mating behaviour of virgin vs. previously mated males and females. It would also be interesting disentangle the magnitude of paternal and maternal effects on the expression of

plasticity. Finally, genomic analyses could be conducted on experimentally evolved males to identify genes under selection and their individual effects. For example, transcriptomic data could be collected from experimentally evolved and wildtype males with and without rival exposure, to compare patterns of gene expression and identify genes of interest for further in-depth study.

Experimental evolution populations maintained on a diet with restricted yeast did not consistently show a reduced investment in either reproductive plasticity, or overall reproductive investment. Males evolved on a 20% yeast diet maintained plasticity in mating duration, and generally did not show reduced mating duration compared to males from 100% yeast regimes. This could suggest that reproductive plasticity and investment in mating duration do not carry substantial costs that would result in resource-limitation. Alternatively, other dietary components such as carbohydrates may play a more important role than protein in limiting male reproductive investment. Previous research has showed that protein availability has important effects on remating frequency, egg production and lifespan in female *D. melanogaster*, and may also mediate male *Drosophila* reproductive fitness (Chapman and Partridge 1996; Droney 1998; Fricke et al. 2008). However, other studies have suggested that the quantity of carbohydrates in the male diet, or the balance of nutritional components, may be more influential on mating investment (Maklakov et al. 2008; Mason et al. 2016). Future research could seek to clarify the relationship between nutrition and male mating investment by quantifying the reproductive investment and fitness of males maintained on varying dietary regimes.

The interpretation of findings from these experimental evolution lines could be strengthened by direct tests of mating allocation, effective population size and operational sex ratio in the sex ratio regimes. Some research has been conducted to observe the behaviour of males and females within the regimes. For example, Rostant et al. (2020) examined courtship frequency, mating frequency and sex-specific lifespan within the sex ratio and diet treatments. Further observations of the behaviour of males and females within the environment of the experimental evolutionary lines could increase understanding of the selective pressures affecting individuals in these lines. For example, such experiments could determine whether males can most effectively increase their mating success by investing in aggressive interactions with rivals, by intensively courting females, or by coercing females to remate. Examining mating allocation in these lines could also allow measurement of the operational sex ratio and effective population size. Snook et al. (2009) calculated that the effective population size of the sex ratio lines does not substantially vary between the MB, EQ and FB populations.

However, it would be useful to supplement this with empirical data. Directly measuring operational sex ratio and effective population size could increase understanding of the role of inbreeding and genetic drift on the evolution of the experimental evolution lines.

6.4 Chapter 4: Fitness consequences of redundant cues of competition in male *D. melanogaster*. Summary of main findings and implications

In Chapter 4, I tested whether olfactory, auditory and tactile components of the cues signalling rival presence to male *D. melanogaster* are redundant in terms of the behavioural response and associated fitness benefits they elicit (Bretman et al. 2011b). I examined cue redundancy in a wider range of social/sexual environments than has been previously tested, by comparing the reproductive fitness of males in both the presence and absence of sperm competition, following exposure to alternative combinations of cue components. Male *D. melanogaster* were exposed to rivals either with the full cue intact, or with the auditory component removed, then mated to a virgin female. Mating duration and offspring production after the first mating were measured. Subsequently, the female was remated and the paternity of resulting offspring was scored, to determine the success of the focal male in sperm defence. I found support for the conclusion that removing one cue component does not prevent males from detecting rivals and responding with significantly longer mating duration, consistent with redundancy. As I discussed in Chapters 1 and 4, such redundancy may facilitate reproductive plasticity by increasing the robustness and/or quantity of the environmental information perceived, or by reducing the time lag between cue and response (Bretman et al. 2011b; Dore et al. 2018). However, I also found that males that mated for longer did not father more offspring or have greater success at sperm defence, either when exposed to all cue components or when one component was removed. This contrasted with previous studies showing that longer mating is associated with increased ejaculate transfer and greater offspring production by male *D. melanogaster* (Bretman et al. 2009; Wigby et al. 2009; Bretman et al. 2011b). Taken with other recent findings (Bretman et al. 2012; Hopkins et al. 2019), this suggests that the relationship between the social/sexual environment, behavioural responses, ejaculate investment and fitness effects is not as direct as previously assumed. Alternatively, the results could suggest that not all elements of cues and their fitness benefits have yet been determined.

To resolve this, future research could seek to better understand the adaptive value of extending mating duration in response to cues of competition. Although extended mating duration did not significantly affect offspring production, female receptivity to remate after 24 h, or paternity under sperm competition, there could be other fitness benefits of this behaviour not measured in Chapter 4. For example, female receptivity to remating was measured only at one point in time, 24 h after the first mating. Extension of mating duration by the focal male could have reduced or delayed female remating within or after the first 24 h, which would not have been detected in my experiments, but might still confer fitness benefits. While many studies of male-induced effects on female receptivity test remating frequency at either 24 or 48 h after the initial mating, research has shown that important changes take place within the first 12 h (Scott 1987; van Vianen and Bijlsma 1993; Bretman et al. 2010b). Although it can take some time for long-term ejaculate-induced effects to establish, reduced receptivity within the first day after mating may be elicited by the act of copulation itself or by particular seminal fluid proteins (Manning 1962; Manning 1967; Scott 1987; Bretman et al. 2010b). If extended mating duration could amplify these short-term effects, significant fitness benefits may be gained. Other potential 'hidden' fitness benefits of longer mating that could be investigated in future research include sperm displacement ability. In my experiments focal males were only tested as the first male to mate. It would be interesting to test the effects of longer mating with previously mated females, to investigate any influence of extended mating on the displacement of sperm from earlier matings (Gilchrist and Partridge 1995).

Identifying redundancy among cue components signalling rival presence to male *D. melanogaster* has been important for better understanding the detection and processing of these cues (Bretman et al. 2011b). Without knowledge of this redundancy, for instance, the observation that removing the auditory cue of rival presence does not inhibit the competitive behavioural response of males may lead to the conclusion that this cue has no role in rival detection. Applying a framework that incorporates principles of redundancy more generally to animal signalling and perception could contribute to better understanding of the intricacies of these systems (Ay et al. 2007; Hebets et al. 2016). Within the *D. melanogaster* system, for example, this could be applied by testing for partial or complete redundancy among visual, auditory, olfactory, gustatory and tactile cues exchanged between males and females during courtship and mating (Greenspan and Ferveur 2000; Ferveur 2005). Tests of redundancy could also be incorporated in the elucidation of cues involved with social learning among female *D. melanogaster*, in which the presence of mated females and the presence of their fertilised eggs both appear to play a role (Sarin and Dukas 2009).

6.5 Chapter 5: Redundant networks and alternative expression pathways: a test case using conspecific competitive responses in male fruit flies. Summary of main findings and implications

In Chapter 5, I addressed the question of whether responses to redundant cue components may be underpinned by alternative pathways of gene expression. I aimed to empirically test the hypothesis that different combinations of cues signalling *D. melanogaster* male rival presence elicit equivalent extensions in mating duration via the expression of alternative, functionally equivalent sets of genes. This built on previous data showing significant differential expression of genes with roles in reproduction among males exposed to rivals, suggesting that there is flexibility in the pathways of gene expression that produce responses to cues of competition (Mohorianu et al. 2017). I found evidence that males exposed to a full repertoire of rival cues vs those with the auditory cue removed exhibited differential expression of genes with likely roles in responses to competition. This provided some support for the idea that alternative transcriptional pathways within redundant genetic networks can contribute to the expression of plastic responses. The data also offered support for the important role of redundancy in biological systems, as a way of increasing robustness at the levels of both sensory perception and gene expression (Edelman and Gally 2001; Greenspan 2012). Furthermore, this study demonstrates the potential for empirically testing the mechanisms linking the perception of social cues and the expression of a plastic phenotype. This could be valuable for broadening understanding of the biological processes involved in plasticity, which may be relevant to identifying costs of its expression and constraints on its evolution.

Future research could seek to expand on this preliminary work by undertaking a more extensive and controlled study of pathways of gene expression in male *D. melanogaster* exposed to alternative cues of rival presence. It would be valuable to reproduce this study with a greater number of biological replicates, and with a control group of males that were not exposed to rivals. Furthermore, some protocols to mechanically remove the olfactory and tactile components of rival cues may have confounding effects on focal males (see Chapter 4 Supplementary Information). The tactile, auditory and olfactory cues of rival presence appear to be interchangeable in terms of eliciting a behavioural response (Bretman et al. 2011b), suggesting that removing the auditory cue alone should be representative of the effects of

removing any single cue component. Nevertheless, future studies could aim to cleanly remove the olfactory and tactile cue components in turn, to determine whether the results presented in Chapters 4 and 5 are generalisable to the removal of any one cue component. This could help to identify possible incomplete redundancy within the cue of rival presence. Alternative methods for removing olfactory and tactile components while avoiding unintended effects on the focal male include the use of genetic mutants and manipulations (e.g. Rouse and Bretman 2016), although this also requires controls for genetic background.

