The role of pathogens and sex ratio in sexual selection

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To my family

The role of pathogens and sex ratio in sexual selection

Abstract

Sexual selection is defined as 'selection of traits associated with competition for mates", where 'mate' is a reproductive partner with which one or more zygotes are formed', providing an explanation for the evolution of colourful and exaggerated traits we see in nature. In this thesis, I explore three key themes of sexual selection in *Drosophila melanogaster*. In Chapter 2, I explore how female choice via "good genes" might lead to population level fitness advantages. In Chapter 3, I investigate sexual dimorphism on a genomic level. In Chapter 4, I test how operational sex ratio- influences sexual selection.

The "good genes" hypothesis related to pathogens predicts that females choose males on the basis of their resistance towards an environmentally relevant pathogen. Populations are expected to harbour ample genetic variation underlying immunity owing to co-evolutionary cycles involving adaptation and counter-adaptation. Therefore, the opportunity for sexual selection based on condition-dependent "good genes" is expected to be large. This gives rise to the expectation that the presence of pathogens and sexual selection might aid adaptation in populations. In this study, I evolved populations of *D. melanogaster* in a design simultaneously manipulating sexual selection and pathogen presence, using a gram-negative insect pathogen *Pseudomonas entomophila*, for 14 generations. I then examined how the presence of sexual selection and the pathogen, and their potential interaction, affected the evolution of pathogen resistance. I found increased resistance to *P. entomophila* in populations that evolved under pathogen pressure, driven primarily by increased female survival after infection despite selection for resistance acting only on males over the course of experimental evolution. I did not find any evidence of sexual selection aiding adaptation to pathogen, a finding contrary to the predictions of "good genes" theory.

In Chapter 3, I studied the genetic basis of sex-specific immunity that may help us to throw light on pathogen-driven sexual selection and its consequences. Immunity has a shared and sexually dimorphic component, the latter can take various forms – from

differential gene expression to different mechanisms that the sexes use to get rid of a pathogen. In the following section, I explored the genetic basis of sexual dimorphism in immunity, including variants with sex-specific and sexually antagonistic effects. For this, I took a pooled-sequencing approach to identify population-level allele frequency differences between baseline individuals (sampled before infection) and survivors of infection. I identified 93 variants associated with stronger immunity in both sexes. Moreover, I also identified 63 candidate variants associated with immunity in only one sex or the other, along with a subset of variants clearly associated with sexually antagonistic effects on survival. I thus determined that the genetic architecture of resistance to pathogens is partially shared and partially distinct in the sexes.

In the penultimate chapter, I move beyond pathogens to understand a seminal prediction about how operational sex ratio influences the strength of sexual selection. This prediction is based on the assumption that stronger competition for mates between members of the more abundant sex leads to higher variance in mating success, and that higher variance results in stronger sexual selection. However, this rationale for the relationship between sex ratio and sexual selection in debatable. In this chapter, I investigated how sex ratio influences the strength of selection on a Mendelian trait in *D. melanogaster*. To this end, I measured competitive mating success of two genotypes, a homozygous mutant of the *ebony* gene, responsible for both sexual and non-sexual fitness, and the wild type, under different sex ratio treatments. The strength of sexual selection against the mutation increased as sex ratios became increasingly male-biased. Moreover, the sex ratio and not the absolute densities of males and females influenced the strength of sexual selection. I also found that the strength of sexual selection waned over consecutive days, highlighting a change in the relative selection on pre- and post-copulatory mate competition. This study suggests that heightened sexual selection in male-biased sex ratios might lead to overall population fitness benefits by selecting against "bad genes".

In summary, my thesis addresses fundamental aspects of sexual selection in *D. melanogaster*. My results challenge the "good genes" hypothesis, putting into question the role of female choice for sexual selection. Moreover, immunity is broad and is maintained by different selection pressures on the genome, indicating that immunity, a sexually selected trait might indicate overall genetic variation. Finally, I confirmed the ability of sexual selection to "purge" bad genes in some demographical scenarios.

