Polyandry and nutrition: key modulators of sexual selection in *Drosophila melanogaster*

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In memory of my aunt Elza

Abstract

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DPhil thesis by Juliano Morimoto, St Hilda's College, submitted in Trinity Term 2016

Sexual selection is responsible for the evolution of formidable adaptations across the tree of life. Traditionally, sexual selection research has focused on male sexual displays and female choice. However, this approach ignores important social and environmental factors that can influence the operation of sexual selection. In this thesis, I aim to contribute to our knowledge of the effects of social and environmental factors, particularly those related to nutrition, on sexual selection. I investigate how the sexual behaviour of females and the nutritional and social environments of both sexes during larval and adult stages modulate sexual selection in the fruit fly *Drosophila melanogaster*.

First, I assess how female promiscuity ("polyandry") affects the strength of sexual selection and patterns of assortative mating in freely interacting populations. There is no current consensus for the conflicting results of previous studies showing that polyandry can have positive, negative or have negligible effect on the opportunity for sexual selection. Using a genetic knockout that increases female sexual receptivity, I show that increasing polyandry reduces the opportunity for sexual selection and shifts the relative role of sexual selection from pre- to post-copulatory in males without affecting assortative mating patterns in freely interacting populations.

Next, I consider how plastic responses to nutritionally poor larval environments and adult social environments modulate sexual selection, an area that has been considerably ignored. I find that although nutritionally poor larval environments reduce individuals' mating and reproductive success, plastic responses to social environments might mitigate against these disadvantages. Moreover, I show that plastic responses to larval and social environments influence the relative role of pre- and post-copulatory sexual selection in males, regulate offspring traits through trans-generational effects and determine population fate.

Finally, I consider whether male reproductive traits have distinct macronutrient (protein and carbohydrate) requirements, and whether males can regulate their feeding to attain a diet that satisfies the requirements for these traits. I find that both a high short-term rate of offspring production when males mate with virgin females, and a high total number of offspring sired when males mate with previously mated females, require carbohydrate-rich diets, whereas male attractiveness requires a balanced (1:1) macronutrient diet. Furthermore, I show that male protein intake can negatively affect female long-term reproduction when males mate with virgins, but not with previously mated females, revealing a novel intersexual effect of male nutrition.

Thus, this thesis deepens our understanding of key evolutionary processes by revealing the negative effects of high polyandry and nutritionally poor larval environments on the operation of sexual selection as well as uncovering male nutritional compromise in the expression of reproductive traits.

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Acknowledgements

If it wasn't for that phrase, at that moment, in that classroom, this thesis would never have been written.

At the age of sixteen, I was tricked into believing that we should pursue what we love. I loved Biology, and I was determined to pursue this dream. However, if you are a low-to-middle-class teenager in Brazil, studying in a public high school with no sufficient credit in your family's bank account to afford a private University, you need more than just effort, you need a breakthrough.

I used to stay after hours at the school, solving additional Maths problems in order to increase the odds of scoring well in the admissions exam of the public Universities. Admissions for public Universities in Brazil are highly competitive, with numbers that can reach more than hundreds of candidates per position available. So I had no choice other than to sit and study. One afternoon, my Maths teacher, Prof Rafael (or *Rafão*) – a tall Afro-American figure, very compassionate, sharp and in love with his profession - handed me a question booklet from the National Maths Competition. I still remember the yellow colour of the pages of the booklet. He instructed me to open it on page seven, and asked me to solve problem nine. It was a question involving geometrical series, where I had to find out what would be the next number of the sequence. That sounds easy, but the sequence was in a spiral, and there was a whole set of constrains such as "the number has to be multiple of all previous numbers in that diagonal". Too hard for me, I wanted the easy stuff. With this mindset, I read the question, thought for five minutes and said:

- I don't know, Rafão.
- But you haven't tried yet – He replied.
- Yeah but I know I don't know.
- Try once more – He said, while preparing his material for his next class.

Another five minutes passed. I wasn't trying. I was just waiting, pretending to think so he could give me the answer and we could move on with our lives.

- Oh come on Rafão, I don't know. What is the correct answer?
- You still haven't tried! – He looked at me firmly.
- But I don't know, it is too hard!

What I did not know was that even Rafão had limited patience, and that the direction of my life would drastically change as a result of that.

He stood up from behind his desk looking firmly at me, grabbed the material he had prepared for his next class and in a powerful voice said:

- *Don't say you can't! You haven't even tried yet. You* ***know*** *the answer. If you want to pass the admission exam, or anything else in your life, you will need to go after it yourself. I won't be there sitting with you in the exam or in life to give you the right answers*!

I felt as if a train had hit me. I was embarrassed, ashamed of myself. But at the same time, I felt powerful and free. *His words freed me.*
But Rafão wasn't done yet. Before leaving the classroom, he turned back and said:

- Only leave this classroom when you have found the right answer.

I tried everything. At that time, I did not have 4G or any of the fancy wifi technology of today's world, so I couldn't "Google" anything. Worse, I did not have a book in the classroom to consult formulas and equations. I had nothing and nobody, just like Rafão told me. But I *knew* the answer, I just needed to realise that.

I was focused. Absorbed.

It took me two hours and fifteen minutes. During this period, I did not go to the restroom or drink water. But in a rather mystical move, I found the answer. I rushed to where Rafão was teaching. He stopped his class because he could see I was so excited that nothing could wait.

- So… did you find the answer?
- Yes!
- Which is?

I gave him the answer. He smiled and asked me about the thought process that led me to that answer.

- Correct! There is a formula for everything you did. But see, you can achieve anything if you put the effort.

This thesis would not have been written if wasn't for Rafão, to whom I will be forever in debt for changing the course of my life.

***

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Declaration and author contributions

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

Chapter 2 - Experimental evidence that polyandry weakens sexual selection on males and increases the relative role of post-copulatory episodes

Authors: Juliano Morimoto*, Grant C. McDonald, Emelia Smith, Tommaso Pizzari, Jennifer Perry, Stuart Wigby

Contributions: JM, TP, JP and SW designed the experiment. JM collected the data. JM, GCM, TP and SW analysed the data. All authors contributed to writing the manuscript. ES was an undergraduate student who helped during data collection of the pilot replicate of the experiments.

Status: In preparation

Chapter 3 - Developmental environment effects on sexual selection in male and female *Drosophila melanogaster*

Authors: Juliano Morimoto*, Tommaso Pizzari, Stuart Wigby

Contributions: JM, SW and TP designed the experiment, analysed the data and wrote the manuscript. JM collected the data.

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Chapter 4 - Interactions between the developmental and adult social environments mediate population dynamics and offspring traits in Drosophila melanogaster

Authors: Juliano Morimoto*, Fleur Ponton, Ilona Tychsen, Jason Cassar, Stuart Wigby

Contributions: JM and SW designed the experiment. JM, FP, IT and JC collected the data. JM, FP and SW wrote the paper.

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Chapter 5 - Differential effects of male nutrient balance on pre- and post-copulatory traits, and consequences for female reproduction in *Drosophila melanogaster*

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Contributions: JM and SW designed the experiment, analysed the data and wrote the manuscript. JM collected the data.

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Collaborations

Gut microbiota modifies olfactory-guided microbial attraction in *Drosophila*

Authors: Adam Chun-Nin Wong, Qiao-Ping Wang, Juliano Morimoto, Alistair Senior, Mathieu Lihoreau, Gregory G. Neely, Stephen J. Simpson and Fleur Ponton

Status: Submitted

Developmental environment and mating status mediate outcomes of female competition

Authors: Eleanor Bath, Juliano Morimoto, Nathalie Seddon, Stuart Wigby

Status: Submitted

Note on references

Harvard reference style is used throughout this thesis. To avoid repetition and save space, there is a single reference list at the end of this thesis.

Chapter 1

General Introduction

Colourful shapes, exuberant forms, and astonishing diversity. Sexual selection is responsible for the evolution of extreme morphological traits across the tree of life (Andersson, 1994). Even though sex carries many costs (Lehtonen et al., 2012, Clutton-Brock, 1994, Maynard-Smith, 1978), sexual reproduction is still ubiquitous (Otto and Lenormand, 2002), and it unlocks another layer of selection in addition to the well-known world of natural selection. Sexual selection was first proposed by Darwin (1859), and later expanded to account for the evolution of human traits (Darwin, 1871). The process of sexual selection was first defined as the struggle of one sex (usually males) for the possession of the limiting sex (usually females) – i.e. "intrasexual competition". The net result of this process is the differential contribution of males and females to the genetic content of the next generation (see (Parker and Pizzari, 2015, Jones and Ratterman, 2009).

For almost a century after its conception, the definition of sexual selection was primarily a verbal argument; no robust quantitative measure of intrasexual selection existed. Consequently, it was impossible to pinpoint differences in selective pressures acting within and between populations or predict how sexes respond to its social and environmental context. However, in 1948, the English geneticist Angus J. Bateman provided a quantitative method for estimating the strength of sexual selection that today is a pillar in sexual selection theory. In a landmark paper, Bateman uncovered what we now recognise as the underlying causes and consequences of intrasexual competition on males and females. Using fruit flies *Drosophila melanogaster*, Bateman investigated how male-male competition influenced the number of mates and offspring of males and females (Bateman, 1948, Snyder and Gowaty, 2007). With an intricate genetic setup, Bateman tracked the number of mates and offspring of each individual in freely mating population (Bateman, 1948) (but see (Gowaty et al., 2013, Gowaty et al., 2012, Snyder and Gowaty, 2007) for flaws of Bateman's original study) and

found three main results, which are now known as the Bateman principles (Arnold, 1994). They are:

1. The variance in the number of mates of individuals tends to be higher for males than females;
2. The variance in the number of offspring tends to be higher for males than females;
3. The correlation between the number of mates and offspring tends to be stronger for males than females – known as "the Bateman gradient" (see (Arnold, 1994);

Principle 3 was regarded as the *cause* of a stronger intrasexual competition in males (Bateman, 1948), whereby males are expected to have a strong positive selective pressure to mate with as many females in the population as possible. This pressure creates a highly competitive environment for males (Arnold, 1994) and only few successful males are likely to secure the majority of matings, leading to an increase in variance in male mating and reproductive success (Principles 1 and 2) (Arnold, 1994). As a result, Principles 1 and 2 are regarded as *consequences* of sex differences in the intensity of intrasexual competition.

The Bateman's principles provided a framework that defined the expected strategies of each sex during reproduction. For instance, after Bateman's work, males and females were expected to vary both in their variance in reproductive and mating success, and also to have distinct selective pressures to obtain multiple mates (see Principle 3). In other words, the Bateman's principles defined the *sex roles* (Knight, 2002) and Bateman's third principle, the correlation between the number of mates and offspring or *the Bateman gradient*, has been particularly useful for predicting sex-specific behaviour. For instance, in species where males compete for the access of choosy females – so-called "traditional sex roles" species – the Bateman gradient is expected to be steep for males (i.e. incremental benefits of multiple mating) and, after the first mating or the first few matings, flat for females (i.e. maximum female fitness is achieved at a lower mating success than male fitness). On the other hand,

when females also compete for the access to males – known as "sex-role reversed" species – the Bateman gradients of both males and females tend to be steep; in some cases, the female Bateman gradient being steeper than male's (e.g. (Jones et al., 2000)). In this thesis I focused on the traditional sex role species *Drosophila melanogaster*.

Polyandry: a revolution in sexual selection theory

Bateman's principles predict that females should gain no direct benefits in terms of offspring number by mating multiply (Arnold, 1994). Yet, in the majority of species across the animal kingdom, female promiscuity ("polyandry") is common (Markow, 1996, Taylor et al., 2014) even though in some cases there is no obvious fitness benefits for females to behave this way or worse, females may pay a high costs for doing so (Chapman et al., 2003a, Arnqvist and Rowe, 2005, Brown et al., 2004, Arnqvist and Nilsson, 2000, Fedorka and Mousseau, 2002, Tregenza and Wedell, 2002, Jennions and Petrie, 2000, Reding, 2015). Although the reasons for the evolution of female polyandry remain debated, the discovery that polyandry is common has profoundly changed the foundations of the field of sexual selection (Taylor et al., 2014, Parker and Birkhead, 2013). This is because polyandry has far-reaching adaptive significance than previously thought (Parker and Birkhead, 2013, Pizzari and Wedell, 2013). One of the consequences of polyandry is that it creates the potential for the ejaculates of different males to interact at the fertilization site within the female tract, where competition can continue after copulation - i.e. "sperm competition" (Birkhead and Pizzari, 2002, Wigby and Chapman, 2004, Simmons, 2001, Parker, 1970). Polyandry can also reduce the likelihood of females experiencing a shortage of sperm, or genetic incompatibilities with their partners (Zeh and Zeh, 1997, Wedell et al., 2002). Furthermore, polyandry may allow females to bias the fertilization of their eggs in favour of certain males (e.g. dominant or

more attractive males) – a process called "female cryptic choice" (Eberhard, 1991, Pizzari and Birkhead, 2000). Therefore, female polyandry has revolutionised the study of sexual selection. It is also important to highlight that the discovery of polyandry revealed the weaknesses on the widely used Bateman's principles. For instance, as explained above, the Bateman gradient predicts that females are not expected to gain fitness benefits, measured in terms of offspring number, from multiple matings (Knight, 2002). However, recent theoretical and empirical studies on the implications of polyandry revealed that multiple matings can benefit females by increasing offspring quality and variability, guaranteeing genetic compatibility with their mates, avoiding sperm depletion and, in systems with nuptial gifts, providing access to nutritionally rich gifts produced and offered by males (Hosken and Stockley, 2003, Zeh and Zeh, 1997, Zeh and Zeh, 2001, Bretman et al., 2004, García-González and Simmons, 2007). Yet, despite these omissions, the Bateman gradient remains a reliable indicator of the roles of each sex during reproduction within species (e.g. (Jones and Ratterman, 2009)). In traditional sex role species, which is the focus of this thesis, it is safe to assume that the main reason for the asymmetries in the roles of male and female during sexual reproduction is the anisogamy, where males produce an almost infinite amount of small motile sperm while females produce few large immotile eggs (Parker and Pizzari, 2015). In this systems, male reproduction is limited by the number of eggs his sperm have access to and fertilize, while female reproductive success is limited by her own resources (e.g. (Parker and Birkhead, 2013)) – hence the positive Bateman gradient for males but not females (Fritzsche and Arnqvist, 2013). Thus, despite the urgent need for a revision on the underlying theory regarding the Bateman's principles, the general conclusions and the interpretations remain a central topic in sexual selection theory (see (Snyder and Gowaty, 2007, Jones, 2003, Jones and Ratterman, 2009, Gowaty et al., 2012, Gowaty et al., 2013)).

Polyandry also revealed the potential for selection at the post-copulatory stage (Pizzari and Wedell, 2013, Simmons, 2001, Birkhead and Pizzari, 2002). As a result of post-copulatory selection, males evolve adaptations that increase their success in fertilization (Birkhead and Pizzari, 2002). These adaptations can occur at the behavioural level (e.g. mate guarding), at the cellular-level in the sperm (e.g. sperm ornamentation, mating plugs) or at the molecular-level in the ejaculate (i.e. seminal fluid proteins) (Lüpold et al., 2016, Bretman et al., 2010, Ross and Crews, 1977, Simmons, 2001, Andersson, 1994, Perry et al., 2013). Seminal fluid proteins influence female physiology and behaviour such as to decrease female receptivity and increase male fertilization success (Kubli, 2003). The best functionally characterised system of male ejaculate mediation of female behaviour and physiology is in *D. melanogaster*, where male ejaculate is cocktail of seminal proteins (Perry et al., 2013) responsible for modulating female mating activity (Wigby and Chapman, 2005, Chen et al., 1988, Soller et al., 1999), food intake (Vargas et al., 2010, Ribeiro and Dickson, 2010), immune responses (Peng et al., 2005b), and sleep patterns (Oh et al., 2014). Thus, female polyandry has far-reaching effects on both female physiology and the evolution of male adaptive traits.

Integrating the effects of polyandry on sexual selection: a long-standing challenge

Given the far-reaching evolutionary implications of polyandry, the original definition of sexual selection had to be expanded to accommodate the distinct episodes of pre- and post-copulatory competition. Today, sexual selection is often defined as the differential offspring production caused by the competition for mates and fertilization (Andersson, 1994). However, although the formal definition of sexual has changed, the incorporation of the effects of

polyandry onto the sexual selection theory still remains largely incomplete. Conflicting results of theoretical and correlational studies have created a debate regarding the general effects of polyandry on sexual selection. Some studies have suggested that polyandry should exacerbate sexual selection, particularly on males (Birkhead and Møller, 1998, Albrecht et al., 2009, Whittingham and Dunn, 2005, Webster et al., 2007, Pischedda and Rice, 2012), while others suggested that polyandry have little effect on the operation of sexual selection (Whittingham and Dunn, 2005, Pischedda and Rice, 2012). Recent theoretical arguments have suggested that polyandry may in fact weaken sexual selection on males by eroding the variance in male mating success and reducing the correlation between male's mating and reproductive success (the Bateman gradient) (Shuster and Wade, 2003, Collet et al., 2012, Collet et al., 2014, Parker and Birkhead, 2013, Kvarnemo and Simmons, 2013). Unfortunately, these theoretical and correlational studies fail to provide evidence of causal effects of polyandry on sexual selection, which in turn compromise our understanding of the mechanisms underlying these effects. This thesis begins with an investigation of the role of polyandry on sexual selection. I address this in Chapter 2, where I take full advantage of the powerful tool of genetic manipulations in *Drosophila melanogaster* to reveal the causal effects of polyandry on the operation of sexual selection in males. Moreover, Chapter 2 scrutinises the underlying mechanisms by which males evolve pre- and post-copulatory traits in response to polyandry. Thus, Chapter 2 advances our understanding of the foundations of sexual selection theory because it is to date the most direct experimental attempt to integrate female polyandry to the framework of sexual selection.

Resource acquisition modulates the expression of sexual traits and influences the operation of sexual selection

The expression of pre- or post-copulation sexually selected traits is costly, and therefore is often influenced by the pool of resources acquired from the environment (Rowe and Houle, 1996). Resources acquisition can determine the plastic responses of individuals and consequently the outcome of individuals' interactions in adult competitive social environments. As a result, resource acquisition can be an essential modulator of the strength of sexual selection (Cotton et al., 2004, Cotton et al., 2006). In insects, the resources acquired during the developmental stage are crucial for determining key phenotypic traits used in male and female interactions in adult social environment such as adult body size, reproductive output, female polyandry and male attractiveness (Amitin and Pitnick, 2007, Pitnick and Garcia-Gonzalez, 2002, Lyimo et al., 1992, Credland et al., 1986, Honek, 1993, Chown and Gaston, 2010, Lindström, 1999). To date, however, there had not been any formal attempts to determine the extent to which the interplay between developmental and adult social environments affects the operation of sexual selection. The second section of my thesis begins by addressing this omission. In Chapter 3, I manipulate *D. melanogaster* larval density to simulate scarcity of resources (primarily nutrients) during developmental stage, which creates adults with different body size phenotypes. I then assemble populations with mix phenotypes of either males, females or both sex(es) to create a range of phenotypically mixed social environments. Using this design, I measure individual's reproductive success and the strength of sexual selection through Bateman gradients (Chapter 3). I then ask whether the effects of the developmental and social environments can also be passed onto next generations (i.e. trans-generation effects) and influence population-level traits such as population growth and survival. These points are addressed in Chapter 4, where I used the same experimental design

of Chapter 3 to assess how the developmental and adult social environments interact and affect offspring traits and population fate. Together, Chapters 3 and 4 advance our knowledge on the ecology of sexual selection and population dynamics and contributes to the growing body of evidences on trans-generational effects.

Nutrient balance and sexual performance: Can males have it all?

The most obvious resources available for allocation on traits are nutrients, which are vital in the molecular homeostasis of the organism and radically shape the physiology, behaviour and the expression of life-history traits (Powell et al., 2012, Chantranupong et al., 2015, Efeyan et al., 2015, Stearns, 1992). In insects, nutrients, particularly carbohydrate and proteins (i.e. “macronutrients”) have been linked to the modulation of lifespan, sexual performance, reproduction and ornamentation (e.g. (Lee et al., 2008, Maklakov et al., 2008, Fanson et al., 2009, Lee et al., 2013, House et al., 2015)). However, it may be difficult for individuals to attain a balanced nutrition that maximises all individual’s traits, and compromises may be made at the expense of fitness. For instance, in polyandrous species, male nutrition has to account for the requirements of both pre- and post-copulatory episodes whereby imbalanced diets can impose constraints on male allocation to each one of these sexual traits (Mehlis et al., 2015). In Chapter 5, I used an innovative method called the Geometric Framework of Nutrition (or Nutritional Geometry (Simpson and Raubenheimer, 1993)) to evaluate adult male nutritional requirements for the expression of pre- and post-copulatory traits as well as the reproduction of male’s mates. Chapter 5 directly compare the nutritional requirements for the expression of behavioural traits involved in the pre- and post-copulatory sexual selection and significantly advances our understanding on the impacts of nutrition on the expression of sexual traits.

In summary, this thesis first reveals the causal links between polyandry and the operation of sexual selection (Chapter 2). The thesis then addresses how resources acquired during the developmental stage, which are crucial for the expression of pre- and post-copulatory traits, interact in mix adult social environment and alter the operation of sexual selection (Chapter 3), offspring traits and population fitness (Chapter 4). Finally, the thesis uncovers how adult nutrition influences the functionality of traits targeted by pre- and post-copoulatory sexual selection (e.g. male attractiveness, fertilization success) (Chapter 5), and its implications for males and females reproduction.

Chapter 2

Experimental evidence that polyandry weakens sexual selection on males and increases the relative role of post-copulatory episodes

Abstract

In polyandrous species, where females mate multiply, sexual selection targets male ability to both mate with more and more fecund females than their competitors (pre-copulation), and fertilise a higher a proportion of their female's eggs (post-copulation). The way in which male sexual selection responds to female polyandry however remains unclear, but recent theoretical and empirical work suggests that under some conditions, increasing polyandry should weaken sexual selection, reducing pre-copulatory episodes while increasing the relative importance of post-copulatory episodes. We genetically manipulated the polyandry of female *Drosophila melanogaster* to demonstrate these effects in freely mating social groups. We show that genetically elevated polyandry erodes variance in male mating success, weakening the overall opportunity for sexual selection and increasing the relative importance of post-copulatory selection on male traits affecting paternity share, such as mating repeatedly with the same female. We also show that this effect arose simply because the saturation of the mating matrix in more polyandrous groups leads to a negative correlation between the mating success of a male and the polyandry of his mates. This study complements recent correlational work, by providing an experimental demonstration of the causal effect of polyandry on male sexual selection. Together, our results reveal the impact of female sexual behaviour on the architecture of male reproduction and provide insights into the far-reaching effects of polyandry on sexual selection and sexual conflict.

Introduction

Darwin (1871) first recognised sexual selection as a fundamental agent of evolutionary change, arising from variation in reproductive success due to intrasexual competition over access to reproductive opportunities. Variation in male reproductive success was traditionally thought to be limited to the number of females with whom a males manages to mate, and the number of eggs (fecundity) of these females (Parker and Pizzari, 2015). However, the discovery that females often mate with multiple males (i.e. polyandry) (Taylor et al., 2014) has drastically changed our view of sexual selection (Parker and Birkhead, 2013). First, by forcing the ejaculates of different males to compete over fertilisation, polyandry prolongs sexual selection after mating creating variation in the proportion of eggs of a female that a male is able to sire (Parker and Birkhead, 2013). Second, by enabling more than one male to mate with a female, polyandry should impact on patterns of variation in male mating success. Together these factors add considerable complexity to the architecture of male reproductive success (Kvarnemo and Simmons, 2013, Shuster et al., 2013, Evans and Garcia-Gonzalez, 2016), and the way in which polyandry modulates the operation of sexual selection on males remains unclear.

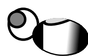
On the one hand, some studies have suggested that polyandry should exacerbate sexual selection on males (Birkhead and Møller, 1998, Albrecht et al., 2009, Whittingham and Dunn, 2005, Webster et al., 2007, Pischedda and Rice, 2012), while others found polyandry to have little effect (Whittingham and Dunn, 2005, Pischedda and Rice, 2012). On the other, recent theoretical arguments have suggested that polyandry may in fact weaken sexual selection on males by eroding the opportunity of pre-copulatory episodes and reduce the correlation between male mating success and the number of offspring sired (Shuster and Wade, 2003, Collet et al., 2012, Collet et al., 2014, Parker and Birkhead, 2013, Kvarnemo and

Simmons, 2013). Consistent with these expectations, Collet et al. (2012) showed that increasing polyandry in groups of red junglefowl (*Gallus gallus*) was associated with a reduction in the overall opportunity of sexual selection on males. This decline was driven by an erosion of variance in male mating success which weakened pre-copulatory sexual selection. As a result, opportunity for post-copulatory sexual selection was relatively more important in high-polyandry groups, and promoted strategies through which males could defend their paternity such as remating repeatedly with the same females (Collet et al., 2012). Similar patterns were found in populations of crickets *(Laupala cerasina*) (Turnell and Shaw, 2015) and hermaphroditic snails (*Physa acuta)* (Pelissie et al., 2014).

One complication is that it is difficult to establish the extent to which these correlational results reflect the causal effect of polyandry on sexual selection, and the specific mechanisms underpinning these effects. For example, changes in female fecundity may simultaneously drive female propensity to mate multiply and patterns of variance in paternity share. In addition, for a given level of average polyandry in a group, the way in which matings are distributed across the mating matrix (i.e. the matrix of male-female matings) of the group can also profoundly modulate the impact of polyandry on sexual selection on males (Sih et al., 2009). When males with higher mating success are more likely to mate with the least polyandrous females, some degree of pre-copulatory sexual selection on mating success will be preserved in virtue of the fact that high mating success is associated with lower sperm competition (and thus higher paternity share (McDonald and Pizzari, 2016). Conversely, a situation in which males with higher mating success preferentially mate with the most polyandrous subset of females will act to weaken pre-copulatory sexual selection beyond what one would expect if the same number of mating combinations were randomly distributed across the group (McDonald and Pizzari, 2016). Studies investigating the causal relationship between polyandry and sexual selection should therefore measure the correlation between the

mating success of a male and the polyandry of his sexual mates (*SCIC*, (McDonald and Pizzari, 2016). Resolving the intricate interplay between female polyandry and male sexual selection is key to understand the complex architecture of reproductive success, the evolutionary ecology of mating systems, patterns of sexual conflict and intersexual coevolution.

In this study, we harness the powerful tool of genetic manipulations in *Drosophila melanogaster* to resolve the causal effects of polyandry on the operation of male sexual selection experimentally. Specifically, we used genetically modified *D. melanogaster* lines in which group polyandry was elevated beyond the natural levels found both in laboratory and wild caught *Drosophila* by abolishing the typical post-mating reduction in female receptivity (high polyandry; Yapici et al. (2008), (Harshman and Clark, 1998); Figure 1).



exual selection indexes

Figure 1 – Summary of the *SPR-* manipulation experiment to increase polyandry levels in freely mating populations.

Results and Discussion

To experimentally elevate the levels of polyandry, we used females with a genetic deficiency covering the *sex-peptide-receptor* gene (henceforth called "*SPR-*"), a manipulation that strongly reduces female post-mating sexual refractoriness (Yapici et al., 2008). We housed individually marked flies in replicate groups of 4 males and 4 females, whereby 3 *white*-eyed males and 1 *spa*-eyed focal male were housed with either 4 *SPR-* females ("*SPR-* treatment") or *white*-eyed females ("Control"). We then measured all matings, reproductive success and the paternity of the genetically identifiable "focal male" from each group. During each day, males and females of both treatments were allowed to interact for 4 hours in the mornings (9am – 1pm) followed by 20 hours of egg laying each day (1pm - 9am) over a 4-day period (see (Bjork and Pitnick, 2006, Morimoto et al., 2016)). *SPR-* females had higher mating success and mating frequency than controls, which confirms the expected high polyandry phenotype of our genetic manipulation (Figure S1A). Focal males in the *SPR-* treatment also had higher mating success and mating frequency than focal males in the control, which –all else being equal- is consistent with the expectation that our genetic manipulation increased polyandry experienced by our focal males in the *SPR-* treatment (Figure 2A and 2B). Together, these results reveal that we successfully increased polyandry levels experienced by both focal males and females beyond that observed in wild-type flies raised in lab conditions as well as wild caught *Drosophila,* which can reach an average of ~1.82 mates per female (see (Harshman and Clark, 1998)) However, as a result of the increased sperm competition, focal males in the *SPR-* treatment sired a lower proportion of the eggs produced by their mates compared to control focal males (Figure 2C), which meant

that they sired just as many offspring overall (measured as number of daughters; see Methods) as control focal males (Figure 2D).

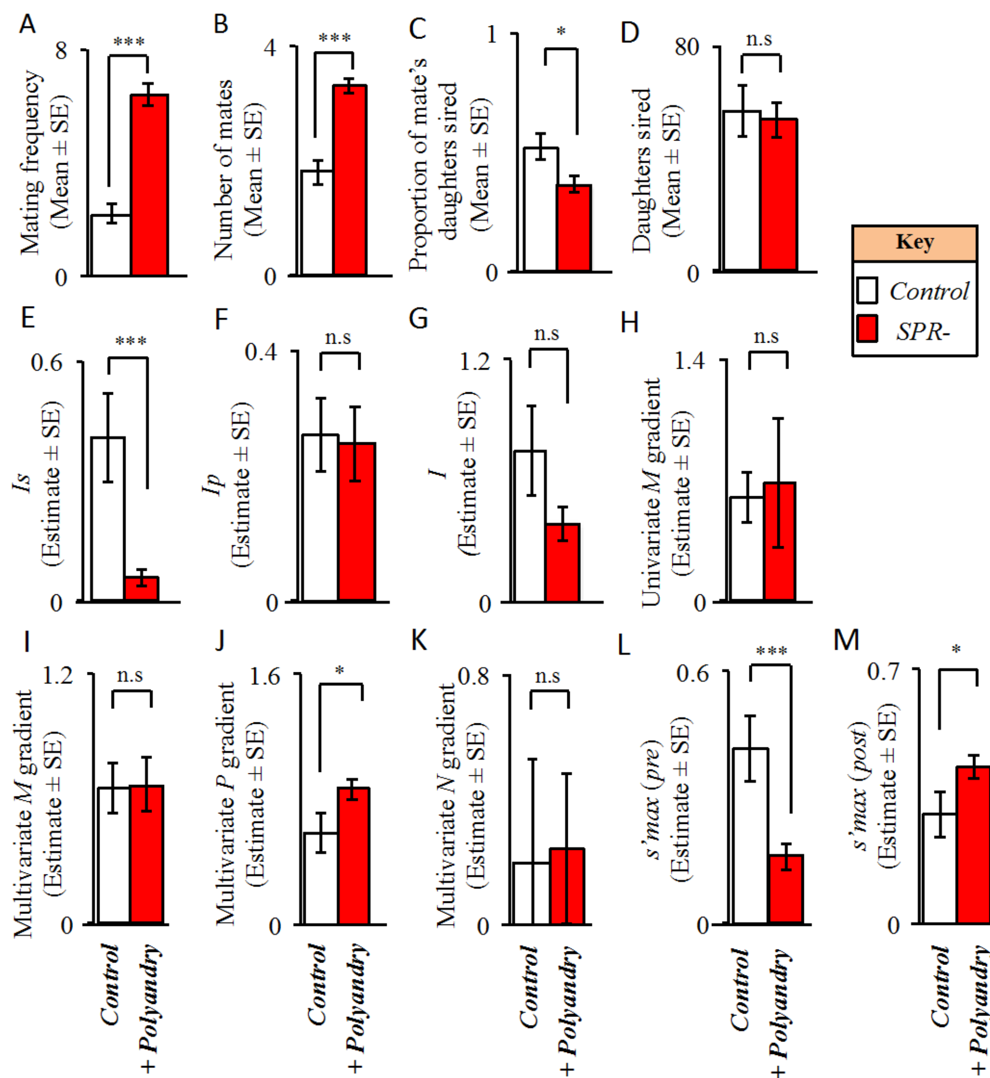


Figure 2 – Effects of elevated polyandry on the operation of sexual selection in males. (A) Focal male mating frequency (number of matings); (B) Focal male mating success (number of mates); (C) Proportion of daughters sired by the focal males with females they mated with; (D) Number of daughters sired by the focal males; (E) the standardized variance in focal male mating success (I_S) ("the opportunity for pre-copulatory sexual selection"); (F) the standardized variance in focal male siring success (I_P) ("the opportunity for post-copulatory sexual selection"); (G) the standardized variance in offspring (daughters) sired by the focal males (I) ("the opportunity for selection"); (H) The univariate gradient of focal male mating success and reproductive success (the univariate M gradient or "the Bateman

gradient”); (I) The multivariate gradient of focal male mating success and reproductive success (the multivariate *M* gradient); (J) The multivariate gradient of focal male paternity share and reproductive success (the multivariate *P* gradient); (K) The multivariate gradient of focal male mate productivity and reproductive success (the multivariate *N* gradient); (L) the maximum standardized pre-mating sexual selection differential index $s'_{max\ (pre)}$ (Jones's index); (M) the maximum standardized post-mating sexual selection differential index $s'_{max\ (post)}$. * $0.01 \leq p < 0.05$; ** $0.001 \leq p < 0.01$; *** $p < 0.001$ except panels (F) where statistics was based on bootstrap and therefore *** - non-overlapping 95% bootstrap confidence intervals. *n.s* – $p > 0.05$ or, in panels E and G, overlapping bootstrap confidence intervals.

It has been argued that increasing average levels of polyandry should result in males achieving similarly high mating success and facing intense sperm competition, reducing variance in male mating success (I_S) while maintaining variance in male fertilization success (I_P) (Jones et al 2001, Shuster & Wade 2003, Collet et al. 2012). Our results provide clear evidence of these patterns: I_S was reduced in the focal males of our *SPR-* treatment compared to control focal males (Figure 2E, Table S1), and there was no effect of treatment on focal male's I_P (Figure 2F). Low variance in mating success should also reduce the variance in total male reproductive success (see Table 1 and 'Methods'). We then investigated whether this reduction in the opportunity for pre-copulatory sexual selection (I_S) in the more polyandrous *SPR-* treatment had an impact on the opportunity for selection, which measures the variance in total male reproductive success (I) (see Table 1). We found a non-significant trend for I to be lower for focal males in the *SPR-* treatment compared to controls (Table S1, Figure 2G), supporting the idea that increasing polyandry tend to reduce variance in total male reproductive success, largely by reducing variation in mating success.

Table 1 – Sexual selection indexes used in our study and the formulas for calculation.

Standardization		
N^{Multi}	*Multivariate partner productivity gradient*	
Repetitive matings with the same females		
SCIC		

T – focal male reproductive success; M – focal male mating success; P – focal male paternity share; N – focal male's mate productivity; *Covariates* – includes "vial fecundity" (except for the 'Remating rate gradient') and "replicate". β_x^{var} = variance-standardized gradient of x, where x is either M (pre-copulatory) or P (post-copulatory). β_x^{Uni} or β_x^{Multi} = uni- and multi-variate mean-standardized gradients of x, where x is either *M, P* or *N.* For *SCI* calculation, *k* is mating success of the jth female mated with the focal male.

To explore this further we dissected male reproductive success into its constituents. Variance in reproductive success among males is determined by three main factors: male mating success (*M),* male fertilization success *(P)* and the productivity (or fecundity) of male's mates (*N*) (Evans and Garcia-Gonzalez, 2016, Collet et al., 2012) and we expected the role of *P* to increase as the polyandry levels of the population increased, whereas the opposite effect should be seen for *M.* To investigate this, we decomposed the variance in focal male reproductive success into its variance-covariance components *M, P* and *N* (Whittingham and Dunn, 2005, Collet et al., 2012, Janicke et al., 2015, Moorad and Wade, 2013, Morimoto et al., 2016, Webster et al., 1995). We confirmed that *M* and *P* were the major sources of variance in male reproductive success, whereas *N* had a negligible contribution (see Table 2). Importantly, we showed that *M* was the main source of variance in focal male reproductive success in controls whereas *P* was the main source of variance in focal male reproductive success in the more polyandrous *SPR-* treatment.

Table 2 – Decomposition of the variance in male reproductive success. Relative contributions of *M, P* and *N* to the variance in male offspring siring (*T*). Delta method of variance decomposition following Webster *et. al.* 1998, Collet *et. al.* 2010 and Morimoto *et. al.* 2016.

Var-Cov Components	Observed contribution to *var(T)*			
	Control		*SPR-*	
var(T)	*24.184*	*%*	*11.064*	*%*
var(M)	11.681	*48.3*	1.527	*13.8*
var(P)	7.570	*31.3*	6.649	*60.1*
var(N)	5.175	*21.4*	1.958	*17.7*
cov(M, P)	-0.726	*3.0*	0.343	*3.1*
cov(M, N)	3.652	*15.1*	0.985	*8.9*
cov(N, P)	2.975	*12.3*	1.361	*12.3*
D	-7.231	*29.9*	-3.142	*28.4*

We then measured the strength of the correlation between *M* and *P* with male reproductive success. Given our results above, we expected *M* to be weakly correlated, and *P* to be strongly correlated with reproductive success in the focal males of the *SPR-* treatment, while we expected the opposite in focal controls. This is because sperm competition associated with polyandry should reduce the reproductive returns associated with gaining additional mates (Kvarnemo and Simmons, 2013, Parker and Birkhead, 2013, Collet et al., 2014, McDonald and Pizzari, 2016). To test these predictions, we fitted two models (see (Collet et al., 2014): i) the traditional univariate linear regression of focal male mating success (*M*) on his reproductive success (*T*) (the "Bateman gradient"; $\beta_{SS}{}^{Uni}$) (Bateman, 1948, Arnold, 1994) and ii) a multivariate linear model of focal male mating success (*M*), paternity share *(P)* and mate productivity (*N*) on reproductive success (*T*) ($\beta_{SS}{}^{Multi}$, $\beta_N{}^{Multi}$ and $\beta_P{}^{Multi}$ respectively)

(Arnold, 1994, Arnold and Duvall, 1994, Collet et al., 2012, Morimoto et al., 2016). Surprisingly, we found no difference between the mating success *(M*) gradient of focal males in the *SPR*- treatment when compared to controls in both the univariate and multivariate models (Univariate: t_{52} = 0.552, p = 0.583; Multivariate: t_{45} = 0.306, p = 0.760, Table S2) (Figure 2H and 2I). However, we found a significantly steeper multivariate paternity (*P*) gradient for *SPR*- treatment compared to controls (Multivariate: t_{45} = 2.569, p = 0.013) (Figure 2J), suggesting that increasing polyandry strengthens the correlation between fertilization success of a male and his reproductive success. There was no difference on the correlation between male reproductive success and mate productivity (*N*) between treatments (Figure 2K).

While the Bateman gradient has been traditionally used as a proxy of the strength of sexual selection (Jones and Ratterman, 2009), this approach has recently been criticised because the Bateman gradient ignores variance in mating success and therefore provides inaccurate measures of sexual selection (Henshaw et al., 2016). To overcome this, an alternative index has been proposed – the maximum standardized sexual selection differential index, which essentially corresponds to the variance-standardized Bateman gradient (also known as Jones's index) (Jones, 2009, Henshaw et al., 2016). We therefore complemented our analysis of univariate and multivariate selection gradients with pre- and post-copulatory Jones's indexes $s'_{max\ (pre)}$ and $s'_{max\ (post)}$ (see Table 1, Table S4). We found that pre-copulatory sexual selection measured as $s'_{max\ (pre)}$ is significantly weaker in focal male of the *SPR*- treatment relative to focal controls (Figure 2L) due to the erosion of the variance in male mating success I_S. We also found that post-copulatory sexual selection ($s'_{max\ (post)}$) is significantly stronger in focal males in the *SPR*- treatment relative to focal controls (Figure 2M), which is a result of the strong correlation between *P* and focal male reproductive success.