Subsequent studies could also adopt a similar approach to the one described in Chapter 5 to examine patterns of gene expression underpinning plastic responses to other complex cues. One instance where this approach could be applied is the multimodal courtship of *Schizocosa* wolf spiders (Taylor et al. 2005; Uetz et al. 2009; Gordon and Uetz 2011). Male spiders increase their use of visual display components when seismic elements are inhibited, suggesting redundant, backup cue components. Although females show responses to isolated visual and seismic cue components, faster responses and increased receptivity are elicited by the multimodal cue (Uetz et al. 2009). Analysing the gene expression of females exposed to isolated cue components vs. the full, multimodal cue could indicate whether the cue components result in increased expression of the same genes when perceived together, or individually elicit the expression of different genes which produce a stronger response when expressed together. This could shed light on the mechanisms of plasticity in this instance, as well as contributing towards testing the broader applicability of the concept of genetic redundancy underpinning phenotypic plasticity.

6.6 The use of proxies for studying reproductive plasticity

An issue highlighted by this thesis is the need for care when selecting proxies for traits such as reproductive effort and interpreting their biological significance. This is particularly pertinent for proxies of complex traits, for example the use of mating duration to infer ejaculate investment. Previous research has found that extended mating duration is associated with increased transfer of two key SFPs, ovulin and sex peptide (Wigby et al. 2009), resulting in higher offspring production (e.g. Bretman et al. 2009). However, throughout Chapters 3 and 4 I found no relationship between longer mating and either offspring production, female receptivity to remating, or sperm defence. While mating duration may be informative to some degree of male reproductive effort, my results suggest it is not a robust and simple proxy for

ejaculate investment. Therefore, a deeper understanding of the adaptive value of mating duration as a behavioural response to competition is needed.

There is a consensus that longer mating duration often correlates with increased sperm transfer (Simmons 2001; Pilastro et al. 2007; Weldingh et al. 2011). However, in *D. melanogaster,* sperm transfer takes place rapidly and is completed by the midpoint of copulation, suggesting that there is no simple relationship between mating duration and sperm investment (Gilchrist and Partridge 2000). Furthermore, mating duration and sperm transfer are subject to distinct neurological controls in *D. melanogaster* and can vary independently of each other in some social contexts (Lüpold et al. 2011; Bretman et al. 2012; Crickmore and Vosshall 2013). Mating duration has also been found to be a poor proxy for compositional changes in seminal fluid proteins associated with exposure to competition (Hopkins 2018; Hopkins et al. 2019). In other taxa, longer copulation has variously been associated with higher fertility and fecundity, increased success under sperm competition, and reduced female remating receptivity (Edvardsson and Canal 2006; Omkar et al. 2006; Weldingh et al. 2011; Klemme and Firman 2013). However, extended mating duration has been found to have a nonsignificant effect on fertilisation rate in *Linyphia triangularis* spiders (Weldingh et al. 2011). In the bruchid beetle *Callosobruchus maculatus*, mating duration had no effect on sperm precedence or immediate female remating propensity, and longer copulations by the first male to mate with a virgin female actually led to lower success under sperm competition (Edvardsson and Canal 2006). Together, these findings suggest that while mating duration is likely have some bearing on reproductive success, there is no simple, direct relationship between mating duration, ejaculate investment and fitness. Measuring mating duration has the benefit of being relatively quick and simple to do. Furthermore, it is non-invasive, allowing further behavioural experiments to be conducted on the same individuals. However, directly measuring the number of sperm transferred during a mating or seminal fluid protein composition of the ejaculate may allow more robust conclusions to be drawn regarding how male ejaculate investment responds to the social environment (e.g. Garbaczewska et al. 2013; Hopkins 2018; Hopkins et al. 2019). Methods such as spectrophotometry, real-time quantitative PCR, microarrays or RNA sequencing could be used to quantify the amount of sperm and SFPs in an ejaculate and to track the expression of individual SFP genes (Perry et al. 2013).

I also encountered problems with the use of L3 wing vein length to represent body size. This measure has been proposed as a simple, robust proxy for body size (e.g. Bretman et al. 2016) but showed an unexpectedly high degree of variability in my experiments. In one

instance, L3 length was found to be significantly affected by the social environment after eclosion (Chapter 2), by which point body size is expected to be fixed (French et al. 1998). This suggests that the relationship between L3 length and body size is not as robust as previously thought and may be partly mediated by environmental factors. This is supported by the finding that the ratio of L3 length: thorax length can vary with nutritional stress and temperature (Barker and Krebs 1995; Loeschcke et al. 2000; Gilchrist and Huey 2004). Furthermore, research suggests that wing size and shape itself may express adaptive plasticity and be under selection (BitnerMathé and Klaczko 1999; Debat et al. 2003; Debat et al. 2009; Menezes et al. 2013), offering further support for the idea that L3 length may not be simply or directly linked to body size. It would be interesting to further investigate the extent to which wing morphology may vary independently of body size in response to features of the social and sexual environment, for example to facilitate courtship song (Menezes et al. 2013). However, for quantifying body size, a measure such as thorax length may be more reliable than using a wing trait as a proxy.

Together, these findings highlight the need to take care in choosing proxies and to be cautious in interpreting their biological meaning. In particular, complex traits that encompass several factors, such as ejaculate investment, may not be accurately and robustly represented by a univariate proxy. The example of L3 length and body size highlights that the relationship between a proxy and a trait of interest may vary across environments, which can complicate the interpretation of results.

6.7 Future directions: cross-generational transfer of social information

Circumstances allowing, I would have conducted a final experiment to test whether social information on the level of male-male competition can be transferred across generations. The experience of males, including social experience, has been found to affect the fitness of their offspring via paternal effects (Adler and Bonduriansky 2013; Crean et al. 2013; Jensen et al. 2014; Guillaume et al. 2016; Evans et al. 2017; Dasgupta et al. 2019). Dasgupta et al. (2016) found that male *D. melanogaster* housed with three other males produced sons that mated for significantly longer than the sons of males that had been housed alone. However, the effects of paternal social experience on the plastic responses of sons to rivals has not been tested. I hypothesised that males exposed to competition may sire sons that are primed to respond more strongly to rivals and increase their reproductive success under cues of competition. Investigating this could shed light on whether the social experience of the previous generation

can be predictive of the level of competition that male offspring will experience, allowing for adaptive parental effects. Alternatively, if no effect of paternal social experience on plastic responses of sons was observed, this could suggest that the rapidly-changing nature of the social environment favours short-term plastic responses based on recent experience.

This experiment could be conducted by assigning males from the parental generation to treatments in which they are either housed with three rival males or housed alone. Subsequently, these males could be mated to produce the focal generation. A subset of male offspring could be dissected to measure the size of the accessory glands and testes. Remaining male offspring could be assigned to treatments in which they would either be exposed to three rival males or housed alone. A mating assay could be conducted to test mating latency and duration – or, given the issues identified with mating duration as a proxy for ejaculate investment, sperm and SFP transfer could be quantified. The number of offspring produced following this mating could be counted. A finding of larger accessory glands and/or testes among sons of males that experienced competition could suggest that social information can be transmitted across generations, resulting in males that are primed to invest more heavily in early reproduction. Identifying a stronger adaptive response to rivals among sons of males that experienced competition, in terms of increased ejaculate investment and/or greater offspring production, would further evidence this. Such a finding would support the hypothesis that male-male competition in the paternal environment is used as a predictor of competition during the adulthood of the offspring, influencing male plastic responses. Conversely, a finding that the social experience of fathers did not influence the plastic morphology and behaviour of their sons would suggest that this social information is not transmitted across generations. This could be explained by the rapidly changing nature of the social environment (Kasumovic et al. 2008; Bretman et al. 2011a), which may mean that the environment that offspring will encounter cannot be predicted based on paternal experience.

6.8 Future directions: the influence of social experience on male plastic responses

Future research could also examine the effect of social experience and learning on plastic male responses to competition. This could build on findings on how the social/sexual environment informs the immediate behaviour of males, by examining the mid- to long-term effects of social cues on plastic responses. Furthermore, it would be interesting to further examine how

remembered social experience in multiple modalities can be compared with incoming information to inform mating decisions (Bailey and Zuk 2009). Research on *Teleogryllus oceanicus* field crickets has shown that social experience, for example auditory cues received during development, can affect adult behaviours such as mate preference, reproductive investment and mating tactics (Bailey and Zuk 2008; Bailey and Zuk 2009; Bailey et al. 2010). Furthermore, social experience has been found to interact with the genotype of male *D. melanogaster* to influence courtship intensity, demonstrating the potential for studying the effects of social experience on reproductive behaviour in this system (Svetec et al. 2005).