Abstract (en Français)

La sélection sexuelle est définie comme "la sélection des caractères associés à la compétition pour obtenir des partenaires", où un "partenaire" est un individu reproducteur avec lequel un ou plusieurs zygote·s sont formé·s", permettant ainsi d'expliquer l'évolution des caractères complexes et exagérés que nous observons dans la nature. Le choix-des-femelles est peut-être primordial pour comprendre la sélection sexuelle. Les modèles "des bons gènes" expliquent le choix des femelles en supposant qu'elles sont favorisées par la sélection parce que les mâles choisis portent des variantes génétiques qui leur confèrent une plus grand fitness. En agissant ainsi, les femelles bénéficient d'avantages génétiques pour leur progéniture. Une version moderne de cette hypothèse prévoit que les ornements sexuels signalent "des bons gènes" liés à la condition de l'individu reflétant honnêtement la qualité génétique globale. La sélection sexuelle devrait donc se traduire par une augmentation de la fitness non-sexuelle.

Les "bons gènes" relatifs aux pathogènes prédisent que les femelles choisissent les mâles sur la base de leur résistance à un pathogène présent dans l'environnement. Les agents pathogènes sont un facteur important de l'évolution du cycle de vie de l'hôte, ils présentent des cycles épidémiologiques et co-évolutifs avec l'hôte, impliquant adaptation et contre-adaptation. Ainsi, les populations présentent une grande variation génétique en relation avec l'immunité et l'on s'attend à ce que la sélection sexuelle basée sur les "bons gènes" dépendant des conditions de l'individu soit importante. Ceci permet de supposer que la présence d'agents pathogènes et la sélection sexuelle pourraient favoriser l'adaptation des populations. Dans cette étude, j'ai fait évoluer des populations de *Drosophila melanogaster* dans un plan expérimental qui a permis de manipuler simultanément la sélection sexuelle et la présence de pathogènes, en utilisant *Pseudomonas entomophila*, un pathogène d'insecte gram-négatif, pendant 14 générations. Ensuite, j'ai examiné comment la présence de la sélection sexuelle et du pathogène, et leur interaction potentielle, influaient sur l'évolution de la résistance au pathogène. J'ai constaté une résistance plus importante à *P. entomophila* dans les

populations qui ont évolué sous la pression du pathogène, due principalement à l'augmentation de la survie des femelles après l'infection, et ce même si la sélection pour la résistance n'a agi que sur les mâles au cours de l'évolution expérimentale. Je n'ai trouvé aucune évidence que la sélection sexuelle favorise l'adaptation au pathogène, un résultat en contradiction avec les prédictions de la théorie "des bons gènes".

Le dimorphisme sexuel en termes d'immunité peut prendre différentes formes - de l'expression différentielle des gènes jusqu'aux différents mécanismes que les sexes utilisent pour éliminer un pathogène. La base génomique de ce dimorphisme est cependant peu explorée. Dans la section suivante, j'ai exploré la base génétique du dimorphisme sexuel en matière d'immunité, notamment les loci spécifiques au sexe et les loci sexuellement antagonistes. Dans ce contexte, j'ai adopté une approche de séquençage groupé pour identifier les différences de fréquences alléliques dans la population entre les individus de référence (échantillonnés avant l'infection) et les survivants de l'infection. J'ai également identifié des variantes candidats spécifiques à chaque sexe associé à l'immunité, ainsi qu'un sous-ensemble de variantes clairement associés à des effets sexuellement antagonistes sur la survie. J'ai ainsi déterminé que l'architecture génétique de la résistance aux pathogènes est partiellement partagée et partiellement distincte chez les deux sexes.