We then explored the mechanisms underpinning variation in these pre- and post-copulatory selection gradients. We first considered pre-copulatory sexual selection. Our results showed that increasing polyandry had no effect on univariate or multivariate Bateman gradients, but had a strong effect on the Jones's index. Taken together these results indicate that mating with additional females results in similar gains in reproductive success across both the *SPR-* treatment and the control (i.e. similarly strong Bateman gradients), but that variation in male mating success was much reduced in the *SPR-* treatment, a difference detected by the variance-standardised Bateman gradients (i.e. higher pre-copulatory Jones's index in the focal males of the control than the *SPR-* treatment). A mechanism that could modulate Bateman gradients in polyandrous populations is patterns of mate sharing (Sih et al., 2009, McDonald et al., 2013, Fisher et al., 2016, Muniz et al., 2015, Wey et al., 2008). As populations become more polyandrous, the matrix of possible male-female matings (i.e. the mating matrix) becomes progressively saturated leading to a positive correlation between the mating success of a male and the polyandry of his mates. This could strengthen the Bateman gradient if males with higher mating success mate preferentially with less polyandrous females (Sih et al., 2009, McDonald and Pizzari, 2016). To investigate whether patterns of mate sharing played a role in the estimates of the Bateman gradient in our groups, we calculated the group sperm competition intensity correlation (*SCIC*), which measures the relationship between male mating success and the intensity of sperm competition faced by his ejaculates (*SCI*) (see Methods, Table 1 and Supplementary Information; (McDonald and Pizzari, 2016). Consistent with our expectations, *SCIC* values were markedly more negative in the *SPR-* treatment than the controls, confirming that polyandry resulted in a saturation of the mating matrix (Figure 3A). Yet, randomisation tests showed that our observed values of *SCIC* were not more extreme than could be expected by chance, given the distribution of mating pairs in the population. (Figure 3A; *SPR-* treatment: p_{rand} = 0.192, *Control*: p_{rand} =

0.97, full details see Methods and Supplementary Information) (Figure 3A), thus revealing no evidence that males with higher mating success mated preferentially with more or less polyandrous females in either treatment. Together, these results demonstrate that polyandry weakens pre-copulatory sexual selection by reducing variance in male mating success and by increasing the level of sperm competition, both of which arise as a simple consequence of saturation of the mating matrix.

We then considered post-copulatory sexual selection. A potential mechanism by which *P* gradients and the post-copulatory Jones's index were be elevated in our *SPR*- treatment is through an increase in male's matings with the same female (see (Collet et al., 2012, Collet et al., 2014)). Focal males in our *SPR*- treatments mated on average more often with the same females than to controls (Figure S1B) and therefore we wondered whether this behaviour was responsible for the stronger correlation between *P* and male reproductive success. To investigate this, we fitted a general linear model of fertilization success (*P*) on the average number of matings with the same female, and found a strong positive correlation between these two factors in the focal males of the *SPR*- treatment but not in the focal controls (Figure 3B, Table S3), suggesting that increasing mating frequency with the same females is the mechanism by which focal males in our *SPR*- treatment attain higher reproductive success.

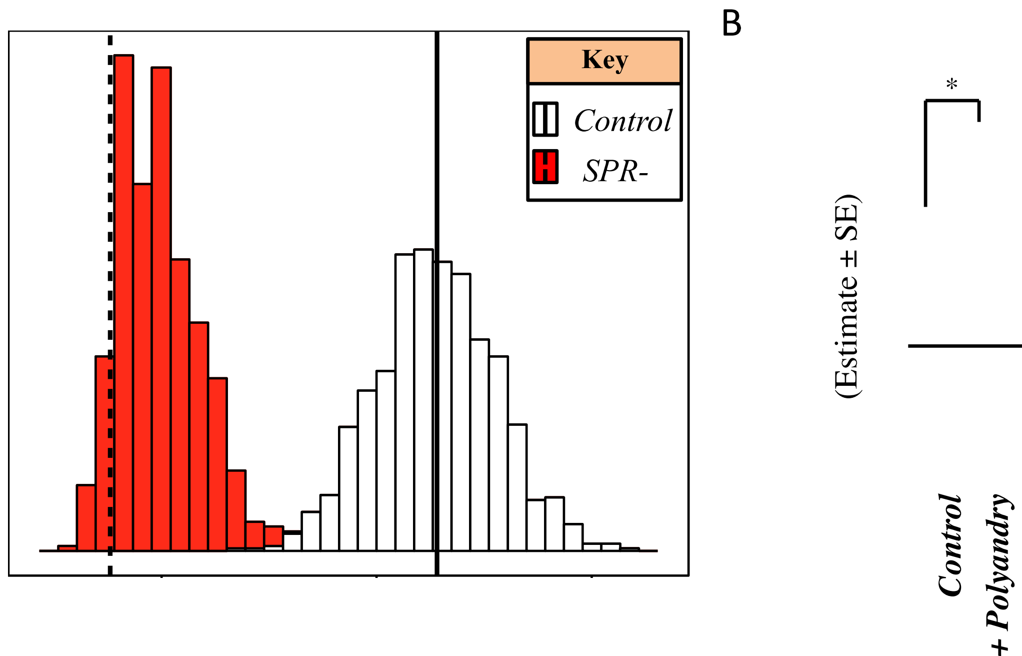


Figure 3 – Mechanisms by which polyandry modulates pre- and post-copulatory episodes. (A) Plot showing the simulated null distributions of sperm competition intensity correlation (*SCIC*) values generated from 1000 randomisations of empirical mating data for *SPR*- and control populations. Vertical lines highlight the observed *SCIC* value in our experiment. Treatments differ in the range of *SCIC* values generated by randomisations, as a result of differing levels of polyandry between treatments. Observed values do not lie outside the range of values expected under the null hypothesis of no assortative mating. (B) The gradient of the average repetitive matings with the same female. * $0.01 \leq p < 0.05$.

An alternative explanation for the results of our *SPR* manipulation is through changes in female productivity. Variation in female productivity can influence our estimates of the sexual selection indexes if, for example, it decreases males' fertilization opportunities and as a result, increases the variance in male fertilization success. Our genetic manipulation of polyandry generated a small but significant reduction in female productivity (*N*) (see Figure S1C). Thus, to test for the effects of low female productivity, we performed an additional experiment with similar design but using a genetic manipulation that reduces the Insulin/Insulin-like signalling (henceforth "*mNSC-ablated"*, full details in the Supplementary Information) (Slack et al., 2011). *mNSC-ablated* females displayed lower productivity but similar polyandry levels to the controls (see Figure S1A and S1C). We found that although the multivariate *M* gradient was significantly steeper for focal males housed with *mNSC-ablated* females, there was no additional effects of reducing female productivity on other sexual selection indexes, which supports our general findings in the *SPR-* experiment (see Figure S2A-N, Table S5-6). Nevertheless, we recognise that there could still exist unaccounted effects of *SPR* knockout. For instance, SPR controls a range of physiological switches in female, including sperm storage and usage (Avila et al., 2015) as well as sleeping patterns and feeding behaviour (Oh et al., 2014, Kubli, 2010). Thus, despite our effort, a perfect control for the off-target phenotypic effects of the *SPR* knockout is currently unavailable, and we acknowledge that our results should be interpreted with some caution.

Our results establish the causal link between the average level of polyandry of a population and the opportunity for sexual selection on the males of such population. We show that increasing polyandry in a moderately polyandrous mating system such as *Drosophila melanogaster* (see (Harshman and Clark, 1998)) causes a reduction in sexual selection and demonstrate that –as pre-copulatory sexual selection is weakened by polyandry- the role of post-copulatory processes becomes progressively more important, particularly through

repetitive matings with the same females. High mating frequency with the same female and intense sperm competition may reduce male ejaculate investment, which in turn could reduce male mating duration in *D. melanogaster* (Wedell et al., 2002). Thus, our findings provide a clear insight into the evolutionary forces underpinning the recent findings that males allowed to evolve in hyper polyandrous populations display lower pre-copulatory performance and had shorter mating duration (Perry et al., 2016). Furthermore, our findings confirm similar patterns of polyandry on sexual selection described by previous correlational studies in a range of taxa, e.g. the sand goby (*Pomatoschistus minutus)* (Jones et al., 2001), Houbara bustard (*Clamydotis undulate undulate*) (Turnell and Shaw, 2015), hermaphroditic snails (*Physa acuta)* (Pelissie et al., 2014)*,* Hawaian sword-tail crickets *(Laupala cerasina)* (Turnell and Shaw, 2015) and the red-jungle fowl (*Gallus gallus*) (Collet et al., 2012) and thus provide a general mechanisms through which polyandry can modulate sexual selection across species.

Previous studies, mostly in socially monogamous birds, have suggested that polyandry has little effect or increase the operation of sexual selection in males (Birkhead and Møller, 1998, Albrecht et al., 2009, Whittingham and Dunn, 2005, Webster et al., 2007). Together with our results, this collection of findings shows that an increase in polyandry in a monogamous mating system increases the opportunity for sexual selection due to extra-pair copulations and high mating success for few individuals whereas increasing polyandry in a polyandrous mating system reduces the opportunity for sexual selection by eroding the skewness in male mating success (see (Collet et al., 2012, Lesobre et al., 2010). Interestingly, we showed that increasing polyandry strengthen the negative correlation of male mating success and his sperm competition by saturating the mating matrix (the matrix of mating interactions between males and females in the population). Yet, increasing polyandry does not create assortative matings between more (or less) promiscuous individuals, which contradicts the assortative matings between highly promiscuous individuals found in a wild population of

Gryllus campestris (Fisher et al., 2016). It is likely that population structure influence the relationship between promiscuous individuals in the wild if, for example, some males are surrounded by highly polyandrous females whereas other males only have access to monogamous mates. In our study, groups were small, and there was limited variation in female receptivity due to the constrains imposed by our genetic manipulation of polyandry. Thus, it is possible that increasing either population size or variance in female receptivity, or both, could generate patterns of assortative mating based on partner promiscuity (i.e. mating success). Future studies should address this question.

Conclusions

Overall, this study provides important experimental contributions to the long-standing debate regarding the implications of polyandry to the operation of sexual selection. By showing that increasing polyandry weakens sexual selection and increases the relative role of post-copulatory processes, we revealed the mechanism by which polyandry can drive male evolutionary responses that lead to behavioural, cellular and molecular adaptations such as mate guarding (Jarrige et al., 2016), ejaculate proteins (reviewed by (Perry et al., 2013)) and unusual sperm morphologies (e.g. (Miller and Pitnick, 2002)).

Experimental Procedures

(a) Fly stocks and culture

All fly genetic mutations were backcrossed into the Dahomey background for >5 generations. Experiments were conducted at 25°C on a 12:12 light:dark cycle in a non-humidified room fed with standard fly food with live yeast *ad libitum*. Flies were marked with

acrylic paint on the thorax, which has been previously shown to have no effect upon fly behaviour so that individuals could be identified during behavioural observations (Nilsen et al., 2004, Tan et al., 2012).

(b) Experimental design

The experimental design was based on (Bjork and Pitnick, 2006, Morimoto et al., 2016) (see Results and Discussion). All offspring was counted as adults 13-15 days after oviposition. Given the genetic constructs used to increase polyandry levels, which comprises a deletion in the X chromosomes (Yapici et al., 2008), we could only assess focal male paternity in daughters (see (Dean et al., 2012)). Therefore, our estimates of focal male and female reproductive success are based on the number of daughters in the progeny.

(c) Data analysis

All analyses were performed in R version 3.2.2 (R Development Core Team, 2015).

Male reproduction - We analysed the response of absolute mating frequencies (i.e. number of copulations), mate number (number of unique mates) (*M)* or offspring production (i.e. number of daughters) (*T*) while controlling for the effects of experimental replicate with a 'quasi Poisson' GLM to account for the overdispersion of the raw data. We used a GLM 'quasibinomial' to test for the effects of increasing polyandry on the proportion of daughter sired by the focal male *(P*). P-values are given from ANOVA.

Opportunity for (pre- and post- copulatory sexual) selection – We calculated I, I_S, and I_P, as described in Table 1. I has been extensively used in a variety of species of birds (e.g. (Whittingham and Dunn, 2005, Collet et al., 2012, Collet et al., 2014, Webster et al., 1995)), newts *Taricha granulosa* (Jones et al., 2002), pronghorn *Antilocapra americana* (Byers and Dunn, 2012), squirrels *Xerospermophilus tereticaudus* (Munroe and Koprowski, 2011),

beetles *Callosobruchus maculatus, C chinensis, Megabruchidius dorsalis* and *M. tonkineus* (Fritzsche and Arnqvist, 2013), hermaphrodite snails *Physa acuta* (Janicke et al., 2015) and also in comparative studies (Jones, 2003), and reflects an estimate of the upper limit of the strength of selection. To test for differences between treatments, we used bootstrap (package "boot") to calculate the confidence interval of our estimates. Only when the confidence interval did not overlap did we consider the differences statistical significant.

Selection gradients and overall strength of pre- and post- copulatory sexual selection – We fitted a general linear model of male reproductive success (*T*) onto a univariate *M* (the Bateman gradients) as well as a multivariate *M*, *P* and *N* to calculate the selection gradients. All variable were mean-standardized (see Table 1). To calculate and test differences in the maximum standardized pre- and post-mating sexual selection differential indexes (also known as Jones's indexes) $s'_{max\ (pre)}$ and $s'_{max\ (post)}$ we fitted a general linear model as mentioned above, but with variance-standardized *M, P* and *N*, which is equivalent to calculating as the mean-standardized selection gradient multiplied by the square-rood of the opportunity for pre- or post-copulatory sexual selection (see Table 1). In all models we tested for differences between treatments by including the interaction Treatment*($M + P + N$) that allowed us to investigate whether treatment affected any of the major components of male reproduction. To calculate the relative importance of increased mating frequency with the same female for post-copulatory sexual selection, we fitted a general linear model of *P* on the average number of matings with the same female, which was also mean-standardized (see Table 1). We tested the significance of the factors in the model with a quasinomial GLM. We square-root boxcox transformed male reproductive success in models of selection gradients for tests of significance; we confirm the fit of the model through inspection of the diagnostic plots.

Sperm competition intensity and assortative mating (SCI) – We calculated a male's sperm competition intensity (*SCI)* as the harmonic mean of male's mates mating success

(Shuster and Wade, 2003, McDonald and Pizzari, 2016, Wey et al., 2008) (see Table 1). To assess the relationship between male reproductive success and *SCI* we fitted a linear model for each treatment of *T* on *M (*or *P)* and *SCI*, while controlling for vial productivity and experimental replicate. To investigate the assortative patterns of mate sharing in our populations, we calculated the sperm competition intensity correlation (*SCIC*) for each treatment according to the formula in Table 1 (see also Supplementary Information for details). To test whether patterns of mating assortment were more or less than could be expected by chance we used randomisations (1,000) of our behavioural mating data to test the significance of the relationship between male *SCI* and *M*. The randomizations shuffled the identity of mating pairs of males and females within each experimental vial (i.e. who mates with who) while holding constant both the number of total copulating pairs and the variation in male and female mating success within each vial (i.e. controlling for average polyandry and the variance in male and female mating success; (Saavedra and Stouffer, 2013). For each randomized data set we calculated *SCIC* as above. Calculations of *SCIC* used data including all males, both focal and non-focal males. All variables used in models were mean-standardized (Table 1).

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Supplementary Information

Supplementary Methods

Sperm competition intensity (SCI) – We calculated a male's sperm competition intensity (*SCI)* as the harmonic mean of male's partners mating success (Shuster and Wade, 2003, McDonald and Pizzari, 2016, Wey et al., 2008). To assess the relationship between male reproductive success and *SCI* we fitted a linear model for each treatment of *T* on *M and SCI*, while controlling for vial fecundity and experimental replicate in a generalized linear model with a quasi Poisson distribution. To assess the role of *SCI* post-copulatory sexual selection we fitted a linear model for each treatment of *P* on *SCI*, while controlling for vial fecundity and experimental replicate in a model with arcsine transformed *P*. To investigate the presence of assortative patterns of mate sharing in our population we calculated the sperm competition intensity correlation (*SCIC*) for each treatment (see main text). This measures the relationship between male mating success (*M*) and the intensity of sperm competition (*SCI*), as the slope of the least-squares regression of *M* on *SCI*. Negative values of *SCIC* describe a tendency for males with high mating success to mate with females with few mating partners and positive values describe a positive correlation between male mating success and the mating success of their female partners. Thus when *SCIC* is positive we predict a reduction in male Bateman gradients, whereas a negative *SCIC* values will steepen the slope of the male Bateman gradient by accentuating the relationship between mating success and male reproductive success. Importantly, non-zero values of *SCIC* are likely to arise through chance (McDonald and Pizzari, 2016). To test whether patterns of mating assortment were more or less than could be expected by chance we used randomisations of our behavioural mating data to test the significance of the relationship between male *SCI* and *M*. Briefly, our

randomisations randomly shuffle the identity of copulating pairs of males and females within each experimental vial (i.e. who mates with who) while holding constant both the number of total copulating pairs and the variation in male and female mating success within each vial (i.e. controlling for average polyandry and the variance in male and female mating success; (Saavedra and Stouffer, 2013). We generated 1,000 randomisations of our behavioural mating data for each treatment. For each randomised data set we calculated *SCIC* as above. Due to the small number of individuals and large number of mating pairs it is important to note that our randomisations were restricted in the number randomized outcomes, some vials could not be shuffled resulting in only one possible *SCIC* value. However, at the population across all vials this process generated a null distribution of *SCIC* values. We then compared our observed values to the simulated distribution of values for each treatment respectively (Farine and Whitehead, 2015). Calculations of *SCIC* used data including all males, both focal and non-focal males. All variables used in models were standardized by dividing by their respective means.

Supplementary Results

Polyandry and the intensity of sperm competition

It is becoming increasingly appreciated that the pattern of mate sharing in a population rather than simply the average polyandry can also affect the strength of selection in males (Sih et al., 2009, McDonald et al., 2013, Fisher et al., 2016, Muniz et al., 2015, Wey et al., 2008). This is because if those males with the highest mating success also mate with the most polyandrous females, they may suffer the highest sperm competition intensity (*SCI*). This is expected to reduce selection on male mating success (i.e. the Bateman gradient) as the benefits of increased mating success are further eroded by increased *SCI*. Similarly, if the

males with the highest mating success enjoy largely exclusive mating access to their females, this may accentuate the relationship between mating success and offspring (Sih et al., 2009, McDonald et al., 2013). To investigate whether such assortative patterns of mating had any additional implication to the estimates of sexual selection in our populations, we first estimated each male's sperm competition intensity (*SCI*), which reflects the average polyandry of a male's mates (see Methods). As expected, we found a negative correlation between male reproductive success (*T*) and sperm competition intensity (*SCI*) (Control: *Estimate: -1.037± 0.407*, p = 0.019; *SPR-*: *Estimate: -1.361± 0.425*, p = 0.004) as well as male paternity share (*P*) and sperm competition intensity (*SCI*) (Control: *Estimate: -0.826± 0.407*, p = 0.013; *SPR-*: *Estimate: -0.559± 0.152*, p = 0.001) in both control and *SPR-* treatment, confirming that male reproductive success and fertilization success are negatively associated with the sperm competition intensity (*SCI*).

The effects of a reduction in female productivity on the operation of sexual selection in males

We manipulated female productivity (Figure S1) by ablating female median neurosecretory cells (Slack et al., 2011); henceforth "*mNSC-ablated*"). There was no effect of our manipulation in either focal males mating frequency, number of mates and on the proportion of mate's daughters sired, although there was a reduction in the number of daughters sired (Figure S2A-D), suggesting a reduction in female productivity. In addition, there was no effect of a reduction in female productivity on male sexual selection indexes except for the Multivariate *M* gradient, which became significantly steeper for focal males in the *mNSC-ablated* treatment compared to focal controls (Figure S2E-N, Table S2-S5).

Supplementary Figures

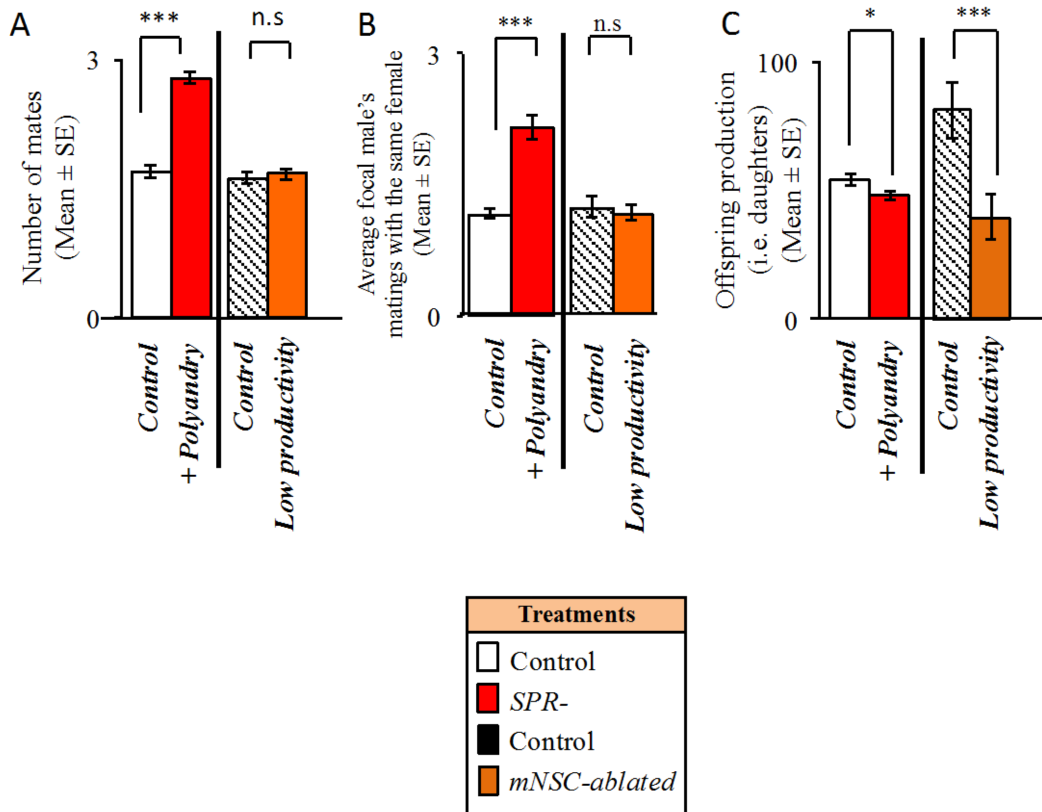

Figure S1 – (A) The number of mates of females of females in both the *SPR-* and *mNSC-ablated* experiments. (B) Average focal male's number of matings with the same female. (C) Offspring (i.e. daughters) production of females in both the *SPR-* and *mNSC-ablated* experiments.. * 0.01 ≤ p < 0.05; ** 0.01 ≤ p < 0.001; *** p < 0.001. *n.s.* – p > 0.05.

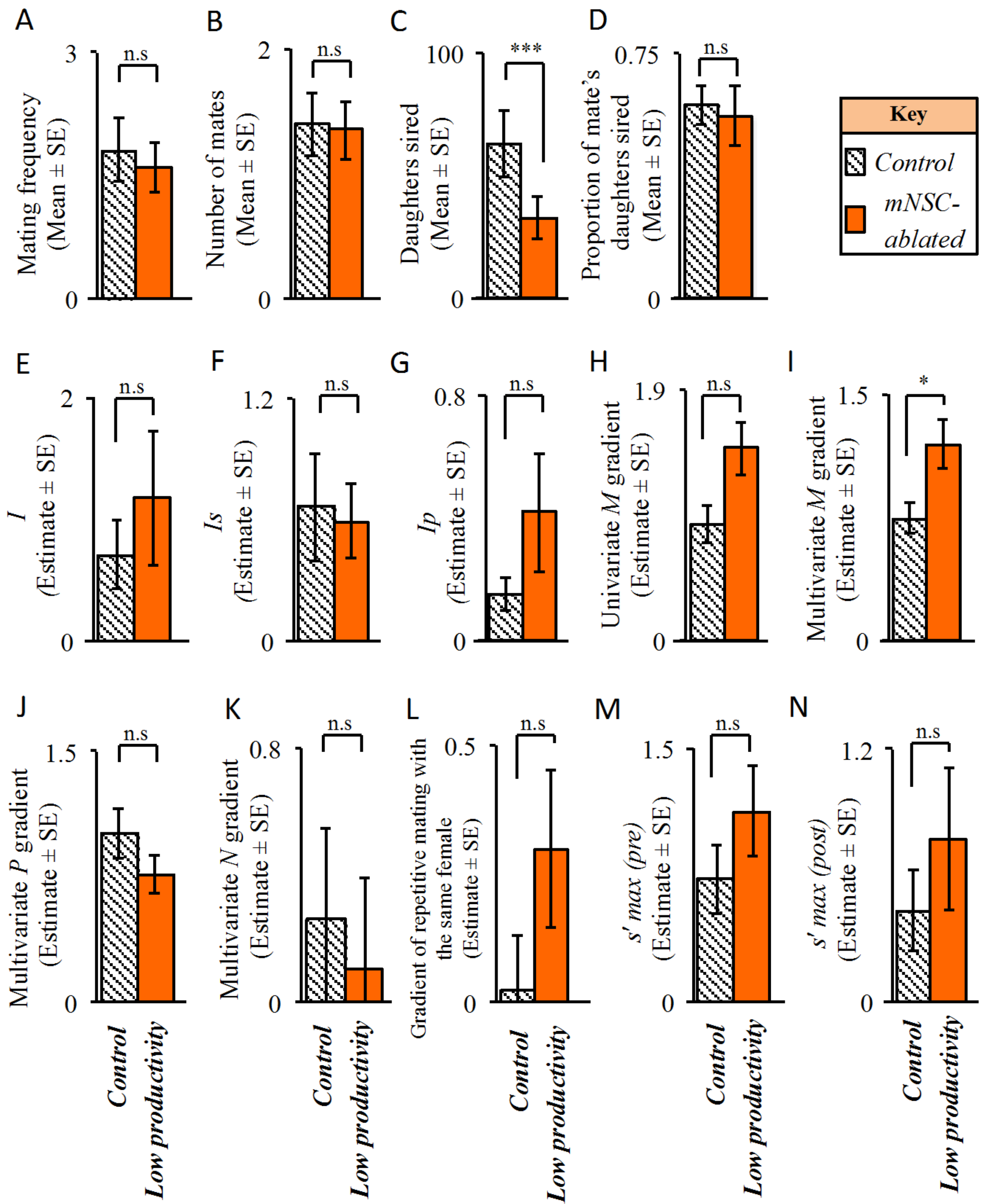
A
B
C
D
Mating frequency (Mean ± SE)
Number of mates (Mean ± SE)
Daughters sired (Mean ± SE)
Proportion of mate's daughters sired (Mean ± SE)
n.s
***
Key
Control
mNSC-ablated
E
F
G
H
I
I (Estimate ± SE)
Is (Estimate ± SE)
Ip (Estimate ± SE)
Univariate M gradient (Estimate ± SE)
Multivariate M gradient (Estimate ± SE)
*
J
K
L
M
N
Multivariate P gradient (Estimate ± SE)
Multivariate N gradient (Estimate ± SE)
Gradient of repetitive mating with the same female (Estimate ± SE)
s' max (pre) (Estimate ± SE)
s' max (post) (Estimate ± SE)
Control
Low productivity

Figure S2 – Effects of low female productivity on the operation of sexual selection in males. (A) Focal male mating frequency (number of matings); (B) Focal male mating success (number of mates); (C) Number of daughters sired by the focal males; (D) Proportion of daughters sired by the focal males with females they mated with; (E) the standardized variance in offspring (daughters) sired by the focal males (I) ("the opportunity for selection"); (F) the standardized variance in focal male mating success (I_S) ("the opportunity for pre-copulatory sexual selection"); (G) the standardized variance in focal male siring success (I_P) ("the opportunity for post-copulatory sexual selection"); (H) The univariate gradient of focal male mating success and offspring (the univariate *M* gradient or "the Bateman gradient"); (I) The multivariate gradient of focal male mating success and offspring (the multivariate *M* gradient); (J) The multivariate gradient of focal male paternity share and offspring (the multivariate *P* gradient); (K) The multivariate gradient of focal male mate productivity and offspring (the multivariate *N* gradient); (L) the Gradient of repetitive matings with the same female. (M) the maximum standardized pre-mating sexual selection differential index $s'_{max\ (pre)}$ (Jones's index); (N) the maximum standardized post-mating sexual selection differential index $s'_{max\ (post)}$. * $0.01 \leq p < 0.05$; ** $0.001 \leq p < 0.01$; *** $p < 0.001$ *n.s* – $p > 0.05$ or overlapping bootstrap confidence intervals.

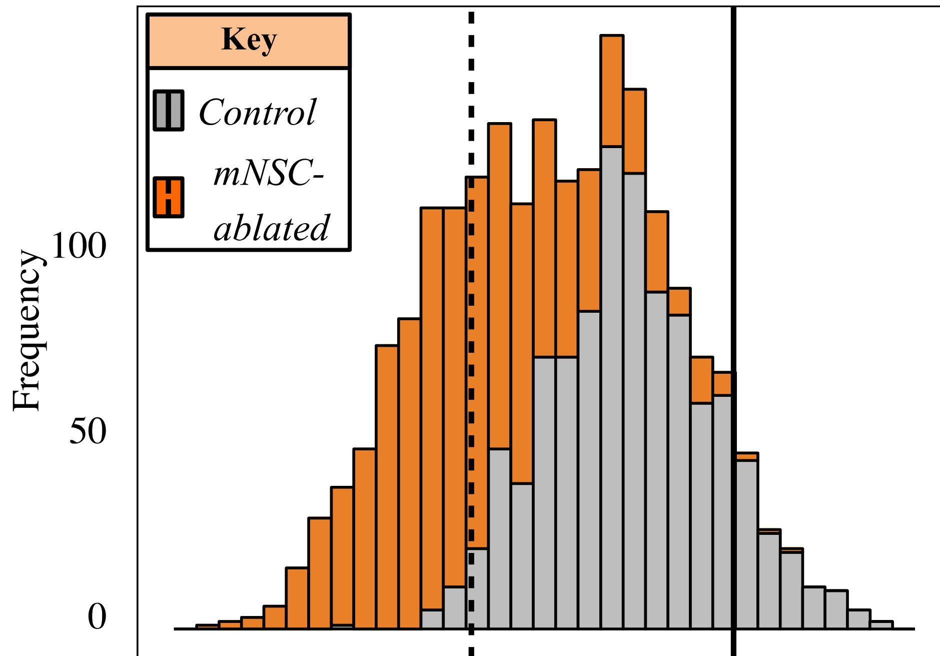

Figure S3 – Randomisations reveal no assortative mating patterns in populations where females have low productivity populations or control populations. Plot showing the simulated null distributions of sperm competition intensity correlation (*SCIC*) values generated from 1000 randomisations of empirical mating data for *mNSC-ablated* and control populations. Vertical lines highlight the observed *SCIC* value for each population. Treatments differ in the range of *SCIC* values generated by randomisations, as a result of differing levels of polyandry between treatments. Observed values do not lie outside the range of values expected under the null hypothesis of no assortative mating.

Table S1 – The opportunity for selection (*I*), pre-copulatory sexual selection *(I_S)* and post-copulatory sexual selection *(I_P).* 95% boostrap confidence intervals (CI).

Index	*Treatment*	*Estimate*	*Lower 95% CI*	*Upper 95% CI*
I	*Control*	0.748	0.447	1.391
	SPR-	0.386	0.257	0.628
	Control	0.712	0.337	1.591
	mNSC-ablated	1.175	0.573	2.736
I_S	*Control*	0.408	0.255	0.730
	SPR-	0.058	0.031	0.131
	Control	0.665	0.318	1.474
	mNSC-ablated	0.593	0.329	1.245
I_P	*Control*	0.264	0.159	0.523
	SPR-	0.251	0.163	0.442
	Control	0.437	0.153	0.904
	mNSC-ablated	0.776	0.319	1.588

Table S2 – Mean standardized univariate and multivariate selection gradients. β_{SS}^{Uni} = Univariate mating success (*M)* gradient; β_{SS}^{Multi} = Multivariate mating success (*M)* gradient; β_{P}^{Multi} = Multivariate paternity (*P)* gradient; β_{N}^{Multi} = multivariate mate productivity (*N*) gradient; *T* = Treatment. Bold = p <0.05.

Factor

$^{Multi} * T$	0.061

Table S3 – The relationship between repetitive matings with the same female and fertilization success (*P*). Bold = p <0.05.

Factor	Controls			*SPR-* (i.e. high polyandrous)			Controls			*mNSC-ablated* (i.e. low productivity)		
	Estimate	**SE**	**p-value**	**Estimate**	**SE**	**p-value**	**Estimate**	**SE**	**p-value**	**Estimate**	**SE**	**p-value**
Same female mating	-0.172	0.205	0.410	0.632	0.155	**<0.001**	0.025	0.106	0.814	0.299	0.153	0.070
Replicate	-0.078	0.423	0.854	0.747	0.320	**0.027**	-	-	-	-	-	-
Slope Comparison							**Slope Comparison**					
	Estimate	**SE**	**p-value**				**Estimate**	**SE**	**p-value**			
Same female mating $*$ ***T***	0.765	0.257	**0.004**				0.274	0.189	0.159			

Table S4 – Variance standardized univariate and multivariate selection gradients. β_{SS}^{Uni} = Univariate mating success (*M)* gradient; β_{SS}^{Multi} = Multivariate mating success (*M)* gradient; β_{P}^{Multi} = Multivariate paternity (*P)* gradient; β_{N}^{Multi} = multivariate mate productivity (*N*) gradient; *T* = Treatment. Bold = p <0.05.

Factor	
$\boldsymbol{_N^{Multi} * T}$	0.061

Table S5– Decomposition of variance in male reproductive success (T) into the relative contributions of M, P and N in the low productivity experiment (*mNSC-ablated*). Delta method of variance decomposition following Webster *et. al.* 1998, Collet *et. al.* 2010 and Morimoto *et. al.* 2016.

Var-Cov Components	Observed contribution to *var(T)*			
	Control		*mNSC-ablated*	
var(T)	*2.813*	*%*	*1.276*	*%*
var(M)	3.128	*111.2*	0.467	*36.6*
var(P)	0.698	*24.8*	0.329	*25.8*
var(N)	0.340	*12.1*	0.077	*6.0*
cov(M, P)	0.267	*9.5*	0.197	*15.4*
cov(M, N)	-0.574	*20.4*	0.129	*10.1*
cov(N, P)	-0.090	*3.2*	0.047	*3.7*
D	-0.956	*34.0*	0.031	*2.4*

Chapter 3

Developmental environment effects on sexual selection in male and female *Drosophila melanogaster*

Abstract

The developmental environment can potentially alter the adult social environment and influence traits targeted by sexual selection such as body size. In this study, we manipulated larval density in male and female *Drosophila melanogaster*, which results in distinct adult size phenotypes – high (low) densities for small (large) adults – and measured sexual selection in experimental groups consisting of adult males and females from high, low, or a mixture of low and high larval densities. Overall, large adult females (those reared at low larval density) had more matings, more mates and produced more offspring than small females (those reared at high larval density). The number of offspring produced by females was positively associated with their number of mates (i.e. there was a positive female Bateman gradient) in social groups where female size was experimentally varied, likely due to the covariance between female productivity and mating rate. For males, we found evidence that the larval environment affected the relative importance of sexual selection via mate number (Bateman gradients), mate productivity, paternity share, and their covariances. Mate number and mate productivity were significantly reduced for small males in social environments where males were of mixed sizes, versus social environments where all males were small, suggesting that social heterogeneity altered selection on this subset of males. Males are commonly assumed to benefit from mating with large females, but in contrast to expectations we found that in groups where both the male and female size varied, males did not gain more offspring per mating with large females. Collectively, our results indicate sex-specific effects of the developmental environment on the operation of sexual selection, via both the phenotype of individuals, and the phenotype of their competitors and mates.

Introduction

Sexual selection favours traits that confer an advantage in intra-sexual competition in both sexes (Andersson, 1994). While historically, sexual selection studies have focused on males, it is now appreciated that intra-sexual competition can also play an important role in the evolution of females, which in turn affect males responses to female adaptations (Clutton-Brock, 2007, Clutton-Brock, 2009). It is therefore important that studies on sexual selection consider both sexes, because resolving the evolution of sex roles and the nature of sexual conflict hinges largely on understanding the mechanisms that cause sex-specific patterns of sexual selection.

Traditionally, intra-sexual competition was considered exclusively over mating opportunities, and the strength of sexual selection has been measured by the slope of the linear univariate regression of offspring number against number of mates - the "Bateman gradient" (Jones, 2003, Jones and Ratterman, 2009, Fritzsche and Arnqvist, 2013, Knight, 2002). Hence, the Bateman gradient explicitly captures only one component of pre-copulatory sexual selection: the number of mates (i.e. "mating success"). By showing that male Bateman gradients are often steeper than female gradients, this approach has been instrumental in defining sex roles (Cunningham and Birkhead, 1998, Trivers, 1972, Brown et al., 2009). Yet, it is becoming increasingly evident that other factors can influence the relationship between mate number and offspring number, particularly for males, where variation in paternity share due to post-copulatory sexual selection and variation in female productivity can be important (e.g. (Collet et al., 2012, Collet et al., 2014, Pelissie et al., 2014)). This indicates that the total number of offspring sired by a male (i.e. his reproductive success) is best described through a multivariate approach as follows:

$$T = M * P * N + \varepsilon \qquad \text{eqn 1}$$

where T is the total number of offspring produced, M is the number of females mated by a male, P is his average paternity share of the offspring produced by his mates, N is the average number of eggs produced by his mates and ε is an error term with 0 mean (Parker and Pizzari, 2015, Collet et al., 2012). In this case, the multivariate model incorporates measures of both pre- and post-copulatory sexual selection and allows the investigation of their relative contribution to the total number of offspring sired by males (Collet et al., 2012). Therefore, the multivariate approach substantially deepens our understanding of the factors that determine variation in the number of offspring sired by different males (Collet et al., 2012, Collet et al., 2014, Webster et al., 1995).

The interpretation of the female Bateman gradient has also attracted considerable debate (Gerlach et al., 2012, Alonzo and Pizzari, 2010, Parker and Tang-Martinez, 2005, Collet et al., 2014). For instance, it is becoming increasingly clear that female Bateman gradients can be steeper than originally assumed (e.g. (Arnold, 1994, Snyder and Gowaty, 2007, Worden and Parker, 2001, Clutton-Brock, 2007, Clutton-Brock, 2009, Ketterson et al., 1998, Collet et al., 2014)). However, the causality of this relationship is not always clear. In principle, a positive female Bateman gradient can measure sexual selection on female mate number, for example when mating provides cumulative direct benefits to females (Wedell and Karlsson, 2003, Alonzo and Pizzari, 2010). However, positive female Bateman gradients can also arise as a result of non-causal or inverse associations between mate number and the number of offspring produced (Parker and Tang-Martinez, 2005, Gerlach et al., 2012, García-Navas et al., 2014), for example when inherently more fecund females either attract or require more mates (Collet et al., 2014, Ketterson et al., 1998).

A likely modulator of sex-specific patterns of sexual selection is the pool of resources available to individuals to allocate to traits (Rowe and Houle, 1996) (i.e. individual's environmental conditions) and the pool of resources available for individual's competitors

(i.e. social condition). Both the individual's environmental and social conditions can influence the strength of sexual selection, for example through the modulation of adult competitive ability, mate preferences, or productivity and body size, if body size is correlated with environmental conditions that affect any of these traits (Bonduriansky, 2001, Cotton et al., 2006). For instance, Janicke et al. (2015) recently showed that fluctuations in adult food availability levels can affect body weight, reproductive traits and the strength of sexual selection on a simultaneously hermaphroditic snail, *Physa acuta*. In food-restricted snails, mating was not significantly associated with increments in offspring number, resulting in non-significant Bateman gradients for both male and female roles, in contrast to larger, food-unrestricted snails where gradients were significantly positive. Adult diet manipulation rather than developmental diet implies relatively rapid plastic responses. However, the study of simultaneous hermaphrodites makes it difficult to disentangle the independent effect that diet may have on male and female roles from its influence on trade-offs in sex allocation within individuals.