Male *D. melanogaster* require a minimum of 24 h exposure to rivals for an extension in mating duration to be elicited, suggesting that this threshold is used to determine whether the current environment accurately represents the prevailing level of sperm competition (Bretman et al. 2010a). It would be interesting to investigate whether this 24 h threshold is fixed or can be influenced by previous social experience. For example, a male that has previously experienced persistent cues of sperm competition may be expected to respond more readily to rivals in the immediate environment after a shorter period of exposure. Once a behavioural response to rivals is elicited in male *D. melanogaster*, this response persists for 12 h after the removal of competition (Rouse and Bretman 2016). Social experience may also be expected to have some influence over the persistence of the extended mating phenotype. For example, a male that does not have previous experience of cues of sperm competition may respond more 'cautiously' by expressing a response that lasts a shorter time, while males that have previous experience of rivals may mount a more persistent response. As many studies on the reproductively plastic behaviour of male *D. melanogaster* are conducted on virgin male and females whose social experience prior to the experiment is controlled (e.g. Bretman et al. 2009; Dore et al. 2020), investigating the potential influence of social experience on mating behaviour would help to determine the extent to which the results of these previous studies can be generalised. Further investigating mechanisms of social learning and memory may also shed light on the role of complete or incomplete redundancy in social cues for producing plastic responses. The modification of mating duration in response to rivals by male *D. melanogaster* is controlled by mechanisms localised to the mushroom bodies, suggesting olfactory cues are particularly important for these processes (Rouse et al. 2018). Furthermore, removing the olfactory or auditory components of rival cues raises the exposure threshold required by males to produce a behavioural response (Rouse and Bretman 2016). Future studies investigating the influence of previous social experience on male responses to rivals could test the effects of experiencing separate combinations of olfactory, auditory and tactile

cue components. This could help to distinguish the roles of these modalities in social experience and learning and expand understanding of the extent of redundancy among these cue components (see Chapter 4).

6.9 Conclusions

I conducted experiments on male reproductive plasticity in response to the social and sexual environment, examining developmental and adult plasticity, the evolution of reproductive plasticity and investment, and the perception and processing of redundant cues to elicit such responses. I presented evidence that the social environment may impact reproductive morphology across developmental stages, possibly up to a later point than previously thought (Chapter 2). I showed that both fixed and plastic behaviours can evolve rapidly in response to the social environment and reported novel plasticity in the courtship behaviour of males evolved in highly competitive environments (Chapter 3). I found evidence for redundancy in cues eliciting behavioural male responses to rivals, but I question the relationship between these behavioural responses and ejaculate investment (Chapter 4). Finally, I investigated the possibility that robust responses to the social environment may be underpinned by redundancy at the genetic level (Chapter 5). Overall, the research in this thesis provides an advance in the field by presenting previously undescribed responses of male reproductive phenotypes to the social and sexual environment, both within individual lifespans and across evolutionary time. This is important because it demonstrates the powerful effects of context-dependence on reproductive behaviour and fitness, with potential implications for the evolution of mating systems and reproductive isolation.

6.10 References

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Appendix

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REVIEW

The role of complex cues in social and reproductive plasticity

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Abstract
Phenotypic plasticity can be a key determinant of fitness. The degree to which the expression of plasticity is adaptive relies upon the accuracy with which information about the state of the environment is integrated. This step might be particularly beneficial when environments, e.g. the social and sexual context, change rapidly. Fluctuating temporal dynamics could increase the difficulty of determining the appropriate level of expression of a plastic response. In this review, we suggest that new insights into plastic responses to the social and sexual environment (social and reproductive plasticity) may be gained by examining the role of complex cues (those comprising multiple, distinct sensory components). Such cues can enable individuals to more accurately monitor their environment in order to respond adaptively to it across the whole life course. We briefly review the hypotheses for the evolution of complex cues and then adapt these ideas to the context of social and sexual plasticity. We propose that the ability to perceive complex cues can facilitate plasticity, increase the associated fitness benefits and decrease the risk of costly 'mismatches' between phenotype and environment by (i) increasing the robustness of information gained from highly variable environments, (ii) fine-tuning responses by using multiple strands of information and (iii) reducing time lags in adaptive responses. We conclude by outlining areas for future research that will help to determine the interplay between complex cues and plasticity.

Keywords Phenotypic plasticity · Sexual selection · Fitness · Reproductive success · Multimodal · Communication

Introduction

Phenotypic plasticity is the extent to which an organism with a given genotype can express alternative phenotypes under different environmental conditions (Gause 1947; Bradshaw 1965). It may represent a key component of adaptation to rapid environmental change (Agrawal 2001; Charmantier et al. 2008). In this review, we discuss how plasticity expressed in response to the social and sexual environment may be facilitated by the perception of environmental cues composed of multiple distinct components ('complex cues'; Hebets and Papaj 2005). We focus on the socio-sexual environment, as it is likely to be both complex (involving multiple individuals) and rapidly changeable (Charmantier et al. 2008; Kasumovic et al. 2008). We identify the factors that influence the proximate expression of plastic responses to the social and sexual environment (social and reproductive plasticity) and discuss how these can ultimately affect the evolution of plasticity. Simple and complex cues are first defined within the context of social and sexual plasticity, and their potential roles across the whole life course are then discussed. We summarise current hypotheses for the evolution of complex cues and adapt these concepts to social and sexual plasticity. We propose that perceiving complex cues may facilitate plasticity and avoid costly phenotype-environment mismatches by (i) maximising information transfer in variable environments, (ii) facilitating the fine-tuning of phenotypes to the environment and (iii) reducing time lags between perception of cues and expression of plasticity.

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Simple and complex cues in assessment of the social environment

In this review, we consider a 'cue' as any kind of indicator that can be used to perceive information about the social or sexual

environment by an individual. Such indicators can be either 'intentionally' or unintentionally transmitted by a signalling individual (Glossary). For instance, body size, which can potentially signal information on aspects of morphology/general condition, versus visual/auditory displays, which give potentially more targeted information, can both be considered as cues. A 'complex cue' comprises two or more distinct subcomponents exchanged during the course of one encounter, which is capable of inducing or influencing a response in a receiver (Hebets and Papaj 2005; Glossary). This contrasts with 'simple cues', in which information received is a single component. In addition, complex cue components can be perceived from one ('unimodal') or multiple ('multimodal') sensory modalities (Hebets and Papaj 2005). Complex cues would be unimodal if female choice was influenced by two or more male sexual ornaments, all processed visually (Møller and Pomiankowski 1993; Auld et al. 2016). An example of multimodal complex cues is the response of male fruit flies to conspecific rivals, with longer mating durations in male *Drosophila* spp. elicited following detection of mating rivals via combinations of three sensory modalities: song, smell and touch (Bretman et al. 2011a, b; Maguire et al. 2015).

Individual components within complex cues may also elicit a receiver response on their own and then interact to alter this response (i.e. 'multiple signals') or elicit a response only if perceived together ('multicomponent signals'; Glossary). We not only focus here mainly on transmission of information between individual signallers and receivers of the same species but also briefly outline the collation of information from multiple signallers. Our emphasis is on the assessment of complex cues by the receiver, the resulting expression of social and reproductive plasticity and associated fitness benefits in the receiver. We do not extensively discuss here the adaptive benefits of complex cues to the signaller, and we make no assumptions about whether information transmission is active or passive. However, a comprehensive understanding of complex cues requires the full roles of signaller and receiver to be evaluated (Hebets and Papaj 2005).

We focus on complex cues in which the components are expressed simultaneously, or near simultaneously, during one reproductive event/encounter and which then directly initiate a receiver response (rather than effects on future phenotypes). However, it should also be noted that the timing of the delivery or perception of different cue components is important (Hebets and Papaj 2005). For example, the successful integration of multiple different cues expressed at different times, potentially across different reproductive events or life stages, may rely on learning or memory. In this way, individuals may employ past experiences and memory to inform their behaviour (Dukas 2006; Bailey and Zuk 2009). Hence, complex cues can also influence the learning and memory abilities of the receiver ('receiver psychology hypothesis'; Table 1). A detailed investigation of learning in the expression of plasticity and the perception of temporally separated complex cues is reviewed elsewhere (Hebets and Papaj 2005).