Dans l'avant-dernier chapitre, je vais au-delà des agents pathogènes pour comprendre une prédiction fondamentale sur la façon dont le sex-ratio opérationnel influence la force de la sélection sexuelle. Cette prédiction est basée sur la supposition qu'une plus forte compétition pour les partenaires, entre les membres du sexe le plus abondant, mène à une plus grande variance dans le succès de l'accouplement, et qu'une plus grande variance se traduit par une plus forte sélection sexuelle. Cependant, la justification de la relation entre le sex-ratio et la sélection sexuelle est discutable. En outre, les différentes métriques utilisées pour étudier la relation entre le RSO (ratio sexuel opérationnel) et la sélection sexuelle ont produit des résultats mitigés. Dans ce chapitre, j'ai étudié les influences du sex-ratio sur la force de la sélection sur un caractère

mendélien chez *Drosophila melanogaster*. Pour ce faire, j'ai mesuré le succès d'accouplement en compétition entre deux génotypes, un mutant homozygote du gène ebony, responsable de l'aptitude sexuelle et non sexuelle, et le type sauvage, sous différents traitements de sex-ratio. La force de la sélection sexuelle contre la mutation a augmenté à mesure que le sex-ratio augmentait en faveur des mâles. Le sex-ratio, et non les densités absolues de mâles et de femelles, influençait la force de la sélection sexuelle. J'ai également constaté que la force de la sélection sexuelle diminuait au cours de jours consécutifs, mettant en évidence un changement de la sélection relative sur la compétition entre partenaires avant et après la copulation. Cette étude renforce l'idée qu'une sélection sexuelle accrue dans des ratios sexuels biaisés en faveur des mâles pourrait entraîner des avantages pour l'ensemble de la population en sélectionnant contre les "mauvais gènes".

Pour résumer, ma thèse traite des aspects fondamentaux de la sélection sexuelle. Premièrement, mes résultats remettent en question l'hypothèse des "bons gènes", car je n'ai trouvé aucun rôle de la sélection sexuelle dans l'adaptation aux agents pathogènes. Deuxièmement, mes résultats suggèrent que l'immunité pourrait effectivement indiquer la qualité génétique, reflétant la variation génétique globale, car la base génétique de l'immunité est large et est maintenue par différentes pressions de sélection qui agissent sur le génome. Enfin, j'ai constaté que la force de la sélection sexuelle augmente avec l'augmentation des sex-ratios biaisés en faveur des mâles, ce qui suggère que ces derniers pourraient contribuer à la capacité de la sélection sexuelle à "purger" les mauvais gènes.

Chapter 1

General Introduction

Sexual selection

The sheer degree of sexual dimorphism we see in many species remains an outstanding evolutionary puzzle. Males are often brightly coloured, loudly vocalising or huge-tusked contrasted with females that seem dull and ascetic. Darwin, like many naturalists of the time, noted this but could not reconcile this observation with natural selection (Darwin, 1871). Why should cumbersome traits that that make the individual more likely to be parasitized or preyed upon be selected for? After many exchanges with his counterparts, Darwin floated an idea that was to spur debate and research in the coming centuries – sexual selection (Darwin, 1871). Darwin's insight combined with his meticulous data keeping and note taking brought him to the conclusion that the question could be reframed 'not [as] a struggle for existence, but [as] a struggle between the males for possession of the females'. In other words, a trait that might lead to enhanced reproductive success, will be favoured by sexual selection.

The definition of sexual selection has undergone many revisions (Alonzo & Servedio, 2019). Darwin's definition was that sexual selection 'depends on the advantage which certain individuals have over other individuals of the same sex and species, in exclusive relation to reproduction' (Darwin, 1871). More definitions range from defining it as selection leading to variance in reproductive success (Arnold & Wade, 1984) to a more specific relation between certain traits leading to differences in competition over mates that ultimately leads to differential reproductive success (Andersson, 1994). More recent definitions (Alonzo & Servedio, 2019), however, include the important roles of post-copulatory mate competition in differential mating success. In this thesis when referring to sexual selection, I refer to the definition by Shuker (2010) wherein he defined sexual selection as "selection of traits associated with competition for mates…" where he defines

a ‘mate’ as a reproductive partner with which one or more zygotes are formed (thereby allowing post-copulatory processes)’.