In insects, including *Drosophila melanogaster*, adult body size is often mediated by the environment during development (Amitin and Pitnick, 2007, Pitnick and Garcia-Gonzalez, 2002, Lyimo et al., 1992, Credland et al., 1986), which in turn tends to positively correlate with female productivity (i.e. large females produce more eggs than small females) and male quality (Bonduriansky, 2001). Adult body size is expected to be under productivity selection (in females) and sexual selection (in males) in adult insects (Bonduriansky, 2001, Honek, 1993, Roff, 2002, Stearns, 1992, Clutton-Brock, 2009). A recent study showed that males with small body size (raised at high larval density) have reduced reserves of seminal fluid but invest proportionally more of this seminal fluid *per* mating than large males (raised at low larval density)(Wigby et al., 2015). Given that seminal fluid is limited in supply in *D. melanogaster* (Hihara, 1981, Linklater et al., 2007, Sirot et al., 2009) small males may have a

reduced ability to transfer multiple full-sized ejaculates. If so, being small could potentially reduce the benefits of multiple matings for males – in which case we would expect to observe reduced Bateman gradients – and could also modulate post-copulatory competitiveness (Bangham et al., 2002). Furthermore, both large and small males invest more seminal fluid when mating with large females, suggesting that males can adjust their ejaculate investment depending on the body size of their mates (Wigby et al., 2015). Importantly, the developmental environment tends to affect male fitness more than female fitness (Zikovitz and Agrawal, 2013), suggesting that environmental conditions can have sex-specific direct (i.e. on the individual) or indirect (i.e. on the individual's mate) effects on selective forces. It is consequently reasonable to expect that there may be links between variation in the developmental environment, adult body size and the strength and form of sexual selection within adult populations.

Together, these previous studies indicate that environmental effects, particularly larval environment-mediated effects on adult body size or on traits associated with body size, have the potential to influence the strength of both pre- and post-copulatory sexual selection within populations. However, despite the growing interest in ecological factors affecting reproduction (Gillespie et al., 2014, Amitin and Pitnick, 2007, Zikovitz and Agrawal, 2013, Long et al., 2009, Cornwallis and Uller, 2010, Janicke et al., 2015), the effects of the developmental environment on patterns of sexual selection have received little attention. In this study, we manipulated adult traits, including body size, by varying larval density in *D. melanogaster*. Increasing larval density limits the quantity and quality of the food available per larvae, results in reduced adult body size, and has far-reaching consequences for male and female reproduction (McGraw et al., 2007, Amitin and Pitnick, 2007, Pitnick, 1991, Pitnick and Garcia-Gonzalez, 2002, Lefranc and Bundgaard, 2000, Byrne and Rice, 2006, Lüpold et al., 2010). Larval density may also signal as an index of population density, which is expected

to play a central role life-history traits in species with high reproductive rates such as *D. melanogaster* and other insects (Stubbs, 1977), and therefore has important ecological and evolutionary implications. For example, high developmental densities might provide cues of intense intrasexual competition in adulthood (Gage, 1995, Lemaitre et al., 2011). Here, we tested how larval density influences reproductive behaviour and the strength of sexual selection, as measured by the Bateman gradient. Although, as described above, a manipulation of larval density is likely to affect multiple traits and impose different selective pressure on the larvae (McGraw et al., 2007, Amitin and Pitnick, 2007, Baldal et al., 2005), for conciseness we refer to the set of experiments and groups according to the body size of adults, because this is the most striking adult phenotype from the larval density manipulation and is consistent with terminology in previous literature (e.g. (Amitin and Pitnick, 2007, Long et al., 2009)).

In principle, the developmental environment can influence the fitness of a focal individual directly by influencing a focal individual's own phenotypes, and by modulating the phenotypes of other group members (i.e. the competitors and potential mates of the focal individual), which can in turn feedback on the fitness of the focal individual. We used an experimental approach to explore these focal and group effects within each sex, by assembling groups of adults in which larval-density manipulations had resulted in body sizes which were constantly large, constantly small or varied in either sex or simultaneously in both sexes. Firstly, we investigated how the larval density manipulations on individuals and on group composition influenced the number of mates, mating frequency and the number of offspring produced by males and females. Then, we investigated how the larval density manipulations on individuals and on group composition affected the strength of sexual selection in females and males. It is important to notice that larval density manipulations likely affected multiple facets of development and physiology in addition to body size such as

ejaculate investment, perceived competition, encounter rate, aggregation and other (e.g.(McGraw et al., 2007, Amitin and Pitnick, 2007, Baldal et al., 2005, Long et al., 2009)). Nonetheless, body size is the most striking phenotypic trait affected by larval density manipulation and, in agreement with the literature, we referred to the social environment by the phenotypic variance in body size caused by our manipulation of the developmental environment.

Predictions

Reproduction

- Overall, based on previous literature, we expected large individuals to mate more frequently, obtain more mates and produce more offspring than small individuals

Sexual selection

- We expected female Bateman gradients to be steeper in female mixed size social environments due to the association between body size, number of mates and offspring production. Because this effect relies solely on female's physiological and behavioural traits, we did not predict effects of male body size variation in this pattern.
- We expected male Bateman gradients to be generally positive regardless of male body size. However, we also expected the social environment to modulate the strength of sexual selection on male size both before and after copulation. For instance, we predicted stronger post-copulatory sexual selection on large males in homogenous social environments (where competitors are similarly large and equally good competitors) than in heterogeneous social environments (where large males may

outcompete small males). Strong post-copulatory sexual selection may increase ejaculate investment and reduce the benefits of multiple copulations. As a result, we also expected the Bateman gradient of large males to be reduced when experiencing homogeneous social environments.

- Finally, when manipulating both sexes body size, we expected to strengthen sexual selection on male size by enabling large males to outcompete small males over large, more fecund females and their eggs, and by enabling large females to outcompete small females over access to large males and their sperm.

Material and Methods

Fly stocks and culture

We used a wild-type stock of *D. melanogaster* that was collected in Dahomey (Benin) in North Africa in 1970 and has been maintained in large (>5,000 individuals) outbred populations in cages with overlapping generations (Wigby et al., 2009). Focal males were wild-type Dahomey, while competitor males and experimental females carried the recessive *sparkling*^{poliert} mutation (*spa*), which had been backcrossed into the Dahomey genetic background for more than 5 generations. The *spa* mutation produces a rough-looking eye phenotype when homozygous (Fu et al., 1998) and is commonly used in sperm competition assays to assign paternity (Fricke et al. (2010b)). All fly stocks were maintained, and all experiments conducted, at 25°C on a 12:12 light:dark cycle in a non-humidified room and were fed with standard sugar-yeast-maize-molasses medium with excess live yeast granules.

Larval density manipulation to vary adult body size

Following the protocol of Clancy and Kennington (2001), we collected eggs from population cages and pipetted eggs. We used the following densities to manipulate the developmental environment: high density ~100 larvae/mL of food (~400 larvae in a 34ml vial containing ~4mL fly food), and low density ~ 4 larvae/mL of food (~200 larvae in a 170ml bottle containing ~50mL fly food), which generated adult flies of small and large body size, respectively. Using this protocol we previously obtained adult females and males of significantly different and non-overlapping body size classes, and with comparable distribution of the variance in the body size (mean mass [mg] ± SE: females, large = 1.60 ± 0.06, small = 0.814 ± 0.08, $F_{1,30}$ = 272.1; males, large = 0.87 ± 0.03, small = 0.60 ± 0.06, $F_{1,30}$ = 85.7; $p < 0.0001$ for all within-sex comparisons (from Wigby et al. (2015)). Using a larger container for the low-density manipulation allowed us to keep the overall population size per container of a similar order of magnitude across the different larval manipulation regimes (i.e. all flies were grown in a container with hundreds of conspecifics) (see [40]). We subsequently refer to the body sizes of adult flies as shorthand for their larval density manipulation: i.e. “large” or “small” to indicate those individuals grown in low and high larval density, respectively. Virgin flies were collected within 8 hours of eclosion and kept in vials of same-sex and same-larval manipulation groups of 15-20 individuals for 2-5 days prior to experiments. In order to track individual flies throughout the behavioural observations we marked all flies of both sexes using 4 colours of acrylic paint - white, yellow, red and orange – one colour *per* individual *per* sex. 24h before experiments began, female flies were randomly allocated to one of these 4 paint colours and were marked on the thorax (Nilsen et al., 2004, Tan et al., 2012); this was done for females across all treatments. Focal males were painted in half of the vials with white colour and with yellow colour in the remaining half of the vials. The three competitor males were assigned the remaining paint colours. After paint

marking, flies were allocated to their experimental groups (see below), and held in single-sex vials prior to the start of experimentation.

Experimental design

Our experimental design for measuring the strength of sexual selection in groups of flies was based on that of Bjork and Pitnick (2006). Briefly, replicate vials contained 4 flies of each sex (i.e. 8 flies per vial) were allowed to interact for 4 hours per day over 4 days, before being discarded. Within each vial, three of the males and all females were *spa*, and one male – the “focal” male – was wild-type, which allowed us to assign the paternity to the focal male (because *spa* is recessive). We conducted three experiments to manipulate social environments. We varied: 1) female adult body size, while keeping male size constant (the “Female Experiment”); 2) male adult body size, while keeping female size constant (the “Male Experiment”); and 3) both male and female body size simultaneously (the “Female-Male Experiment”, see Fig 1).

Experiment	Social manipulation	♂ Large	♂ Small	♀ Large	♀ Small
Female	*Homogeneous Large ($HomL^{F}$)*	4		4	
	Homogeneous Small ($HomS^{F}$)	4			4
	Heterogeneous (Het^{F})	4		2	2
Male	*Homogeneous Large ($HomL^{M}$)*	4		4	
	Homogeneous Small ($HomS^{M}$)		4	4	
	Heterogeneous (Het^{M})	2	2	4	
Female-Male	*Homogeneous Large ($HomL^{FM}$)*	4		4	
	Homogeneous Small ($HomS^{FM}$)		4		4
	Heterogeneous (Het^{FM})	2	2	2	2

Figure 1 – Diagram of the experimental design. We investigated the effects of the developmental environment on the strength of sexual selection. Because larval density strongly influences adult body size we refer to low-larval density flies as “large” and high larval density flies as “small”. *The Female Experiment* – experiment varying female larval density, keeping males constant (low larval density). Homogeneous social environments consisted of 4 large or 4 small females in addition to 4 large males. Heterogeneous social environments consisted of 2 large *and* 2 small females in addition to 4 large males. *The Male Experiment* – experiment varying male larval density, keeping females constant. Homogeneous social environments consisted of 4 large or 4 small males in addition to 4 large females. Heterogeneous social environments consisted of 2 large *and* 2 small males. *The*

Female -Male Experiment – experiment varying both male and female larval density. Homogeneous social environments consisted of 4 large males and females or 4 small males and females. Heterogeneous social environments consisted of 2 large males and females *and* 2 small males and females. In all experiments one male, out of each group of four, was the focal individual for which we obtained paternity data (see methods).

The Female Experiment – In this experiment, female body size was manipulated while male body size was kept constant (i.e. large body size). The heterogeneous social environment (Het^F) comprised of two large ($HetL^F$) and two small ($HetS^L$) females with the four large males. The homogeneous social environment groups consisted of females with constant body size class (i.e. all from the same larval density rearing environments) – i.e. either all large ($HomL^F$) or all small ($HomS^F$) females.

The Male Experiment – In this experiment, male body size was manipulated while female body size was kept constant (i.e. large body size). The heterogeneous social environment (Het^M) comprised of two large ($HetL^M$) and two small ($HetS^M$) males with the four large females. For the heterogeneous social environments, focal males had small body size in half of the replicates (N = 10) and large body size in the other half (N=10) of the replicates. The homogeneous social environment groups consisted of males with constant body size class (i.e. all from the same larval density rearing environments) – i.e. either all large ($HomL^M$) or all small ($HomS^M$) males.

The Female-Male Experiment – In this experiment, both male and female body size was manipulated. The heterogeneous social environment (Het^{FM}) comprised of two large males and two large females ($HetL^{FM}$) and two small males and two small females ($HetS^{FM}$).

For the heterogeneous social environments, focal males had small body size in half of the replicates (N = 10) and large body size in the other half (N=10) of the replicates. The homogeneous social environment groups consisted of males and females with constant body size class (i.e. all from the same larval density rearing environments) – i.e. either all large ($HomL^{M}$) or all small ($HomS^{M}$) individuals.

Note that $HomL^{F}$, $HomL^{M}$, and $HomL^{FM}$ are identical treatments across each experiment, and the label differences simply represent different experiments they were part of (i.e. Female Experiment, Male Experiment, and Female-Male Experiment, respectively).

Therefore, throughout our experiments, individuals in the homogeneous environments were exposed to individuals with the same body size of the sex being manipulated, whereas individuals in the heterogeneous environments were exposed to both small and large individuals. We made 20 replicate vials for each heterogeneous social environment (see below), and 10 for each homogeneous social environment group (i.e. 40 vials for each experiment, 120 in total).

We recorded all matings during the 4-hour interaction periods on each of the 4 days of the experiment. Thus, we scored every mating for every individual, allowing us to calculate both the mating frequency and number of mates for each individual. Between interaction periods females were separated from males and placed individually in fresh oviposition vials, and were allowed to lay eggs for twenty hours (i.e. until the interaction period the following day). Flies were discarded in the fifth day. Oviposition vials were retained and all emerging adults were counted after eclosion (13-15 days after the day of oviposition to ensure all flies had sufficient development time) to measure the number of offspring produced by females. Because all females and the three competitor males carried the recessive *spa* mutation, we could assess the number of offspring sired by focal males: by counting how many offspring were *spa* and how many were wild-type we could calculate the focal (wild-type) male's

paternity share. Paternity share was calculated as the sum of wild-type offspring produced by all the mates of the focal male divided by the total offspring (*spa* plus wild-type) produced by those females. Because the three competitor males were all *spa,* we did not have individual paternity data for these males – thus, only the competitor male's paternity share could be measured. We also calculated "*per mating"* female offspring production as the total number of offspring produced by a female divided by the total number times that female mated. We used female *per mating* offspring production as an estimate of the expected average reproductive value of each additional mating for males (Note that offspring *per* mating is not equivalent to the Bateman gradient, which measures the slope of the observed relationship between number of *mates* and number of offspring – see below). Final sample sizes were as follows: The Female Experiment, n = 156 females ($HomL^F$ = 40, $HomS^F$ = 40, Het^F = 76), n = 40 focal males ($HomL^F$ = 10, $HomS^F$ = 10, Het^F = 20); The Male Experiment, n = 149 females ($HomL^M$ = 37, $HomS^M$ = 38, Het^M = 74), n = 40 males ($HomL^M$ = 10, $HomS^M$ = 10 and Het^M = 20); The Female-Male Experiment, n = 155 females ($HomL^{FM}$ = 39, $HomS^{FM}$ = 39, Het^{FM} = 77), n = 40 males ($HomL^{FM}$ = 10, $HomS^{FM}$ = 10, Het^{FM} = 20). In some vials a single focal female (but no males) died during the experiment: the Female Experiment, Het^F group (4 vials); Male Experiment, $HomL^M$ (3 vials), $HomS^M$ (2 vials) and Het^M (6 vials); Female-Male Experiment, $HomL^{FM}$ (1 vial), $HomS^{FM}$ (1 vial) and Het^{FM} (3 vials). Males that failed to mate were kept in the analysis because failing to mate is likely a consequence of sexual selection. There were no non-mating females.

Data analysis

The three experiments were conducted independently so we carried out analyses separately for each experiment. Because we had complete paternity data for one focal male per replicate group (see methods above), whereas we had data for every female in each

replicate group, the analyses were also conducted separately for each sex. Therefore, for males, we used the single focal individual per vial as the unit of replication, whereas for females we analysed data from all individuals but included the vial as a covariate in all models, to account for pseudoreplication. We first characterised the effects of developmental environment on female and male reproductive traits, and subsequently measured the effects on sexual selection.

Male and female reproductive trait analyses

First we investigated the potential effects of female deaths ("*DF*", dead females) on the results. We pooled the data of our three experiments and created a binary variable *DF,* which contained information on the occurrence (value = 1) or not (value = 0) of a female death in the social environment of a particular individual. A significant effect of *DF* means that the death of an experimental female increased or decreased reproduction of the remaining individuals in that social group. We used quasi Poisson GLMs, which account for overdispersion of the data, to evaluate whether *DF* had a significant effect on the number of mates, mating frequency and number of offspring of both females and focal males. There was a significant effect of *DF* on focal male estimates of number of mates and offspring (see 'S1 Text') and, therefore, *DF* was retained in all analyses of focal male reproduction and sexual selection. Then, we tested for the effect of paint marking, but found no effect of colours on either females or focal male reproduction (see 'S1 Text'); we therefore excluded colours from the final analysis.

We then focused on both females and focal male reproduction. We used generalised linear models (GLM) (family = 'quasi Poisson') to test for differences between levels (see below) in male and female mating frequencies and mate numbers, in the number of offspring produced by females, and in female *per mating* offspring (the number of offspring produced

by a female divided by the number of times that female mated). We used a 'quasibinomial' GLM to investigate the average proportion of offspring sired by a focal male. In our models, the explanatory variables were social environment ("social", which was either homogenous or heterogeneous: see experimental design above), the body size of the sex under consideration, and the interaction between social environment group and body size (i.e. social*body size) whenever the sex under consideration varied in body size (i.e. females in the Female Experiment, males in the Male Experiment, and both sexes in the Female-Male Experiment). If the sex being analysed did not vary in body size (i.e. males in the Female Experiment, females in the Male Experiment), we used GLMs with "social" environment treated as the variable with three levels (e.g. Hom^L, Hom^S and Het). For models in which "social" was the explanatory variable with three levels and the analysis showed p-value ≤ 0.05, we performed a post-hoc "SNK-test" to investigate the difference between the means of the three levels (Zar, 1999). P-values are based on F-statistics for all GLM models.

Sexual selection analyses

For females, we measured the strength of sexual selection on mate number, as **β**, the slope of a linear regression of standardised offspring number (*T*) on standardised number of mates (*M*):

$$T(M) = (\beta * M) + \boldsymbol{\varepsilon} \qquad \text{eqn 2}$$

We standardised *T* by dividing the offspring number of each individual by the mean number of offspring produced by all members of that sex in the group (to give relative reproductive success; see Arnold 1994). Similarly, we standardised *M* as the following: we subtracted the mean number of mates of all individuals of that sex in the group from the number of mates of each individual, and divided the resultant value by the standard deviation of the number of mates of all individuals of that sex in the group. In doing so, we scaled *M* to have a mean of

zero and a standard deviation of unity and *T* to have a mean of 1 (Arnold 1994). We also investigated quadratic effects (see S1 Text) on female Bateman gradients (Collet et al., 2014) because *T* might peak at a given number of males mated followed by a plateau or decline (Jones 2009). Whenever the quadratic term was non-significant, it was removed from the analysis.

For males we first characterised sexual selection in a qualitative way, by decomposing variance in *T* as follows:

$$Var(M * N * P) = \bar{N}^2\,\bar{P}^2\,Var(M) + \bar{M}^2\bar{P}^2\,Var(N) + \bar{N}^2\,\bar{M}^2\,Var(P) + 2\,\bar{M}\,\bar{N}\,\bar{P}^2\,Cov(M,N) + 2\,\bar{M}\,\bar{P}\,\bar{N}^2\,Cov(M,P) + 2\,\bar{P}\,\bar{N}\,\bar{M}^2\,Cov(P,N) + D$$

eqn 3

where $\overline{M}, \overline{P},$ and $\overline{N}$ are the mean values of *M*, *P* (average paternity share) and *N* (average number of adult offspring produced by a male's mates), and D includes the variance in the error term **ε** (Collet et al., 2012, Webster et al., 1995, Bohrnstedt and Goldberger, 1969). This approach enables us to measure the relative contributions of *M*, *P* and *N* and their covariances to *T* (see (Collet et al., 2012, Janicke et al., 2015)). We also used commonality analysis (CA) (Seibold and McPhee, 1979), which decomposes the explained variance of the multivariate linear model (see below) into percentage of variance explained by one (i.e. *unique* explained variance) or a set of variables (i.e. *common* explained variance) (Ray-Mukherjee et al., 2014, Rowell, 1991), to explore the relative contributions of *M, P*, *N*, and their covariate to variation in the number of offspring sired by males (see S1 Text).

Although the decomposition of variances is useful to make qualitative comparisons across social treatments, the quantitative interpretation of variances and covariances can be difficult (Lebigre et al., 2012). To overcome this limitation, we measured linear selection

gradients on male M, P and N using a multivariate model based on eqn 1, which was composed of T (dependent variable), M, P and N (independent variables) as well as "total vial productivity" (VP), which accounted for differences in within-vial offspring productivity, and DF, which accounted for female deaths in the social environment (see above). The model was (in 'R' notation):

$$st(T) \sim st(M) + st(P) + st(N) + VP + DF \qquad \text{eqn 4}$$

where *st( )* indicates the standardised values of the variables T, M, P and N (see above). This multivariate approach allowed us to investigate the effects of the larval manipulation on the Bateman gradient (i.e. gradient of T regressed over M, controlling for all covariates), the paternity share gradient (i.e. gradient of T regressed over P, controlling for all covariates) and the mate productivity gradient (i.e. T regressed over N, controlling for all covariates). We standardised P and N in the multivariate model dividing P and N of each individual focal male by the mean number of P and N of all focal males in the group, respectively.

For both males and females we first tested whether the Bateman gradients of the homogenous and heterogeneous groups significantly differed from 0 in each experiment. We also analysed the gradients of small and large flies within heterogeneous groups (e.g. Het^{F}, Het^{M}, Het^{FM}) separately. This allowed us to determine whether selection was acting differently on small *versus* large flies within heterogeneous groups. For males we additionally tested paternity and mate productivity gradients, using the same approach as for Bateman gradients above (i.e. for both homogenous and heterogeneous groups, and for small and large males separately in heterogeneous groups). Finally, we tested the influence of the social environment by comparing the selection gradients on small and large individuals in homogenous versus heterogeneous groups. To do this we fitted a multivariate linear model for large and small individuals separately that included interaction terms of M, P and N and social

environment composition (*SEC*) (i.e. *M*SEC, P*SEC and N*SEC*), in addition to our covariates of *VP* and *DF*. We present p-values from *t*-statistics for all multivariate linear models. All analyses were performed using the R software (version 3.0.2, (R Development Core Team, 2015)).

Results

Patterns of female reproduction

Large adult females (those reared at low larval density) mated significantly more frequently, mated with significantly more mates, and produced significantly more offspring, than small females (those reared at high larval density) across all experiments in which female body size was manipulated (i.e. Female Experiment and Female-Male experiment) (Table 1, Fig 2a-f, S1 Fig). Large females had higher offspring *per mating* in the Female Experiment (i.e. where female body sized varied), but this difference was absent in the Female-Male Experiment (where both sexes varied in body size; Table 1, Fig 2g-i). Social environment (i.e. homogenous or heterogeneous) affected female mating behaviour in the Female Experiment, where females in the homogeneous social environment mated significantly more frequently, and with more mates, than females in heterogeneous environment (Table 1, Fig 2a, S1 Fig). The social environment also affected female offspring production in the Male Experiment, where females in the heterogeneous male size environment (i.e. exposed to large and small males) produced significantly fewer offspring than females in a homogeneous male social environment (i.e where all males were had large body size; Table 1, Fig 2b, post-hoc SNK test HomL > HomS > Het (α = 0.05). We also found a significant interaction between social environment and female body size (i.e. social*body size) on female mating frequency in the Female Experiment, which was driven by a stronger reduction in mating frequency for small females in heterogeneous social environments relative to homogeneous social environments (Table 1, Fig 2a). We did not detect an effect of social environment or an interaction between social environment and body size on offspring production or offspring per mating of females in the Female Experiment, or on mating frequency, number of mates, or offspring per mating

in the Male Experiment, or on any of the reproductive measures in the Female-Male Experiment (Table 1). The exclusion of a single female in the Female Experiment that mated but did not produce offspring does not qualitatively change the results except for total offspring production, in which the interaction term social * body size, that was marginally non-significant ($p = 0.081$), becomes significant ($p = 0.024$). This result would indicate that the reduction in offspring production in the heterogeneous groups was stronger for small than for large females.

Table 1 – Development effects on reproduction. Analysis of focal male and female mating frequency, number of mates, absolute offspring production, proportion of focal male's offspring production and female *per-mating* offspring production.

F-values and p-values are given. "Social" refers to the composition of the social environment (i.e. homogeneous or heterogeneous) (see Methods). The Female Experiment – varying female body size; The Male Experiment – varying male body size; The Female-Male Experiment – varying both male and female body size. Bold – p – value ≤ 0.05.

Response variable	Factor	Female experiment Varying ♀ body size				Male experiment Varying ♂ body size				Female-Male experiment Varying ♂ &♀ body size			
		♂		♀		♂		♀		♂		♀	
		F-value	p-value	F-value	p-value	F-value	p-value	F-value	p-value	F-value	p-value	F-value	p-value
Mating frequency	Social	0.384	0.683	5.157	**0.024**	0.094	0.760	2.838	0.061	0.485	0.490	2.537	0.113
	Size	-	-	21.551	**<0.001**	0.377	0.542	-	-	0.019	0.890	14.175	**<0.001**
	Vial	-	-	1.087	0.298	-	-	1.178	0.279	-	-	0.079	0.779
	DF	0.568	0.455	-	-	0.141	0.708	-	-	0.819	0.371	-	-
	Social*Size	-	-	5.687	**0.018**	8.373	**0.006**	-	-	0.832	0.367	1.394	0.239
Number of mates	Social	1.105	0.342	6.616	**0.011**	2.994	0.092	0.392	0.676	0.419	0.521	0.216	0.642
	Size	-	-	9.929	**0.001**	0.478	0.493	-	-	0.046	0.830	9.948	**0.001**
	Vial	-	-	4.924	**0.027**	-	-	2.121	0.147	-	-	0.081	0.775
	DF	0.070	0.792	-	-	0.033	0.854	-	-	10.884	**0.002**	-	-
	Social*Size	-	-	0.885	0.348	1.171	0.286	-	-	0.205	0.653	0.255	0.614
Offspring production	Social	3.356	**0.046**	0.882	0.349	0.132	0.717	4.571	0.011	4.622	**0.038**	0.995	0.320
	Size	-	-	268.830	**<0.001**	0.324	0.572	-	-	2.459	0.125	52.286	**<0.001**
	Vial	-	-	1.531	0.217	-	-	0.073	0.786	-	-	1.315	0.253
	DF	1.547	0.221	-	-	0.019	0.889	-	-	6.065	**0.018**	-	-
	Social*Size	-	-	3.081	0.081	4.883	**0.033**	-	-	0.766	0.387	0.281	0.596
Proportion of focal male's offspring	Social	1.533	0.230	-	-	0.060	0.807	-	-	0.626	0.434	-	-
	Size	-	-	-	-	10.812	**0.002**	-	-	0.385	0.538	-	-
	DF	1.496	0.229	-	-	0.000	0.982	-	-	0.049	0.825	-	-
	Social*Size	-	-	-	-	5.296	**0.027**	-		0.070	0.792	-	-
Offspring *per* mating	Social	-	-	3.443	0.065	-	-	0.482	0.618	-	-	0.480	0.489
	Size	-	-	5.079	**0.025**	-	-	-	-	-	-	0.002	0.764
	Vial	-	-	0.249	0.618	-	-	0.318	0.573	-	-	0.010	0.918
	Social*Size	-	-	0.006	0.936	-	-	-	-	-	-	0.872	0.351

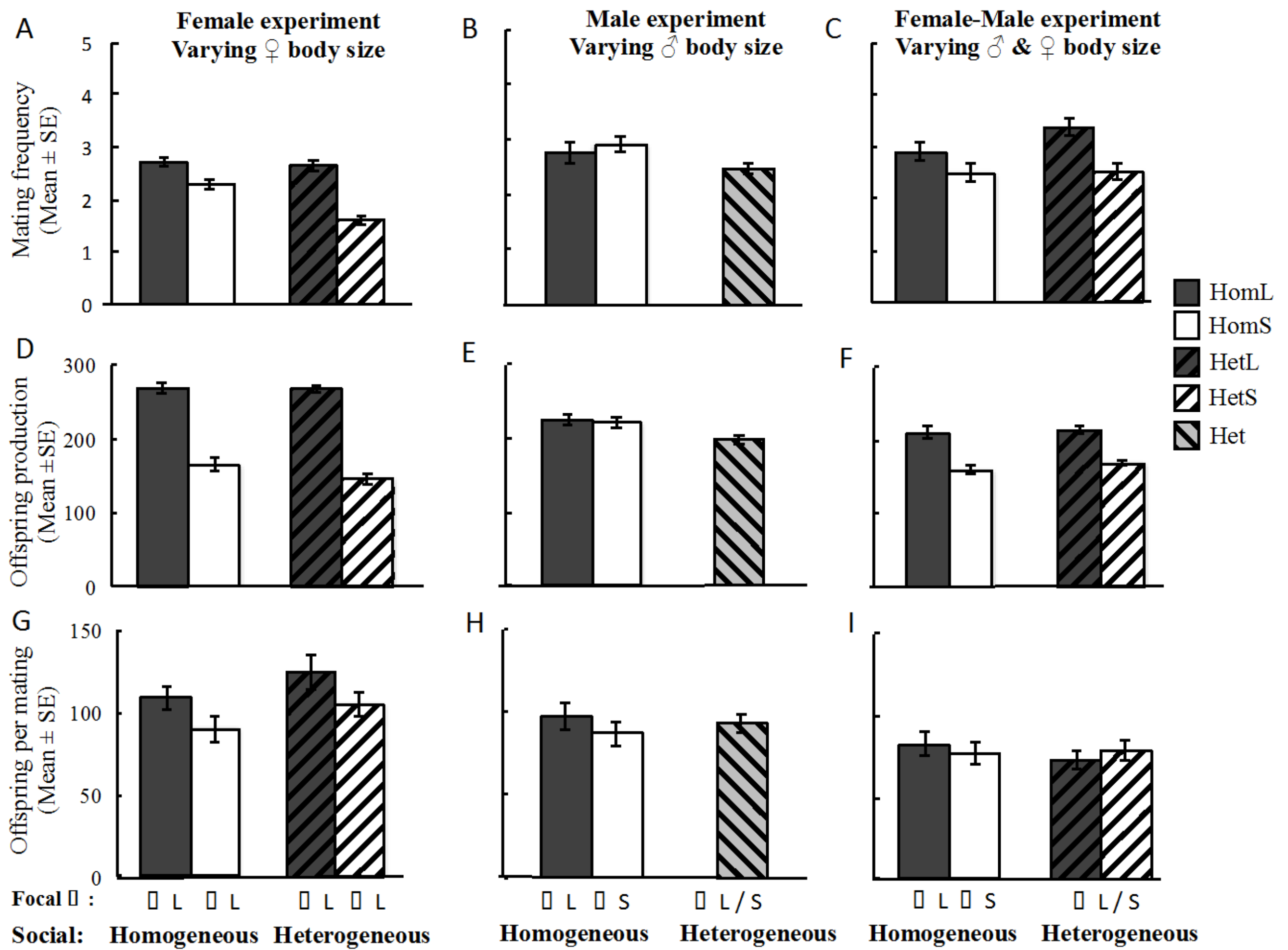

Figure 2 – Female developmental environment and adult reproduction. (a – c) Female mating frequency: (a) Female Experiment; (b) Male Experiment and (c) Female-Male experiments. (d – f) Female offspring production: (d) Female Experiment; (e) Male Experiment and (f) Female-Male experiments. (g-i) *per mating* female offspring production: (g) Female Experiment; (h) Male Experiment and (i) Female-Male experiments. Solid dark grey – Homogeneous Large, Solid white – Homogeneous Small, Dark grey striped from bottom left to upper right – Heterogeneous Large, White striped from bottom left to upper right – Heterogeneous Small, Light grey striped from bottom right to upper left – Heterogeneous (combined Large and Small).

Sexual selection on females

Female Bateman gradients did not significantly differ from zero for most female groups, with the following exceptions. The two heterogeneous environments in which female body size varied were significantly positive (i.e. Het^{F} and Het^{FM}, where both small and large females are considered as a single population). Also, HetS in the Female-Male Experiment (small females in the heterogeneous group, considered separately from large females), and HomL in the Male Experiment (large females with large males) were significantly positive (see Table 2 & S2 Table; Fig 3 & S2 and S1 Table).

We then tested whether the Bateman gradient of large and small females in heterogeneous social environments significantly differed from the Bateman gradient of large and small females in homogeneous social environments (Female and Female-Male Experiments), and found no significant differences (Fig 3, Table 2, S2 Table). The exclusion of a single female in the $HomS^{F}$ group that mated but did not produce any offspring does not qualitatively change the results.

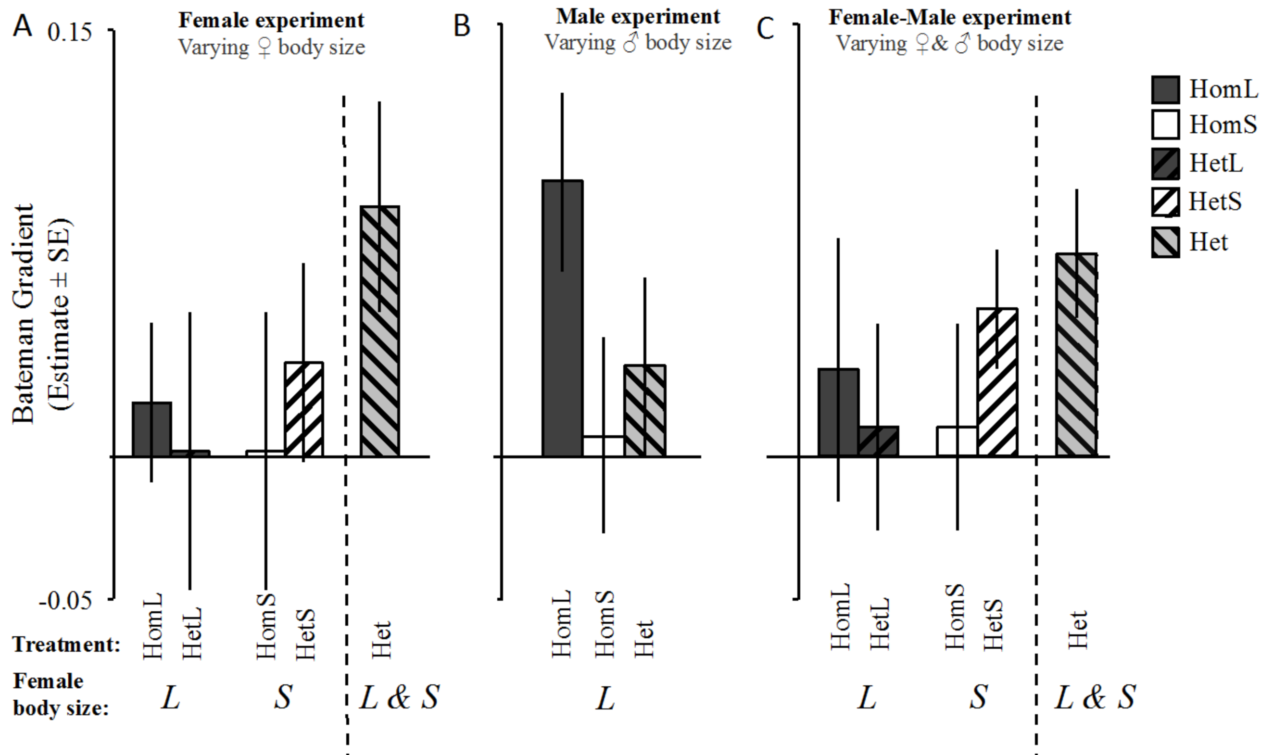

Figure 3 – Female Bateman gradients. Estimate (± SE) extracted from the univariate models. Female Bateman gradients in (a) the Female Experiment; (b) the Male Experiment and (c) the Female -Male Experiment. Solid dark grey – Homogeneous Large, Solid white – Homogeneous Small, Dark grey striped from bottom left to upper right – Heterogeneous Large, White striped from bottom left to upper right – Heterogeneous Small, Light grey striped from bottom right to upper left – Heterogeneous (combined Large and Small).L – Large; S – Small.

Table 2 – Female and focal male Bateman gradients. Estimate ± SE (p-value) are shown. Female estimates of the Bateman gradient are extracted from the univariate model. Male Bateman gradients, paternity share gradients and mate productivity gradients are extracted from the multivariate models. L – Large body size; S – Small body size. Bold – p-value ≤ 0.05. HomL – Homogeneous Large; HomS – Homogeneous Small; HetL – Heterogeneous Large, HetS – Heterogeneous Small, Het – Heterogeneous (combined large and small). The Female Experiment – varying female body size; The Male Experiment – varying male body size and the Female -Male Experiment – varying both female and male body size.