The perception of complex cues may play an important role in the expression of adaptive plasticity in response to varied and rapidly changing social and sexual environments (Charlat et al. 2007; Bretman et al. 2011a) according to factors such as the density of conspecifics and characteristics of competitors and mates (Oliveira 2012). Phenotypic plasticity is predicted to evolve in environments that vary rapidly, with intermediate to high predictability—where predictability refers to the degree to which a cue is correlated with future environmental conditions (Botero et al. 2015). Social environments may often meet these criteria, as they can be variable over a short timescale (Bretman et al. 2011a). Features of the future social environment (e.g. sperm competition) can often be at least partly predicted by current conditions (e.g. the presence of other males in the vicinity). Moreover, responses, such as choosing mates or strategically allocating reproductive investment are directly linked to fitness. Hence, complex cues might be particularly relevant in a social/sexual context if, as predicted, they can increase accuracy and/or speed of the response to environmental change and hence minimise the effects of mismatches in phenotypes directly linked to fitness (Charlat et al. 2007; Bretman et al. 2011a; Fig. 1).

Plastic responses can be modelled as a reaction norm—a function describing the expression of an individual's phenotype across an environmental gradient (Via et al. 1995; see Nussey et al. 2007 for discussion of reaction norm approach). A plastic response can be characterised by the elevation (the degree of expression of the response) and the slope (the extent to which the expression of the response changes across the environmental gradient, i.e. the degree of plasticity). Individuals may vary in both the elevation and slope of a response due to genetic and non-genetic factors, such as experience and condition (Blumstein and Bouskila 1996; Nussey et al. 2007). Furthermore, social interactions are proposed to influence the evolution of reaction norms, affecting both between-individual and within-individual variation in the elevation and slope of a social response (see Dingemanse and Araya-Ajoy 2015). Theory on complex cues can be applied to reaction norms, with the environmental gradient of the reaction norm referring to the composite message that the complex cue confers. For example, an environmental gradient of potential sperm competition can be assessed through a complex cue of rival presence consisting of olfactory, auditory and tactile stimuli (Bretman et al. 2011b). Plastic responses to such gradients can occur on a spectrum of specialist to generalist (Gabriel et al. 2005). A more specialist strategy may be characterised by a steeper gradient, resulting in a highly expressed response in some environments and a low level of expression in others, whereas a generalist strategy may be modelled by a flatter reaction norm. Time lags between environmental

Table 1 Hypotheses for the evolution of complex cues in animal communication, developed in the context of social/sexual plasticity. Evidence for the potential selective advantage of each hypothesis is given

Hypothesis	Theory	Evidence	Possible role in social/sexual plasticity
'Backup signal' 'Redundant signal'	Multiple cues convey one message. The receiver benefits by assessing the message with increased accuracy. The signaller may benefit when the cost of signalling is reduced by spreading investment across multiple components (Møller and Pomiankowski 1993; Johnstone 1996).	Female swordtail fish distinguish hetero- and con-specific males more accurately based on both chemical and visual cues (Hankison and Morris 2003); male wolf spiders use more visual courtship displays when seismic components are inhibited (Gordon and Uetz 2011).	Improved robustness of information transmission in fluctuating social environments (Bro-Jørgensen 2010) and/or accelerated passing of a stimulus threshold (Rouse and Bretman 2016), resulting in phenotypes better suited to the current environment.
'Multiple messages'	Each cue conveys a different message to one receiver. For example, different sexual ornaments could reflect different aspects of male quality. The signaller and/or the receiver may benefit by increasing the scope of information that can be exchanged (Møller and Pomiankowski 1993).	Components of great tit (*Xiphophorus pygmaeus*) birdsong are related to different measures of male quality (Rivera-Gutierrez et al. 2010); agonistic male-male signalling in eland antelopes (*Tragelaphus oryx*) reflect separate aspects of fighting ability (Bro-Jørgensen and Dabelsteen 2008).	Plastic responses can be fine-tuned to multiple features of the environment.
'Unreliable signal'	Only one cue is a reliable indicator of quality. Any other signals are maintained because they are not costly to produce and are subject to weak Fisherian runaway selection (Fisher 1930). The signaller gains some benefit from the additional, more minor mate preference. The receiver does not gain any increase in the accuracy of the message (Møller and Pomiankowski 1993; Hankison and Morris 2003).	Bill brightness is significantly correlated with male mating success in mallards (*Anas platyrhynchos*) and plumage only loosely correlated (Omland 1996); female red jungle fowl (*Gallus gallus*) show a primary preference for male comb colour and weaker preferences for other ornaments (Johnsen and Zuk 1996).	No likely application to social/sexual plasticity.
'Emergent message'	A single message emerges through the combination of non-redundant cue components. May benefit the receiver by conveying a more general and informative message based on multiple factors (Partan and Marler 2005; Bro-Jørgensen 2010).	Multiple species of songbirds account for a trade-off between trill rate and frequency bandwidth when assessing trills (Ballentine et al. 2004; Illes et al. 2006; Bro-Jørgensen 2010).	Plastic responses can be fine-tuned to multiple features of the environment, and information transmission is more robust.
'Alerting signal'	One cue component may catch the attention of the receiver and direct it towards one or more other, informative signals. The signaller and the receiver may benefit from improved transmission of the message(s) (Hebets and Papaj 2005; Bro-Jørgensen 2010).	Bornean ranid frog (*Staurois guttatus*) calls direct the attention of conspecifics towards a visual foot-flagging display (Grafe and Wanger 2007); olfactory signals from male Gasterosteidae sticklebacks may make females alert to subsequent visual signals and increase detection (McLennan 2003).	Informative cues are made more salient, resulting in a phenotype better-matched to the social environment.
'Receiver psychology'	Complex cues may benefit the signaller and the receiver by enhancing detection, discrimination, learning and memory of the message (Guilford and Dawkins 1993; Candolin 2003; Hebets and Papaj 2005; Bro-Jørgensen 2010).	The presence of auditory signals improves the speed of colour discrimination in domestic chicks (*Gallus Gallus domesticus*; Rowe 2002); audiovisual stimuli enhance song acquisition and quality in nightingales (*Luscinia megarhynchos*; Hultsch et al. 1999).	Informative cues are more salient, influential or efficiently processed; as in 'alerting signal'.
'Sensory overload'	In agonistic interactions, the signaller may benefit from the transmission of complex cues by reducing the accuracy and/or speed of message transmission (Hebets and Papaj 2005; Bro-Jørgensen 2010).	Dark-eyed juncos (*Junco hyemalis*) react more slowly when exposed to alarm calls in addition to a visual cue, compared to the visual cue alone (Randolet et al. 2014).	The response of the receiver may be rendered less advantageous or more costly due to time lags (Padilla and Adolph 1996; DeWitt et al. 1998) and phenotype-environment mismatches to the benefit of the signaller.

change and response, and receiving incomplete information on the environment, are predicted to result in a trade-off between specialist and generalist strategies of reversible plasticity (Gabriel et al. 2005). As discussed below, complex cues may confer more complete environmental information and elicit faster responses, allowing for higher

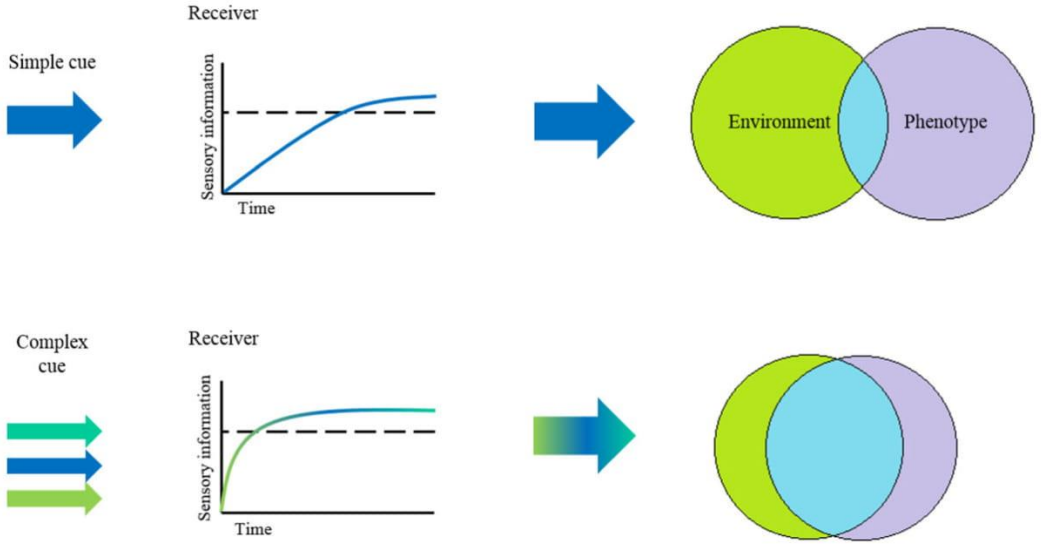

Fig. 1 Complex cues reduce time lags. The perception of cues from the social and sexual environment comprising multiple distinct sensory components (complex cues in multiple colours versus simple cues in one colour) can decrease the time taken to reach sensory thresholds required to initiate a response (dotted line), resulting in a shorter time lag between environmental change and phenotypic change and hence a better-adapted phenotype

maximum fitness in the current environment at reduced cost to future fitness when the environment changes.