In this thesis, I explore central themes in sexual selection using *Drosophila melanogaster*. In Chapter 2, I explore how female choice, one of the main pillars on which sexual selection, acts via “good genes” might lead to population level fitness advantages. In Chapter 3, I investigate sexual dimorphism on a genomic level, an important evolutionary consequence of a shared genome yet with different selective pressures for the sexes. In Chapter 4, I test how an important demographical factor - operational sex ratio- influences sexual selection.

Sexual selection and female choice – “good genes” models and parasites

Darwin’s treatment of sexual selection did not go much further and his explanation of sexual selection led to more questions than answers. For instance, he implied that females exercise choice and that this is an important context in which sexual selection operates (Rosenthal & Ryan, 2022). However, the idea of female choice was ahead of its time given the Victorian era and its misogynistic ideals, and even Darwin’s co-discoverer Wallace, rejected it. However, neither Darwin nor his peers could - and were probably not prepared in the social context – to work out how female choice was realised, what were the mechanisms that led to it and how it evolved, explaining the evolution of exaggerated ornaments leading to sexual dimorphism we see in nature (Rosenthal & Ryan, 2022). More fundamentally, the question of what traits females were choosing, and why, has remained a work in progress even to this day.

While female choice for male traits is established (Kirkpatrick & Ryan, 1991; Andersson, 1994), uncertainty remains over the benefits to females of choosing the most extravagant male. Most simply, a female could choose a mate for some direct benefits such as nuptial gifts, a good territory to breed in, increased access to food, protection from harassing males, or avoid getting infected with parasites or diseases by choosing healthy males

(Andersson & Iwasa, 1996). However, females do not always benefit directly, the most extreme examples being lekking species, where females gain nothing more than fertilisation. This raises a big question – on what basis then do females discriminate males when there seem to be no direct benefits? A resolution to this conundrum could lie in the fact that females could be benefiting indirectly from their choice, by accruing genetic benefits for her offspring.

The "good genes" hypothesis predicts that females choose males who carry genetic variants that confer higher fitness (Hunt *et al.*, 2004). A modern version of the "good genes" hypothesis essentially aligns with Darwin's prediction about sexual selection having an all-pervading influence on population fitness: "the strongest and most vigorous males, or those provided with the best weapons, have prevailed under nature, and have led to the improvement of the natural breed or species' (Darwin 1871). However, the "good genes" models have a shortcoming. Female preference for traits can lead to directional and persistent selection on them, which is expected to exhaust genetic variation bringing selection to halt. For selection to continue, sexually selected traits must continue to reflect overall genetic variation, that might control how organisms acquire and allocate resources, also called "condition" (Hill, 2011). Condition-dependant sexually-selected traits are then expected to be honest signals of fitness (Rowe & Houle, 1996). This concept marries sexual and natural selection and can explain how sexual selection can lead to increased non-sexual fitness, as originally predicted by Darwin (1871). For instance, condition-dependence of sexually selected traits can lead to accelerated rates of adaptation (Lorch *et al.*, 2003) and purging of deleterious mutations (Whitlock & Agrawal, 2009).

In line with the condition-dependent beneficial effects of sexual selection, Hamilton and Zuk (1982) proposed that females choose males based on "good genes" or genetic quality that is reflected honestly in sexual ornaments that mirror immunity towards a certain pathogen or parasite (Hamilton & Zuk, 1982; Martin, 1990). Pathogens, due to their prevalence, diversity, and ability to adapt to the host, represent a moving target.

This means that immunity to a pathogen is an ever-moving optimum which by extension indicates that populations harbour ample genetic variation underlying immunity and the opportunity for sexual selection based on condition-dependent "good genes" is expected to be large. Hamilton and Zuk (1982), in their "good genes" model, predicted additive genetic covariance between paternal and offspring immunity, thereby explaining how sexual selection could lead to increased resistance towards a certain pathogen. However, despite being a decades old hypothesis (Hamilton and Zuk 1982), no study has directly tested if the additive genetic covariance between paternal resistance and offspring immunity evolves readily in populations that also experience sexual selection (alongside pathogen presence). This leaves an open question about the role of pathogens in sexual selection, and the population-level consequences this might have.