Test	Social manipulation	Factors	Sex analysed	Female experiment varying ♀ body size				Male experiment varying ♂ body size				Female-Male experiment varying ♂ &♀ body size			
				Size	***Estimate***	***SE***	***p-value***	***Size***	***Estimate***	***SE***	***p-value***	***Size***	***Estimate***	***SE***	***p-value***
Social environment	*Hom Large*	*Mates*	Female	*L*	0.019	0.028	0.496	*L*	0.112	0.034	**0.003**	*L*	0.031	0.051	0.538
		Vial ID			0.006	0.009	0.524		0.016	0.016	0.349		0.032	0.020	0.121
	Hom Small	*Mates*	Female	*S*	0.002	0.049	0.968	*L*	0.012	0.041	0.768	*S*	0.014	0.034	0.681
		Vial ID			-0.024	0.017	0.156		0.011	0.012	0.394		0.023	0.012	0.072
	Het	*Mates*	Female	*L / S*	0.098	0.04	**0.018**	*L*	0.024	0.031	0.455	*L / S*	0.072	0.026	**0.008**
		Vial ID			-0.001	0.006	0.785		-0.002	0.005	0.693		0.001	0.004	0.777
Size within Het groups	*Het Large*	*Mates*	Female	*L*	0.01	0.026	0.686	-	-	-	-	*L*	0.018	0.038	0.638
		Vial ID			-0.001	0.004	0.694		-	-	-		0.008	0.006	0.186
	Het Small	*Mates*	Female	*S*	-0.004	0.039	0.913	-	-	-	-	*S*	0.047	0.026	0.077
		Vial ID			-0.007	0.005	0.132		-	-	-		-0.005	0.003	0.131
Social env	*Hom x Het Large*	*Mates * Social*	Female	-	-0.001	0.047	0.770	-	-	-	-	-	-0.018	0.067	0.788
within size	*Hom x Het Small*	*Mates * Social*	Female	-	-0.015	0.057	0.786	-	-	-	-	-	0.035	0.046	0.451

Table 2 – *Cont.*

Test	**Social manipulation**	**Factors**	**Sex analysed**	**Female experiment varying ♀ body size**				**Male experiment varying ♂ body size**				**Female-Male experiment varying ♂ &♀ body size**			
				Size	***Estimate***	***SE***	***p-value***	***Size***	***Estimate***	***SE***	***p-value***	***Size***	***Estimate***	***SE***	***p-value***
Social environment	*Hom Large*	*Mates*	Male	*L*	0.104	0.06	0.146	*L*	-0.001	0.108	0.932	*L*	0.169	0.030	**0.004**
		Paternity			0.259	0.066	**0.011**		0.411	0.066	**0.008**		0.161	0.026	**0.003**
		Partner productivity			0.239	0.064	**0.014**		0.061	0.040	0.223		-0.018	0.037	0.636
		Vial productivity			0	0	0.730		0	0	0.965		0	0	0.300
		DF			NA	NA	-		0.075	0.219	0.062		0.168	0.091	0.139
	Hom Small	*Mates*	Male	*L*	0.822	0.245	**0.028**	*S*	0.134	0.047	**0.045**	*S*	0.339	0.037	**<0.001**
		Paternity			0.084	0.087	0.386		0.216	0.03	**0.002**		0.12	0.03	**0.016**
		Partner productivity			-0.149	0.159	0.402		0.093	0.027	**0.026**		0.211	0.034	**0.003**
		Vial productivity			-0.001	0.001	0.258		-0.001	0	**0.036**		0.001	0	**0.014**
		DF			NA	NA	-		0.143	0.083	0.160		-0.112	0.106	0.348
	Het	*Mates*	Male	*L*	0.038	0.029	0.205	*L / S*	0.082	0.043	0.081	*L / S*	0.231	0.033	**<0.001**
		Paternity			0.259	0.03	**<0.001**		0.295	0.044	**<0.001**		0.236	0.029	**<0.001**
		Partner productivity			0.004	0.026	0.875		0.138	0.041	**0.005**		0.046	0.026	0.108
		Vial productivity			0	0	**0.011**		0	0	0.148		0	0	0.169
		DF			-0.062	0.058	0.309		-0.177	0.085	0.056		-0.106	0.093	0.275

Table 2 – *Cont.*

Test	Social manipulation	Factors	Sex analysed	Female experiment varying ♀ body size				Male experiment varying ♂ body size				Female-Male experiment varying ♂ &♀ body size			
				Size	***Estimate***	***SE***	***p-value***	***Size***	***Estimate***	***SE***	***p-value***	***Size***	***Estimate***	***SE***	***p-value***
Size within Het groups	*Het Large*	*Mates*	Male	-	-	-	-	*L*	0.169	0.076	0.091	*L*	0.258	0.037	**0.002**
		Paternity			-	-	-		0.197	0.188	0.354		0.252	0.02	**<0.001**
		Partner productivity							0.149	0.217	0.529		0.097	0.072	0.247
		Vial productivity			-	-	-		0	0	0.659		0	0	0.672
		DF							-0.127	0.268	0.66		-0.376	0.196	0.128
	Het Small	*Mates*	Male	-	-	-	-	*S*	0.025	0.067	0.726	*S*	0.257	0.047	**0.005**
		Paternity			-	-	-		0.300	0.087	0.025		0.223	0.080	0.050
		Partner productivity			-	-	-		0.159	0.054	0.049		0.066	0.043	0.199
		Vial productivity			-	-	-		0	0	0.195		-0.001	0	0.066
		DF			-	-	-		-0.198	0.147	0.250		0.030	0.118	0.809

Table 2 – *Cont.*

Test	Social manipulation	Factors	Sex analysed	Female experiment varying ♀ body size				Male experiment varying ♂ body size				Female-Male experiment varying ♂ &♀ body size			
				Size	***Estimate***	***SE***	***p-value***	***Size***	***Estimate***	***SE***	***p-value***	***Size***	***Estimate***	***SE***	***p-value***
Social env within size	*Hom x Het Large*	*Mates * Social*	Male	-	-	-	-	*L*	0.137	0.196	0.501	*L*	0.020	0.045	0.659
		*Paternity * Social*			-	-	-		-0.287	0.188	0.161		0.069	0.037	0.090
		*Partner productivity * Social*			-	-	-		0.233	0.144	0.139		-0.061	0.054	0.283
		DF							0.119	0.117	0.333		0.106	0.080	0.252
	Hom x Het Small	*Mates * Social*	Male	-	-	-	-	*S*	-0.127	0.082	0.150	*S*	-0.179	0.076	**0.041**
		*Paternity * Social*			-	-	-		0.115	0.098	0.268		-0.052	0.100	0.613
		*Partner productivity * Social*			-	-	-		0.030	0.075	0.662		-0.257	0.067	**0.003**
		DF			-	-	-		-0.028	0.092	0.259		-0.052	0.118	0.667

Patterns of male reproduction

The social environment affected the number of offspring sired by focal males in the Female Experiment with males producing more offspring in the HomL than in the Het followed by the HomS groups (post-hoc SNK test, $\alpha = 0.05$), and in the Female-Male experiment with focal males in homogeneous groups producing more offspring than focal males in the heterogeneous group (Table 1, Fig 4d). Focal male mating frequency, number of mates or proportion of offspring sired across did not significantly differ with social environment in any experiment (Table 1, Fig 4a-f, S4 Fig). In the Male Experiment, the proportion of offspring sired by large males (i.e. reared at low larval density) was significantly higher than for than small (high larval density) males (Table 1, S5 Fig). We found a significant interaction between social environment and focal male body size (i.e. social * body size) on focal male mating frequency, the proportion of offspring sired by focal males, and on the total number of offspring sired by focal males in the Male Experiment: small focal males in a heterogeneous social environment mated less frequently and sired fewer offspring than small males in a homogenous social environment (Table 1, Fig 4b and 4e, S5 Fig). We did not detect an effect of body size or the interaction body size and social environment in any of the focal male reproductive measures in the Female-Male Experiment (Table 1). The exclusion of one male in $HomS^F$ and one male in $HomL^M$ that failed to obtain any mates changes the analyses for number of offspring: the effect of social environment became non-significant in the Female Experiment (the p-value changed from 0.046 to 0.068) and, the interaction social * body size of the focal male on the number of offspring sired by males in the Male Experiment becomes marginally non-significant ($p = 0.061$) where it had previously been significant ($p = 0.033$).

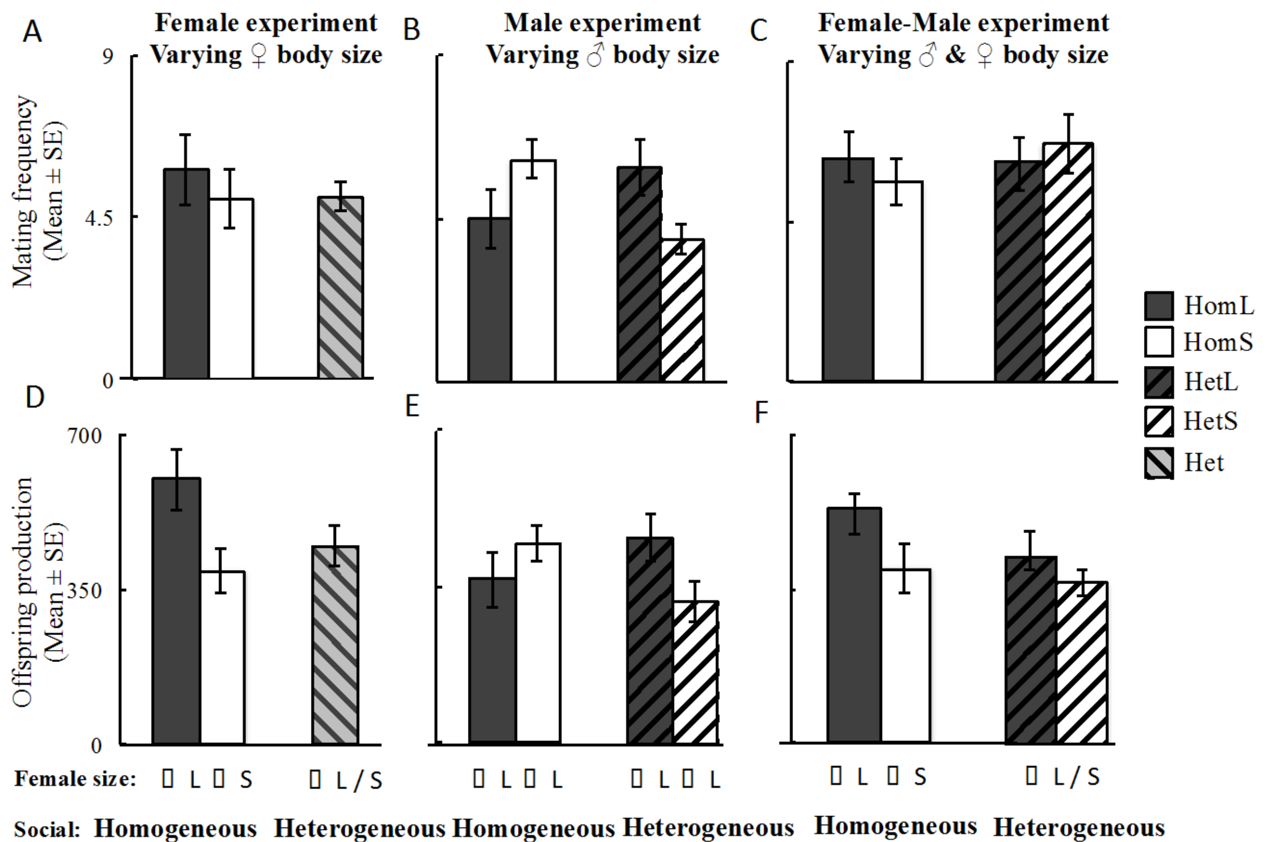

Figure 4 – Effects of larval environment on focal male reproduction. (a – c) Mean mating frequency of the focal male: (a) Female Experiment; (b) Male Experiment and (c) Female-Male experiment. (d-f) The number of offspring sired by focal males: (a) Female Experiment; (b) Male Experiment and (c) Female-Male experiment. The size of females is represented below the barplot. $♀_L$ - Large females; $♀_S$ - Small females. Solid dark grey – Homogeneous Large, Solid white – Homogeneous Small, Dark grey striped from bottom left to upper right – Heterogeneous Large, White striped from bottom left to upper right – Heterogeneous Small, Light grey striped from bottom right to upper left – Heterogeneous (combined Large and Small).

Sexual selection on males

Firstly, we applied eqn 3 to investigate the relative contribution of male mate number (*M*), paternity share (*P*), mate productivity (*N*) and their covariances in explaining variance in offspring sired (*T*). Overall, variance in *P* explained the largest proportion of variance in *T*, followed by variance in *M* and the covariance between *M* and *P* (Table 3, see Methods). We then qualitatively investigated whether the social environment changed the relative contributions of *M, P, N* and their covariates. The decomposition of the variance in *T* for large and small focal male in a heterogeneous social environment had broadly the same characteristics of the decomposition of variance in *T* for focal males in a homogeneous social environment – i.e. variation in *T* was largely explained by variance in *P* followed by variation in *M* and the covariance between *M* and *P* (Table 3). A Commonality Analysis (CA), which uses an alternative method to decompose variance in *T,* qualitatively supports the overall contribution of variances and covariances related to *M, P* and *N* for male reproduction (S3 Table).

Table 3 – Variance decomposition: observed contributions of the variances and covariances in *M, N* and *P* in explaining the variance in the number of offspring sired by males (*T*). The decomposition is based on the approach proposed by Webster et al. (1995). *Var(x)* represents the variance component of the factor *x* (i.e. *M, N or P*), and *cov(x, y)* represents the covariance components between the factors *x* and *y* (e.g. covariance between *M* and *P* – cov(*M, P*)). *D* represents the variance in the error term ε. HomL – Homogeneous Large; HomS – Homogeneous Small; HetL – Heterogeneous Large, HetS – Heterogeneous Small, Het – Heterogeneous. The Female Experiment – varying female body size; The Male Experiment – varying male body size and The Female-Male Experiment – varying both female and male body size.

Experiment	Var-Cov Components	Observed contribution to var(T)									
		HomL	%	HomS	%	HetL	%	HetS	%	Het	%
Female	*var(T)*	47.14		46.03		-		-		21.34	
	var(M)	13.52	28.67	17.2	37.36	-	-	-	-	4.37	20.48
	var(P)	15.17	32.18	10.22	22.21	-	-	-	-	12.36	57.92
	var(N)	5.46	11.59	4.61	10.01	-	-	-	-	3.49	16.34
	cov(M, P)	4.84	10.26	-0.59	-1.28	-	-	-	-	9.21	43.16
	cov(M, N)	5.08	10.78	9.8	21.29	-	-	-	-	0.66	3.08
	cov(N, P)	-5.42	-11.5	1.81	3.94	-	-	-	-	2.39	11.22
	D	8.5	18.02	2.98	6.47	-	-	-	-	-11.14	-52.2
Male	*var(T)*	40.32		16.2		29.5		20.04		29	
	var(M)	17.78	44.09	4.59	28.36	11.04	37.42	4.31	21.5	6.96	23.99
	var(P)	17.48	43.35	4.88	30.1	12.43	42.15	10.15	50.64	16.69	57.57
	var(N)	0.42	1.03	1.76	10.85	2.38	8.08	1.89	9.42	2.25	7.76
	cov(M, P)	18.75	46.49	0.73	4.49	11.03	37.39	5.81	29	8.69	29.95
	cov(M, N)	1.94	4.82	0.92	5.7	2.38	8.08	1.85	9.24	2.02	6.97
	cov(N, P)	2.25	5.59	2.52	15.56	8.97	30.42	0.82	4.11	3.74	12.9
	D	-18.29	-45.37	0.8	4.93	-18.74	-63.53	-4.79	-23.91	-11.35	-39.14
Female-Male	*var(T)*	14.77		32.26		31.22		9.17		19.39	
	var(M)	10.51	71.14	5.16	15.98	13.96	44.72	11	119.96	11.84	61.05
	var(P)	8.25	55.84	6.92	21.45	18.76	60.09	6.62	72.14	11.85	61.12
	var(N)	0.99	6.67	2.16	6.7	0.44	1.42	1.69	18.47	1.01	5.22
	cov(M, P)	-3.19	-21.57	-2.65	-8.2	17.4	55.74	6.24	68.02	11.24	57.96
	cov(M, N)	3.06	20.73	-2.68	-8.31	1.44	4.62	-2.72	-29.67	-0.6	-3.07
	cov(N, P)	-0.34	-2.31	1.19	3.67	-0.17	-0.55	-4.26	-46.43	-2.09	-10.78
	D	-4.51	-30.49	22.17	68.71	-20.62	-66.04	-9.4	-102.5	-13.86	-71.49

We then used a predictive multivariate linear model (eqn 4) to test whether the slope of the gradient of a focal male's offspring number regressed over mate number (*M)*, paternity share (*P)*, and mate productivity gradients (*N)* controlling for the other gradients were influenced by our social manipulations. First, we tested whether the focal male multivariate *M, P* and *N* gradients were significantly different from zero in the homogenous and heterogeneous groups. Significantly positive multivariate *M* gradients (Bateman gradients) were observed for large males in the $HomS^F$ group of the Female Experiment (where large males were exposed to small females), for small males in the $HomS^M$ group of the Male Experiment (where small males were exposed to large females), and in all groups of the Female-Male Experiment (i.e. $HomL^{FM}$, $HomS^{FM}$ and Het^{FM}; Table 2; Fig 5a-i). However, multivariate *M* gradients were positive but not significantly different from zero in the $HomL^F$ group of the Female Experiment (where large males were exposed to large females), in the Het^F group of the Female Experiment (where large males were exposed to females of mixed body sizes), and in the Het^M group of the Male Experiment (where mixed size males were exposed to large females) (Fig 5a, Table 2, S6 Fig). Multivariate *M* gradient did not show a positive trend in the $HomL^M$ group of the Male Experiment (where large males were exposed to large females; see Fig 5a, Table 2, S6 Fig). Multivariate *P* gradients were highly significant in all social environment manipulations except $HomS^F$ (Table 2). Significantly positive multivariate *N* gradients were limited to $HomL^F$, $HomS^M$, Het^M, and $HomS^{FM}$ (Table 2).

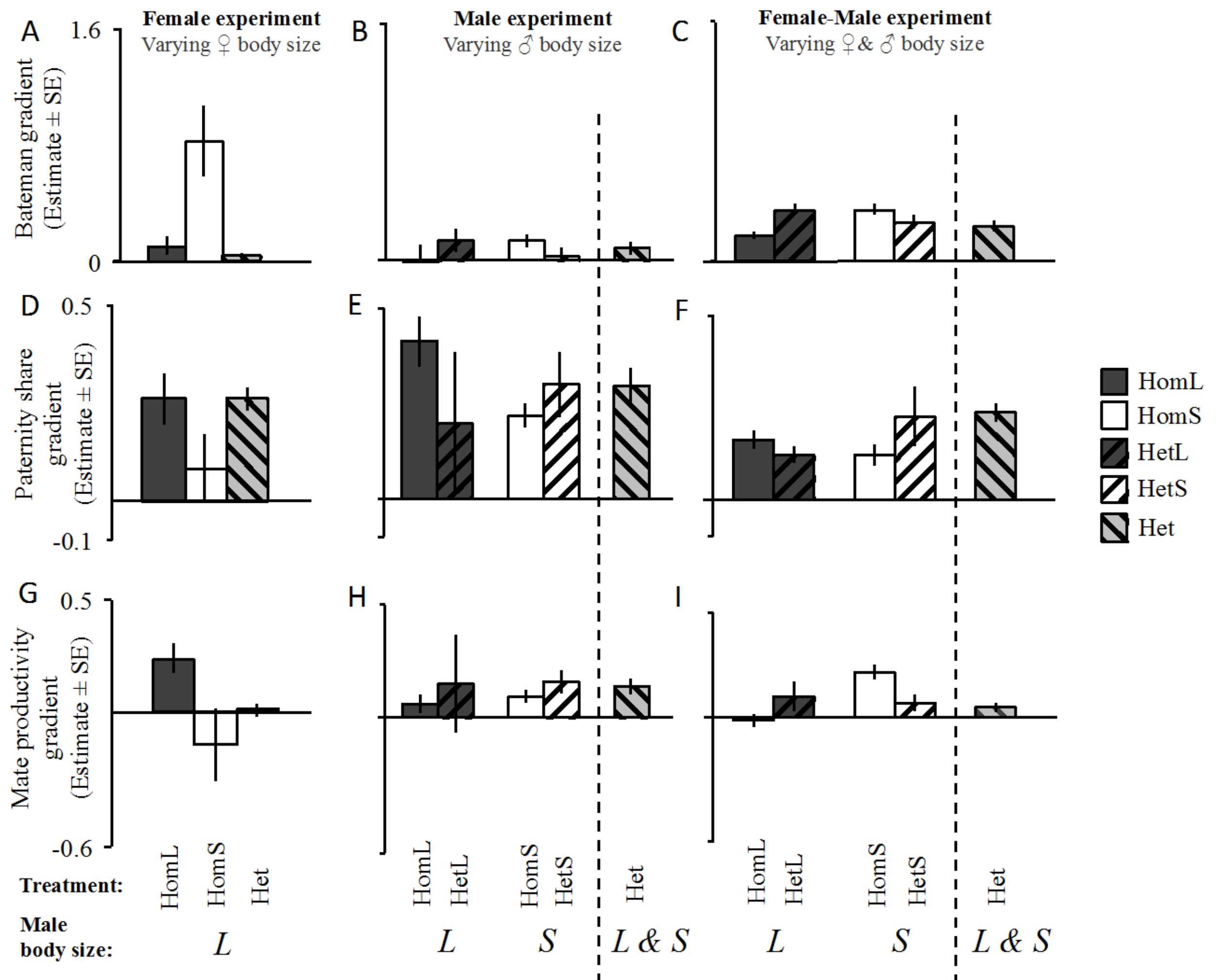

Figure 5 – Focal male Bateman gradients, paternity share gradients and mate productivity gradients. Estimate (±SE) extracted from the multivariate models. (a-c) the focal male Bateman gradients in (a) the Female Experiment; (b) the Male Experiment and (c) the Female-Male Experiment. (d-f) paternity share gradients in (d) the Female Experiment; (e) the Male Experiment and (f) the Female-Male Experiment. (g-i) mate productivity gradients in (g) the Female Experiment; (h) the Male Experiment and (i) the Female-Male Experiment. Solid dark grey – Homogeneous Large, Solid white – Homogeneous Small, Dark grey striped from bottom left to upper right – Heterogeneous Large, White striped from bottom left to upper right – Heterogeneous Small, Light grey striped from bottom right to upper left – Heterogeneous (combined Large and Small). L – Large; S – Small.

We next investigated whether *M, P* and *N* gradients were altered when focal males of different body sizes experienced a heterogeneous versus a homogenous social environment. We did not find a significantly greater than zero *M* gradients for either large or small focal males in a heterogeneous environment of the Male Experiment (i.e. $HetL^{M}$ and $HetS^{M}$). However, the *M* gradient was significantly greater than zero for both large and small focal males of the Female-Male Experiment (i.e. $HetL^{FM}$ and $HetS^{FM}$; Table 2, S7 Fig). *P* gradient was significantly positive for small focal males but not large focal males in the heterogeneous environment of the Male Experiment, and was significantly positive for both large and small focal males in a heterogeneous environment of the Female-Male Experiment (Table 2). Multivariate *N* gradients were not significantly different from zero for small or large males in any of the heterogeneous groups (Table 2).

We then compared *M, P* and *N* gradients of large and small males in a heterogeneous environment (considering each size class separately) with the gradients of large and small males in a homogeneous environment, respectively. We found for small focal males that both *M* and *N* gradients were strongly reduced in a heterogeneous social environment of the Female-Male Experiment ($HetS^{FM}$) relative to small males in a homogeneous social environment (Fig 5c, 5i & S7 Fig, Table 2 & S2 Table). These effects were not observed for either small focal males of the Male Experiment ($HetS^{M}$) or large focal males of both Male and Female-Male Experiments, where *M, P* or *N* did not significantly differ between heterogeneous and homogeneous social environments (Table 2, S2 Table).

Discussion

- We confirmed our predictions that larval density generate large body sized individuals that are more successful in obtaining mates and matings as well as producing offspring.
- We confirmed that the positive relationship between the number of mates and offspring of females arise from differences in female body size (and total productivity) in a group. This result highlights the potential for misleading interpretations of a positive female Bateman gradient.
- We partially confirmed our expectations of an overall positive Bateman gradient for both large and small males, although this was not true for all treatments.
- We found that, in a mixed environment where both sexes varied in body size, neither female body size nor social environment affected the number of offspring *per* mating produced by females. This finding may open a new perspective on the evolution of male mate choice by showing that males do not necessarily gain additional offspring from mating with large (more fecund) females.
- We also found that small, but not large focal males have a significantly reduced Bateman gradient and partner fecundity gradient in a mixed social environment when both sexes varied in body size, suggesting that small individuals suffer costs from competing with large individuals.

Below, we discuss the main findings of our study in detail.

Sexual selection in females

Our data suggest that the larval environment experienced by individuals within groups can influence adult Bateman gradients in female *D. melanogaster*. For instance, we found that the Bateman gradients were significantly positive in heterogeneous social environments whereas gradients were less steep, and not significantly different from 0 in most (though not all) homogenous social environments (Table 2, Fig 3). As predicted, the steep Bateman gradients in heterogeneous social environments likely arise as a consequence of associations between larval density effects on the number of offspring produced by adult females (likely linked to body size), and their mating frequency. As expected, females grown at high larval density eclose smaller, produce fewer offspring, and have lower mating frequencies relative to females grown at low density (i.e. large adult females). Thus, in populations containing both large and small females, there is inevitably a correlation between mate number and number of offspring produced, which is ultimately driven by differences in the larval environments. This idea is supported by the fact that, when we compared the heterogeneous social environment Bateman gradients for small and large females considered separately, we did not see significant differences from small and large females in homogeneous social environments. This suggests that it is the heterogeneity of females *per se*, not changes in selection on small and large females that generates the positive Bateman gradients. In other words, the positive female Bateman gradients in heterogeneous groups are an emergent property of the heterogeneity in larval conditions experienced by female individuals within the group. Our results therefore highlight the fact that Bateman gradients should be interpreted cautiously as a measure of sexual selection on mate number in females, because the correlation between number of offspring produced and number of mates does not necessarily reflect causality (Collet et al., 2014, Parker and Tang-Martinez, 2005, Gerlach et al., 2012, García-Navas et al., 2014, Chapman and Partridge, 1996, Amitin and Pitnick, 2007, Pitnick and Garcia-

Gonzalez, 2002, Prosser et al., 2002). We also saw unexplained variation in Bateman gradients between experiments. For example in the HomL group of the Male Experiment the Bateman gradient was significantly different from 0, but in the Female or Female-Male experiments HomL was much less steep, and not significantly different from 0, despite the fact that all HomL groups were treated identically. Thus, we should exhibit some caution when interpreting the data.

Sexual selection in males

Our results suggest a complex architecture of male reproductive success, and highlight the importance of decomposing the offspring number sired by males into its *M, P* and *N* components (Collet et al., 2012, Collet et al., 2014, Janicke et al., 2015, Fritzsche and Arnqvist, 2013, Pelissie et al., 2014). For example, the covariance between *M* and *P* was mostly positive across experiments (except in the $HomL^{FM}$ and $HomS^{FM}$), which suggests that males that mate with more females obtain higher paternity share with their mates (Table 3). Conversely, a negative covariance between *M* and *P* (as in $HomS^{FM}$ and $HomL^{FM}$) reflects a potential trade-off between pre- and post-copulatory success, in which males that attract more mates are poor post-copulatory competitors (Table 3). Whilst males are expected to gain from additional mates, the primary mechanisms by which males gain fitness might in some cases be via mate choice for high productivity females (but see below), or via success in post-copulatory competition, and these traits may or may not be correlated with pre-copulatory success (Table 2) (Collet et al., 2012, Collet et al., 2014, Hosken et al., 2008). Our finding that *P* explains a large proportion of variance is broadly consistent with the findings in other promiscuous species such as the red jungle fowl *Gallus gallus* (Collet et al., 2012, Collet et al., 2014), guppies *Poecilia reticulata* (Devigili et al., 2015) and the hermaphrodite snail *Physa acuta* (Janicke et al., 2015). However, it is important to interpret our results for *P* with caution. For instance, in a study on semelparous-adapted *D. melanogaster* population – which

was cultured each generation with a single short reproductive bout – most post-copulatory success was explained by last male sperm precedence, leaving only a further ~2% of variance in reproductive success explained by additional post-copulatory processes (Manier et al., 2010, Pischedda and Rice, 2012). This previous study show that last male precedence can significantly contribute to the overall strength of post-copulatory sexual selection, and could be a confounding effect when analysing post-copulatory processes (*P)*. Other mechanisms that might also contributes to variance in *P* include cryptic female choice (Eberhard, 1991) and sperm viability (Bonduriansky, 2001, García-González and Simmons, 2005, Hunter and Birkhead, 2002), which skew male fertilization success similar to male precedence. Interestingly, however, Pischedda and Rice (2012) showed that the total variance in male reproductive success explained by either general post-copulatory processes or by post-copulatory processes after controlling for male precedence was the same, suggesting that male precedence is a component of, instead of additional to, *P* (see Fig 2 in (Pischedda and Rice, 2012)). Thus, it is unlikely that considering male precedence (or other processes) would explain additional variance than that already captured by *P* and, consequently, it would not alter the relative contributions of *M* and *N*. Therefore, although male precedence, female cryptic choice and sperm viability are important, their relative contributions to the variance in *P* lie outside the scope of this paper and should be the focus of future analysis. It will also be particularly informative for future studies to determine how much last-male sperm precedence explains post-copulatory success in other iteroparous populations.

We found that small males in groups consisting of both males and females from different larval densities (i.e. the heterogeneous group in the Female-Male Experiment.) had significantly reduced Bateman and mate productivity gradients relative to small males in the homogeneous social environment (Table 2, Fig 5). This suggests that the operation of sexual selection – the benefits of obtaining multiple mates, and obtaining mates of high productivity

– are modified for small males when in the presence of large males and a mix of female phenotypes. This is likely explained by a combination of factors. When in competition with large males, small males have a lower share of paternity because they are outcompeted over access to females and possibly because females favour inseminations by large males during or after mating. Such loss of paternity would erode the benefits of mating with multiple females (Parker and Birkhead, 2013), thus weakening the Bateman gradient of small males. These factors may also mean that small males may have a relatively higher share of paternity with smaller, less fecund females leading to weaker gradients of sexual selection on mate productivity and the large negative covariance between *N* and *P* found in small males in the $HetS^{FM}$ treatment (Table 3).

Our results – and previous studies – show that *D. melanogaster* males who experience favourable developmental environments, that lead to large adult size, display higher success in pre and post-copulatory competition (Fig 4a-c, Table 2) (Pitnick, 1991, Pitnick and Garcia-Gonzalez, 2002). Large adult males can obtain higher proportion of offspring for several reasons: they may be able to either monopolize most of the mates, elicit mating behaviour more frequently or effectively (Pitnick, 1991), win sperm competition, or be preferred in cryptic female choice (Amitin and Pitnick, 2007). Moreover, the larval developmental environment influences ejaculate investment, whereby males with small body size invest relatively more ejaculate to each mating relative to large males (Wigby et al., 2015) . Thus, small males experiencing a heterogeneous environment might have adjusted their behaviour to accommodate the competition with large males, leading to an alternative path to reproductive success.

Variation in female productivity is expected to be a major driving force in the evolution of male mate choice, and current theory predicts that male mate choice for large adult females should evolve because large females produce more eggs (reviewed by

Bonduriansky (2001)). In *D. melanogaster*, some studies indicate that males preferentially court large, highly fecund females (Byrne and Rice, 2006, Long et al., 2009) to gain fitness as a consequence of mate choice (Edward and Chapman, 2012). However, other studies suggest that male *D. melanogaster* can prefer small females, raising questions over the consistency of male preferences (Edward and Chapman, 2012, Edward and Chapman, 2013). We found that the number of offspring that a female produced *per* mating was not higher for large (low larval density) females when both sexes varied in size (The Female -Male Experiment, Fig 2g-i). Large females remate more frequently (Amitin and Pitnick, 2007, Wigby et al., 2015) meaning that sperm competition may be more intense for mates of large females, potentially offsetting the benefits of mating with high fecundity large females. The positive correlation between developmental environment-induced changes in body size and productivity (Bonduriansky, 2001) may mean that large females are more receptive to mating simply because they require greater quantities of ejaculate in order to fertilize the large number of eggs that are produced (Trevitt et al., 1988). Similarly, large females may have lower sensitivity to male receptivity-inhibiting seminal proteins resulting in a more rapid return to receptivity. In addition, the low larval density might have served as cue of low mating opportunities, hence priming females to have higher receptivity (or lower resistance) to matings (Wigby et al., 2015). Males might prefer and target large females simply because large females are more willing to remate, or represent larger and slower targets for courtship. The idea that males do not always gain an advantage by mating with large females has received empirical support in the golden-orb web spider *Neuphila plumipes*, in which males changed their mate preference as the levels of intrasexual competition increased, but not necessarily targeted larger females (Jordan et al., 2014). Thus, male mate preference can be thought of as analogous to the Ideal-Free Distribution Model (IFD) where females are seen as patches and males tend to distribute themselves according to "female's quality" (Jordan et al.,

2014). Therefore, male choice for large females might arise because body size is correlated with the rate of egg production, not just total egg production, in which fast reproduction is likely beneficial in stable and expanding populations (Edward et al., 2011a, Stearns, 1992).

In conclusion, our results suggest that the developmental environment can influence the operation of sexual selection during adulthood. Our study also adds to the growing body of evidence that shows the importance of considering more than simply the number of mates and offspring in measures of the strength of sexual selection on males (Collet et al., 2014, Collet et al., 2012, Pelissie et al., 2014). Key questions for future studies include 1) how commonly does variation in female developmental environment or adult condition generate positive associations between mate and offspring number? 2) Do males often gain similar numbers of progeny per mating from low and high condition females? 3) How commonly do environmental conditions influence the benefits of additional mates for males? 4) To what extent do mechanisms other than mate number (e.g. paternity share) explain variation in the number of offspring sired by males?

Acknowledgments

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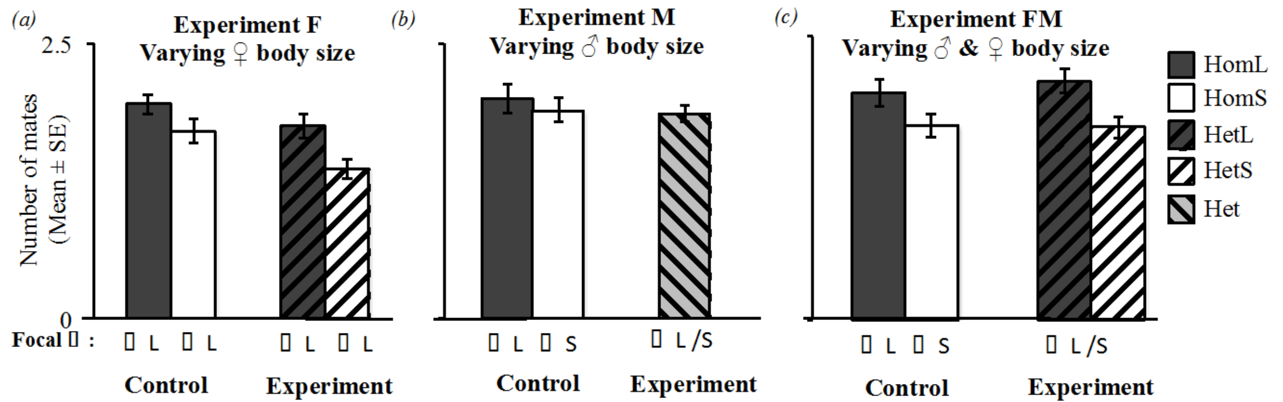
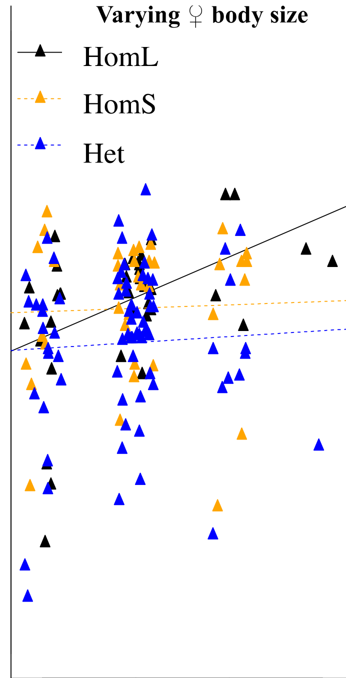
Supplementary Information



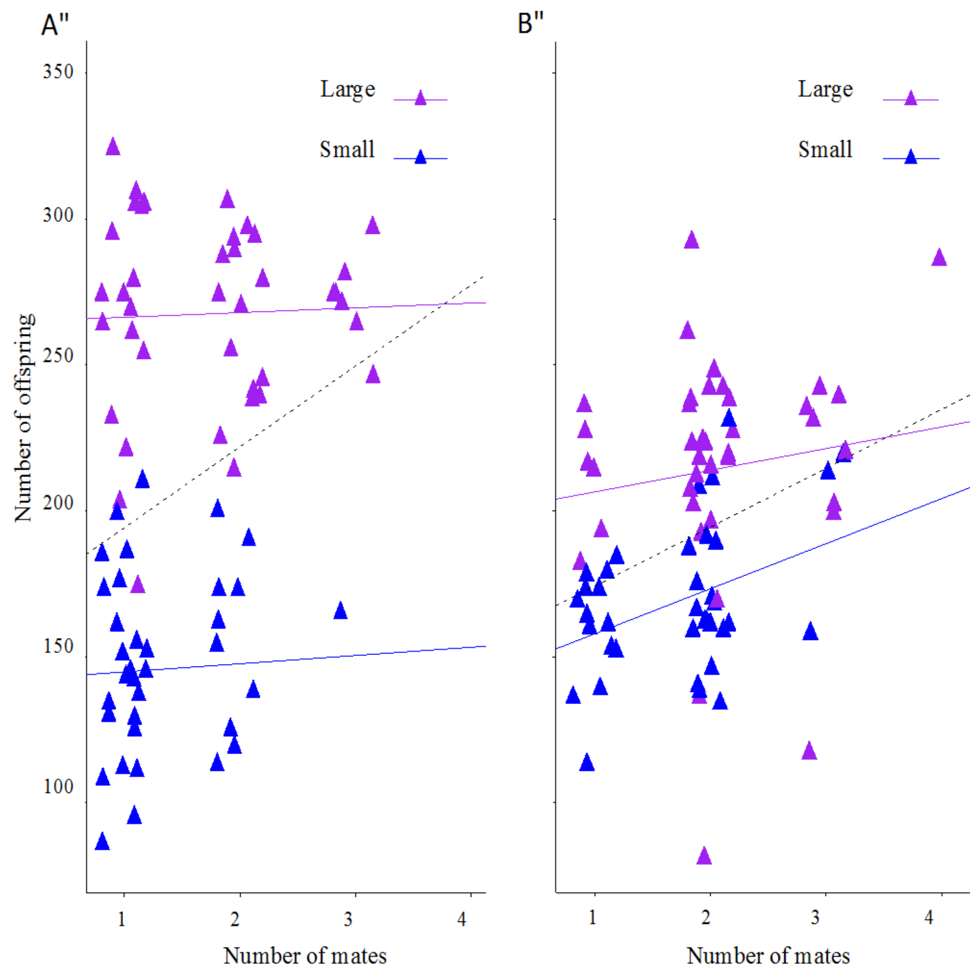
S1 Fig - Mean number of mates of females. (a) The Female Experiment; (b) The Male Experiment; (c) The Female-Male Experiment. Error bars = ±SE. Solid dark grey – Homogeneous Large, Solid white – Homogeneous Small, Dark grey striped from bottom left to upper right – Heterogeneous Large, White striped from bottom left to upper right – Heterogeneous Small, Light grey striped from bottom right to upper left – Heterogeneous (combined Large and Small).

periment

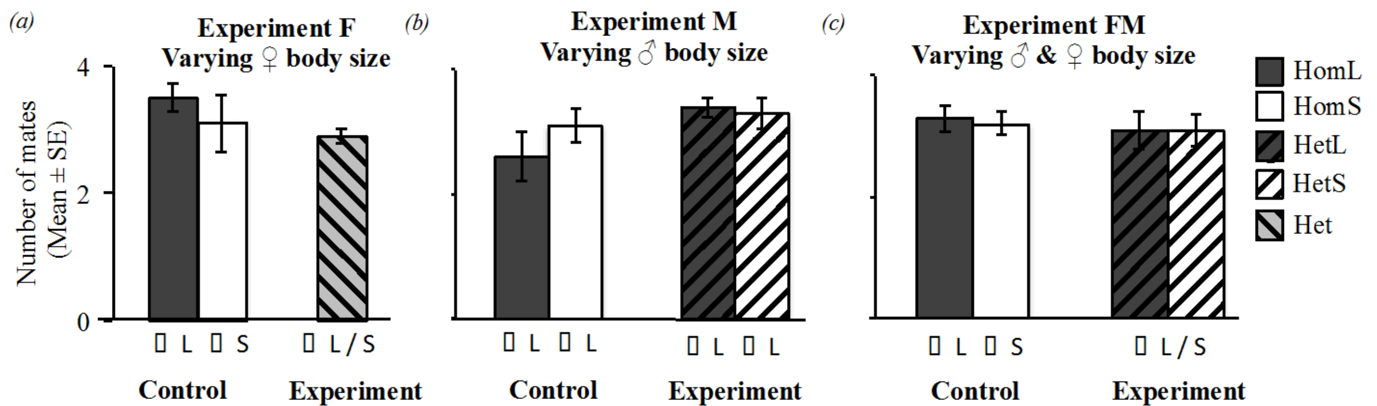
Varying ♀ body size



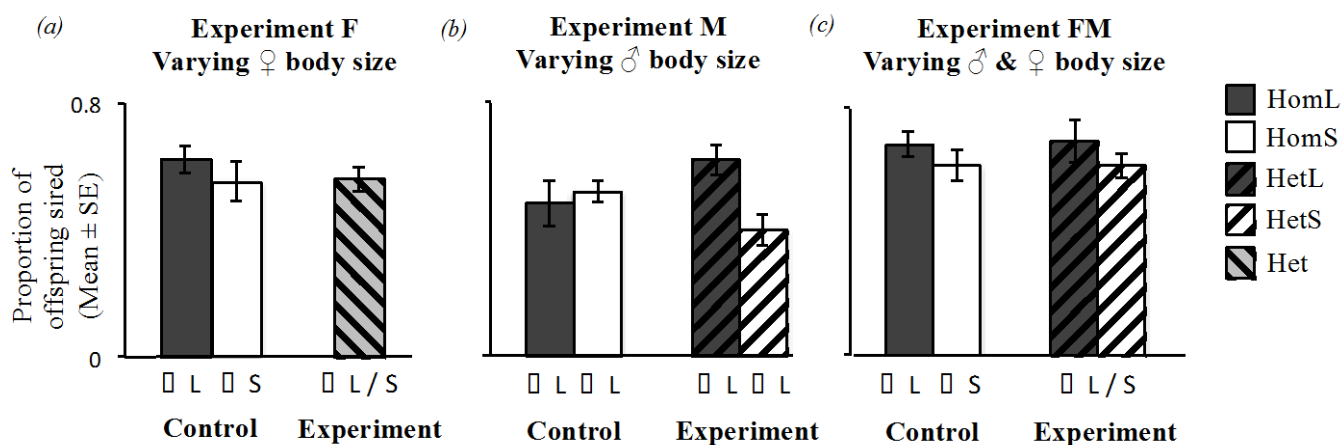
S2 Fig – (a-c) **Univariate Bateman gradients of females**. (a) The Female Experiment; (b) The Male Experiment and (c) the Female-Male Experiment. Homogeneous Large (HomL), Homogeneous small (HomS) and Hetergoeneous (Het) groups.