Social and sexual plasticity is expressed by both sexes

Adaptive reproductive plasticity is expected whenever reproductive investment or resources are limiting. Individuals might respond to the presence, density, local sex ratio, quality or relatedness of conspecifics, and this may affect both behavioural and physiological investment in reproduction such as mate choice, displaying or mate guarding, fecundity, oviposition site choice or parental care (Glossary). We expect social plasticity to be common in females, because reproductive investment is often substantial in this sex. The empirical data support this view, with female mate choice often observed as highly socially plastic (Rodriguez et al. 2013; Lyons et al. 2014). Other female reproductive behaviours also exhibit social plasticity. For example, oviposition by *Drosophila melanogaster* females can be tailored to the social environment. When encountering a new site, females show a preference to oviposit in the presence of another female who has already laid eggs at that site (Sarin and Dukas 2009). However, it is becoming increasingly evident that males may also often exhibit reproductive plasticity. This is because, contrary to the traditional view that a male's reproductive investment is much lower than that of females (Bateman 1948; Clutton-Brock and Parker 1992; Ahnesjo et al. 2001), it is now known that ejaculate production can incur considerable costs (Dewsbury 1982; Parker 1982; Wedell et al. 2002). Hence, a trade-off between investing in current and future mating opportunities can shape the optimum ejaculate transfer for any given reproductive episode and hence select for plasticity (Dewsbury 1982; Parker 1982; Wedell et al. 2002). There is abundant evidence that males can adapt their ejaculate investment to features of their social environment, for example, to the presence of rivals and to female quality (Wedell et al. 2002; Bretman et al. 2011a). This suggests that social plasticity in reproductive phenotypes can confer substantial benefits and conversely that fixed or 'trial-and-error' responses may result in lower fitness. However, in order for such plasticity to be adaptive, it should be based on accurate information (DeWitt et al. 1998; Auld et al. 2010). Hence, the role of complex cues in increasing the quantity and quality of social information, in comparison to simple cues, can be important for both sexes.

Complex cues may have a particular role in intersexual conflict. For example, there can be a selective advantage to males conveying deceptive cues to females regarding their individual quality, while females are selected to detect honest information (Holland and Rice 1998). As females evolve resistance to one deceptive male trait, males may evolve new cues to manipulate female perception of quality, resulting in multicomponent mating displays. In this way, sexual conflict has been proposed to promote the evolution of complex cues (Candolin 2003; Bro-Jørgensen 2010). Further research on the relationship between sexual conflict and complex cues is required to fully understand these dynamics.

The importance of cues varies across the life course

The perception and processing of complex and simple cues, as well as the fitness consequences of social and sexual plasticity, are likely to vary across life stages (Groothuis and Taborsky 2015). The level of plastic responses can be fixed irreversibly during development (developmental plasticity), either in anticipation of the future environment (anticipatory plasticity) or as a response to the current conditions (reactive developmental plasticity; Kasumovic and Brooks 2011; Kasumovic 2013; Snell-Rood 2013). Alternatively, plasticity can occur as a rapid, flexible and potentially reversible response at any life stage (activational plasticity; Snell-Rood 2013). In activational and reactive developmental plasticity, the use of complex cues to produce faster responses (Fig. 1) may be particularly beneficial in avoiding time lags between cue detection and phenotype expression and hence poor environment matching. Complex cues may also be beneficial in providing more and higher quality information to fine-tune plastic phenotypes (Kasumovic 2013; Table 2). The way complex cues are processed, and the relative importance of the separate components for determining the response, may vary across different kinds of plasticity. Cue components that predict the future environment and/or are fairly stable over time may be more important for determining irreversibly plastic responses. In the case of relatively quick and reversible responses, cue components that fluctuate in relation to the immediate environment may be more pertinent.

Table 2 Hypotheses to explain the benefits of the use of complex cues and their underlying assumptions

Hypothesis	Underlying assumptions
1. Complex cues can prevent information loss in variable environments	(i) Simple cues are significantly compromised in variable environments.
	(ii) This loss of information Leads to phenotypes mismatched to the environment. The resulting deleterious effects reduce fitness and hence exert selection for processing complex rather than simple cues.
	(iii) Cue components can convey equivalent information and are interchangeable to the extent that the overall message is intact if one or more components are compromised.
2. Complex cues can fine-tune plastic responses based on multiple features of the environment	(i) Cue components provide at least partially different information.
	(ii) Perception of a greater quantity of environmental information results in a better phenotype-environment match.
3. Complex cues can reduce time lags between environmental change and response	(i) Sensory thresholds for initiating responses exist.
	(ii) Complex cue components additively or synergistically contribute to meeting these thresholds.
	(iii) The information transferred by each cue component is correlated.
	(iv) A fast-responding phenotype confers adaptive benefits.
	(v) These benefits outweigh potential costs of changing the phenotype in response to an ephemeral environmental fluctuation.
All the above	(i) Genetic and phenotypic variation in ancestral populations existed, upon which natural selection acted to promote the processing of complex cues.
	(ii) Organisms have the sensory and cognitive capacity to receive, process and integrate more than one cue component.
	(iii) The production of complex cues either Directly benefits the signaller, or occurs for other purposes and is co-opted by the receiver.
	(iv) A phenotype that is more closely matched to the social/sexual environment confers fitness benefits.
	(v) These benefits outweigh the potential costs of processing complex cues.

There are also specific periods of development, known as sensitive windows, during which phenotypes can be strongly influenced by the environment. The number, strength and consistency of complex cues may be particularly important in accurately translating this environment (Fawcett and Frankenhuis 2015), particularly if it is highly variable (Bro-Jørgensen 2010). The fitness consequences of receiving accurate and informative cues from the immediate environment may be higher during early life because an individual has less experience of the longer-term prevailing environment (Frankenhuis and Panchanathan 2011). Later in life, this may be less important; for instance, as the probability of re-mating in the future declines with age it may become more advantageous to invest heavily in current mating opportunities regardless of the environment (Rebar and Greenfield 2017). In this scenario, the perception of complex cues will become less advantageous with age as the fitness benefits of environment-phenotype matching diminish. Complex cues and the potential for cue redundancy might also become increasingly advantageous with age and increasing senescence in sensory perception and cognitive processing.

The evolution of complex cues

To date, the evolution of complex cues has mostly been discussed in the broader context of animal communication. We explore here how the predominant hypotheses for the selective advantages conferred by the use of complex cues can also be applied to the context of social and reproductive plasticity (Table 1). The evidence to support these hypotheses in this new context is variable, and so we also suggest ways in which the perception and production of complex cues could evolve under these scenarios.

The 'backup signal' and 'multiple messages' hypotheses are currently the best known (Table 1; Johnstone 1996; Partan and Marler 2005; McElroy et al. 2007; Girard et al. 2015). They emphasise the predominance of redundant and non-redundant cues, respectively (Johnstone 1996; Partan and Marler 2005; Stynoski and Noble 2012). Redundancy could ensure that responses are based on robust information, while non-redundancy could increase the range of the environmental information available to inform optimal phenotype expression (Table 1). Evidence for the backup signal hypothesis comes from the expression of reproductive plasticity by male *D. melanogaster* in response to intrasexual competitors, via the perception of auditory, olfactory and tactile cues. Any two of these cues in combination, or all three, result in the same magnitude of response, implying redundancy or incomplete redundancy (Hypothesis 3 below; Bretman et al. 2011b). In the multiple message hypothesis (Box 1), each cue conveys a different message. This is supported in the context of reproductive plasticity by the example of the detection of pheromones by *D. melanogaster* males, in which separate cues signal female presence versus mating status, respectively (Siwicki et al. 2005; Lacaille et al. 2007).