Given this significant gap in explaining sexual selection, in Chapter 2, I experimentally test the "good genes" hypothesis relating to pathogens. Here, I test if pathogens and sexual selection interact or act independently to lead to elevated population fitness with respect to adaptation to pathogen.

Parasites and sexual dimorphism

Sexual dimorphism in many traits can be explained by sexual selection, as rightly noted by Darwin (1871). However, sexual dimorphism can also be attributed simply to physiological differences between males and females and the fact that they have different life-history strategies (Rolff, 2002).

Sexual dimorphism in immunity is well established (Belmonte *et al.*, 2020). This dimorphism could take various forms. For instance, the two sexes might differ in their relative investment towards the different components of the adaptive and innate arms of the immune system. For example, in arthropods, females show higher bactericidal activity and melanisation, whilst males have higher interleukin responses to bacterial pathogens. The two sexes also differ in how major immune pathways, such as Toll and IMD are

regulated (Klein & Flanagan, 2016), leading to differences in how quickly each sex will clear a pathogen (Duneau *et al.* 2017; reviewed in Belmonte *et al.* 2020). Globally, the two sexes also show sexually dimorphic gene expression changes in response to pathogens, indicating broad intrinsic differences in how resistance functions in males and females (Duneau *et al.*, 2017).

Avoiding pathogens is of mutual interest in males and females. However, given that the sexes have different life histories and immunity is an energetically expensive trait, fitness optima for the two sexes are not fully aligned, which could lead to the evolution of sexual dimorphism (Rolff, 2002). A less appreciated aspect of this dimorphism is genomic basis. On the one hand, a shared genome would be expected to result in shared immune responses to a particular pathogen, but on the other, opposing interests might play out as sex-specific or sexually antagonistic selection over a shared genome.

Then studying sexual dimorphism in immunity is important to understand what selection pressures might be acting on the genome, which might ultimately bear consequences for pathogen driven sexual selection. An immune response requires interactions between products of several genes across the genome (Lemaitre & Hoffmann, 2007), indicating that any selection on the genome for immunity - shared or sex-specific - is likely to have a broad genomic signature. In Chapter 3, I explore the genetic basis of sexual dimorphism wherein I identify genomic loci associated with a shared and sex-specific basis of immunity.

Operational sex ratio and strength of sexual selection

Darwin was puzzled and amazed to see the sheer degree of sexual dimorphism, which led him to recognise sexual selection. He quickly noted that animals had different mating systems, ranging from monogamous to polygamous, and the degree of sexual dimorphism differed depending on the mating system. He noted extreme degrees of dimorphism in mating systems with a skew in sex ratio - particularly in polygynous

systems (Darwin, 1871). Almost a century later, Emlen and Oring (1977) were one of the first to classify mating systems where they described two concepts. First, they posited that the intensity of sexual selection would depend on the potential for mate monopolization i.e. the extent to which certain individuals of a given sex can 'control' or dominate mating opportunities compared to their counterparts. Second, they also pinpointed another contributor to mating systems, the operational sex ratio.

It was theorised that competition between members of the abundant sex in a sex-biased mating system might lead to variance in mating success, which would lead to stronger sexual selection on the abundant sex (Shuster, 2009; Jennions *et al.*, 2012). However, the use of sex ratio along with other variance-based metrics to measure sexual selection have a major problem. Rather than directly quantifying the strength of selection on particular traits, they reflect the "opportunity" for sexual selection (Klug *et al.*, 2010).

A large body of literature is dedicated to understanding and validating the relationship between sex ratio and sexual selection. Theoretical and empirical work using variance based metrics to quantify sexual selection under different sex ratios has found mixed results. While some studies indicate that sexual selection increases as variance in mating success increases, some pointing in the opposite direction (Klug *et al.*, 2010; Jennions *et al.*, 2012). Moreover, even studies using trait-based measures (e.g. selection gradient, selection differential) to directly measure selection under different sex ratios, are conflicted about the relationship it has with sexual selection (Klug *et al.*, 2010); some literature points towards higher sexual selection on traits in male biased ratios, while the inverse is found to be true in some studies.