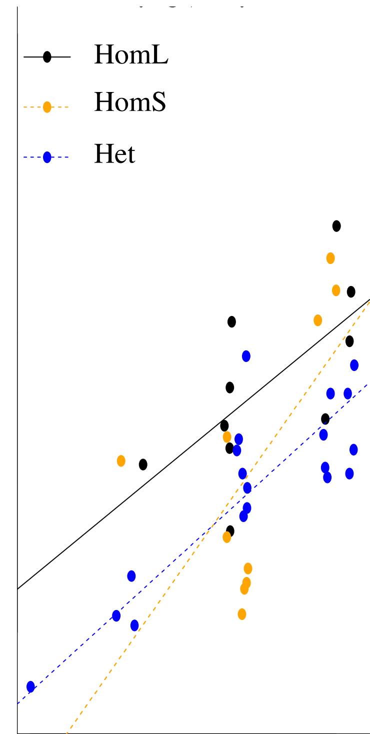
S3 Fig – Female Bateman gradients of the heterogeneous group in which female varied in body size (Famale and Female-Male Experiments). Purple – Large body size; Blue – Small body size; Dashed line – Univariate Bateman gradient of the whole experimental treatment. (a) The Female Experiment, varying female body size. (b) The Female-Male Experiment, varying male and female body size.



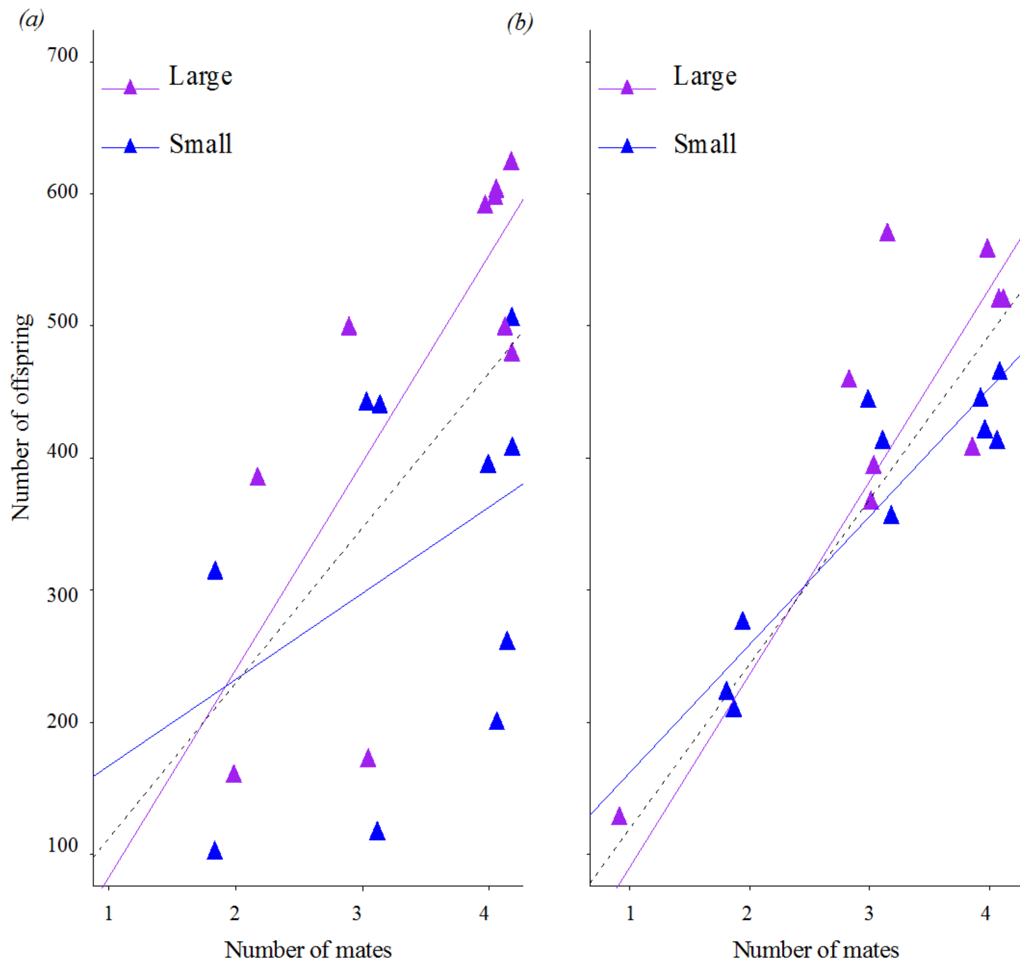
S4 Fig – Mean number of mates of focal males. (a) The Female Experiment; (b) The Male Experiment; (c) The Female-Male Experiment. Means ±SE are shown. Solid dark grey – Homogeneous Large, Solid white – Homogeneous Small, Dark grey striped from bottom left to upper right – Heterogeneous Large, White striped from bottom left to upper right – Heterogeneous Small, Light grey striped from bottom right to upper left – Heterogeneous (combined Large and Small).



S5 Fig – Mean proportion of focal male's offspring production. (a) The Female Experiment; (b) The Male Experiment; (c) The Female-Male Experiment. Means ±SE are shown. Solid dark grey – Homogeneous Large, Solid white – Homogeneous Small, Dark grey striped from bottom left to upper right – Heterogeneous Large, White striped from bottom left to upper right – Heterogeneous Small, Light grey striped from bottom right to upper left – Heterogeneous (combined Large and Small).



S6 Fig– Focal male univariate Bateman gradients. (a) The Female Experiment; (b) The Male Experiment and (c) The Female-Male Experiment. Homogeneous Large (HomL), Homogeneous small (HomS) and Hetergoeneous (Het) groups.



S7 Fig– Focal male's Bateman gradient of the Heterogeneous group in which male (the Male Experiment), or both sexes varied in body size (the Female-Male Experiment). Purple – Large body size; Blue – Small body size; Dashed line – Univariate Bateman gradient. (a) The Male Experiment (b) The Female-Male Experiment; Small (blue) –Large (purple).

S1 Table – Univariate analysis of male Bateman gradients. Bold – p – value ≤ 0.1.

Treatment	Factors	Female experiment ♀ body size				Male experiment ♂ body size				Female-Male experiment ♂ &♀ body size			
		Size	*Estimate*	*SE*	*p-value*	*Size*	*Estimate*	*SE*	*p-value*	*Size*	*Estimate*	*SE*	*p-value*
Homogeneous Large (HomL)	*Mates*	L	0.207	0.106	0.092	L	0.470	0.082	**0.001**	L	0.133	0.069	0.097
	Vial productivity		0.000	0.001	0.593		0.000	0.000	0.199		0.001	0.001	0.377
	DF		NA	NA	-		0.012	0.166	0.944		0.110	0.215	0.624
Homogenous Small (HomS)	*Mates*	L	0.494	0.076	**<0.001**	S	-0.007	0.185	0.969	S	0.192	0.102	**0.109**
	Vial productivity		0.000	0.001	0.370		0.000	0.001	0.681		0.001	0.001	0.070
	DF		NA	NA	-		0.335	0.337	0.358		-0.186	0.333	0.596
Heterogenous (Het)	*Mates*	L	0.196	0.052	**0.001**	L / S	0.238	0.089	**0.017**	L / S	0.304	0.072	**<0.001**
	Vial productivity		0.001	0.001	0.075		0.000	0.001	0.783		0.000	0.001	0.906
	DF		-0.198	0.129	0.147		-0.125	0.193	0.527		-0.037	0.206	0.858

S2 Table – Comparison of the selection gradients of individuals with different body sizes in homogenous or heterogeneous social environments. Estimates ± SE (*t- value* and p-values) are shown. Bold – *t*-statistics p – value ≤ 0.1. The Female Experiment – varying female body size; The Male Experiment – varying male body size and the Female-Male Experiment – varying both female and male body size. M – Mating success; P – Paternity share, N – Mate productivity; SEC – Social environment; VP – Vial productivity, DF – dead individuals

Body size	**Factors**	**Sex**	**Female experiment** ♀ body size				**Male experiment** ♂ body size				**Female-Male experiment** ♂ &♀ body size			
			Estimate	***SE***	***t-value***	***p-value***	***Estimate***	***SE***	***t-value***	***p-value***	***Estimate***	***SE***	***t-value***	***p-value***
L	M	Male	-	-	-	-	0.050	0.182	0.280	0.780	0.165	0.030	5.421	**<0.001**
	P		-	-	-	-	0.378	0.111	3.410	**0.007**	0.163	0.027	6.022	**<0.001**
	N		-	-	-	-	0.055	0.069	0.790	0.447	-0.002	0.035	-0.082	0.939
	SEC		-	-	-	-	0.010	0.108	0.100	0.921	0.011	0.044	0.261	0.799
	VP		-	-	-	-	0.000	0.000	-0.010	0.337	0.000	0.000	1.007	0.337
	DF		-	-	-	-	0.106	0.087	1.213	0.991	0.106	0.087	1.213	0.252
	M*SEC		-	-	-	-	0.137	0.196	0.700	0.501	0.021	0.045	0.454	0.659
	P*SEC		-	-	-	-	-0.287	0.188	-1.530	0.161	0.069	0.037	1.872	0.090
	N*SEC						0.233	0.144	1.620	0.139	-0.061	0.054	-1.133	0.283

Table S2 – *(cont.)*

Body size	Factors	Sex	Female experiment ♀ body size				Male experiment ♂ body size				Female-Male experiment ♂ &♀ body size			
			Estimate	***SE***	***t-value***	***p-value***	***Estimate***	***SE***	***t-value***	***p-value***	***Estimate***	***SE***	***t-value***	***p-value***
S	M	Male	-	-	-	-	0.150	0.059	2.522	**0.030**	0.365	0.055	6.623	**<0.001**
	P		-	-	-	-	0.181	0.062	2.910	**0.015**	0.118	0.044	2.661	**0.024**
	N		-	-	-	-	0.118	0.060	1.947	0.080	0.228	0.051	4.523	**0.001**
	SEC		-	-	-	-	-0.109	0.087	-1.218	0.259	0.011	0.062	0.180	0.860
	VP		-	-	-	-	-0.001	0.000	-2.694	0.022	0.001	0.000	2.114	0.060
	DF		-	-	-	-	-0.028	0.092	-0.305	0.765	-0.052	0.118	-0.442	0.667
	M*SEC		-	-	-	-	-0.127	0.082	-1.556	-0.179	-0.067	0.076	-2.342	**0.041**
	P*SEC		-	-	-	-	0.115	0.098	1.170	0.268	-0.052	0.100	-0.521	0.613
	N*SEC						0.034	0.075	0.450	0.662	-0.257	0.067	-3.798	**0.003**
L	M	Female	0.018	0.026	0.688	0.494	-	-	-	-	0.034	0.040	0.847	0.400
	SEC		0.274	0.044	6.229	**<0.001**	-	-	-	-	0.061	0.064	0.955	0.343
	Vial ID		0.000	0.004	-0.048	0.962	-	-	-	-	0.009	0.006	1.565	0.122
	M*SEC		-0.013	0.036	-0.366	0.715	-	-	-	-	-0.013	0.056	-0.237	0.813
S	M	Female	0.014	0.039	0.362	0.718	-	-	-	-	0.009	0.030	0.310	0.757
	SEC		-0.255	0.065	-3.880	**<0.001**	-	-	-	-	-0.102	0.050	-2.025	**0.046**
	Vial ID		-0.009	0.006	-1.569	0.121	-	-	-	-	-0.001	0.004	-0.213	0.831
	M*SEC		-0.014	0.065	-0.225	0.822	-	-	-	-	0.043	0.045	0.961	0.340

S3 Table – Commonality analysis (CA) to partition the explained variance in the number of offspring sired by males. The results are given in percentage of explained variance. *SEC* – social environment composition (i.e. homogeneous vs. heterogeneous). *M* – number of mates, *P* –paternity share, *N*- mate productivity, *VP* –vial productivity. HomL – Homogeneous large, HomS – Homogeneous small, HetL – Heterogeneous large, HetS – Heterogeneous small, Het – Heterogeneous.

Var-Cov Components	**Observed contribution to *var(T)***												
	Female experiment			**Male experiment**					**Female-Male experiment**				
	HomL	*HomS*	***Het***	*HomL*	*HomS*	*HetL*	*HetS*	***Het***	*HomL*	*HomS*	*HetL*	*HetS*	***Het***
R-squared	*0.882*	*0.879*	*0.933*	*0.986*	*0.972*	*0.913*	*0.888*	*0.888*	*0.949*	*0.981*	0.992	0.948	*0.942*
% of R-squared explained by:													
U(M)	7.83	38.63	0.89	0.00	5.72	11.59	0.44	3.16	41.54	38.07	8.61	39.59	20.31
U(P)	39.94	3.25	36.59	17.98	34.01	2.60	37.41	40.24	47.60	7.58	26.14	10.33	28.48
U(N)	36.71	2.77	0.01	1.09	8.17	1.12	26.85	9.90	0.35	17.62	0.33	3.20	1.28
C(M,P)	14.17	-3.24	36.94	48.19	-5.35	3.16	13.48	15.88	-9.47	-3.75	1.32	-9.98	13.42
C(M,N)	21.28	51.4	0.00	0.32	0.46	-0.02	0.03	2.04	7.7	-16.03	1.94	0.67	-1.07
C(N,P)	-22.54	-1.45	0.17	-1.06	28.71	27.31	7.72	11.8	0.83	2.22	3.14	-1.08	-0.34
C(M,P,N)	-6.81	2.66	0.39	18.89	-0.81	29.1	2.73	11.82	-5.07	-1.13	25.98	11.72	6.71
Remaining (i.e. VF, DF)	9.42	5.98	25.01	14.59	29.09	25.14	11.34	5.16	16.52	55.42	32.54	45.55	31.21
Total % of R-squared explained	100	100	100	100	100	100	100	100	100	100	100	100	100

S1 Text _Supplementary_Results. Analyses of the effects of colour and changes of sex ratio due to female deaths.

Univariate Bateman gradient – Here, we measured male Bateman gradients using a univariate model. Traditionally, the Bateman gradient β was measured as the slope of a linear regression of standardised offspring number on standardised number of mates for both sexes (univariate Bateman gradient; (Arnold and Duvall, 1994), as thus:

$$T(M) = (\beta * M) + \varepsilon$$

where ε is an error term of zero mean. However, the univariate approach tends to overestimate the Bateman gradient, and therefore a multivariate model is required in order to investigate the relative contributions of pre- and prost-copulatory sexual selection for male reproductive success (Collet et al., 2012, Collet et al., 2014) (see main text). Nonetheless, we present the results of the univariate analyses of our data to be consistent with previous literature.

Commonality analysis (CA) – CA was performed as suggested by (Ray-Mukherjee et al., 2014) with the package 'yhat' in R software version 3.0.2 (R Development Core Team, 2015).

Sex ratio effects cause by deaths of experimental females (DF) - Some focal females (but not males) died. To investigate potential effects of these deaths on our results, we pooled the data of our three experiments and created a binary variable called *DF* (dead females), which contained information on the occurrence (value = 1) or not (value = 0) of a female death in the social environment of a particular individual. A significant effect of *DF* means that deaths of experimental females increased or decreased reproduction of the remaining individuals in their social group. We performed GLMs (quasi Poisson error distribution to

account for overdispersion) to evaluate whether *DF* had a significant effect on the number of mates, mating frequency and number of offspring of both females and focal males (see Table 2 of S1 Text below). Because *DF* had a significant effect on the estimates of the number of mates and offspring of focal males, we included *DF* in all focal male models of reproduction and sexual selection (see Main Text).

Paint marking – Colour had no overall effect on the number of offspring and number of mates of either females or focal males (Table 1 of S1 Text).

Table 1 of S1 Text – The effects of paint marking on male and female reproduction.

Sex	**Mating frequency**		**Number of mates**		**Number of offspring**	
	F-value	*p-value*	*F-value*	*p-value*	*F-value*	*p-value*
Female	*0.992*	*0.396*	*0.649*	*0.584*	*1.720*	*0.162*
Focal male	*1.785*	*0.184*	*2.296*	*0.134*	*3.416*	*0.067*

Univariate Male Bateman gradients:

Standardised univariate male Bateman gradients (Arnold and Duvall, 1994, Bateman, 1948) were positive, and mostly significant or close to significant across the experiments, with three exceptions: the Bateman gradient for small males with large females in the Male Experiment ($HomS^{M}$), and the homogenous environment with large focal males competing with large male competitors in both the Female and Female-Male Experiments (i.e. $HomL^{F}$ and $HomL^{FM}$) (S1 Table).

Quadratic effects – We measured the quadratic effect by adding a quadratic term to the linear model of standard female Bateman gradients. The relationship between offspring production and number of mates might be non-linear for females, suggesting that females maximise offspring production at a given number of mates. Here, we show that non-linear (i.e. negative

quadratic effect) relationship between mates and offspring of females. We found a significant negative quadratic effect on the female Bateman gradient in the experimental group Het^M of the Male Experiment (Estimate [p-value]; M^2- -0.046 [0.020]). Negative female quadratic effect was also found in female jungle-fowl (Collet et al., 2014) and suggests that offspring production peaks at a certain number of mates, decreasing thereafter. This pattern is consistent with females having an optimum number of mates.

Sex ratio effects cause by deaths of experimental females:

Table 2 of S1 Text – The effects of changes in sex ratio due to the deaths of experimental females (*DF*).

Sex	Factor	Mating frequency		Number of mates		Number of offspring	
		F-value	*p-value*	*F-value*	*p-value*	*F-value*	*p-value*
Female		*0.008*	*0.924*	*1.411*	*0.235*	*1.233*	*0.267*
Focal male		*2.263*	*0.135*	*7.907*	***0.005***	*8.875*	***0.003***

Chapter 4

Interactions between the developmental and adult social environments mediate group dynamics and offspring traits in *Drosophila melanogaster*

Summary

1. Competition over resources during developmental stages can strongly influence adult phenotypes, with potent consequences for reproduction. Individuals of different phenotypes then interact in social groups in adulthood, and the specific mix of phenotypes has the potential to mediate the processes of sexual selection and sexual conflict.

2. Social interactions in phenotypically mixed populations theoretically alter the costs or benefits of developmental environments if those interactions selectively favour or disfavour individuals from specific developmental backgrounds. However, the implications of these social interactions for the dynamics of groups, and for subsequent generations, are poorly understood.

3. To address this, we raised *Drosophila melanogaster* at low and high larval density and created adults with large and small body size, respectively. We then manipulated the phenotypic composition of adult populations by mixing males, females, or both sexes from these two larval densities and measured group courtship activity, reproductive output, and offspring traits.

4. Groups where both sexes experienced high larval density had high early reproductive rates (and population growth rates, *r*), high offspring mass and offspring eclosion success, but died rapidly as compared to groups where both sexes experienced low larval density.

5. Phenotypic variation amongst either sex was sufficient to lower group-level courtship and rescue the eclosion success and body mass of offspring, suggesting interacting effects of developmental and adult social environments. Total offspring production was greater in populations consisting of females from low larval density and males from both low and high larval densities.

6. Groups with mixed male phenotypes had lower rates of increase in offspring mass, and reached peak reproductive rate earlier (6-9 days) than groups where all males were phenotypically equal (9-13 days); female phenotype had no effect on these traits suggesting a sex-specific effect.

7. Our results suggest that many group traits are influenced by an interplay between the developmental and adult social environments, with effects transferred onto offspring. The presence of at least some individuals from the high larval density environment appears sufficient to improve group reproductive rates and offspring mass suggesting that group benefit from containing a mix of phenotypes.

Introduction

In natural populations, the interactions between individuals with different phenotypes can be modulated by the availability of resources in the environment (Svanbäck and Bolnick, 2007, Bolnick et al., 2007). The pool of resources acquired by organisms is often referred to as the organism's condition (Rowe and Houle, 1996), and all traits are expected to depend on the allocation of these resources (Boggs, 2009). Resource allocation modulates the expression of male and female sexually-selected traits that in turn influences key evolutionary processes such as sexual conflict and sexual selection (Rowe and Houle, 1996, Wigby et al., 2015, Morimoto et al., 2016, Janicke et al., 2015, Fricke et al., 2010a, Mason et al., 2016, Zikovitz and Agrawal, 2013). These processes can potentially influence speciation and extinction rates and thus shape biodiversity (Arnqvist et al., 2000, Lumley et al., 2015, Lorch et al., 2003, Radwan, 2004, Rankin et al., 2011, Kokko and Brooks, 2003, Van Doorn et al., 1998).

Resource acquisition and competition experienced during the developmental stage can modulate the social interactions of individuals with their potential mates and rivals in adulthood, both in laboratory and field populations and social groups (Atkinson, 1979, Lemaitre et al., 2011, Gage, 1995, Zikovitz and Agrawal, 2013, Wigby et al., 2015). In insects, the developmental environment influences adult body size (Amitin and Pitnick, 2007, Pitnick and Garcia-Gonzalez, 2002, Lyimo et al., 1992, Credland et al., 1986), which tends to positively correlate with female fecundity (i.e. large females produce more eggs than small females) and male competiveness (Bonduriansky, 2001). Large individuals tend to have large reproductive organs, high courtship activity (in males), high mating frequency, and high reproductive output compared to small individuals (Partridge et al., 1987, Amitin and Pitnick, 2007, Morimoto et al., 2016, Lyimo et al., 1992, Pitnick and Garcia-Gonzalez, 2002, Wigby et al., 2015, Lüpold et al., 2010, Parker and Pizzari, 2010, Chapman, 1998, Honek, 1993,

Shine et al., 2000). Thus, adult body size is expected to be under fecundity selection in females and sexual selection in males (Bonduriansky, 2001, Honek, 1993, Roff, 2002, Stearns, 1992, Clutton-Brock, 2009, Morimoto et al., 2016).

Despite the far-reaching effects on the physiology and behaviour of individuals, the extent to which the developmental and social environments of individuals interact to shape survival and fitness of populations and groups remains largely unexplored. Most studies on the developmental environment have focused on the plastic responses of focal individuals (e.g. (Wigby et al., 2015, McGraw et al., 2007)), and only recently have studies in *Drosophila melanogaster* begun to investigate the dynamics within mixed-phenotype social groups (Long et al., 2009, Morimoto et al., 2016, Zikovitz and Agrawal, 2013). In *D. melanogaster,* high larval density generally results in smaller body-size adults: males that are less competitive with rivals, and females that produce fewer offspring (Morimoto et al., 2016, Pitnick and Garcia-Gonzalez, 2002, Amitin and Pitnick, 2007, Wigby et al., 2015). However, the magnitude of these larval density effects on adult reproduction can be mitigated in mixed-phenotype social groups (Long et al., 2009, Morimoto et al., 2016). For instance, males direct their courtship efforts preferentially to large females in mix female size environments, which in turn reduces the fecundity advantages of large over small females (Long et al., 2009). Moreover, variation in female body size can affect male siring success whereby large males sire a higher proportion of offspring than small males when females are large, but this effect is lost when females vary in body size (Morimoto et al., 2016). These findings attest to the need of considering mixed-phenotypes social groups in order to fully understand the consequences of developmental environments for fitness in adulthood and the resulting patterns of selection and evolution. Key questions remain unanswered: do interactions between the developmental and adult social environments influence the survival and reproductive output of populations? Can any of the effects of the interaction between

developmental and adult social environments be passed onto the next generation and affect population likelihood of persistence in an environment?

To address these questions, we manipulated the larval density of *D. melanogaster* to obtain large and small adult phenotypes of both sexes. Five treatments consisting in groups with either equal or unequal body size groups of males, females, or both were assembled and used to measure group survival, courtship levels, reproductive output and fitness as well as effects on offspring eclosion success and body size. To our knowledge, this is the first study to explore effects of developmental and social environments at a social group level.

Predictions

Our predictions were:

1. If males adopt canalized strategies based on their own developmental environment, groups with large males (from low density larval environments) will tend to have higher courtship levels because large males are more sexually active than small males;
2. If females adopt canalized reproductive strategies based on their own developmental environment, groups with large females (from low density larval environments) will tend to have higher offspring production because female body size is positively correlated with female productivity. Moreover, large females should have more resources to expend on provisioning eggs, which might benefit offspring.
3. However, if males and female reproductive traits respond to changes in the phenotype of their social rivals and mates over and above the strategies set by their own developmental environment, then we will expect to see deviations from the patterns predicted above.

Material and Methods

Fly stocks and culture

We used wild-type inbred *OregonR*, which was the only stock of *D. melanogaster* available from Bloomington Drosophila Stock Centre maintained by Greg Neely's Lab at the University of Sydney. This change in strain from Dahomey (see other Chapters) to *OregonR* was necessary because the present work was performed in Australia whereby the Dahomey strain was not available. *OregonR* is an inbred line with limited genetic variation, which can lead to phenotypic and behavioural effects on the individuals (e.g. see (Tan et al., 2012) for a review of the consequences of inbreeding). Thus, although the results are likely to be of general significance, we recognise that they should be interpreted with some caution. The *OregonR* stock was maintained in populations (>5,000 individuals) in cages with overlapping generations for >10 generations. All fly stocks were maintained and all experiments conducted, at 25°C on a 14:10 light:dark cycle in a controlled humidified room (humidity = 68%) and fed with standard sugar-yeast-maize-molasses medium with excess live yeast granules (See Supplementary Information for Recipe).

Larval density manipulation and adult body size

Following the protocol of Clancy and Kennington (2001), we collected eggs from population cages and manipulated larval density of flies in the following way: for the high larval density treatment (small body size adults) we placed ~200 larvae/mL of food (~100 larvae per 34ml vial containing ~ 4 mL fly food); for the low larval density treatment (large body size) we placed ~ 4 larvae/mL of food (~40 larvae in per 34ml vial containing ~ 10 mL fly food). A similar approach has been extensively used to create significant differences in the average

body size of males and females (e.g. (Amitin and Pitnick, 2007, Wigby et al., 2015, Morimoto et al., 2016). We used these categorical variations in body size to manipulate our population social composition (see Fig 1). Virgin flies of both sexes were collected within 8 hours of eclosion and kept in vials of same-sex and same-larval manipulation groups of 15-20 individuals for 2-5 days prior to experiments. To minimise any potential effects of larval familiarity, we setup a large number of vials (>50) per larval density manipulation, in which we pooled all adult individuals together before collecting virgin flies used in the experiment. This methodology reduced the likelihood of placing familiar individuals in the same treatments.

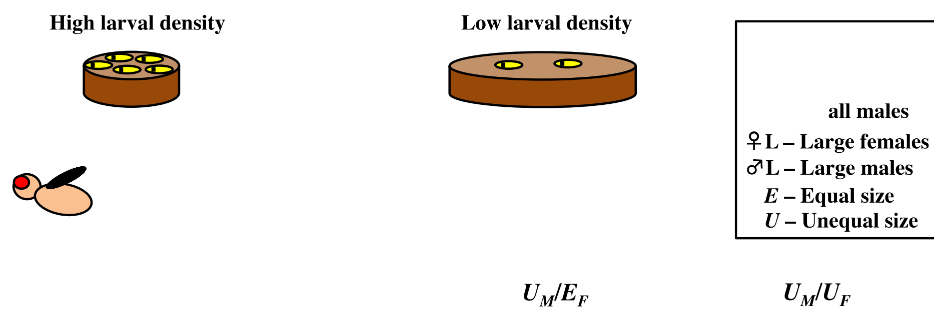


Figure 1 – Summary of the experimental design. We manipulated larval density to obtain males and females of large and small body size. We then setup populations composed of individuals with different combinations of body size. $E_M/E_F^{(high)}$ – populations where all individuals experienced high larval density; $E_M/E_F^{(low)}$– populations where all individuals experienced low larval density; E_M/U_F – populations where all males were phenotypically equal, from a low density larval density developmental environment and females had mixed

phenotypes; U_M/E_F – populations where all females were phenotypically equal, from a low density larval density developmental environment, and males had mixed phenotypes; U_M/U_F – populations where both males and females had mixed phenotypes.

Experimental design

We created populations with 'Equal' (E) or 'Unequal' (U) phenotypes of male (M), female (F), or both (Fig 1). The design was based on Morimoto et al. (2016). Briefly, replicate populations containing 4 flies of each sex were placed in vials (i.e. 1 population of 8 flies per vial), and flies were allowed to interact continuously throughout their lifespan. Populations were transferred to fresh vials with standard maize-molasses food and yeast *ad libitum* in discrete time intervals throughout their lifespan. We assembled 17 replicate populations *per* treatment, in five treatments (see Fig 1):

1. Populations with equal-sized males and equal-sized females in which males and females experienced high larval density (small adult body size) ($E_M/E_F^{(high)}$);
2. Populations with equal-sized males and equal-sized females, in which males and females experienced low larval density (large adult body size) ($E_M/E_F^{(low)}$);
3. Populations with phenotypically equal-sized males but unequal-sized females, in which all 4 males experienced low larval density, 2 females experienced high and 2 females experienced low larval density (E_M/U_F);
4. Populations with phenotypically unequal-sized males but equal-sized females, in which 2 males experienced high larval density and 2 males experienced low larval density, all 4 females experienced low larval density (U_M/E_F);
5. Populations with phenotypically unequal-sized males and unequal-sized females (U_M/U_F).

Courtship level

Courtship levels in groups were assessed based on the count of male courtship behaviours towards females (e.g. chasing, wing-extension, attempting copulation (Sokolowski, 2001)) during 1h observations every morning when groups were transferred to fresh vials with standard maize-molasses food and yeast *ad libitum*. We scored deaths and calculated the number of males and females alive in in each group at each time interval used for the behavioural and reproductive measurements. Groups were considered "dead" when all 4 females died, in which case males, if still alive, were discarded. After the behavioural observations of courtship, we transferred the groups to vials with fresh standard maize-molasses food and yeast *ad libitum* until the next vial replacement. We stored all interaction vials for 13-15 days, until all the adult offspring had emerged. We scored the number of eclosing offspring produced in each vial and calculated the rate of offspring production and total offspring production of each group. We also scored the number of non-emerging pupae to measure eclosion success. We scored courtship and offspring for the first 35 days by which time reproduction had ceased, and from this point onwards only scored survival. In total, we counted 35,881 adult offspring and 5,203 non-emergent pupae.

Offspring mass

We randomly sampled ~25 female and ~25 male offspring *per* group *per* day ($N_{total} = 1,458$) and measured their wet body mass on a fine scale Sartorius® with precision of 0.0001g.

Data analysis

Larval density effects on body size

We used one-way ANOVA after checking for the normality of the data to evaluate the effects of larval density on male and female body sizes.

Courtship and survival

To analyse courtship we used a Generalized Linear Model (GLM) with a quasi Poisson distribution to account for overdispersion of the data. The model included the linear and quadratic effects of time, treatment and their interactions. As individuals died, they were not replaced and thus the number of individuals contributing to group fitness and survival declined. To control for this, we included covariates in the models to account for the number of females and males in the group at the time of the courtship activity measurements. p-values are given from F-tests. We also included “vial” as a fixed effect to control for pseudoreplication in courtship models. We reckon that “vial” could be fitted as either fixed or random effect. We chose to fit “vial” as a fixed effect because it allowed us to use a ‘quasi’ Poisson model to account for the overdispersion of the data (Note that ‘quasi’ parameter cannot be used in Generalized Mixed Models with random factors). We used Student-Newman-Keuls (‘SNK’) post-hoc test to determine differences in mean courtship levels between groups. For survival analysis, we tested for differences in survival of the group by fitting a proportional hazardous model using the phaz function of the ‘survival’ package in R; p-values are given from the χ^2 test.

Offspring production

To analyse offspring production, we fitted a general linear model on the full dataset that included – in addition to the variables described above – the interactions between the linear and quadratic effect of time and treatment (i.e. Time*Group and $Time^2$*Group). This allowed us to compare how the slope and the peak reproductive success of groups differ depending on the phenotype composition of males and females in the group. For the group total offspring production, we used SNK post-hoc test to check for differences between treatments. We checked the normality assumption of the data for all models.

Group fitness

We estimated group growth rates by calculating (r), an index obtained from the age-specific offspring production and survival which better represent fitness than lifetime offspring production in *D. melanogaster* (see (Stearns, 1992, Edward et al., 2011b, Wigby and Chapman, 2005). We calculated r for each vial, and tested differences between treatments with a non-parametric Kruskal-Wallis Rank Sum Test (with p-values reported from a χ^2 test) followed by a Student-Newman-Keuls (SNK) post-hoc test to assign differences in means.

The timing of reproduction has a large impact on group growth rates. We therefore investigated whether social and developmental environments interact to alter age-specific group reproductive rates. To do this, we fitted general linear models for each treatment individually, which included linear and quadratic effects of time while controlling for the number of females and males in the group as described above. When the quadratic effect of time was statistically significant, we calculated the peak reproductive success of the group with the derivative of the quadratic model as follows:

$$F(x) = \alpha x^2 + \beta x + \gamma \qquad \text{eq 1}$$

where α and β are the quadratic and linear coefficients of the linear model, respectively, after controlling for confounding covariates and *x* is our time intervals. We then took a derivative of eq 1

$$\frac{\partial F(x)}{\partial x} = 2\alpha x + \beta \qquad \text{eq 2}$$

where $\frac{\partial F(x)}{\partial x}$ is the first derivative of eq 1 in time. If $\alpha < 0$, then solving eq 2 for $\frac{\partial F(x)}{\partial x} = 0$ defines the point in which eq 1 is maximised – i.e. it defines the day when the rate of offspring production peaked.

We also investigated whether the variance in group total reproductive success was affected by our treatments using the 'leveneTest' function of the 'car' package in R, which allowed us to test for homogeneity of variance between our treatments.

Pupal eclosion

To analyse the absolute number of non-emergent pupae, we used a general linear model with treatment while controlling for the linear and quadratic effects of day and the total number of pupae in the group (i.e. adult offspring (emergent pupae) + non-emergent pupae). Courtship level was also included as a covariate in the model to investigate whether courtship levels affected eclosion failure. In addition to the absolute number of non-emergent pupae, we also analysed the proportion of non-emergent pupae in relation to the total number of pupae with a GLM with a quasibinomial distribution to account for overdispersion of the data; p-values are given from F-tests. We used the SNK post-hoc test to test for differences in mean absolute count of non-emergent pupae between our treatments.

Offspring body mass

To test for effects on offspring body mass we used a general linear model with treatment, sex of the offspring and their interaction while controlling for the effects of courtship, the density of offspring per vial and the linear and quadratic effects of time and their interaction with treatment. We Boxcox transformed offspring mass (i.e. mass^0.5) to fit the normality assumption. We used the SNK post-hoc test in each sex separately to test for differences in mean offspring body mass between our treatments.

All figures are of non-transformed (raw) data, and all analyses were performed in R version 3. 2. 2.

Results

Larval development manipulation and body size

Males and females from high and low larval density treatments had significantly different body sizes (*Male:* $F_{1,68} = 18.336$, $p < 0.001$; *Female:* $F_{1,74} = 14.725$, $p < 0.001$) whereby individuals from high larval density had smaller body size than individuals from low larval density treatments (Fig S1). The variance in body size was not affected by our larval manipulation in either males ($F_{var36,33} = 0.806$, $p = 0.526$) or females ($F_{var36,39} = 1.006$, $p = 0.979$).

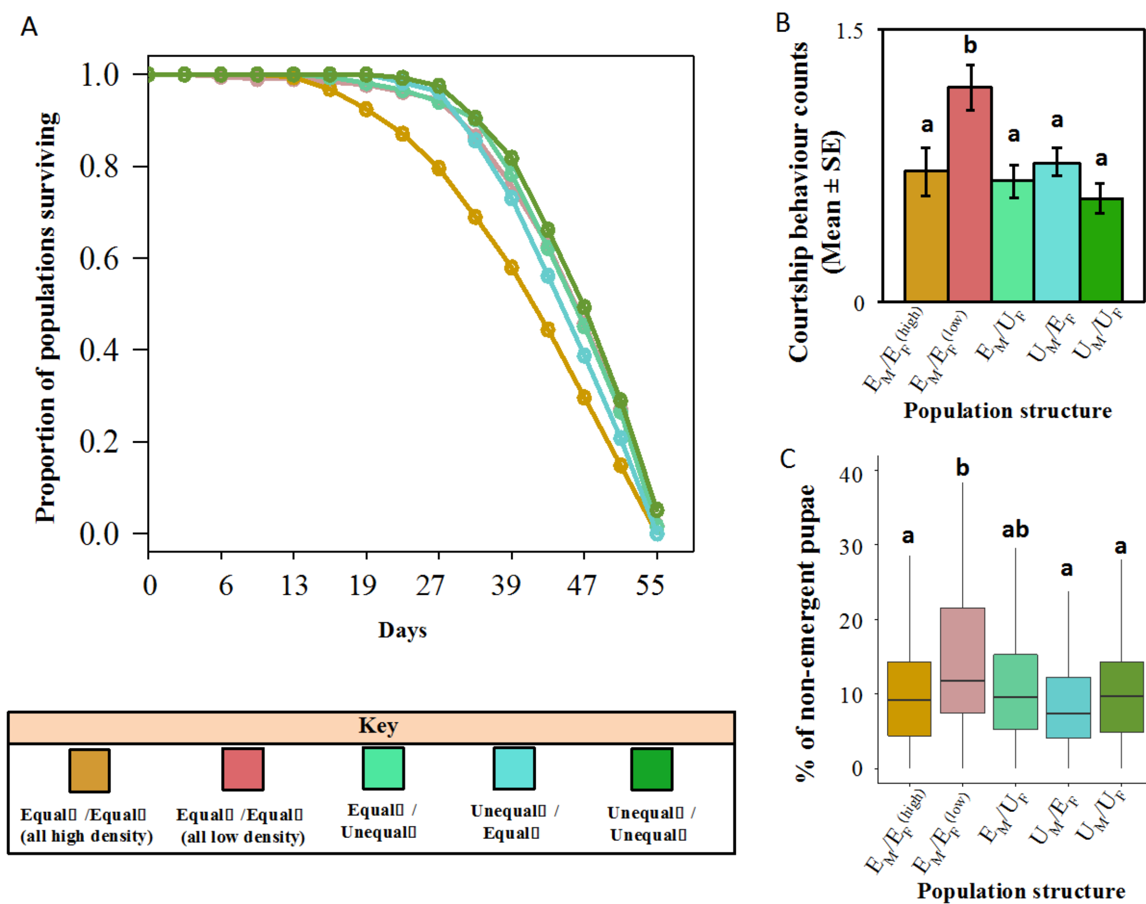
Group survival

Treatment had a significant effect on survival (Treatment: $\chi^2_4 = 20.660$, $p <0.001$). Groups in which both males and females experienced high larval density (i.e. E_M/E_F $^{(high)}$) died significantly faster than groups in which both males and females experienced low larval

density (i.e. E_M/E_F $^{(low)}$). There was no difference in survival between U_M/E_F and U_M/U_F treatments relative to E_M/E_F $^{(low)}$ (Fig 2A).

Courtship levels

Groups in which both males and females experienced low larval density (i.e. $E_M/E_F^{(low)}$) had significantly higher courtship behaviour counts than all other groups which did not significantly differ from one another ($F_{4,528} = 4.949$, $p < 0.001$, Fig 2B). There was no



evidence for a linear nor a quadratic effect of time (Time: $F_{1,532} = 1.465$, $p = 0.226$, Time^2: $F_{1,527} = 0.869$, $p = 0.351$, Table S1).

Figure 2 – Survival, courtship levels and pupae eclosion. (**A**) Proportion of groups surviving throughout the experiment. Likelihood ratio test = 20.74, df = 5, $p < 0.001$. (**B**) Courtship levels in the treatments. **(C)** Pupae eclosion success (in %) measured as the number of non-emergent pupae. $E_M/E_F^{(high)}$ – groups where all individuals experienced high larval

density (gold); $E_M/E_F^{(low)}$– groups where all individuals experienced low larval density (red); E_M/U_F – groups where all males were phenotypically equal, from a low density larval density developmental environment and females had mixed phenotypes (light green); U_M/E_F – groups where all females were phenotypically equal, from a low density larval density developmental environment, and males had mixed phenotypes (blue); U_M/U_F – groups where both males and females had mixed phenotypes (dark green). Post-hoc SNK test ($\alpha = 0.05$).