Box 1 Mechanisms underlying signal integration

Integral to understanding the ultimate consequence of social and sexual cues is resolving how the mechanisms mediating cues and behaviour control plastic responses to dynamic environmental information. Short-term plastic responses to transient stimuli may be achieved via different mechanisms than those underlying longer-lasting responses. For example, rapid responses may be achieved by switches in neural state and persistent responses by changes in gene expression (Winbush et al. 2012; Montague and Baker 2016). There is evidence for this from courtship suppression in *Drosophila melanogaster*, in which multiple signal components are integrated into pathways resulting in a plastic response. In this system, males exposed to a previously mated female for 1 h plastically reduce their courtship effort towards any female encountered in the next 2–3 h (Siegel and Hall 1979). The chemical signal 9-pentacosene, which indicates the presence of a female (Siwicki et al. 2005), is integrated with stimuli that signal the female's recent mating status, via components of the male cuticular hydrocarbon profile, which are transferred to the female during courtship and copulation (Lacaille et al. 2007). These stimuli initiate the cyclic adenosine monophosphate (cAMP) pathway, which is associated with learning and memory and involves a molecular cascade in the mushroom bodies of the brain (centres of olfactory learning in *Drosophila*; Siwicki and Ladewski 2003; Keene and Waddell 2007; Montague and Baker 2016). In addition to these neurological effects, expression changes in genes associated with long-term memory of courtship rejection were observed 24 h after exposure to a mated female (Winbush et al. 2012). This suggests that longer-term plastic responses can be induced by differential gene expression when neural correlates of signals are continually reinforced. This example also exemplifies the integration of multiple signal components into one pathway, potentially leading to signal 'thresholds' required to generate a response being met more quickly (Griffith and Ejima 2009).

It is likely that the backup signal and multiple messages hypotheses, and their associated benefits to plasticity, are not mutually exclusive. Cues could be partially overlapping in information content or may convey different information via alternative combinations (Johnstone 1996; Ay et al. 2007). Evidence for this idea comes from ornate tree lizards (*Urosaurus ornatus*), in which male quality, which can affect plastic responses of competitors and potential mates (Kolm 2001; Swierk and Langkilde 2013), is communicated by a complex cue of multiple morphological and behavioural characteristics. Some characteristics are correlated, indicating a repertoire of partially overlapping cue components (McElroy et al. 2007). This may confer benefits to the receiver in terms of the robustness of the cue and the range of information transmitted. Nevertheless, the possibility for multimodal cues to act in a redundant or compensatory way likely depends on flexibility in cue production and in the cue components that can initiate a receiver response. It is possible that this may impose substantial evolutionary constraints (Gray et al. 2014), an idea tested in *Teleogryllus oceanicus* field crickets, in which female choice is based both on male song and CHC

composition. A flatwing male morph, unable to produce song, has recently evolved in some Hawaiian populations. However, there has been no concomitant increase in the attractiveness of cuticular hydrocarbons, suggesting that the reduced ability to attract females via acoustic cues is not compensated through other sensory modalities (Gray et al. 2014). Therefore, insights into the evolution of complex cues could be gained by considering a spectrum from fully redundant to fully non-redundant cues, as well as recognising that cues may combine in different ways to convey different messages (Ay et al. 2007).

Whether the benefits of receiving complex cues fall under the multiple messages or backup signal hypotheses may also depend on the extent to which the social/sexual environment is predictable (Botero et al. 2015). In scenarios where the future conditions are closely correlated with current cues, the selective pressure to receive redundant complex cues as 'back-up' for cue components with poor predictive accuracy is likely to be weaker. On the other hand, receiving multiple, highly predictive cue components may increase the amount of environmental information on which a future phenotype can be based, as described by the multiple messages hypothesis. When the environment is moderately predictable, redundant complex cues may allow for robust information to be received and an appropriate response to be expressed, even if one or more cue component has declined in predictive accuracy.

Potential advantages to the signaller are also key to the evolution of complex cues (Table 1). For example, in mutualistic interactions when the fitness interests of the signaller and the receiver are correlated, both may benefit from the increased robustness of information derived from complex cues. On the other hand, if there is a conflict of interests between the signaller and the receiver, the perception of complex cues could also evolve in scenarios in which only the receiver benefits, if cues arising in another context are intercepted by receivers. For example, components of cuticular hydrocarbon profiles transferred from males to females during courtship and mating are likely to have evolved prior to the capacity of later-arriving males to detect such cues to infer a female's mating status (Siwicki et al. 2005; Lacaille et al. 2007). Selection for such detection by late-arriving males would not depend on benefits to the female. Transferring information via complex cues may also evolve in situations in which the signaller, but not the receiver, benefits (sensory overload hypothesis; Table 1). This can occur if the receiver has a pre-existing sensory and/or cognitive capacity to detect and process cues that the signaller can exploit (Valkonen et al. 2014).

The integration of multiple cue components to inform a response to the social and sexual environment may be subject to cognitive constraints (Table 2). However, even apparently 'simple' organisms can achieve this, as such neurological integration of multiple sensory inputs appears common in insects (Wessnitzer and Webb 2006; Leonard and Masek 2014). In *D. melanogaster*, male plasticity in courtship effort (Box 1; Siwicki and Ladewski 2003; Keene and Waddell 2007; Montague and Baker 2016), responses to rival males (Rouse et al. 2018) and female mate choice and oviposition decisions (Dickson 2008; Joseph et al. 2009; Sarin and Dukas 2009; Joseph and Heberlein 2012; Lin et al. 2015) all use multicomponent sensory information, in various combinations, and all utilise common genetic and neural pathways and brain regions. These studies are revealing that disparate behaviours relying on different combinations of cues can utilise similar neural mechanisms. Thus, once evolved, the neural costs of novel plasticity may actually be relatively low.

Studying the perception and processing of complex cues, and plasticity itself, is complicated by the fact that the detection and discrimination of cues does not always lead to an observable response. In addition to the necessary fitness benefits and mechanisms for plastic responses to complex social cues to evolve, resource availability and trade-offs may limit when, and to what degree, a response is expressed. Furthermore, the assessment and decision-making processes linking the perception of cues to the expression of a response may vary depending on the characteristics and experience of the individual (Blumstein and Bouskila 1996). Variation in how cue perception translates to a response may occur both between and within individuals depending on sex, age, experience, state and genotype (Blumstein and Bouskila 1996). For example, there may be individual variation in sensitivity to particular cue components. Redundancy in complex cues could override this variation such that the same message is received regardless of which cue components are strongly or weakly perceived. Alternatively, under the multiple messages theory, a complex cue may confer a different meaning to a male versus a female, or a young individual versus an old individual, depending on the differential sensitivity of each individual to specific cue components.

Hypotheses for the benefits of complex cue use in social and reproductive plasticity

The evolution of complex cue perception relies on the assumption that the resulting fitness benefits outweigh costs (DeWitt et al. 1998; Auld et al. 2010) and on the absence of cognitive and evolutionary constraints (Table 2). Costs incurred by signallers producing complex over simple cues could include greater energy expenditure, higher risk of predation and disease, and increased potential for eavesdropping (Hebets and Papaj 2005; Bro-Jørgensen 2010). Receiver costs are less well studied but are likely to be associated with the increased energetic and cognitive effort of processing a greater quantity of information (DeWitt et al. 1998). It is also possible that components of complex cues may be unreliable or even

contradictory, leading to receiver costs associated with the potential to be misled about an environment.

There may also be instances where the perception of complex cues increases uncertainty about the environment, rather than reducing it (Munoz and Blumstein 2012). One potential example of this is when the components of a complex cue vary over different temporal scales. Under the multiple messages hypothesis, this may be advantageous in some scenarios by conferring multiple strands of information about features of the environment that similarly vary differentially over time. For example, a display signalling male quality may include fixed cue components indicating good genes and temporally variable signal components indicating current state, e.g. parasite load (Hebets and Papaj 2005). However, in some cases, the overall meaning of the message may depend on synchrony between cue components. When these cue components become decoupled, the message may be disrupted, and the optimum receiver response may not be expressed (Taylor et al. 2011). Even when cue components are in synchrony, if multiple components each convey environmental information with a margin of error, perceiving a complex cue may increase the potential for incorrect information to be perceived.

Despite these potential costs of receiving complex cues, the many examples of social and reproductive behaviour in which there are significant disadvantages of phenotype-environment mismatches (Gwynne and Rentz 1983; Preston et al. 2001; Presgraves et al. 2003; Bretman et al. 2013) suggest that such costs can be outweighed, giving a selective advantage to the perception of robust and informative complex cues (Candolin 2003; Hebets and Papaj 2005; McElroy et al. 2007; Bro-Jørgensen 2010; Bretman et al. 2011b; Auld et al. 2016). Whether the benefits of processing complex cues outweigh the costs is likely to depend upon the individual's experience, the 'missed opportunity' cost of not responding to the environmental change and the features of the current social environment (Munoz and Blumstein 2012; Munoz 2015). For example, if a female first processes one cue component which indicates that a nearby male is likely to be a heterospecific, there would be little benefit to processing additional components to determine the quality of the male. However, if the first cue component indicated that the male was a conspecific, the benefits of synthesising additional cues to further discriminate the type of male, and subsequent effects on reproductive fitness, may outweigh the costs of further information processing. Thus, processing complex cues may result in a net fitness gain or loss, depending on individual and environmental factors (Munoz and Blumstein 2012; Munoz 2015).

Below, we explore further the benefits of receiving complex cues in facilitating the adaptive expression of social and sexual plasticity, leading to fitness benefits through the production of more robust phenotypes that can be fine-tuned more rapidly to the prevailing environment, thus avoiding the costs of phenotype-environment mismatches (Fig. 1).

Complex cues can provide robust information in variable environments

In a social and sexual environment that varies significantly through time and space (Charlat et al. 2007; Kasumovic et al. 2008), complex cues have the potential to confer more robust information than is true for simple cues, because they can contain back-up components. They may also prevent information loss in variable environments, under the assumption that a simple cue cannot accurately convey information across all environments (Hebets and Papaj 2005; Kaczorowski et al. 2012; Cole and Endler 2015; Table 2). There are several scenarios in which the efficiency of simple cues could be compromised by environmental fluctuations. The first is when the content of the message to be transmitted is spatiotemporally variable, but the cue is fixed (Bro-Jørgensen 2010). For example, the horn length of male sheep (*Ovis aries*) can function as a fixed signal of quality to females, which may influence female mate choice. Because it is an indicator phenotype expressed at a fixed level, it best predicts reproductive success when climates and environmental conditions are stable across generations (Robinson et al. 2008; Bro-Jørgensen 2010). In the second scenario, simple cues cease to be informative if they converge. Consider a scenario in which male quality is indicated by a cue linked to a handicap (Zahavi 1975; Iwasa and Pomiankowski 1991). In favourable environments, the accuracy of the information conferred by this cue could be compromised because all individuals may have the resources to fully express the handicap, irrespective of male quality. Conversely, in poor environments, no individuals can express the handicap. Hence, the handicap ceases to be an informative indicator of quality and the simple cue carries no information. Finally, simple cues will convey only limited information if receiver preferences vary with the environment due to changes in the relevance or accuracy of cues or their assessment costs (Bro-Jørgensen 2010).

Under the above scenarios, individuals that receive simple cues would obtain incomplete or inaccurate information concerning the social or sexual environment. However, if complex cues are received, alternative cue components can be available for scrutiny even if one is compromised. A complex cue may also comprise various components, with some being particularly informative in different environments. This could allow receivers to more accurately track a range of environments (Lyons et al. 2014; Reparaz et al. 2014; Rhebergen et al. 2015). For example, in the swordtail fish *Xiphophorus multilineatus*, female choice seems to be based upon the component of a complex visual cue that is a better indicator of male quality under the environmental conditions to which the female has been previously exposed (Lyons et al. 2014). In this instance, the use of complex cues allows females to match their responses more tightly to their developmental environment. Moreover, in the green swordtail *X. helleri*, female

choice, which uses multicomponent cues, is dependent on social context, with a female's preference changing according to the range of male types with which she is presented (Royle et al. 2008). Thus, complex cues may enhance the benefits of plasticity by providing more accurate information concerning variable social environments, to which plastic responses can then be tailored with increased efficiency. The perception of complex cues is likely to be particularly important in cases where plasticity is expressed as a permanent change in an individual's lifetime. These kinds of responses may have greater long-term fitness consequences (Fawcett and Frankenhuis 2015).

As well as enhancing the benefits of plasticity by allowing specific responses to variable conditions, the receipt of complex cues may avoid costly mismatches between phenotypes and fluctuating social environments. Bretman et al. (2011b) proposed that receiving complex cues may increase the reliability of information perceived, allowing plastic responses to be better suited to the social-sexual environment and thus avoiding 'off-target' reproductive investment. For complex cues to be selected, these 'mistakes' must impose significant fitness costs (Table 2). Evidence for such costs comes from the finding that prolonged high-level investment in reproduction under extended exposure to competition leads to sperm depletion, fewer later mating opportunities and shorter lifespan (Preston et al. 2001; Bretman et al. 2013). Thus, significant costs can arise if inaccurate or incomplete information on the social environment leads to an individual responding by increasing investment in current reproduction when it is not advantageous. Other costly mistakes can occur if incorrect assessments of cues lead to mating or attempted matings with the same sex, close relatives, individuals of a different species or with inanimate objects (Gwynne and Rentz 1983; Keller and Waller 2002; Presgraves et al. 2003). Accurate transmission of social information using complex cues can avoid these scenarios and reduce fitness costs.

The hypothesis that complex cues can prevent information loss when environments vary assumes that their components are equivalent or interchangeable to the extent that the message remains intact even if one or more of the components is compromised. Support for this idea comes from Bretman et al. (2011b) in which it was found that the removal of single cues indicating the presence of male rivals in *D. melanogaster* led to no apparent reduction in response from males, in comparison to scenarios in which males could detect a full sensory repertoire. Similarly, in eland antelopes (*Tragelaphus oryx*), while some components of male displays were non-redundant, three separate cue components act as backup signals of androgen-related aggression (Bro-Jørgensen and Dabelsteen 2008). In addition, several aspects of male mating displays are correlated and redundant in peacock spiders (*Maratus volans*; Girard et al. 2015). These studies support the hypothesis that complex cues benefit receivers through the robust transmission of information that would otherwise be compromised in variable environments.

Perception of complex cues can fine-tune plastic responses based on multiple features of the environment

A second hypothesis is that the perception of complex cues provides fitness benefits by producing a phenotype that is better-matched to the social and sexual environment. This is due to the ability of complex cues to provide a greater volume of information about multiple environmental factors. The expression of such benefits relies upon the assumption that cue components relay at least some partially distinct information and that this greater range of social and sexual information leads the plastic responses of receivers to better match the environment (Table 2). There is some empirical support for this idea. As discussed above, female mating preference in *T. oceanicus* crickets is based both on song components and CHCs. These traits are not correlated in males and are likely to convey distinct information about condition (via song) versus genetic compatibility (via CHCs; Simmons et al. 2013). Similarly, male great tit (*Parus major*) mating displays consist of multiple components, some of which overlap in the information content, while others apparently communicate separate facets of male quality (Rivera-Gutierrez et al. 2010). These data show that complex cue components have the capacity to confer partially distinct strands of information. This will increase the range of environmental information that can be received in comparison to simple cues and influence the plastic responses of receivers. The increased information content of complex cues may explain why female swordtail fish (*Xiphophorus nigrensis*) have faster reaction times in response to males when presented with variation in multiple cue components (Reding and Cummings 2017). Furthermore, Bretman et al. (2011b) suggested that in order for *D. melanogaster* males to appropriately respond to the presence of rivals, information must be perceived regarding the species, sex and prevalence of potential rivals. This could be achieved by the processing of multiple cue components. Subsequently, it has been shown that interfering with one cue alters the magnitude of off-target responses to heterospecific males (Bretman et al. 2017), providing some evidence that multiple cues indeed enable better environmental matching.

Complex cues are likely to be particularly pertinent in scenarios where information needs to be gathered from multiple individuals in order to inform adaptive choice. For example, female ocellated wrasse, *Symphodus ocellatus*, use indicators of a male's prior mating success and of the presence of other spawning females to inform their mate choice (Alonzo 2008). Information about the level of sperm competition (both risk and intensity) can be assessed through the presence of rival males together with information on the mating status of

females. For example, *T. oceanicus* crickets alter their ejaculate in response to cues gathered directly from other males and indirectly from females. Experience of rival male song is reported to increase the number of viable sperm in the ejaculate (Gray and Simmons 2013), whereas sperm numbers decrease when males are exposed to females experimentally exposed to a blend of CHCs extracted from multiple males (Thomas and Simmons 2009). Likewise, *D. melanogaster* males use information from males and females to strategically invest in different components of reproductive behaviour, i.e. alterations to mating duration and courting effort (Box 1). Thus, the perception of complex social and sexual cues may be adaptive in allowing the reproductive behaviours of the receiver to be calibrated to the presence of potential mates and the risk and intensity of competition.

A well-studied example of the integration of multiple stimuli to inform a response is that of courtship suppression in *D. melanogaster* (Griffith and Ejima 2009). In this, complex cues comprising information on different features of the social environment are consolidated into one physiological pathway (Box 1), allowing the resulting response to be fine-tuned to these multiple information strands. In this way, complex cues have the capacity to convey a greater range of environmental information than is the case for simple cues, increasing the benefits of plasticity. These data do not necessarily indicate a 'one-to-one' association between cues and messages, but instead, owing to the interchangeability of cues, that information detected by different sensory modalities can be partially overlapping, or combined in a degenerate manner (Ay et al. 2007).