Pupae eclosion

Treatment had a significant effect on both the absolute and the proportion of non-emergent pupae (*Absolute:* $F_{4,524} = 6.862$, $p < 0.001$; *Proportion:* $F_{4,489} = 2.745$, $p = 0.027$; see Table S2, Fig S2). Groups in which both males and females experienced low larval density (i.e. $E_M/E_F^{(low)}$) had significantly more non-emergent pupae than treatments $E_M/E_F^{(high)}$, U_M/E_F, U_M/U_F, and a non-significant trend in the same direction for E_M/U_F (Fig 2C). This effect was above and beyond the effects of offspring density in each vial (*Absolute*: $F_{1, 522} = 210.64$, $p < 0.001$). In addition, these effects were independent of courtship levels because there was no significant correlation between courtship levels and pupae eclosion (*Absolute:* $F_{1, 522} = 2.316$, $p = 0.128$, *Proportion:* $F_{1, 488} = 2.235$, $p = 0.135$, Table S2).

Total offspring production

Treatment had a significant effect on total offspring production ($F_{4,76} = 4.021$, $p = 0.005$, Table S3). A post-hoc analysis revealed that groups in which males and females experienced high larval density (i.e. $E_M/E_F^{(high)}$) produced significantly fewer offspring than U_M/E_F

groups, and all other groups were intermediate and not significantly different from any another (Fig 3A).

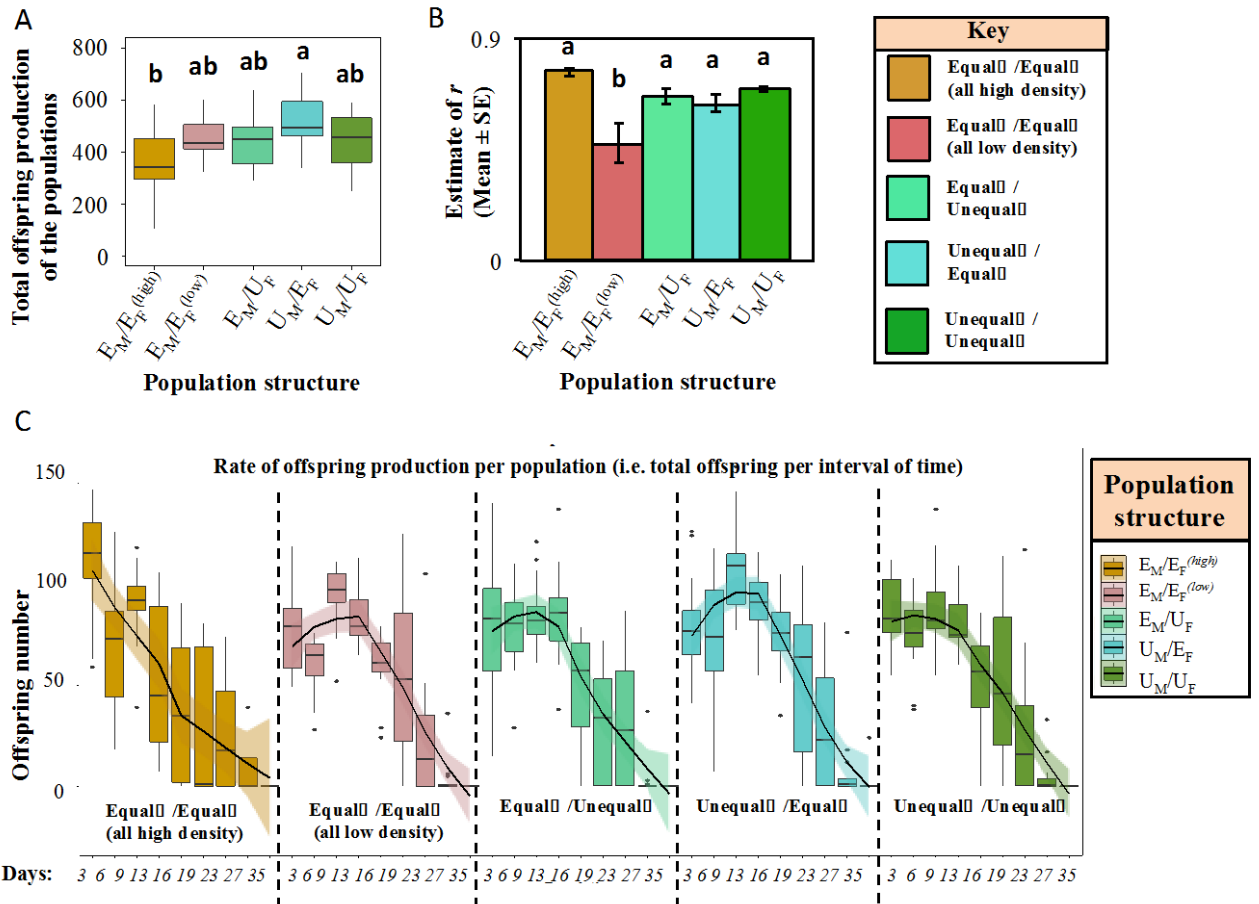


Figure 3 – Estimates of group reproductive success, fitness and offspring production rate. (**A**) Total lifetime offspring production. (**B**) Age-specific fitness of groups (*r*). (**C**) Offspring production *per* interval of time. $E_M/E_F^{(high)}$ – groups where all individuals experienced high larval density (gold); $E_M/E_F^{(low)}$– groups where all individuals experienced low larval density (red); E_M/U_F – groups where all males were phenotypically equal, from a low density larval density developmental environment and females had mixed phenotypes (light green); U_M/E_F – groups where all females were phenotypically equal, from a low density larval density developmental environment, and males had mixed phenotypes (blue); U_M/U_F – groups where both males and females had mixed phenotypes (dark green). Post-hoc SNK test ($\alpha = 0.05$)

Group fitness, r

We calculated *r*, an age-specific offspring production index often used as a proxy for group fitness (see e.g. (Wigby and Chapman, 2005, Charlesworth, 1994, Stearns, 1992)). Treatment had a significant effect on estimates of *r* (Treatment: $\chi^2_4 = 23.191$, p <0.001) whereby groups composed of males and females from low larval density developmental environment (i.e. E_M/E_F $^{(low)}$) had significantly lower estimates of *r* (highest fitness) than other treatments (Fig 3B).

Offspring production rate

Time had a significant negative linear and quadratic effect on the rate of offspring production of the groups (Time: $F_{1, 608}$= 767.552, p < 0.001; Time^2: $F_{1, 608}$ = 72,207, p < 0.001, Fig 3C, Table S4), which was expected since females reduce (and eventually stop) producing eggs through time. There was no significant interaction between linear Time and Group ($F_{4, 608}$ = 1.842, p = 0.119). However, we found a significant quadratic interaction, Time^2*Group, on offspring production ($F_{4,608}$ = 7.935 , p < 0.001, Fig 3C), suggesting that the peak reproductive success differed between groups. Relative comparison between treatments revealed that this effect was mainly driven by groups where males and females were small (i.e. E_M/E_F $^{(high)}$), in which the estimates of the concavity of the curve did not reach statistical significance (Time^2: *t-value = 0.462*, p = 0.645). This pattern suggests that E_M/E_F $^{(high)}$ reached maximum reproductive success between days 0-3, after which reproduction declined linearly (Fig 3C). The number of males and females in the group at the time of the measurement was significantly positively correlated with the rate of offspring production (see also Table S4 and Fig S3 for cumulative offspring production).

We then used eq 1 and eq 2 to calculate the peak of the rate of offspring production in our groups. For groups with males from low larval density environments ($E_M/E_F^{(low)}$ and E_M/U_F) the estimates of peak reproductive success laid in between days 6-9 of group survival, whereas groups with mixed male phenotypes (i.e. U_M/E_F and U_M/U_F) reached peak reproductive success at around days 9-13. For groups with males and females from high larval density developmental environments ($E_M/E_F^{(high)}$) the peak reproductive success was the earliest among our treatments (0-3 days) (Fig 3C, see Table S5).

Offspring body mass

Treatment had a significant effect on offspring weight ($F_{4,330} = 5.895$, $p < 0.001$). Post-hoc analysis revealed that this effect is caused by a reduction in offspring weight in groups composed of all large males and females individuals (i.e. $E_M/E_F^{(low)}$) compared to all other treatments (Fig 4, Table S6). There was a linear and a quadratic effect of Time (Time: $F_{1,330} = 147.213$, $p < 0.001$; Time^2: $F_{1,330} = 126.141$, $p < 0.001$) suggesting that offspring body mass increased after the first day of egg-laying and plateaued after ~9-13 days after the beginning of the experiment. There was a significant interaction of Treatment*Time ($F_{1,330} = 5.438$, $p < 0.001$), which showed that the linear increase in offspring body mass differ between groups (Fig S4; Table S7). Comparison of the slopes of offspring body mass on time revealed that the slope of U_M/U_F and the U_M/E_F groups increased at a slower rate than $E_M/E_F^{(low)}$, although this effect was not found for $E_M/E_F^{(high)}$ and E_M/U_F groups. These data indicate that in groups with mixed male phenotypes the body mass of offspring increases a slower rate over time. Courtship levels were not correlated with offspring body mass ($F_{1,330} = 0.468$, $p = 0.494$), suggesting that the reduction in offspring body mass was not driven solely by group courtship levels. The interaction Sex*Treatment and Time^2*Treatment were not significant (Sex*Treatment: $F_{4,330} = 1.660$, $p = 0.158$; Time^2*Treatment: $F_{4,330} = 2.318$, $p = 0.056$).

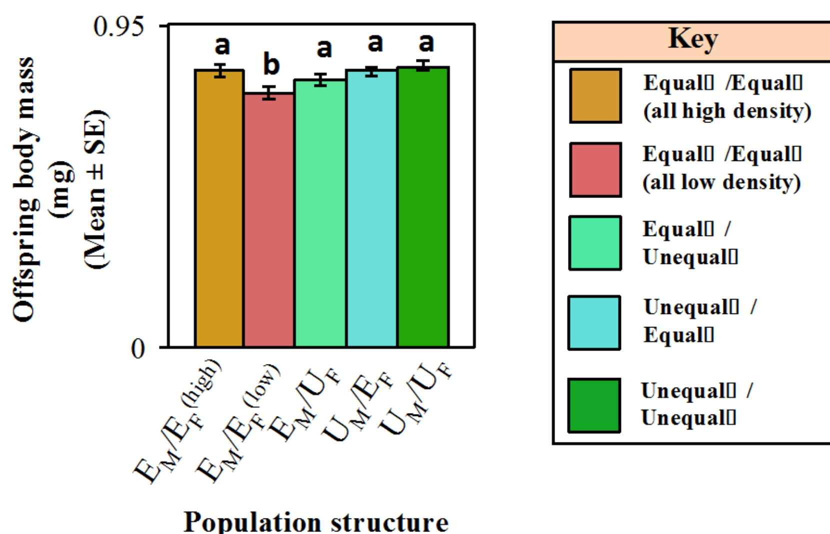


Figure 4 – Offspring body mass. Average offspring body mass (in mg) (Mean ± SE). $E_M/E_F^{(high)}$ – groups where all individuals experienced high larval density (gold); $E_M/E_F^{(low)}$– groups where all individuals experienced low larval density (red); E_M/U_F – groups where all males were phenotypically equal, from a low density larval density developmental environment and females had mixed phenotypes (light green); U_M/E_F – groups where all females were phenotypically equal, from a low density larval density developmental environment, and males had mixed phenotypes (blue); U_M/U_F – groups where both males and females had mixed phenotypes (dark green). Post-hoc SNK test ($\alpha = 0.05$).

Discussion

The data presented here show that phenotypically homogeneous groups where individuals of both sexes experienced high larval density (i.e. $E_M/E_F^{(high)}$) had high early reproductive rates (see *r*) and offspring eclosion success, but died rapidly relative to other treatments. When both males and females experienced low larval density (i.e. $E_M/E_F^{(low)}$), groups had low early reproductive rates (see *r*), produced offspring with lower body mass and lower eclosion success, which were rescued in groups consisted of mixed phenotypes. In groups where

males, but not females, were phenotypically mixed, there was a delayed peak in reproduction and reduced rates of increasing offspring body mass. Overall, these results suggest far-reaching effects of the interaction between developmental and social environments on offspring and group traits. Below, we discuss the main findings of our study in more detail.

Courtship levels and trans-generational effects

We found that groups composed of males and females from the low larval density developmental environment (large body size phenotype) had higher courtship levels (Fig 2B) and produced offspring with lower body mass (Fig 4). Moreover, we showed that the presence of some individuals from low larval density (i.e. phenotypically mixed groups) was sufficient to lower courtship levels and increase offspring body mass. These results contrast with predictions 1 and 2 in which we would expect courtship to be significantly higher in all groups consisted of males from the low larval density developmental environment (i.e. $E_M/E_F^{(low)}$ and E_M/U_F) (prediction 1), and offspring body mass to be significantly higher in all groups consisted of females also from the low larval density environment (i.e. $E_M/E_F^{(low)}$ and U_M/E_F) (prediction 2). Thus, the data suggest prediction 3 to be true: the social and developmental environments interact to shape courtship activity and offspring traits. There are several possible explanations for our findings. Female phenotypic variation may trigger male adaptive male choice, whereby males direct their courtship efforts towards large and more attractive females (Byrne and Rice, 2006, Long et al., 2009), which in turn might reduce the average courtship in the group. Moreover, female phenotypic variation may also increase the uncertainty of females in assessing the level of intra-sexual competition for resources, which may trigger female responses to increase investment in their progeny in order to guarantee their offspring's success in the next generation of unpredictable competition. In parallel, male phenotypic variation is also likely to have influenced group dynamics. In groups consisted of

mixed male phenotypes, large males (from low larval density) might have perceived weaker intra-sexual competition (due to the presence of small males), and therefore may decrease their courtship investment to allocate resources in ejaculate and offspring size. Furthermore, the larval manipulation could have signalled the level of intra-sexual competition that individuals were likely to face in adulthood (Gage, 1995, Lemaitre et al., 2011) which in turn could have altered male and female offspring resource allocation in offspring (Allen et al., 2008, Crean and Bonduriansky, 2014) and life-history strategies (Stearns, 1992). Scrutinising these hypotheses lies beyond the scope of this paper, but remains an important topic of research for future studies.

We also found that offspring body mass increased with group age and plateaued at ~9-13 days of group survival, which was approximately coincident with peak reproductive success (except for $E_M/E_F^{(high)}$, see discussion below) (Fig S4). Although the significance of this pattern is unclear, differential female offspring allocation and male accessory gland maturation could have influenced offspring body mass (Mousseau and Fox, 1998, Crean and Bonduriansky, 2014, Ruhmann et al., 2016). In *Daphnia magna*, maternal age is positively associated with offspring size (Glazier, 1992). On the other hand, female age delays development and is negatively associated with egg size and hatchability in *Collosobruchus maculatus* (Fox, 1993). In *D.* melanogaster, the quantity of seminal fluid and the size of male accessory gland increase with male age (Ruhmann et al., 2016), which could alter male ejaculate investment and account, at least partly, for the increased offspring body mass with increased group age. Moreover, theory suggests that maternal investment in offspring might increase with age (Moorad and Nussey, 2016) although evidences of this effect in *D. melanogaster* are still lacking. More studies are needed to investigate the significance of the increase in offspring body mass as the group aged.

Group reproduction and fitness

If the developmental environment was the sole factor determining group fitness, we would have observed higher group fitness for groups consisted of females from the low larval density developmental environment (i.e. $E_M/E_F^{(low)}$ and U_M/E_F) (prediction 2) because these females are larger and more fecund. However, our results suggest that groups consisting of males and female from low larval density developmental environments (i.e. $E_M/E_F^{(low)}$) had lower estimates of group fitness (*r)*, which was fully rescued in phenotypically mixed groups of either (or both) sex(es). (Fig 3A and 3B). These findings corroborate prediction 3, and shows that the interaction between the developmental and social environments shape group fitness (see 'Predictions'). We also showed that the peak reproductive success for groups composed of males from low larval density was earlier (6-9 day of survival) than for groups with mixed male phenotypes (9-13 day of survival). Social and developmental environments can potentially modulate group reproductive success and fitness by altering female reproductive investment as a response to social context as well as male ejaculate investment based on competition levels or accessory gland maturation, or both (Ruhmann et al., 2016, Sirot et al., 2011). We also found that groups consisting of males and females from high larval density (i.e. $E_M/E_F^{(high)}$) had the earliest peak reproductive success amongst our treatments (0-3 days of group survival), and group reproduction rate became a negative linear function in time (Fig 3C). This pattern might arise from possible differences in life-history strategies at the individual level caused by social interactions, whereby males from high larval density environments, when surrounded by rivals with the same background experiences of stressful developmental environment adopted a strategy to invest heavily on current reproduction at the expense of survival and future reproduction (Stearns, 1992, Stearns, 1989, Kirkwood and Rose, 1991). Future studies should investigate the relationship between larval

density and male maturation as well as individual's strategies (as opposed to group's responses) adopted in different social contexts.

Group survival

Groups that consisted of males and females from high larval density environments (small body size) died significantly faster than other treatments (Fig 2A), corroborating previous findings that small individuals have shorter lifespan (Partridge et al., 1987, Partridge and Farquhar, 1981). Phenotypically mixed groups of either males or females, or both, showed similar survival to groups consisted of individuals from low larval density environments, which supports our prediction 3 that social and developmental environments also interact to shape group survival (see discussion above). Had the developmental environment been the sole factor affecting group survival, we might expect groups consisted of males from low larval density environment to died faster because of the higher costs incurred by the elevated courtship activity of these males (prediction 1). Instead, group survival was likely to have been influenced by a myriad of traits of an individual (e.g. female reproductive output) and the interaction between traits among individuals (e.g. female post-mating responses to male seminal fluid proteins) and therefore, further studies are required to fully comprehend the survival patterns observed here. Interestingly, however, Adler et al. (2016) recently showed that the developmental and social environments modulate somatic deterioration of males neriid fly *Telostylinus angusticollis*, whereby somatic deterioration is significantly elevated for high condition males in social groups to the point in which high condition males did not outlive low condition males. Together, our results and the results of Adler et al. (2016) open a new avenue for future research on the interaction between ecological and social factors affecting group survival.

Interactions between developmental and social environments

The plastic responses of different phenotypes to the interactions with their surrounding environment are crucial for the success of individuals in nature (Price, 2006). This plasticity may allow individuals and, thus groups, to survive and persist in new environments when facing unprecedented challenges (Robinson and Dukas, 1999, West-Eberhard, 2003). These patterns are revealed in our data whereby plastic responses to social and developmental environments modulates group survival and trans-generational effects in eclosion success and body mass, which might influence long-term group persistence in challenging environments. Plastic responses to developmental and social environments can shape mating and non-mating behaviour of individuals, affecting the distribution, the quality, and the availability of mates and rivals in the group (Kokko and Rankin, 2006, Han and Brooks, 2014, Morimoto et al., 2016, Lackey and Boughman, 2013). As a result, plastic responses to the developmental and social environments can modulate the operation of sexual selection in groups, which in turn can shape group survival, growth and speciation patterns (Agrawal, 2001, West-Eberhard, 2003, Forsman, 2015, Morimoto et al., 2016, Arnqvist et al., 2000). This study found that courtship levels, reproduction and fitness of groups are determined by plastic responses to social and developmental environments, which lend support to the idea that plastic responses to the surrounding environmental conditions can modulate sexual and reproductive behaviours and ultimately influence evolutionary processes. Thus, future ecological and evolutionary studies should acknowledge the implications of the multi-level interactions between the early-life experiences (e.g. larval density, larval nutrition) and the social context of competing adults in order to fully comprehend the magnitude of key evolutionary processes such as sexual selection and sexual conflict.

Conclusion

In conclusion, our results show important effects of the developmental and social environments on group fitness in *D. melanogaster* and add to the growing body of research on environmentally-mediated trans-generational effects. All species face challenges in their developmental environment (i.e. physical or nutritional constraints), and in most species individuals are required to interact during their lifetime both for survival and reproduction. Thus, the patterns revealed here could potentially be widespread, and future studies should expand and test our results and prediction in different species across the animal kingdom. Important questions still remain, such as 1) What are the individual level responses to the interactions between developmental and social environments? 2) What are the mechanisms by which individuals discriminate rivals and mates from similar or different developmental environment? 3) What are the molecular mechanisms by which the developmental and social environments interact and lead to the trans-generational effects observed in this study? 4) What are the long-term fitness consequences for the offspring for having lower body mass? 5) For how many generations do the trans-generational effects of the development and social environment persist? The answer to these questions will advance our understanding of the evolutionary and ecological processes affected by the structure, composition and social dynamics of groups.

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Supplementary Information

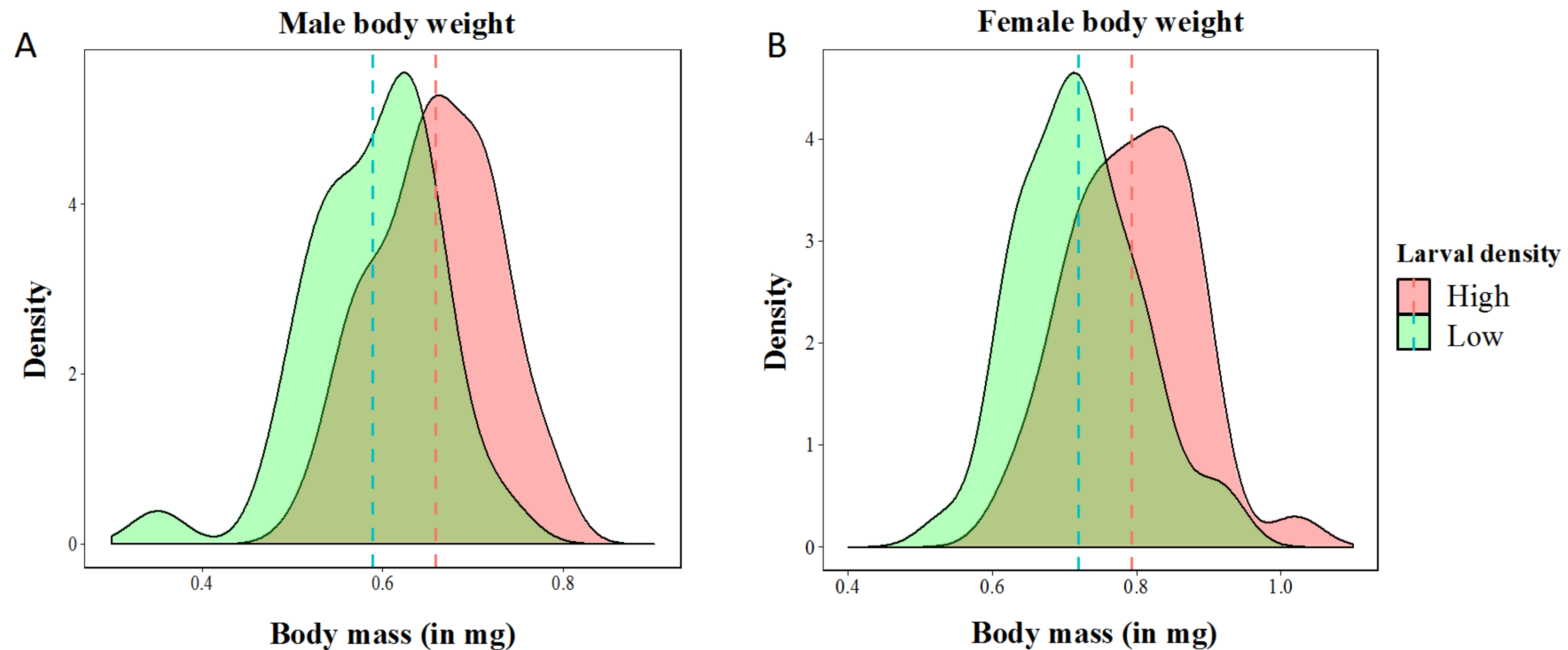

Figure S1 – Differences in body size caused by manipulations in the larval density (in mg). (**A**) Density plot of male weight and (**B**) Density plot of female weight. Green – High larval density; Red – Low larval density. Green dashed line – average body size of males and females raised at high larval density; Red dashed line - average body size of males and females raised at low larval density.

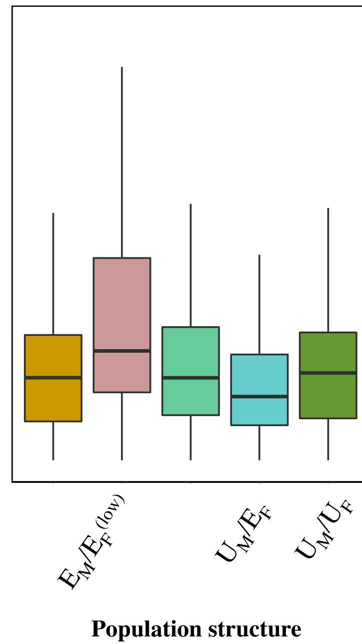

Figure S2 – Proportion of non-emergent pupae. $E_M/E_F^{(high)}$ – groups where all individuals experienced high larval density (gold); $E_M/E_F^{(low)}$– groups where all individuals experienced low larval density (red); E_M/U_F – groups where all males were phenotypically equal, from a low density larval density developmental environment and females had mixed phenotypes (light green); U_M/E_F – groups where all females were phenotypically equal, from a low density larval density developmental environment, and males had mixed phenotypes (blue); U_M/U_F – groups where both males and females had mixed phenotypes (dark green).

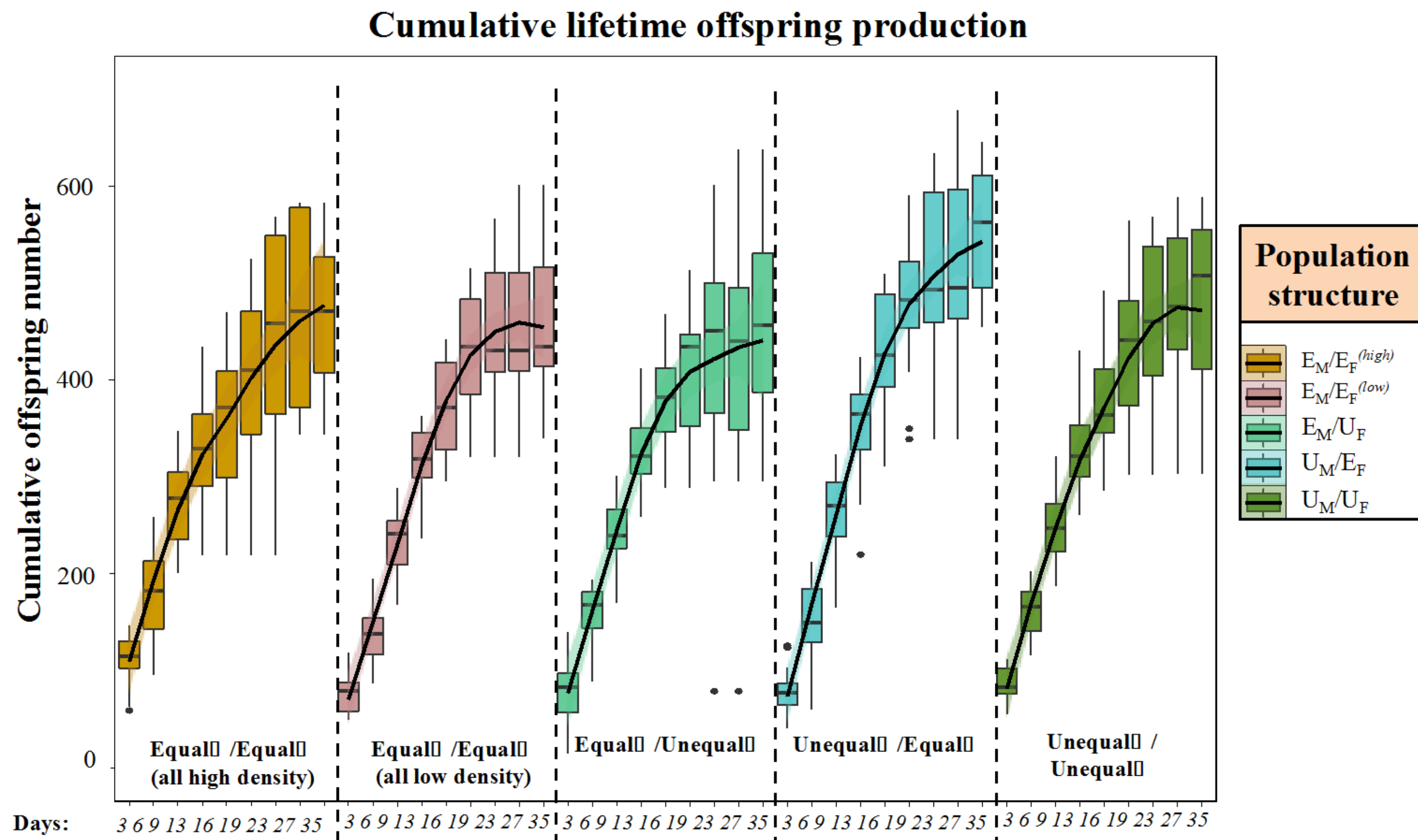

Figure S3 – Cumulative offspring production. $E_M/E_F^{(high)}$ – groups where all individuals experienced high larval density (gold); $E_M/E_F^{(low)}$– groups where all individuals experienced low larval density (red); E_M/U_F – groups where all males were phenotypically equal, from a low density larval density developmental environment and females had mixed phenotypes (light green); U_M/E_F – groups where all females were

phenotypically equal, from a low density larval density developmental environment, and males had mixed phenotypes (blue); U_M/U_F – groups where both males and females had mixed phenotypes (dark green).

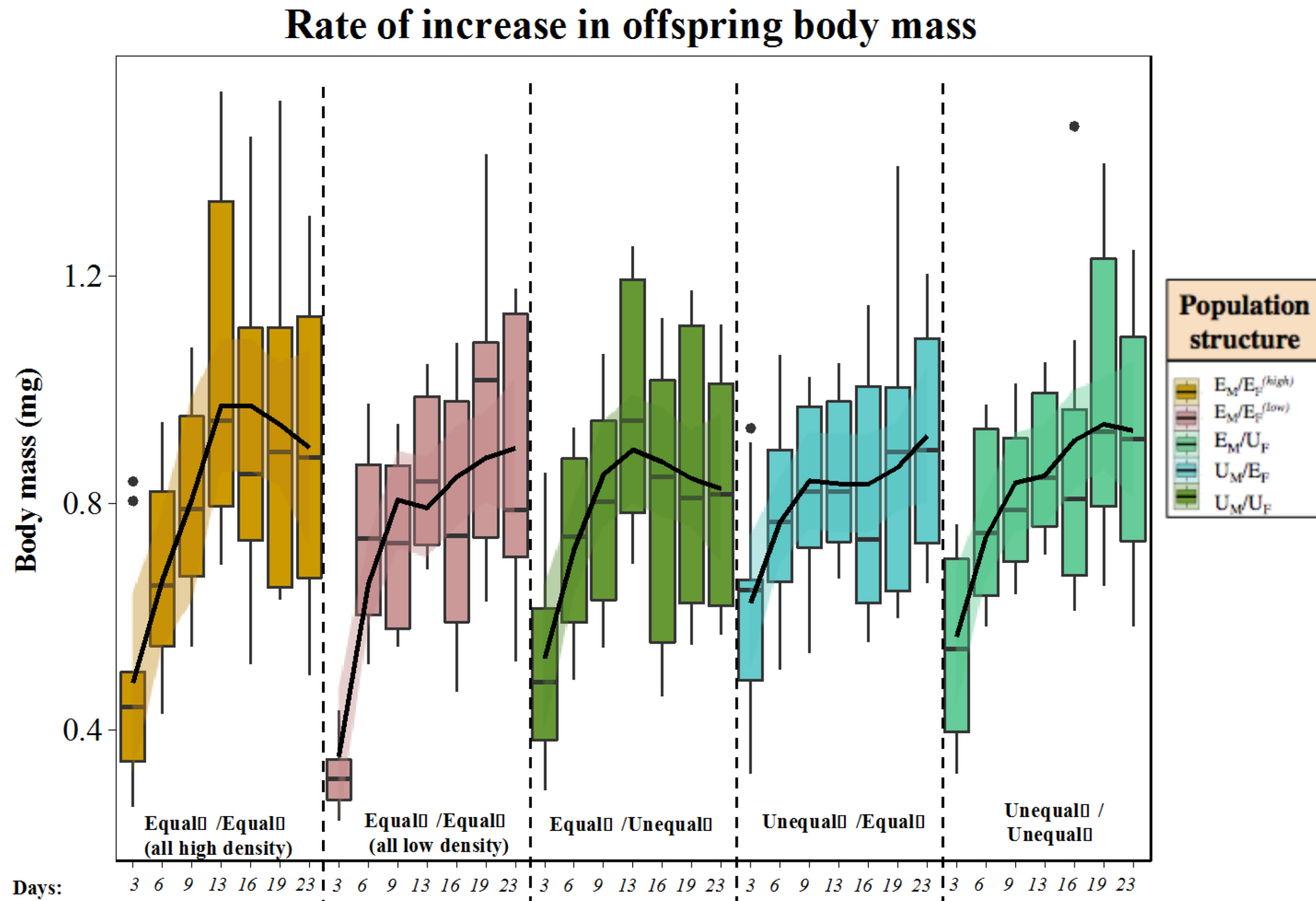

Figure S4 – Rate of increase in offspring body mass (in mg). $E_M/E_F^{(high)}$ – groups where all individuals experienced high larval density (gold); $E_M/E_F^{(low)}$– groups where all individuals experienced low larval density (red); E_M/U_F – groups where all males were phenotypically equal, from a low density larval density developmental environment and females had mixed phenotypes (light green); U_M/E_F – groups where all females

were phenotypically equal, from a low density larval density developmental environment, and males had mixed phenotypes (blue); U_M/U_F – groups where both males and females had mixed phenotypes (dark green).

Table S1 – Complete analysis of group courtship levels. **Bold:** $p < 0.05$.

Factors	**Courtship levels**	
	F-value	***p-value***
Treatment	4.949	**<0.001**
Time	1.465	0.226
Time^2	0.869	0.351
Number of Males	0.311	0.576
Number of females	2.838	0.092
Treatment*Time	1.006	0.403
Treatment*Time^2	2.043	0.087
Dispersion	*1.410*	

Table S2 – Complete analysis pupae eclosion (both absolute and proportion). **Bold:** $p < 0.05$.

Factor	Pupal eclosion (Absolute)		Pupal eclosion (Proportion)	
	F-value	***p-value***	***F-value***	***p-value***
Treatment	6.862	**<0.001**	2.745	**0.027**
Density of offspring in each vial	210.643	**<0.001**	-	-
Courtship levels	2.316	0.128	2.235	0.135

Table S3 – Complete analysis of total reproductive success and offspring body mass. **Bold:** $p < 0.05$.

Factor	**Total reproductive success**		**Offspring body mass**	
	F-value	***p-value***	***F-value***	***p-value***
Treatment	4.021	**0.005**	6.458	**<0.001**
Time	-	-	159.008	**<0.001**
Time^2	-	-	150.526	**<0.001**
Density of offspring in each vial	-	-	198.165	**<0.001**
Sex	-	-	735.117	**<0.001**
Courtship levels	-	-	1.303	0.254
Sex*Treatment	-	-	1.308	0.266

Table S4 – Complete analysis of the rate of offspring production. **Bold:** $p < 0.05$.

Factors	**Rate of offspring production**	
	F-value	***p-value***
Treatment	3.266	**0.011**
Time	767.555	**<0.001**
Time^2	72.207	**<0.001**
Number of males	22.889	**<0.001**
Number of females	14.808	**<0.001**
Treatment*Time	1.842	0.119
Treatment*Time^2	7.935	**<0.001**

Table S5– Complete analysis of the effects of time (both linear and quadratic) on offspring production rate within each group treatment, while controlling for the effects of the number of males and females contributing to the group at the time interval. Peak reproductive success was calculated as described in the main text. **Bold:** $p < 0.05$.

Group treatment	**Factor**								
	Time		**Time^2**		**Number of males**		**Number of females**		**Peak**
	F-value	***p-value***	***F-value***	***p-value***	***F-value***	***p-value***	***F-value***	***p-value***	
$E_M/E_F^{(high)}$	107.921	**<0.001**	1.997	0.161	5.092	**0.026**	10.089	**0.002**	0-3 days
$E_M/E_F^{(low)}$	155.410	**<0.001**	37.280	**<0.001**	0.733	0.393	0.953	0.330	6-9 days
E_M/U_F	143.590	**<0.001**	14.690	**<0.001**	4.642	0.331	2.686	0.103	6-9 days
U_M/E_F	155.550	**<0.001**	43.602	**<0.001**	2.841	0.094	2.526	0.114	9-13 days
U_M/U_F	245.417	**<0.001**	29,527	**<0.001**	17.013	**<0.001**	3.110	0.080	9-13 days

Table S6 – Complete analysis of the rate of increase in offspring mass. **Bold:** $p < 0.05$.

Factors	**Rate of increase in offspring body mass**	
	F-value	***p-value***
Density of offspring in each vial	210.443	**<0.001**
Courtship levels	0.468	0.494
Treatment	5.895	**<0.001**
Time	147.213	**<0.001**
Time^2	126.141	**<0.001**
Sex (offspring)	803.927	**<0.001**
Treatment*Time	5.438	**<0.001**
Treatment*Time^2	2.318	0.056

Ingredient	Quantity
Agar	
Water	
Maize Flour	3600 g
Yeast Powder	732 g
Soya	432 g
Molasses Mix	
Malt	3600 g
Molasses	1050 g
Water	2 L
Nipagin mix	
Methyl 4-Hydroxybenzoate	148 g
Ethanol	1300 mL
Water	200 mL
Acid Mix	
Propionic acid	1 L
Orthophosphoric acid	64 mL

Standard fly food recipe - Recipe for the standard fly food used in this study.

Chapter 5

Differential effects of male nutrient balance on pre- and post-copulatory traits, and consequences for female reproduction in *Drosophila melanogaster*

Abstract

Male fitness depends on the expression of costly traits involved in obtaining mates (pre-copulatory) and fertilization (post-copulatory). However, very little is known about the nutrient requirements for these traits and whether males compromise their diet to maximize one trait at the expense of another. Here we used Nutritional Geometry to investigate macronutrient requirements for pre- and post-copulatory traits in *Drosophila melanogaster,* when males were the first or second to mate with females. We found no significant effects of male diet on sperm competitiveness. However, although males self-regulate their macronutrient intake at a protein-to-carbohydrate ratio ("P:C ratio") of 1:1.5, this ratio does not coincide with their optima for several key reproductive traits: both the short-term (~24hr) rate of offspring production after a female's first mating, as well as the total offspring number sired when males were second to mate were maximized at a P:C ratio of 1:9, whereas male attractiveness (latency to mate), were maximised at a P:C ratio of 1:1. These results suggest a compromised optimum diet, and no single diet that simultaneously maximizes all male reproductive traits. The protein intake of first males also negatively affected female offspring production following remating, suggesting a long-term intersexual effect of male nutrition.

Introduction

In most species, females are polyandrous (females copulate with multiple males) and, as a result, males are likely to encounter both competition over access to mates (pre-copulation) and fertilization (post-copulation) (Pizzari and Wedell, 2013, Parker and Pizzari, 2015). These two competitive episodes strongly influence male fitness (Kvarnemo and Simmons, 2013, Simmons, 2001). However, the expression of male sexual traits involved in both pre- and post-copulatory competition is thought to be costly, meaning that male fitness is expected to depend on resource availability, including nutrients (Simpson and Raubenheimer, 2012, Rowe and Houle, 1996).