Complex cues can reduce time lags between environmental change and response

Complex cues may enable more rapid responses to the environment, allowing individuals to better experience the benefits of short-term plasticity. As noted above, a male's response to conspecific rivals in *D. melanogaster* is highly sensitive to the social environment. It is also fully reversible and exhibits the capacity to switch on and off several times over several days (Bretman et al. 2012). A contributing factor to this ability to respond to short-term changes in rival presence may be the use of complex cues in the detection of competitors (Bretman et al. 2011b). This idea relies on the existence of sensory thresholds that need to be exceeded in order to initiate a response (Fig. 1) and that complex cue components can additively or synergistically contribute to reaching these thresholds such that receiving a complex cue results in a faster response than is the case for a simple cue (Table 2; Partan and Marler 2005; Bretman et al. 2011b; Smith and Evans 2013). Consistent with this, there is evidence for the existence of sensory thresholds to initiate responses across species (Blaxter 1968; Page et al. 1998; Brown et al. 2006). Additive or synergistic effects of cue components have been observed on female choice in field crickets and wolf spiders (Scheuber et al. 2004; Uetz et al. 2009). Thus, a response threshold may be exceeded more quickly when there are multiple sensory components acting as an input, enabling individuals to adapt more rapidly to novel environments (Fig. 1). Studies on *D. melanogaster* rival responses support this, with males with only one sensory cue removed being able to extend mating duration after exposure to rivals, but requiring a longer exposure time to exhibit the same magnitude of response (Rouse and Bretman 2016). This is paralleled by studies of female mate choice in gryllid crickets, whereby mating decisions are accelerated by the combination of acoustic and chemical cues. In *T. oceanicus*, females respond more quickly to male song if they are simultaneously presented with male CHCs (Bailey 2011). Conversely, removal of the ability to detect CHCs in *Gryllodes sigillatus* increases the time taken for females to mount males (Ryan and Sakaluk 2009). These lines of evidence suggest an important role for complex cues in securing the benefits of a plastic phenotype.

Performing costly adjustments to an environmental stimulus that is too short term to allow considerable fitness benefits to be achieved may negate the advantages of a rapidly changing phenotype (Table 2). However, this will be avoided if there are minimum periods of exposure to stimuli required to elicit responses, as observed in male *D. melanogaster* responses to rivals (Bretman et al. 2010). Beyond an adaptive minimum period, time lags between environmental change and phenotypic adaptation can reduce or negate the benefits of plasticity (Padilla and Adolph 1996; DeWitt et al. 1998). Rapid responses to the environment may be particularly important in a social context due to the dynamic nature of social environments (Charlat et al. 2007; Bretman et al. 2011a). Thus, the potential for complex cues to reduce time lags could be an important factor in facilitating social plasticity.

Conclusion

Social and sexual plasticity are ecologically important processes, whose benefits depend upon accurate and reliable monitoring of the environment. Complex cues may have a key role in facilitating social and sexual plasticity and maintaining associated fitness benefits, particularly in the context of dynamic environments that can otherwise disrupt signal transmission and hinder adaptive responses. We have outlined how this could occur via the availability of alternative signals when one is compromised, via the opportunity to integrate multiple strands of environmental information, via the reduction of time lags in adaptive responses and through the avoidance of costly 'mismatches' between phenotype and environment. It is clear that the investigation of the roles and evolution of complex cues will significantly enhance our understanding

of the existence and pattern of plasticity. For example, if the existence of complex cues was not known, one would investigate the effects of simple cues in the detection of the social and sexual environment by individual males of *D. melanogaster* and conclude that none were responsible, hence that the correct cue had not yet been identified. Rather than being able to conclude that combinations of these cues are actually used by males (Bretman et al. 2011b), the search for mechanisms involved would become confused. Further research into the costs, benefits and evolution of complex cues is expected to provide a significant advance in our general understanding of adaptive plasticity, as outlined below.

Measurement of the benefits and costs of receiving complex cues

There is evidence that complex cues convey benefits by preserving information transmission across variable environments and by producing receiver phenotypes better matched to the environment (Taylor et al. 2005; Wilgers and Hebets 2011; Rhebergen et al. 2015). Though the study of complex cues has begun in the context of social and sexual plasticity (Bretman et al. 2011b; Rouse and Bretman 2016; Rouse et al. 2018), the benefits and costs are yet to be fully experimentally explored, especially in longitudinal studies. Such data would improve our understanding of how the perception of complex cues to inform social and reproductive plasticity evolved. Experiments on the effects of the systematic manipulation of social and sexual cues on phenotypes and fitness could be carried out under a broader range of conditions and taxa to test the hypotheses proposed above. For example, determining the effects of individual condition (e.g. by varying nutrition; Mason et al. 2016) on the ability to process complex cues may shed light on when the costs of receiving complex cues become limiting. It would also be beneficial to measure genetic, phenotypic and environmental variation within the populations in which the perception of complex cues has evolved. This could be achieved through genetic or phylogenetic analysis or by using artificial selection. Such research would yield a greater awareness of the conditions in which the perception of complex cues to inform social and reproductive plasticity is advantageous and when and how the perception of complex cues should evolve.

The effects of age and experience on the role of complex cues in social and sexual plasticity

The effect of age on the expression of plasticity has begun to be explored, and this has already yielded useful information on how the influence of environmental cues changes across life stages (Rebar and Greenfield 2017). Such an approach could also be adopted in the context of complex social and sexual cues to give a greater understanding of how individuals respond depending on their own intrinsic state as well as environmental factors. Studies of expressed phenotypes and associated fitness effects under systematic manipulation of sensory cues could be performed on individuals of different ages. Longitudinal studies could also be conducted to establish a longer-term picture of the effects of complex social and sexual cues. Furthermore, studies of the role of complex cues on how experience influences social and reproductive behaviour via learning and memory would be useful. One approach could be to expose individuals to simple or complex cues and monitor their future social and reproductive behaviour. This would advance our understanding of how the perception of complex cues interacts with experience, learning and memory to affect phenotypic flexibility on a longer-term basis.

The effects of additional features of cue information on the expression of plasticity and associated fitness outcomes

In addition to considering the presence and effects of separate components of complex cues, other features of social and sexual information might also be important for understanding how social cues evolve and function. Research into the influence of multimodal versus unimodal complex cues on the expression and fitness effects of social and reproductive plasticity would be valuable. Identifying the effects of different sources of information might also be important, for example whether directed signalling versus incidental cues, or honest versus deceptive information, translate into differences in responses and fitness effects. This could increase the resolution of our understanding of how the features of social and sexual information influence phenotypic and fitness outcomes and hence provide a useful basis for classifying plasticity and predicting its effects. Experimental manipulations of signallers could also be useful to determine whether the signaller's ability to detect the receiver influences the transmission of information and receiver responses.

The benefits and costs to the signaller of the production of complex social cues

The role of both the signaller and the receiver should be considered to give a full understanding of the role and evolution of complex cues in social/sexual plasticity (Hebets and Papaj 2005). The costs and benefits of producing complex cues could be investigated by examining the fitness outcomes of individuals able to produce complex cues in a social interaction or manipulated so as only to produce simple cues. Potential costs could further be uncovered by examining condition dependence in the production of complex cues by manipulating nutrition.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

Glossary

Complex cue — A package of information assessed by an individual within one social encounter, consisting of two or more distinct components, which can be either multimodal or unimodal (see below). Adapted from Hebets and Papaj (2005), but here, 'cue' encompasses both deliberate and incidental communication. Complex cues ('signals' of Hebets and Papaj (2005)) can be further subdivided as follows:

- Multicomponent cue—a complex cue in which none of the components alone result in a receiver response, but do when received together. Generally unimodal.
- Multiple cues—a complex signal in which each of the components alone can elicit a receiver response, but when received simultaneously, the response may differ ('emergent message hypothesis'; Table 1)
- Multiple traits—a complex cue in which one component confers species identity and another signals other information, such as individual quality.

Cue components — The elements of a complex cue.
Multimodal — A cue derived across multiple sensory modalities, perceived by multiple sensory systems.
Receiver response — The plastic change in behaviour, physiology or morphology, initiated by a cue in the individual that receives it.
Simple cue — Information assessed by an individual, consisting of a single component of information.
Social/sexual cues — Cues relating to the social and sexual environment, encompassing information *directly* signalled by conspecifics or heterospecifics (such as individual quality signalled to potential mates) or *incidental* social information (such as the population density, or the physical characteristics of other individuals).
Social/sexual/reproductive plasticity — Variable phenotypic responses to social and sexual cues, in an individual's social behaviour, mating strategies, reproductive behaviour or morphology.
Unimodal — A cue derived from within one sensory modality (e.g. smell) and perceived by one sensory system.

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