Macronutrients (i.e. protein, carbohydrates and fats) are vital in the molecular homeostasis of the organism and radically shape the physiology, behaviour and the expression of life-history traits (Powell et al., 2012, Chantranupong et al., 2015, Efeyan et al., 2015, Stearns, 1992). In insects, the intake of specific macronutrients can affect both lifespan and reproduction in a sex-specific fashion (e.g. (Lee et al., 2008, Maklakov et al., 2008, Fanson et al., 2009, Lee et al., 2013)). For instance, in both *Drosophila melanogaster* (Lee et al., 2008, Jensen et al., 2015) and *Teleogrylus commodus* (Maklakov et al., 2008) female lifespan increases under high-carbohydrate and low-protein diets whereas maximal female reproduction generally requires high-protein diets. On the other hand, male lifespan and reproduction can align: in addition to longer lifespan, high carbohydrate intake also increases male sexual performance in the cockroach *Nauphoeta cinerea* (Bunning et al., 2015, South et al., 2011), in the broad-horned beetle *Gnatocerus cornutus* (House et al., 2015) and in *D. melanogaster* (Jensen et al., 2015). However, male nutrition has to integrate the needs of both pre- and post-copulatory episodes, and imbalanced diets can impose constraints on male allocation to sexual traits (Mehlis et al., 2015). Although recent studies have attempted to investigate dietary effects on the interrelation between pre- and post-copulatory traits (e.g.

(Mehlis et al., 2015, Janicke et al., 2015, Devigili et al., 2015)), few have attempted to characterise the specific macronutrient requirements that maximize male expression of these traits (House et al., 2015, South et al., 2011, Jensen et al., 2015, Bunning et al., 2015, Maklakov et al., 2008, Reddiex et al., 2013, Rapkin et al., 2015). Most studies in this field have focused primarily on pre-copulatory traits (e.g. (Maklakov et al., 2008, Rapkin et al., 2015, South et al., 2011)), and only recently have researchers attempted to address the effects of macronutrient balance on male post-copulatory traits (Jensen et al., 2015, Bunning et al., 2015, Reddiex et al., 2013, Fricke et al., 2008). For instance, recent work indicates that a protein:carbohydrate ratio of 1:2 maximises sperm number and fertility in the cockroach *Nauphoeta cinerea* (Rapkin et al., 2015). Although sperm number can be important for male sperm competitiveness, it does not necessarily predict the outcome of post-copulatory competition (see (Snook, 2005)). Positive linear relationships between carbohydrate intake and fitness have been demonstrated in male fruitflies *D. melanogaster* (Reddiex et al., 2013, Jensen et al., 2015). Yet, it is not clear whether carbohydrate is beneficial to males in pre- or post-copulatory sexual selection, or both. This raises important questions: what are the specific nutritional requirements for competitive male pre- and post-copulatory traits? Can male diet maximize pre- and post-copulatory traits simultaneously or do different traits require different diets, meaning that males have to compromise in their dietary choices?

To address these questions, we used the geometric framework of nutrition (Nutritional Geometry (Simpson and Raubenheimer, 1993)) to experimentally investigate the nutritional requirements across male *Drosophila melanogaster* sexual selection episodes. Nutritional geometry is a state-space model to investigate the effects of multiple nutrients on fitness (reviewed in (Simpson and Raubenheimer, 2012)). We had two main motivations for this study: i) to investigate the specific nutritional requirement for male success in both pre- and post-copulatory sexual selection and ii) to determine the nutritional balance sought by males

when given a choice of diets. We first depleted male ejaculate reserves (e.g. accessory gland products) by mating males 3 times in succession (see (Sirot et al., 2009, Leiblich et al., 2012, Linklater et al., 2007, Hihara, 1981)) - which we expected to place males in a condition in which macronutrient acquisition is necessary to replenish their ejaculate reserves. We gave a subset of these males a choice between complementary diets with different concentrations of macronutrient and measured the protein-to-carbohydrate ratio (P:C ratio) males aimed to achieve by self-regulating their diet intake – the “target ratio”. The remaining males were allocated to one of 15 defined diets (“no choice”), which varied in both the P:C ratio and in the concentration of macronutrients (Simpson and Raubenheimer, 2012). Males in these no-choice diet treatments then assumed either one of two roles: 1) the first male to mate with a virgin female, in which the female was subsequently mated with a competitor male (P1 experiment) or 2) the second male to mate with a non-virgin female who had previously mated with a competitor male (P2 experiment). This design allowed us to scrutinise whether male dietary self-regulation matches the macronutrient requirements of both pre- and post-copulatory episodes of sexual selection.

Methods

(a) Fly stocks and culture

We used a wild-type *D. melanogaster* stock collected from Dahomey (now Benin) in 1970, and maintained in large outbred groups with overlapping generations (Wigby et al., 2009). We also used a recessive *sparkling*^{poliert} mutation (*spa*) strain, which produces a rough-looking eye phenotype when homozygous (Fu et al., 1998). The *spa* strain was back-crossed into Dahomey for >5 generations to ensure a standard genetic background (Fricke et al., 2010b). Using wild-type focal males, and homozygous *spa* females and competitor males,

allowed us to assign paternity through the progeny's eye phenotype (Fricke et al., 2010b). All fly stocks were maintained, and all experiments conducted, at 25°C on a 12:12 light:dark cycle in a non-humidified room (i.e. natural humidity). We used standard sugar-yeast-maize-molasses medium with *ad libitum* yeast to feed all *spa* females and *spa* male competitors (see Supplemental Information for standard fly food recipe). We controlled larval density to avoid effects on adult phenotypes (Clancy and Kennington, 2001): all experimental flies were raised at a density of ~200 eggs in 75ml bottles with ~45ml of standard maize-molasses fly food (see Supplementary Information). Virgin flies were collected on ice anaesthesia within 8 hours of eclosion and kept in single-sex vials of 15-20 individuals for 3 days with *ad libitum* yeast prior to experiments.

(b) Male ejaculate depletion

The first steps of all experiments were identical and therefore are explained together until the point in which the experimental procedures diverge (Figure 1). We allowed virgin wild-type males to mature in standard maize-molasses fly food with *ad libitum* yeast for 3d after emerging from pupae in same sex groups of 15 individuals. We then transferred focal males to fresh vials also with standard food and *ad libitum* yeast and conduced 3 matings to deplete male ejaculate reserves, as follows. We placed each male with one wild-type virgin female and allowed him to mate. After the first mating, we substituted the female for a fresh wild-type virgin female. This process was repeated until our focal males had mated exactly 3 times. Males were given up to 10h to complete the 3 matings. Three consecutive matings can dramatically reduce the amount of seminal fluid transferred to females due to ejaculate depletion (Sirot et al., 2009). Therefore our treatment was likely place males in a condition in which nutrient acquisition is necessary to replenish male's ejaculate reserves, and possibly also energy reserves for courtship. Males that successfully mated with 3 females (i.e. henceforth "focal males") at the end of 10h were randomly allocated to one of the three

experiments (see below). Males that failed to mate with 3 females were discarded. For the P1 and P2 experiments, focal males were randomly allocated to one of the 15 diet treatments described above and maintained on these diets for 4 days before the experimental matings.

(c) Diet preparation

Focal males were maintained in vials containing agar/water/nipagin medium sealed with Parafilm into which a 5µL capillary with liquid diet was placed (adapted from the CAFE assay (Lee et al., 2008, Ja et al., 2007). To prepare the diets, we used sucrose as source of carbohydrate (MP Biomedicals, cat. 194018) and hydrolysed yeast as source of protein (cat. 103304). We used sterilized distilled water, filtered in micro-filter of Merck Millipore with pores of 0.22 µm in a sterilized flow, and replaced from the capillaries every ~18h to minimize bacteria contamination. Diet intake was measured using a digital calliper.

(d) Experimental design

We performed 3 experiments: (1) a *dietary choice experiment* - where focal males, held singly, were given a choice between complementary diets, and dietary choice was assessed. This experiment allowed us to investigate the P:C ratio males choose when given the opportunity to balance their diet; (2) *P1 experiment* - where focal males were the first to mate with a virgin *spa* female, and females were subsequently given the opportunity to remate with a competitor *spa* male; (3) *P2 experiment* - where *spa* females were first mated to a *spa* male and subsequently given the opportunity to mate with a focal male (Figure 1) (Simpson and Raubenheimer, 2012). For all experiments, the evaporation rate was measured as the average loss of diet in three empty vials (i.e. no flies) *per* diet *per* concentration.

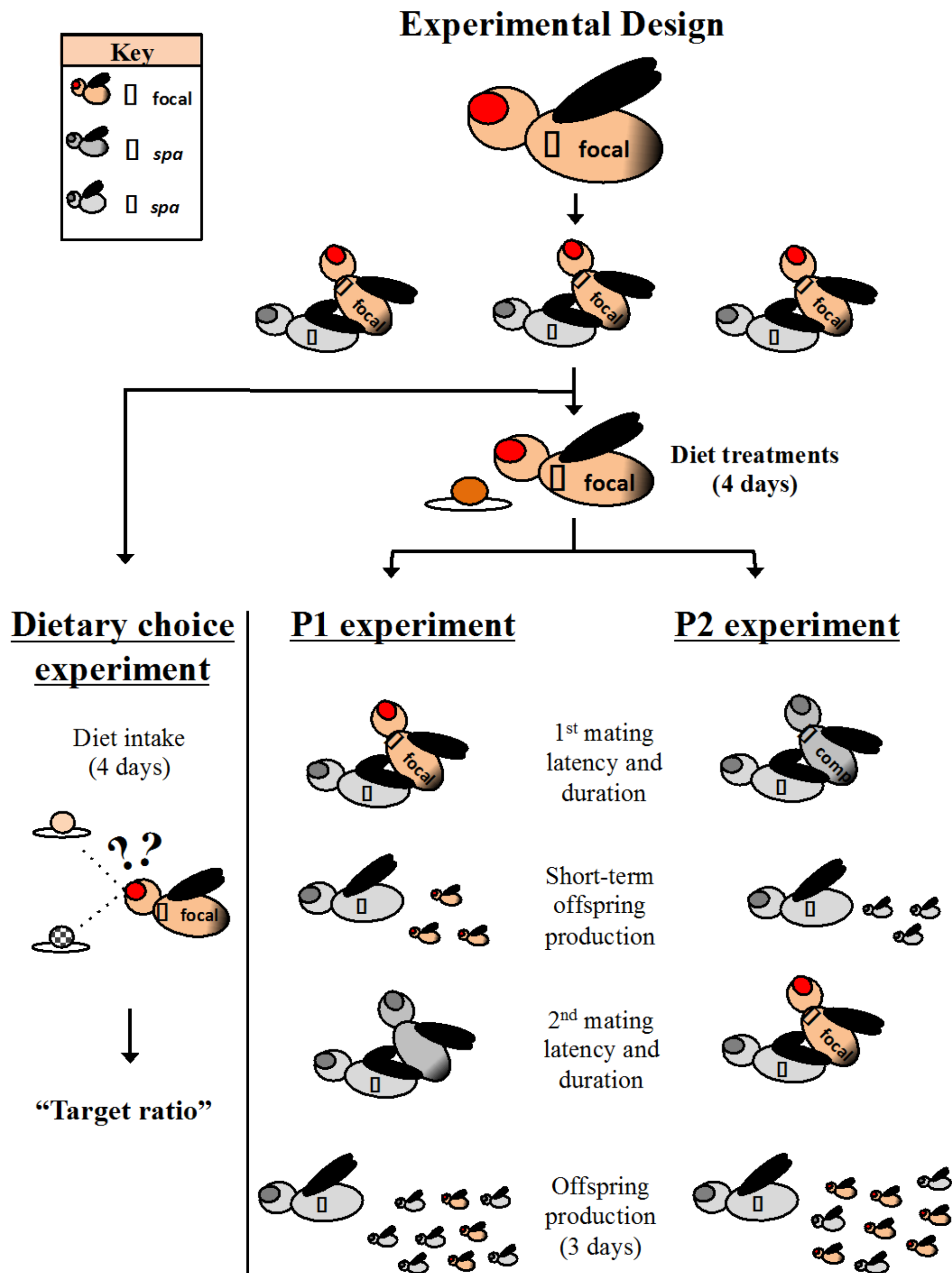

Figure 1 – Schematic overview of the experimental design. Virgin wild-type males were kept 3d in standard food with ad libitum yeast for maturation, and then mated with three virgin females consecutively to deplete their ejaculate reserves (i.e. "focal males"). Focal males were then assigned to either a Choice experiment or to 15 distinct defined diets for the P1 and P2 experiments (see Methods for details).

Dietary choice experiment - Focal males were given a choice between two capillaries with diets with P:C ratios of 1:16 and 3:1, both diets at one of the three concentrations (45g/L, 90g/L and 180g/L). The cumulative intake of both diets were measured every ~18h for 4d, after which focal males were discarded. The experiment was conducted in two replicates (total N = 68 males). The P:C ratio that males aimed to reach is referred to as 'the target ratio' (see (Simpson and Raubenheimer, 2012) for a review of the method).

P1 experiment - We used P:C ratios of: 1:16, 1:9, 1:3, 1:1 and 3:1, each with 3 different concentrations (45g/L, 90g/L and 180g/L) for a total of 15 diets prepared as described above (see "Dietary choice Experiment"). The average intake of protein and carbohydrate in all diets is given in Table S1. After 4 days on one of the 15 the diet treatments, focal males were transferred to fresh vials containing one virgin *spa* female on standard maize-molasses food with *ad libitum* yeast. Flies were allowed to interact until mating was observed. Immediately after a single mating, or if no mating occurred in 4 hours, focal males were discarded. The latency of females to mate with the focal male – a measure of male pre-copulatory attractiveness (Bretman et al., 2009) and copulation duration were recorded. Mated females were retained and allowed to lay eggs until the following day. 24hrs after their initial mating the *spa* females were moved to fresh vials with *ad libitum* yeast granules, and 1 *spa* male (competitor male) was added to each vial. The pair were allowed to interact for up to 8 hours or until a single copulation was observed. *spa* males, and females that failed to remate, were discarded. Females that mated with *spa* males (89 out of the initial 210) were immediately transferred into fresh vials with standard maize-molasses and yeast *ad libitum* for egg-laying every 24h for 3 days, after which females were then discarded. Females that did not remate to *spa* males were discarded. The offspring produced from eggs laid in vials in which females spent the period between matings were used as an estimate of short-

term offspring production (including offspring produced by females that did not remate), which is an important component of both male and female reproductive success, since both sexes potentially benefit from a high rate of offspring production soon after the first mating (Edward et al., 2011a). We also measured the total offspring produced by females that successfully mated with both the 1st and 2nd males, as well the total offspring sired by the focal male (i.e. the wild-type-eyed offspring) over the total of 4 days (i.e. the "short term" offspring, plus those offspring produced after the female remated). We calculated P1, the proportion of offspring sired by the focal males relative to all offspring produced by females after their second mating (see (Fricke et al., 2008)) as an measure of focal male success in sperm competition. All adult offspring were counted 15-17 days after oviposition to allow ample time for development and eclosion at 25°C.

P2 experiment – The design of the P2 experiment was conducted identically to the P1 experiment (see above), except that the *spa* competitor male was the 1st to mate with the female and the focal male was the 2nd to mate with the female. 105 out of 221 females mated with both the *spa* and the focal male. We counted the number of wild-type eyed offspring (sired by the focal male) and *spa* offspring (sired by the *spa* male) produced by females that copulated with focal males, and calculated P2 - the proportion of offspring sired by focal males as the second male to mate - as a measure of sperm competitiveness.

(e) Data analysis

We first standardised the intake measure of both macronutrients as follows:

$$St = \frac{I_x - \bar{I}_x}{sd(I_x)} \qquad \text{eq 1.}$$

where I_x is the individual intake of the macronutrient (protein or carbohydrate), $\bar{I}_x$ is the mean male intake of that macronutrient and $sd(I_x)$ is the standard deviation of the male intake of that macronutrient (Bunning et al., 2015). For the P1 experiment we tested latency

of females to mate with focal males, duration of mating with the focal males, the short-term offspring production (between the first and second matings), the proportion of females that remated, P1 paternity share, total offspring sired by the focal male, and total offspring production of the females. We analysed the same factors for the P2 experiment except without the short-term offspring production, and P2 paternity share instead of P1.

We used a general linear model for the analysis of duration of the focal male matings. We used a generalized model (GLM) with quasi Poisson errors for the analysis of count data such as the short-term offspring production, total offspring sired by the focal male, total offspring production of female (P1 experiment), and offspring production of females after the 2nd mating (P2 experiment). For proportion data (i.e. P1 and P2 paternity share, the proportion of females that remated), we used a GLM with quasibinomial errors. We fitted a GLM with a gamma error to evaluate the effects of latency of females to mate with focal males; latency was square-root transformed to improve model fit in the P2 experiment. For the P1 latency analysis we included all females (including those that subsequently failed to remate). We Box-Cox transformed the mating duration data for the focal male mating in the P1 experiment to best approximate normality of residuals. In addition to the main effects of P and C intake, we controlled for confounding variables by adding the following covariates in models where appropriate: intermating period (i.e. period between the 1st and 2nd mating), duration of 1st and 2nd mating, latency to 1st mating, short-term productivity, and total female productivity (see Supplemental Tables for details). We provide the output of the analyses in Tables S2-S7 together with the raw data of the reproductive traits measured in this study (Figure S1 and S2) in the Supplementary Information. To visualise the nutritional landscapes, we used the functions ‘Tps’ and ‘surface’ of the ‘fields’ package in R, which is a standard method for visualization of nutritional geometry data (e.g. (Jensen et al., 2015, Lee et al., 2008, Simpson and Raubenheimer, 2012)). All nutritional landscapes are of raw (non-transformed) data. For

the dietary choice experiment, we measured male total volume intake and total macronutrient intake using ANOVA with diet concentration (i.e. 45g/L, 90g/L and 180g/L) controlling for experimental replicate, followed by a post-hoc Student-Newman-Keuls test ($\alpha = 0.05$) using the 'agricolae' package in R. We confirmed that the ANOVA residuals approximated normality. We used paired *t-test* to compare the observed and the predicted intake of carbohydrates. The predicted carbohydrate intake was calculated as the total individual macronutrient intake – i.e. sum of the intakes of protein and carbohydrates - multiplied by the P:C ratio of interest (e.g. predicted carbohydrate intake in a P:C ratio of 1:1 = total male macronutrient intake * 0.5). All analyses were performed using R version 3.2.2 (R Development Core Team, 2015). No statistical significant outliers were detected in the variables measured in both the P1 and P2 experiments (see Figures S1 and S2).

Results

(a) Dietary choice experiment

We first investigated the P:C ratio regulated by males when given the opportunity to balance their macronutrient intake. Despite having a significantly higher volume intake in the 45g/L diets (*1:16 food source*: $ANOVA_{Concentration}$: $F_{2,64} = 11.459$, $p < 0.001$; *3:1 food source:* $ANOVA_{Concentration}$: $F_{2,64} = 9.240$, $p < 0.001$; *Total volume intake:* $ANOVA_{Concentration}$: $F_{2,64} = 12.267$, $p < 0.001$, Figure 2a), the total macronutrient intake was still lower when males fed on a 45g/L diet ($ANOVA_{Concentration}$: $F_{2, 64} = 10.17$, $p < 0.001$, Figure 2b). In diet concentrations of 90g/L and 180g/L, males reached similar macronutrient intake, but compensated the difference in diet concentration by eating a higher volume of food when fed a 90g/L diet (Figure 2a and 2b); this volume was similar to the volume eaten by males in 45g/L diets (Figure 2a). Together, these results suggest that males can compensate the low

food concentration by eating more, but there is a point beyond which the food is too diluted for compensation to be attained (i.e. males are not physically or physiologically able to eat more). Importantly, across all concentrations males consumed a P:C ratio ~ 1:1.5 (45g/L, P:C = 1:1.57; 90g/L, P:C = 1:1.3; 180g/L, P:C = 1:1.58; Mean P:C = ~1:1.5; Figure 2c) suggesting that males biased their macronutrient intake to high carbohydrates. In total, males ate an average of approximately 590μg of carbohydrates and 400μg of protein. Males had a higher carbohydrate intake than expected in a 1:1 ratio ($t_{67} = 6.544$, $p < 0.001$), and a lower carbohydrate intake than expected in a 1:9 ratio ($t_{67} = -12.784$, $p < 0.001$), confirming that male dietary choice tends to have a slightly higher carbohydrate intake than a balanced diet of P:C ratio 1:1. Knowing the P:C ratio males aimed to achieve, we hypothesized that this ratio maximized either pre- or post-copulatory traits, or both. We then fed males on 15 diets with fixed P:C ratios and performed P1 and P2 experiments (see Methods).

(b) P1 experiment – focal male 1st to mate

Dietary effects on pre-copulatory traits: There was a marginally non-significant quadratic effect of focal male protein intake on our measure of male attractiveness to virgin females (the latency of virgin females to mate with the focal male), in which the nutritional landscape peaked at an intermediate protein intake of ~500μg and a low-to-intermediate carbohydrate consumption (~150μg to ~1500μg; Dispersion: 0.218; Protein*Protein: $F_{1,206} = 3.454$, p-value = 0.064; Table S2) with P:C ratios lying between 3:1 and 1:3 (Figure 3a).

Dietary effects on mating duration and post-copulatory traits: We found that mating duration was negatively influenced by carbohydrate intake (Dispersion: 62.455; Carbohydrate: $F_{1,102} = 4.134$, p-value = 0.043; Table S3, Figure 3b). Maximum mating durations corresponded to the low carbohydrate and protein intakes and P:C ratios between

1:4 and 1:9. We found no significant effect of macronutrients on the latency of females to mate with the 2nd male or proportion of females that subsequently remated with the 2nd male (Tables S2 and S4). Increasing carbohydrate intake had a significant effect on short-term offspring production (offspring produced before remating; Dispersion: 6.38; Carbohydrate: $F_{1,86} = 4.293$, p-value = 0.041; Table S5), and the nutritional landscape peaked at a high carbohydrate intake (~3500μg) and a P:C ratio of ~ 1:9 (Figure 3c). We did not find a significant effect of macronutrient intake on the total offspring sired by males over the entire period of the experiment (Table S5). However, the total number of offspring produced by females (which includes both those sired by the focal and competitor male) was negatively influenced by focal (1st male) dietary protein intake (Dispersion: 14.24; Protein, $F_{1,87} = 6.830$, p-value = 0.010; Table S5), and there was a marginally non-significant quadratic trend for a carbohydrate effect (Carbohydrate* Carbohydrate: $F_{1,84} = 3.595$, p-value = 0.061; see Table S5); inspection of the nutritional landscape showed that the total number of offspring produced by females was the greatest at high male carbohydrate intake (~3250μg) and low male protein intake (~360μg) also in a P:C ratio of ~1:9 (Figure 2d). However, neither protein nor carbohydrate significantly influenced P1 (i.e. the proportion of offspring sired by focal males as the first male to mate; Table S6).

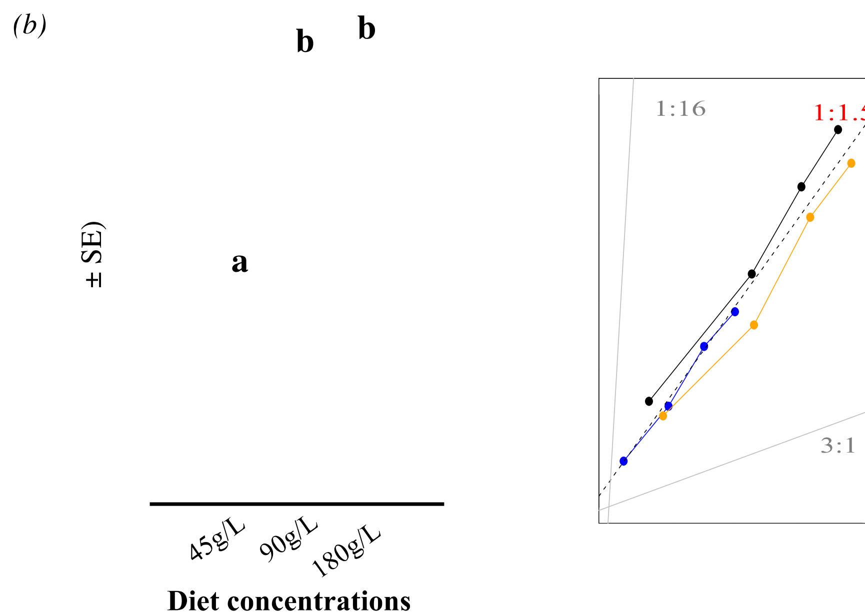

Figure 2 – Male dietary choice experiment. *(a) Solid bars:* Male volume intake (in µL) of the 1:16 and 3:1 food sources across the three experimental concentrations (i.e. 45g/L, 90g/L and 180g/L). *Striped bards:* The sum of the intake of both food sources ("total intake of both food sources"). Note that male volume intake is higher in 45g/L and 90g/L diets (post-hoc SNK-test (α = 0.05)). Blue bars – diet concentration of 45g/L; Orange bars – diet concentration of 90g/L; Dark grey bars – diet concentration of 180g/L. *(b)* Total male macronutrient intake (in µg) across the three different concentrations (i.e. 45g/L, 90g/L and

180g/L) in the dietary choice experiment. Post-hoc SNK-test (α = 0.05). Blue bar – diet concentration of 45g/L; Orange bar – diet concentration of 90g/L; Dark grey bar – diet concentration of 180g/L. *(c)* Cumulative intake of protein and carbohydrate (in μg) and the target ratio of 1:1.5 of male in our experiment. Dark-grey solid lines represent the two dietary choices available for males (i.e. 1:16 and 3:1 P:C ratios). Black dashed line – Target ratio of 1:1.5. Blue line – diet concentration of 45g/L, Orange line – diet concentration of 90g/L and Black line – diet concentration of 180g/L.

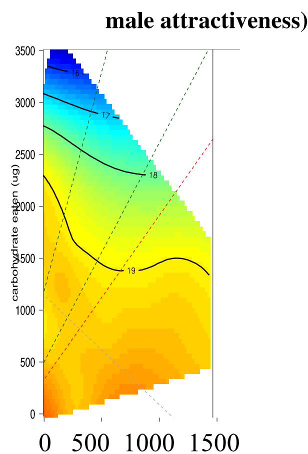

Figure 3 – Nutritional landscapes of the P1 experiment, in which male mated with virgin females. *(a)* Female latency to mate with the focal male (min) – i.e. focal male attractiveness.

Note that higher male attractiveness (low latency) is indicated by hotter colours. *(b)* Duration of the focal male mating. *(c)* Short-term (~24h) offspring production after mating with the focal male. *(d)* Total offspring production of females (including offspring of the 1st and 2nd males). For guidance, we included reference lines in the graph: Grey dashed line is the caloric isocline (i.e. line in which the calories are the same); Black dashed line represents the most carbohydrate-rich diet used in our experiment (i.e. P:C ratio of 1:16); Red dashed line stress the P:C ratio of 1:1.5 or the "target ratio" and green dashed lines show the P:C ratio of 1:4 and 1:2.

(c) P2 experiment – focal male 2nd to mate

Dietary effects on pre-copulatory traits – We found a non-significant trend for increased carbohydrate intake to reduce the latency of females to mate with the focal male (i.e. an increase in male attractiveness; Dispersion: 0.333; Carbohydrate: $F_{1,104} = 3.044$, p-value = 0.084; Table S2), which measures male attractiveness when focal males encounter an unreceptive females; there was also a borderline non-significant interaction protein*carbohydrate on the latency of females to mate with the focal male (Protein*Carbohydrate: $F_{1,99} = 3.799$, p-value = 0.054, Table S2). Together, these results showed that the nutritional landscape for male attractiveness as a 2nd male to mate with unreceptive females peaked at relatively high macronutrient intakes (~1100μg of each of the macronutrients) and a P:C ratio of ~1:1 (Figure 4a). We found no effect of focal male macronutrient intake on the proportion of females that remated with the focal male (Table S4).

Dietary effects on mating duration and post-copulatory traits: Focal male macronutrient intake had no significant effect on the duration of the focal male mating (Table S3). There was a significant interaction between protein and carbohydrate intake on the

number of offspring sired by the focal male (Dispersion: 25.03; Protein*Carbohydrate: $F_{1,97}$ = 4.598, p-value = 0.034, Table S7), in which the number of offspring sired by the focal male was the greatest at a low protein intake (~60µg) but at an intermediate carbohydrate intake (~550µg) in a P:C ratio of ~1:9 (Figure 4b). However, there was no significant effect of focal male macronutrient intake on the total number of offspring produced by females (including those sired by both the *spa* 1st male and the focal 2nd male; Table S7), or on the proportion of wild-type offspring sired (P2) by the focal males (Table S6).

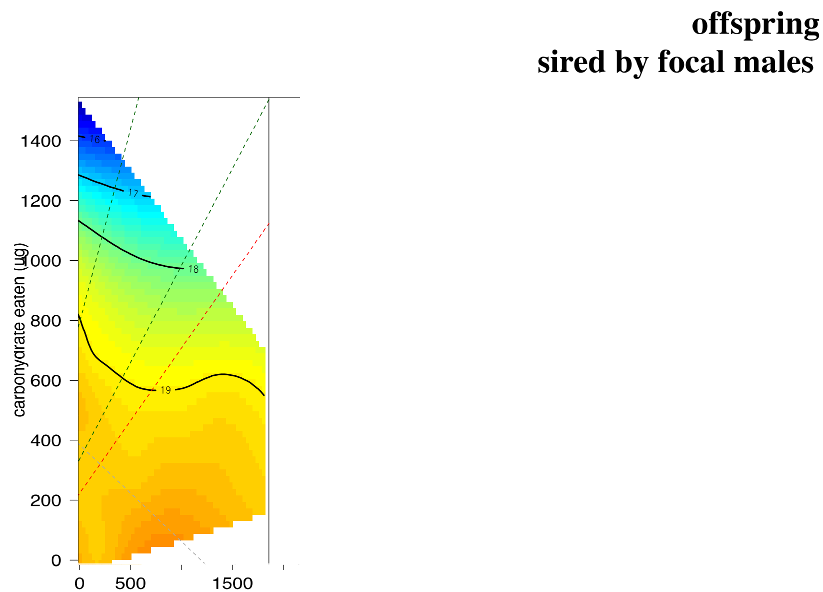

Figure 4 – Nutritional landscapes of the P2 experiment, in which males mated with non-virgin females. *(a)* Female latency to mate with the focal male (min) – i.e. focal male attractiveness. Note that higher male attractiveness (low latency) is indicated by hotter colours. *(b)* the number of offspring sired by the focal male. For guidance, we included reference lines in the graph: Grey dashed line is the caloric isocline (i.e. line in which the calories are the same); Black dashed line represents the most carbohydrate-rich diet used in

our experiment (i.e. P:C ratio of 1:16); Red dashed line stress the P:C ratio of 1:1.5 or the "target ratio" and green dashed lines show the P:C ratio of 1:4 and 1:2.

Discussion

Our results reveal that macronutrient intake, particularly of carbohydrates, can influence male pre and post-copulatory reproductive traits. The data suggest that there is a mismatch between male dietary choice and the dietary requirement for specific reproductive traits. When given the opportunity to self-regulate their diet, males seek a P:C ratio of ~1:1.5. However, this ratio is different from the P:C ratios that maximized offspring production (~1:9; Figure 3c) and male attractiveness (~1.1; Figure 3a). These results show that males may be compromising their dietary intake to maximize different pre- and post-copulatory traits since there is not a single diet that maximizes all male reproductive traits. We also found that increasing male protein intake significantly reduced the total offspring productivity of their mates, suggesting an intersexual effect of male diet on female offspring production. Below, we discuss the main findings of this study.

(a) Male dietary choice

The data show that males compensate the macronutrient dilution in the diet by eating more food, as found previously for female *D. melanogaster* (Lee et al., 2008). However, there is likely either a physical or physiological constraint (or both) that constrains male maximum food intake. If diets are too diluted, as in the case of our 45g/L concentration, males may not be able to consume sufficient liquid to reach their optimum macronutrient intake (Figure 2a and 2b). Despite this, males in our experiment still balanced their nutrient intake to a ~1:1.5 P:C ratio even when they failed to reach their optimum total macronutrient intake (Figure 2c). This carbohydrate-rich diet is likely to have fitness benefits but it does not coincide exactly

with the peaks of the nutritional landscapes of the reproductive traits measured in this study (see 'Results'). These results, together with the findings of (Jensen et al., 2015, Reddiex et al., 2013) in *D. melanogaster,* provide evidence that males may have to compromise their diet balance intake, because there is not a unique diet capable of maximizing all male traits.

There have been several explanations for why males do not balance their diet to maximize their fitness traits (e.g. (Maklakov et al., 2008) and (Jensen et al., 2015)). First, males might maximize a trait that has not been measured in the study (Jensen et al., 2015) or regulate nutrient intake to maximize multiple, competing traits (South et al., 2011, Bunning et al., 2015). However, males might also be constrained in their dietary balance for the expression of fitness traits because of their shared genome and dietary preferences with females ("sexual conflict over nutrition"), which might shift male choice to a diet balance that is not optimal for male's fitness (e.g. see discussion in (Maklakov et al., 2008)). Sexual conflict over nutrition was proposed as one of the explanation for male non-optimal dietary balance in *D. melanogaster* (Jensen et al., 2015) (but see (Lee et al., 2013)), *T. commodus* (Maklakov et al., 2008) and *Gryllodes sigillatus* (Rapkin et al., 2015).

(b) Dietary effects on the production of offspring

The data show that male carbohydrate intake in a P:C ratio of ~1:9 significantly maximizes both short-term offspring productivity after mating with virgin females, and the number wild-type offspring sired by males after mating with non-virgin females (Figure 3c and 4b). These results are consistent with the broader results of (Jensen et al., 2015), in which male *D. melanogaster* lifetime offspring siring increases with male carbohydrate intake. The mechanisms underlying the effects of macronutrient on offspring siring are not known, although several potential routes for this effect are possible. For instance, in adult male cockroaches *Nauphoeta cinerea,* carbohydrate intake in a P:C ratio of ~1:2 maximizes male sperm number (Bunning et al., 2015), showing that adult male carbohydrate can influence

ejaculate components. In *D. melanogaster*, sex-peptide (SP), a seminal fluid protein present in the male ejaculate and known for increasing egg laying and reducing female receptivity (Avila et al., 2010, Chapman et al., 2003b, Kubli, 2003, Liu and Kubli, 2003, Wigby and Chapman, 2005), binds to and is gradually released from sperm (Kubli and Bopp, 2012, Chapman et al., 2003b, Liu and Kubli, 2003, Peng et al., 2005a). Therefore, it is possible that the effects found in our study might be linked directly to SP or sperm, or to an interaction between sperm number and SP transference and release. Other seminal fluid proteins could also be affected by male macronutrient intake. For instance, *D. melanogaster* ovulin is a peptide present in the male ejaculate that controls female egg laying in the first day after mating (Herndon and Wolfner, 1995, Heifetz et al., 2000), and a suite of other seminal proteins are required for long-term female post-mating responses (Ram and Wolfner, 2007, Gligorov et al., 2013). If the expression of any of these proteins is under dietary regulation then diet will inevitably have consequences for male reproductive success.

We also found that high protein intake has a significantly strong negative effect on the total offspring productivity of their mates in the P1, but not P2, experiment. Although there are several mechanisms that can underlie intersexual effects of macronutrient intake, a change in ejaculate traits is again a likely candidate (Crean et al., 2014). For instance, if male macronutrient intake alters the production or transference of SP, ovulin, or other proteins required for full female post-mating responses, this could ultimately affect female productivity (see discussion above). In addition, a surplus of protein intake has been hypothesised to be toxic for both males and females, even though it maximises female egg production (Jensen et al., 2015, Lee et al., 2008, Fanson and Taylor, 2012, Fanson et al., 2009, Maklakov et al., 2008). Thus, high protein intake might impair male ejaculate production or reduce ejaculate quality, which in turn could reduce offspring production of females. These results also suggest an intersexual effect of male nutrition on the offspring

production of females. Future studies should address whether male macronutrient intake can also influence offspring traits as well as number, and the potential underlying ejaculate mechanisms.

(c) Dietary effects on sperm competitiveness

We found no evidence that the intake of macronutrient affected male sperm competitive ability, which was measured as the proportion of offspring sired by the focal males (i.e. P1 or P2) (see (Fricke et al., 2008) for similar approach). This is consistent with the previous findings of in adult male *D. melanogaster*, which showed that yeast concentration (i.e. varying from 20% up to 200% of dietary yeast) did not have any effect on either P1 or P2 (Fricke et al., 2008). Nonetheless, diet can affect the proportion of offspring sired by males if changes in the availability of macronutrients occur during development. For instance, showed that larvae *D. melanogaster* reared in low yeast (i.e. 10% of the normal yeast content) developed into smaller adult males that had significantly lower P1 (McGraw et al., 2007). This may suggest a critical window for the development or maturation of male traits involved in sperm competition, in which the lack of essential macronutrients at this critical point affects male adult success in sperm competition, whereas adult dietary changes have a lesser influence on sperm competitiveness.

It should also be noted that in our study both the sperm competition and offspring production measures following female remating were neccesarily taken from females that successfully remated under controlled conditions (i.e. on the day following the initial mating), in both the P1 and P2 experiments. Thus, these females may be particularly susceptible to fast remating. It will be important in future studies to investigate the influences on overall male reproductive success when all females (not just those that remate the following day) are given opportunities to remate multiply, in free-mating conditions and over

longer periods, to more closely reflect the conditions under which sexual selection acts in nature (as well as our lab-adapted groups).

(d) Dietary effects on male pre-copulatory success and mating duration

The results suggest that male dietary requirements to maximize pre-copulatory success are different from the requirements for offspring siring and short-term productivity. We found that male carbohydrate intake has a significant negative effect on the duration of the 1st mating (with the focal male) in the P1 experiment, and that mating duration was maximized at low macronutrient intake (Figure 3b). In addition, there was a marginal non-significant trend for the interaction of carbohydrate and protein to affect male pre-copulatory attractiveness to previously mated, unreceptive females, whereby males performed better under a high macronutrient with a P:C ratio of ~1:1 (Figure 4a). Encountering unreceptive females is likely common to males *D. melanogaster* as females in this species are polyandrous (Markow, 1996). Therefore, male attractiveness to unreceptive females is expected to play a significant role in determining male fitness. This is consistent with findings for the cockroach, *Nauphoeta cinerea*, for which a positive effect of carbohydrate intake on pheromone production and male attractiveness has been reported (South et al., 2011). In males *D. melanogaster,* sexual attractiveness and female recognition are influenced by volatile pheromones and cuticular hydrocarbons (Kurtovic et al., 2007, Datta et al., 2008, Ferveur, 2005), and future studies will reveal whether male carbohydrate intake increases male sexual attractiveness by modulating the production of these compounds. Carbohydrate intake might also contribute to male energetic supply during courtship. For instance, male carbohydrate intake is positively associated with male calling effort in *Teleogryllus commodus*, which is a trait used by males to attract females, suggesting that dietary carbohydrate may be used as a

direct source of energy for courtship (Maklakov et al., 2008). It is important to note, however, that we only detected a marginally non-significant trend in this study, that the macronutrient effects on mating latency in the P2 experiment are for females that were willing to remate in the experiment (as with the sperm competition measures – see discussion above), and that we detected no significant effects of macronutrients on the proportion of females that remated (Table S4). Thus, the overall importance to male reproductive success of macronutrient intake effects on mating latency with previously mated females requires further investigation. We also found a non-significant trend for intermediate intake of protein (~500μg) to increase male attractiveness to receptive (virgin) females in P:C ratios from 1:3 and 3:1. This result is consistent with the idea that male diet can influence pre-copulatory attractiveness to both virgin and previously mated females, and the data from both experiments are consistent with the idea that macronutrient intake has different effects on male pre- and post-copulatory traits.

It is important to note that the data in our experiment was taken from males under a specific set of conditions. Males were 8 days old at the time of measurements, and had been experimentally depleted of ejaculate prior to treatments. The effect of previous mating history on dietary effects and dietary choices remains to be tested, although there is currently no evidence that age alters the P:C ratio chosen by male *D. melanogaster* (see (Jensen et al., 2015)). More generally it will be interesting for future studies to test whether flies make dynamic choices throughout their lives based on social experience, and whether there are affects of different types of social interactions (e.g. mating, male-male aggression) Furthermore, it will be important to determine whether the ability to choose diets *per se* influences male performance, by comparing individuals given diet choice *versus* no-choice.

The specific mechanisms that link male diet to the reproductive traits measured in this study remain to be elucidated. Some effects may be part of a more general behavioural and physiological response to diet. For example, non-optimal diets in general should reduce the

pool of resources available to males to allocate to specific traits. This could potentially lead to an overall change in male locomotor activity which might explain the effects on copulation duration and the trends in latency to mating. Similarly, changes to post-copulatory traits might map onto a more general physiological response to diet. However, that fact that our results show macronutrient intake having effects on different traits (or no effects on some traits) suggests that the link between macronutrients and male reproductive traits are more complex than simply a non-specific loss of optimal condition under non-optimal diets. Our results support the idea that some traits are much more sensitive to diets than other traits, and that different diets maximise different traits.

Conclusions

Overall, our results show male dietary compromise, whereby male dietary choice does not coincide with specific male nutritional requirements for traits involved in pre- and post-copulatory sexual selection. Our study also supports previous findings on the strong effects of carbohydrate intake on male reproduction, which seems to be consistent across insect species (South et al., 2011, Bunning et al., 2015, Jensen et al., 2015, Rapkin et al., 2015, Maklakov et al., 2008). This taxonomic consistency suggests a tentative possibility of a widespread effect of carbohydrate on male reproduction in invertebrates. It will be important to know how male nutrition affects the abundance of seminal fluid proteins in the ejaculate and its implications to male fitness, which in turn will likely affect the intensity and operation of sexual selection and sexual conflict. Most species of vertebrates also undergo pre- and post-copulatory sexual selection (Birkhead and Pizzari, 2002, Birkhead, 2000, Andersson, 1994), and future investigations should seek to reveal whether macronutrients have similar effects in these taxa.

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Supplementary Information

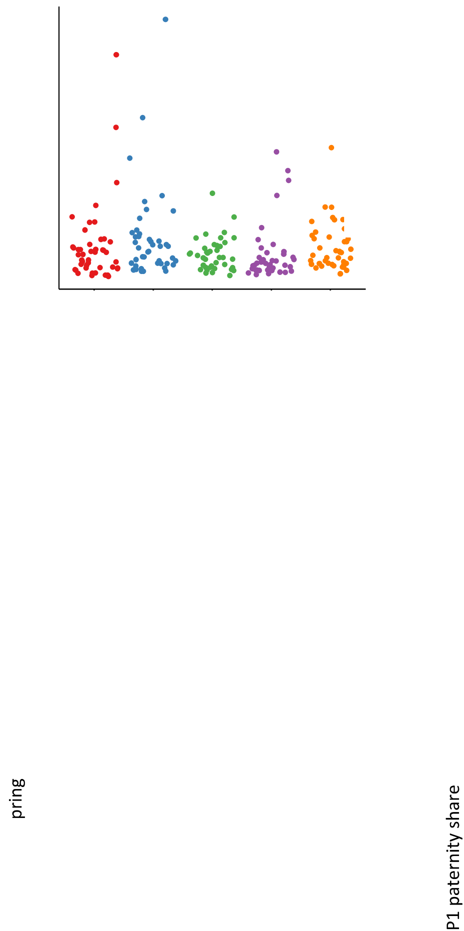

Figure S1 – Raw data of the reproductive traits measured in the P1 experiment. Individual data points are shown. *(a)* Latency of females to mate with the focal male. *(b)* The duration of the focal male mating. *(c)* Short-term offspring production. *(d)* The total offspring sired by males. *(e)* The total offspring produced by females that successfully remated. *(f)* The proportion of offspring sired by focal males with females remated with a competitor male (i.e. P1 experiment).

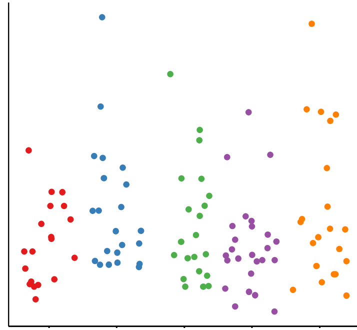

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Figure S2 –Raw data of the reproductive traits measured in the P2 experiment.

Individual data points are shown. *(a)* Latency of females to remate with the focal male. *(b)* The duration of the focal male mating. *(c)* The total offspring produced by females after remating with focal males. *(d)* The total offspring sired by males. *(e)* The proportion of offspring sired by focal males with unreceptive (previously mated) females (i.e. P2 experiment).

Table S1 – Mean intake of protein and carbohydrate in each nutritional rail of both P1 and P2 experiments.

		P1 experiment				P2 experiment			
P:C ratio	**Concentration**	**Carbohydrate**	**±SE**	**Protein**	**±SE**	**Carbohydrate**	**±SE**	**Protein**	**±SE**
1:16	**45g/L**	528.23	55.55	33.01	3.47	378.12	65.05	23.63	4.06
	90g/L	656.80	75.97	41.05	4.74	971.18	118.19	60.69	7.38
	180g/L	1251.52	157.10	78.22	9.81	1100.21	118.54	68.76	7.40
1:9	**45g/L**	333.99	36.04	37.11	4.00	378.51	41.95	42.05	4.66
	90g/L	1153.67	176.75	128.18	19.63	507.06	88.65	56.34	9.85
	180g/L	2400.57	172.40	266.73	19.15	524.44	139.18	58.27	15.46
1:3	**45g/L**	531.77	43.47	177.25	14.49	156.55	30.74	52.18	10.24
	90g/L	1533.84	92.46	511.28	30.82	315.64	55.20	105.21	18.40
	180g/L	912.42	124.83	304.14	41.61	243.41	83.00	81.13	27.66
1:1	**45g/L**	130.19	25.33	130.19	25.33	210.76	28.72	210.76	28.72
	90g/L	340.35	42.32	340.35	42.32	425.31	56.55	425.31	56.55
	180g/L	899.35	108.84	899.35	108.84	388.51	60.73	388.51	60.73
3:1	**45g/L**	167.75	16.12	503.25	48.37	222.79	23.76	668.39	71.30
	90g/L	244.73	42.65	734.20	127.97	272.24	22.69	816.72	68.09
	180g/L	283.70	33.97	851.12	101.92	530.47	32.60	1591.42	97.81

Table S2 - Complete table of the analyses of the female latency to mate with the focal male (i.e. P1 and P2 experiments) and female latency to mate with *a* spa competitor male (P1 experiment). $- Analysis performed with females that successfully mated with the 2nd male, £- Statistical analysis performed with transformed response variable (response^0.5). Bold – p <0.1. * – p < 0.05. ** – p < 0.01

Covariate	Latency analysis											
	Female latency to mate with the focal male (P1 experiment)£				Female latency to remate (with a *spa* competitor male) (P1 experiment)				Female latency to mate with the focal male (P2 experiment)$			
	Estimate	*SE*	*F-value*	*p-value*	*Estimate*	*SE*	*F-value*	*p-value*	*Estimate*	*SE*	*F-value*	*p-value*
(Intercept)	3.6220	0.015	-	-	-0.0050	0.0019	-	-	0.0097	0.0016	-	-
P	0.0220	0.016	0.4257	0.514	-0.0000	0.0008	0.052	0.820	0.0012	0.0011	0.105	0.746
C	-0.0070	0.016	0.617	0.433	-0.0006	0.0007	0.129	0.720	0.0019	0.0009	**3.044**	**0.084**
P*P	-0.0150	0.006	3.454	**0.064**	0.0002	0.0004	0.383	0.537	-0.0007	0.0006	1.742	0.189
C*C	-0.0020	0.007	0.092	0.761	0.0003	0.0003	0.899	0.345	-0.0000	0.0004	0.226	0.635
Duration of the 1st mating	-	-	-	-	0.0000	0.0000	0.249	0.619	-0.0000	0.0000	2.100	0.150
Latency to the 1st mating	-	-	-	-	0.0000	0.0000	0.793	0.375	-0.0000	0.0000	**7.983**	**0.005****
P*C	0.0130	0.014	0.979	0.323	0.0007	0.0010	0.497	0.482	0.0030	0.0017	**3.799**	**0.054**
Dispersion:	*0.218*				*0.503*				*0.333*			

Table S3 - Complete table of the analyses of the duration of the mating of the focal male in both P1 and P2 experiments. Bold – p <0.1. * – p < 0.05. @- Statistical analysis performed with transformed response variable (response^0.15).

	Duration of the mating of the focal male							
Covariate	**P1 experiment@**				**P2 experiment**			
	Estimate	*SE*	*F-value*	*p-value*	*Estimate*	*SE*	*F-value*	*p-value*
(Intercept)	0.1567	0.0070	-	-	103.922	103.937	-	-
P	-0.008	0.0070	0.432	0.511	1.7258	1.4052	0.649	0.423
C	-0,0002	0.0060	**4.134**	**0.043***	-0.8406	0.9255	0.135	0.713
PxP	0.0030	0.0030	1.066	0.302	-0.2557	0.5440	1.208	0.274
CxC	-0.005	0.0030	2.356	0.126	-0.4226	0.4781	0.439	0.508
Duration of the 1st mating	-	-	-	-	-0.0792	0.0768	0.776	0.380
Intermating period	-	-	-	-	-0.0583	0.0712	0.989	0.322
Latency to the 1st mate	-0.0008	0.0003	**6.070**	**0.014***	-	-	-	-
PxC	0.0006	0.0060	0.008	0.926	-1.1078	1.3724	0.651	0.421
Dispersion:	*62.455*				*20.021*			

Table S4 - Proportion of females that remated with the *spa* male (P1 experiment) or the focal male (P2 experiment). The analyses were performed with a GLM model with a quasibinomial error. * – p < 0.05.

	Proportion of females that remated							
Covariate	**P1 experiment (remating with *spa* male)**				**P2 experiment (rematings with focal male)**			
	Estimate	*SE*	*F-value*	*p-value*	*Estimate*	*SE*	*F-value*	*p-value*
(Intercept)	0.624	0.529	-	-	0.8900	0.5530	-	-
P	-0.173	0.241	0.008	0.927	-0.0977	0.3235	0.377	0.539
C	0.033	0.226	0.996	0.319	0.3371	0.2829	0.919	0.338
PxP	0.068	0.115	0.049	0.823	-0.0060	0.1662	1.363	0.244
CxC	0.086	0.113	0.254	0.614	-0.1715	0.1284	1.633	0.202
Latency to the 1st mating	-0.008	0011	0.137	0.711	-0.0011	0.0038	0.024	0.875
Short-term offspring production	-0.013	0.006	**3.983**	**0.047***	-0.0141	0.0076	4.046	**0.045***
PxC	-0.316	0.227	2.017	0.157	0.4868	0.4560	1.161	0.282
Dispersion:	*1.038*				*1.030*			

Table S5 – Complete table of the analysis of short-term offspring production, total offspring sired by the focal male and total offspring produced by females in the P1 experiment. The analyses were performed with a GLM model with a quasi Poisson error. Bold – p <0.1. * p <0.05. ** – p < 0.01.

Covariate	P1 experiment reproductive analyses											
	Short-term offspring production				Total offspring sired by the focal male				Total offspring production of females			
	Estimate	*SE*	*F-value*	*p-value*	*Estimate*	*SE*	*F-value*	*p-value*	*Estimate*	*SE*	*F-value*	*p-value*
(Intercept)	-0.162	5.498	-	-	-30.383	21.172	-	-	-1.770	4.643	-	-
P	-0.001	0.071	0.035	0.850	-0.210	0.253	0.0001	0.993	-0.110	0.060	**6.830**	**0.010****
C	0.020	0.052	**4.293**	**0.041***	0.153	0.185	0.007	0.930	-0.022	0.044	1.360	0.246
P*P	-0.015	0.028	0.835	0.363	0.111	0.093	0.012	0.910	-0.005	0.025	1.868	0.175
C*C	0.019	0.205	0.811	0.370	-0.131	0.086	0.326	0.569	0.021	0.017	**3.595**	**0.061**
Duration of the 1st mating	0.019	0.004	0.710	0.401	-0.009	0.019	0.888	0.348	-0.005	0.003	2.330	0.130
Intermating period	0.002	0.003	0.604	0.439	0.022	0.014	**5.446**	**0.022***	0.005	0.003	2.210	0.140
Duration of the 2nd mating	0.001	0.004	0.190	0.663	-0.042	0.024	**5.005**	**0.028***	-0.003	0.003	0.971	0.327
Total offspring production of females	-	-	**-**	**-**	0.006	0.002	**9.090**	**0.003****	-	-	-	-
P*C	-0.035	0.067	0.269	0.604	0.053	0.243	0.047	0.827	-0.019	0.058	0.105	0.745
Dispersion:	*6.38*				*31.31*				*14.24*			

Table S6 - Complete table of the analysis of the proportion of offspring sired by the focal male in both P1 and P2 experiments.

The analyses were performed with a GLM model with a quasibinomial error. Bold – p <0.1. * p <0.05. [&]- Analysis performed with all females that successfully mated.

	Proportion of offspring sired by the focal male							
Covariate	**P1 experiment**				**P2 experiment**			
	Estimate	*SE*	*F-value*	*p-value*	*Estimate*	*SE*	*F-value*	*p-value*
(Intercept)	-0.470	0.270	-	-	0.914	30.77	-	-
P	-0.292	0.328	0.953	0.331	-0.076	0.4257	2.306	0.132
C	0.264	0.239	0.052	0.819	0.3107	0.2784	0.469	0.494
PxP	0.168	0.132	0.146	0.703	-0.119	0.1723	0.018	0.890
CxC	-0.159	0.105	1.081	0.301	-0.073	0.1522	0.081	0.776
Duration of the 1st mating	-0.070	0.024	0.135	0713	0.0185	0.0242	0.169	0.681
Intermating period	0.030	0.0018	**3.958**	**0.050**	-0.000	0.0210	0.045	0.831
Duration of the 2nd mating	-0.052	0.0352	**3.609**	**0.061**	0.0396	0.0289	1.530	0.219
Total offspring production of females	0.0006	0.003	0.041	0.838	0.0004	0.0028	0.0101	0.920
Short-term offspring production	-	-	-	-	-0.0052	0.0092	0.592	0.443
PxC	0.063	0.311	0.040	0.840	0.410	0.454	0.984	0.323
Dispersion:	*39.950*				*37.99*			

Table S7 - Complete table of the analysis of offspring sired by the focal male and offspring production of females after mating with the focal male in the P2 experiment. The analyses were performed with a GLM model with a quasi Poisson error. Bold – p <0.1. * p <0.05. ** – p < 0.01. *** p <0.001. $- Analysis performed with females that successfully mated with our focal males.

	P2 experiment$							
Covariate	**Offspring sired by the focal male**				**Offspring production of females after mating with the focal male**			
	Estimate	*SE*	*F-value*	*p-value*	*Estimate*	*SE*	*F-value*	*p-value*
(Intercept)	3.445	9.231	-	-	6.4600	8.887	-	-
P	0.0045	0.123	0.623	0.431	0.0034	0.117	0.001	0.973
C	0.0885	0.081	0.0005	0.982	0.0598	0.077	0.078	0.779
P*P	-0.0406	0.045	0.044	0.833	-0.0186	0.043	0.177	0.674
C*C	-0.0193	0.044	0.926	0.338	-0.0312	0.041	0.740	0.391
Duration of the 1st mating	0.0178	0.006	**6.649**	**0.011***	-	-	**-**	**-**
Intermating period	0.0004	0.006	0.019	0.888	-0.0008	0.006	0.0001	0.992
Duration of the 2nd mating (i.e. remating)	0.0089	0.009	1.446	0.231	0.0042	0.008	0.197	0.657
Short-term offspring production of the competitor male	0.0079	0.002	**14.966**	**<0.001*****	-	-	-	-
P*C	0.2521	0.114	**4.598**	**0.034***	0.114	0.109	1.006	0.304
Dispersion:	*25.03*				*25.94*			

Standard fly food recipe - Recipe for the standard fly food used in this study.

Ingredient	Quantity
Agar	
Water	
Maize Flour	3600 g
Yeast Powder	732 g
Soya	432 g
Molasses Mix	
Malt	3600 g
Molasses	1050 g
Water	2 L
Nipagin mix	
Methyl 4-Hydroxybenzoate	148 g
Ethanol	1300 mL
Water	200 mL
Acid Mix	
Propionic acid	1 L
Orthophosphoric acid	64 mL

Chapter 6

General Discussion

This thesis has contributed to our understanding of the effects of nutrition, both at larval and adult stages, on group fate and the operation of sexual selection. Below, I discuss the main findings of my work and propose the future directions that should follow from my results.

Increasing female promiscuity reduces sexual selection

In the first section of my thesis, I tested how sexual selection responded to increasing polyandry, which is a ubiquitous behaviour in nature (Taylor et al., 2014). The implications of polyandry for sexual selection have been the focus of intense debate, with previous theoretical and correlational studies arguing that increasing polyandry could increase, decrease, or have no effect on sexual selection (Birkhead and Møller, 1998, Albrecht et al., 2009, Whittingham and Dunn, 2005, Webster et al., 2007, Pischedda and Rice, 2012) (Shuster and Wade, 2003, Collet et al., 2012, Collet et al., 2014, Parker and Birkhead, 2013, Kvarnemo and Simmons, 2013). To experimentally test the relationship between polyandry and sexual selection, I genetically manipulated female receptivity through the knockout of the Sex-Peptide Receptor (SPR; (Yapici et al., 2008)) and allowed individuals to freely interact (see Chapter 2). The novelty of this study was two-fold. First, I used a targeted manipulation of polyandry, which allowed me to assess causality and mechanisms by which polyandry affect sexual selection. Second, although the groups were small, I measured sexual selection in freely mating groups, which a social environment that more closely resembles natural population. Together, these novel experimental manipulations allowed me to measure a range of sexual selection indexes and showed clear evidence of the causal link between increasing polyandry and a reduction in the operation of sexual selection in males. The results of Chapter 2 provide important experimental evidence for the long-standing debate of the effects of polyandry on the operation of sexual selection.

Developmental nutrition and adult social environment: key modulators of sexual selection, offspring quality and group fate

Nutrition is vital for the molecular homeostasis of organisms and shapes the physiology, behaviour and expression of life-history traits (Powell et al., 2012, Chantranupong et al., 2015, Efeyan et al., 2015, Stearns, 1992). In the second section of my thesis, I focused on the effects of nutrition and social interactions in altering individual's sexual and reproductive plastic responses as well as group dynamics. In Chapter 3, I showed how nutritional stress early in life and social interactions in adulthood alter individual's plastic responses and the operation sexual selection. For instance, when individuals experienced a stressful nutritional environment during the larval stage, both males and females developed smaller bodies (Amitin and Pitnick, 2007, Pitnick and Garcia-Gonzalez, 2002, Lyimo et al., 1992, Credland et al., 1986), which in turn reduced individual's mating and reproductive success. In addition, small males had reduced post-copulatory competitiveness, agreeing with results of previous studies (Bonduriansky, 2001, Honek, 1993, Roff, 2002, Stearns, 1992, Clutton-Brock, 2009) (see Chapter 3). However, I showed that when both males and females vary in body size in a group, the disadvantages caused by the nutritional stress in the developmental environment could be mitigated (see Chapter 3). The extent to which the results of Chapter 3 represent a general effect remains subject of scrutiny. However, nutritional stresses in early life have similar negative implications for health in mammals (Fernandez‐Twinn and Ozanne, 2010, Vickers, 2014, Gluckman et al., 2008, Kuzawa, 2007). Poor early nutrition has been linked to lower number of stem cells in muscles, increased risk of obesity, insulin resistance, affection disorders and other health conditions in rodents and humans (Fernandez‐Twinn and Ozanne, 2010, Vickers, 2014, Yajnik, 2004, Woo et al., 2011), which can severely impact individual's life quality. Given

the results of Chapters 3, it may be possible to mitigate the negative effects of poor nutrition in early life through manipulations of the social environment. An enriched social environment – i.e. presence of other individuals, physical challenges - can reduce anxiety and depression in rats, likely due to plastic remodelling and rewiring of brain connections in response to social interactions (Fowler et al., 2002, Sáenz et al., 2006, Lieberwirth and Wang, 2012). Therefore, social environment interactions in mammals can induce potent anatomical, physiological and behavioural plastic responses that may be sufficient to overcome, at least partly, the disabilities caused by poor nutrition during early development.

Plastic responses to stresses and social interactions are essential for the survival and reproduction of individuals in nature (Price, 2006). As a result, the plasticity at the individual level may allow groups to overcome environmental challenges, which in turn can affect the maintenance of groups (Robinson and Dukas, 1999, West-Eberhard, 2003). In Chapter 4, I showed how plastic responses to the developmental and social environments determined group fate. I showed that groups where males and females experienced nutritionally favourable (low larval density) developmental environments displayed higher courtship activity and lower fitness, as well as reduced offspring eclosion success and body mass; these results suggest novel trans-generational effects of the developmental and social environments that remain to be explored.

Together, Chapters 3 and 4 provided important insights into the ecology of sexual selection, and showed how multi-level interactions between the developmental and social environments determine sexual selection and the evolutionary fate of groups.

Male dietary compromise: “You can’t have it both ways!”

In polyandrous species, where a male’s reproductive success depends on his ability to mate (pre-copulatory) and fertilize (post-copulatory) his mate’s eggs, male immediate plastic

responses to fluctuations in nutrient availability can profoundly modulate the outcome of reproduction. Since pre- and post-copulatory sexual selection are distinct selective forces that may act independently (Mehlis et al., 2015), it was reasonable to expect that the nutritional requirements for traits under pre- or post-copulatory sexual selection differed. Although several studies had shown the differences in macronutrient requirement for male sexually selected traits (Mehlis et al., 2015, Janicke et al., 2015, Devigili et al., 2015, House et al., 2015, South et al., 2011, Jensen et al., 2015, Bunning et al., 2015, Maklakov et al., 2008, Reddiex et al., 2013, Rapkin et al., 2015), no studies had yet investigated whether male nutrient balance could maximise all male reproductive traits simultaneously. In Chapter 5, I explored this omission by investigating the nutritional requirements for male reproductive success in both pre- and post-copulatory episodes, and how males balance these requirements when given the opportunity to choose between complementary diets. I showed that the nutritional requirements for all male's reproductive traits may vary, and that a balance that satisfies all traits cannot be attained simultaneously. As a result, males compromise their intake of macronutrients (Chapter 5). The novelty of my approach revealed the necessity of a nutritional compromise, whereby the nutritional requirements of male's reproductive traits cannot be reached in a single diet. These results reveal the nutritional complexity created by pre- and post-copulatory sexual selection on the architecture of male reproduction.

The link between nutrition and male reproductive performance extends beyond invertebrate species (Mosley, 2012, Tremellen and Pearce, 2015, Robinson et al., 2006, Simpson and Raubenheimer, 2012). For instance, a wide range of dietary interventions have been used as means to treat infertility in humans and other mammals (Robinson et al., 2006, Zhou et al., 2007, Agarwal et al., 2004, Agarwal and Sekhon, 2010). Moreover, studies on metabolic diseases related to nutrition, such as obesity and diabetes, have also revealed a

negative effect of these disorders on male fertility (Cabler et al., 2012, Pasquali and Gambineri, 2006, Sallmén et al., 2006), suggesting a role of nutritional disorders on fertility.

Unfortunately, previous studies in humans and mammals have focused primarily on the beneficial role of antioxidants on improving male fertility (Agarwal et al., 2004, Sheweita et al., 2005, Gharagozloo et al., 2016) and only recently have studies focus on other nutrients such as protein and carbohydrate (Simpson and Raubenheimer, 2012, Gosby et al., 2014). Yet, my results in Chapter 5 showed that protein and carbohydrate intakes modulate male and female reproductive performance, and revealed a more general effect of common nutrients on reproduction. Thus, future studies in mammals should focus on protein and carbohydrate intakes in order to establish the link between the intake of these nutrients and male reproductive performance.

Future directions

The role of population size and proportion of promiscuous individuals in creating assortative mating patterns

Recent studies have highlighted the importance of social interactions in determining the operation of sexual selection. A positive assortative mating between highly promiscuous individuals can reduce variance in male reproductive success (Sih et al., 2009) – i.e. males with high mating success should lose reproductive success due to high sperm competition – and thus reduce the scope of selection. My results in Chapter 2 suggest that increasing polyandry saturates the mating matrix (i.e. matrix of mating interactions between individuals in the population), although it does not create assortative mating patterns based on individual promiscuity. In the wild, however, Fisher et al. (2016) showed positive assortative mating between highly promiscuous field crickets *Gryllus campestris.* It is possible that the

distribution of promiscuous individuals in the population and the population size can influence patterns of assortative mating if, for example, more (or less) promiscuous individuals become concentrated in a defined area within the population distribution range. This should constrain the available mating options for individuals of the opposite sex, resulting in a seemingly positive assortative mating pattern based on promiscuity. My populations in Chapter 2 were small, and variation in female polyandry was reduced due to our genetic manipulation. Therefore, it remains unknown whether variation in polyandry within the population and the population size can generate patterns of assortative mating based on individual promiscuity. Thus, it will be interesting for future studies to explore the powerful *D. melanogaster* genetic tool to increase female polyandry (*SPR knockout*; see Chapter 2; (Yapici et al., 2008)) while varying population size and the proportion of promiscuous individuals in the population (i.e. variation in promiscuity) (Figure 1). I predict that, for a given proportion of promiscuous individuals, population size should be positively associated with positive assortative mating based on individual's promiscuity. This is because individuals in large populations have a limited capacity to sample potential mates, and thus more promiscuous individuals mate with only a subset of the available mates in the population. Conversely, for a fixed population size, the proportion of promiscuous individuals composing the population should be negatively correlated with assortative mating patterns because high promiscuity should saturate the mating matrix but not create mating assortativity (see Chapter 2). The results of this study will shed light on the mechanisms by which population size and individual variation in polyandry influences assortative mating patterns based on promiscuity, and will allow future studies to predict more accurately potential patterns of assortative mating in wild populations. These assortative mating patterns emerging from individual's sexual behaviour, whereby more (or less) promiscuous individuals preferentially mate, can reduce the strength of selection and hence have important

implications for evolutionary processes (Sih et al., 2009, McDonald et al., 2013, McDonald and Pizzari, 2016).

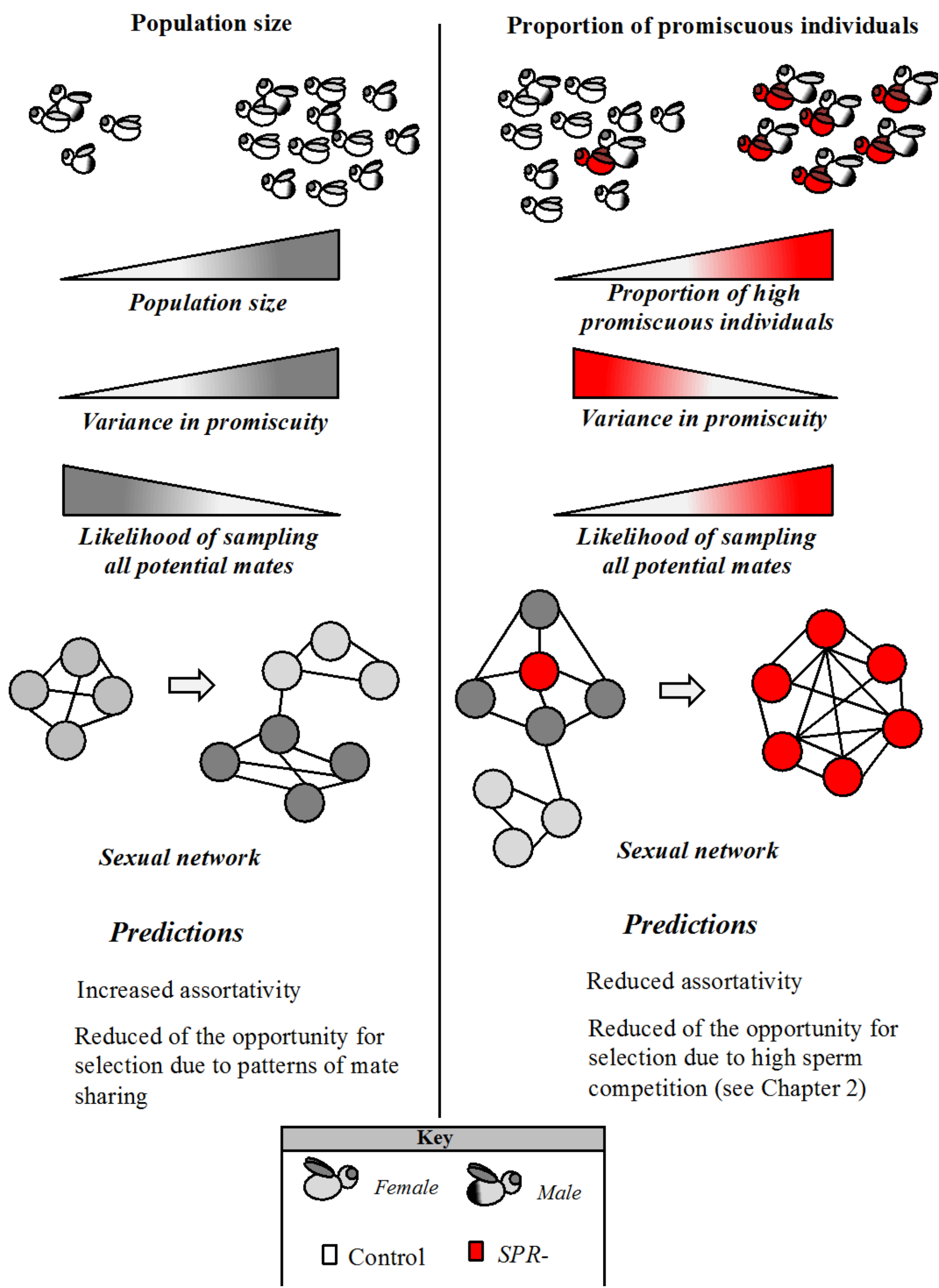

Figure 1 – The effects of population size and the proportion of promiscuous individuals in a population in generating patterns of assortative mating

Nutrient stress in early life and its effects on sexual maturity of male accessory glands

In male *D. melanogaster,* the maturation and functionality of accessory glands are essential for male reproductive success because they produce seminal fluid proteins, which determine male ability to trigger female physiological responses to mating and produce important seminal proteins that help male in sperm competition (Perry et al., 2013). Recently, Ruhmann et al. (2016) showed that the quantity of seminal fluid and the size of male accessory gland increases with male age, which could alter male seminal fluid investment *per* mating. Wigby et al. (2015) showed that the developmental environment can alter male ejaculate reserves and investment. For instance, males raised in high larval density have reduced reserves of seminal fluid although invest proportionally more of this seminal fluid *per* mating than males raised at low larval density (Wigby et al., 2015). However, the extent to which nutritional stresses during development affect accessory glands maturation and seminal fluid protein profile in the ejaculate of adult males remains unknown. There are numerous unanswered questions, such as ‘ are the accessory glands of males raised in high larval density fully equipped to produce a functional ejaculate with the same timing as the accessory glands of males raised in low larval density?’ and ‘Do males raised in high (stressful) larval densities invest more of the ejaculate with the same profile of seminal fluid proteins than males raised in low larval densities?’ Essentially, work remains to be done to answer ‘how nutritional stress during development alters the male seminal fluid protein profile in the ejaculate?’ It will be interesting to combine proteomics and analysis of gene expression to investigate how the developmental environment affect accessory gland maturation rate and seminal fluid production, as well as characterise the time-dependent seminal fluid profile of accessory glands from males of different developmental environment

treatments (Figure 2). Answering these questions will shed light on the mechanisms by which development modulates male reproductive success.

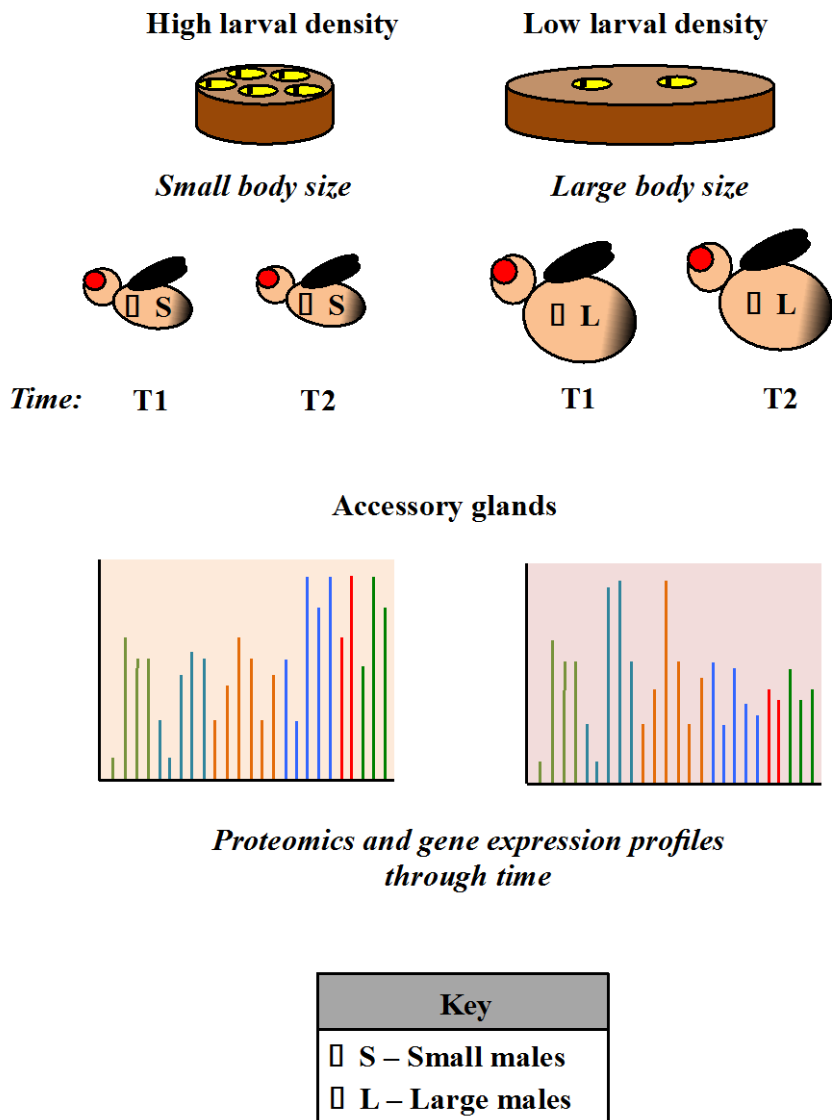

Figure 2 – Nutrient stress in early life and its effects on sexual maturity of male accessory glands

The effects of nutrient intake on the seminal fluid protein profile and offspring quality

The expression of male sexual traits involved in both pre- and post-copulatory competition is costly but essential for males to increase their reproductive success (Pizzari and Wedell, 2013, Parker and Pizzari, 2015). Because of the costs, the expression of sexually selected traits is expected to depend on resource acquisition such as nutrients (Simpson and Raubenheimer, 2012, Rowe and Houle, 1996). In Chapter 5, I showed that males compromise their macronutrient intake of protein and carbohydrate at an intermediate point between the diets that maximise male reproductive traits. Interestingly, I showed that the protein intake of the first male to mate with a virgin female was negatively correlated with female offspring production even after females remated with a nutritionally standard male (see Chapter 5). A potential mechanism by which male diet can modulate female offspring production is through changes in the ejaculate composition and seminal fluid protein profile. It is possible that the ejaculate of the first male to mate with a female imprints that male's nutritional status onto female physiology via post-mating responses to seminal fluid proteins and, as a result, female reproductive success is determined by the nutritional status of their first mates. However, evidence of nutritional effects on the complete male seminal fluid profile is still missing. To address this, future studies should combine the Geometric Framework of Nutrition (see Chapter 5) and Proteomics analysis of male seminal fluid proteins to determine how different ratios of macronutrients affect male seminal fluid composition, and how these changes in the male ejaculate can become key modulators of female reproduction.

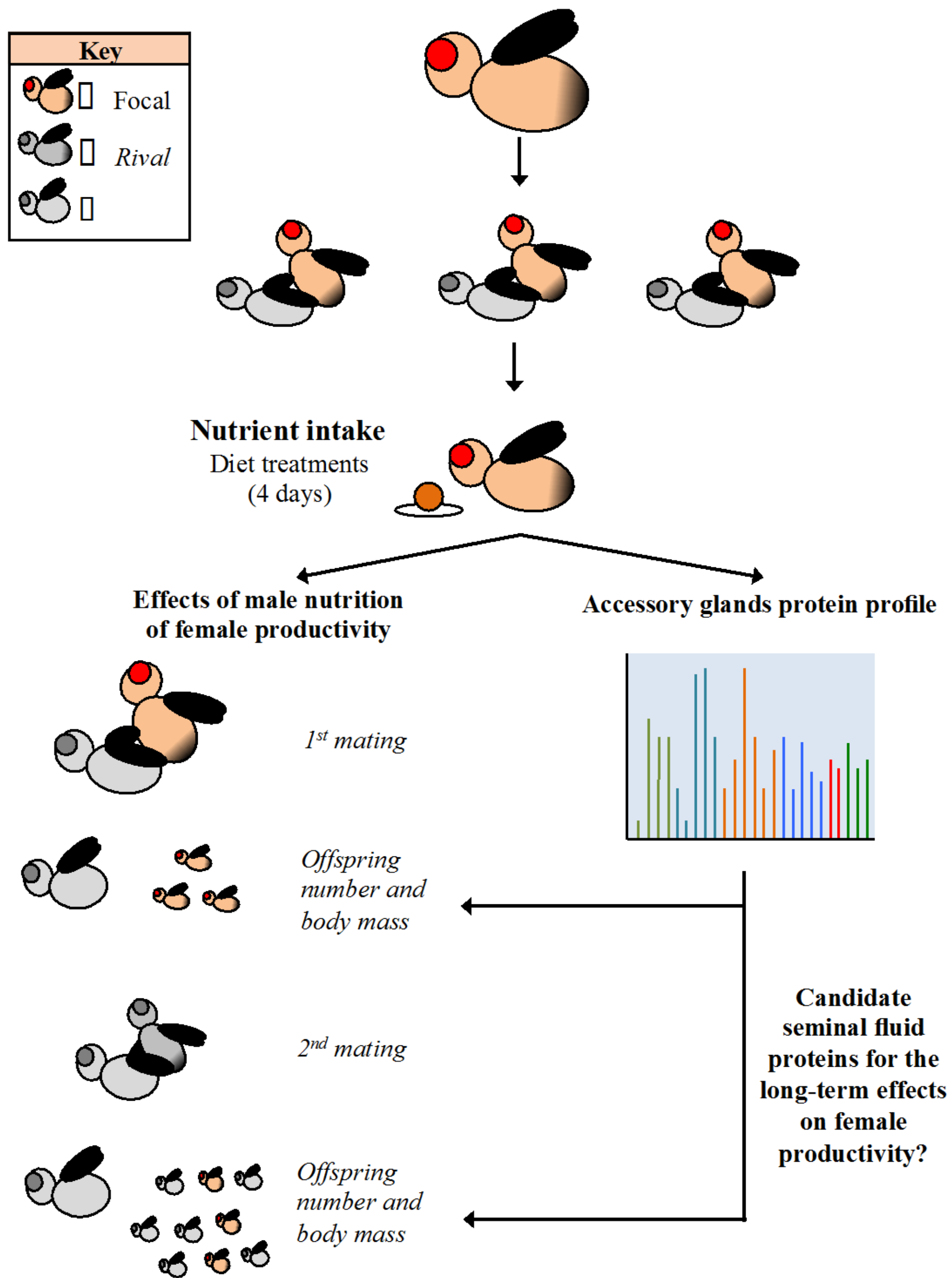

Figure 3 – The effects of nutrient intake on the seminal fluid protein profile and long-term effects on female productivity.

Conclusion

In conclusion, this thesis makes significant advances to the integration of nutrition, ecology and evolutionary biology by revealing how polyandry and nutrition affect the operation of sexual selection, offspring quality, and population fate in *D. melanogaster*, with potential relevance to species throughout the animal kingdom. Thus, this thesis extends our knowledge of the scope of sexual selection, and provides invaluable insight on its ability to explain the astounding beauty and complexity of nature.

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