Quantifying how major demographical variables such as sex ratio influence the strength of sexual selection is of paramount importance. This might help to understand the evolutionary consequences of sexual selection, such as trade-offs associated with the development, and maintenance of sexual traits (Rowe & Houle, 1996; Bonduriansky & Chenoweth, 2009). One way to resolve and validate the relationship between sex ratio

and sexual selection could be measuring selection on something more fundamental than traits – genes, which form the basis of selection. In Chapter 4, I look at how sex ratios influence the strength of sexual selection by directly measuring selection against an allele that negatively influences both sexual and non-sexual traits.

Study system

In this thesis, I used *D. melanogaster,* an amenable study system for evolutionary biology, to explore sexual selection. Scores of studies have used *D. melanogaster* to study sexual selection (Promislow *et al.*, 1998; Hollis & Houle, 2011) and sexual conflict (Hollis *et al.*, 2019). While *D. melanogaster* lacks the extreme ornaments, it still exhibits an elaborate and energetically expensive courtship behaviour (Greenspan & Ferveur, 2000). The courtship song along with other visual and olfactory cues from males eventually influence whether a female takes the male as a mating partner or not (Immonen & Ritchie, 2012; Billeter & Wolfner, 2018). Males engage in aggressive mate competition for access to females (Saltz & Foley, 2011). Moreover, the reaches of sexual selection also extend to post-copulatory sperm competition where seminal fluid proteins are known to take a major role (Hopkins *et al.*, 2019).

The low cost of maintenance and a rapid generation time also makes the fly an ideal system to use for experimental evolution to study for instance, the consequences of sexual selection on population fitness (Hollis *et al.*, 2009; Hollis & Houle, 2011). In the context of pathogens, *D. melanogaster* has been used to study the trade-offs of life-history traits with immunity (McKean & Nunney, 2008) and sexual selection on pathogen resistance (Joye & Kawecki, 2019).

In Chapters 2 and 3, I used a generalist gram-negative pathogen, *Pseudomonas entomophila* along with *D. melanogaster* as a host. This pathogen is now well characterised with respect to how it targets the host and what immune responses the host uses in defence. For instance, the bacteria leads to the activation of both local and

systemic immune responses, including the secretion of specific anti-microbial peptides such as aprA (Vodovar *et al.*, 2005; Liehl *et al.*, 2006). Moreover, studies have also elucidated various candidate loci - associated with cell stress and repair - that underpin immunity to the pathogen and the gene expression changes (such as those associated with oxidative stress) that follow an immune challenge (Bou Sleiman *et al.*, 2015). *P. entomophila* has also been used in an evolutionary context in work looking at life-history trade-offs (Vijendravarma *et al.*, 2015) and sexual selection (Joye & Kawecki, 2019).

Thus, both *D. melanogaster* and *P. entomophila* are ideal to study sexual selection.

Thesis outline

My thesis is at the crossroads of central themes of sexual selection.

In Chapter 2, I explore the role of parasites in sexual selection, specifically how female choice through "good genes" might lead to population-level fitness advantages. For this, I experimentally evolved *D. melanogaster* for fourteen generations with a full factorial design (with and without pathogen pressure and with and without sexual selection). I found a clear signature of adaptation to pathogen in regimes that encountered the pathogen in comparison to regimes that never encountered the pathogen. Surprisingly, despite only infecting males over the course of experimental evolution, adaptation to pathogen was more prominent in females. Contrary to predictions from sexual selection theory, however, we did not find any evidence that sexual selection can aid adaptation to pathogen.

In Chapter 3, I investigate the genomic basis of sexual dimorphism in resistance to pathogens i.e. the genetic basis of sex-specific and sexually antagonistic immunity. For this, I took a sequencing approach to identify allele frequency differences between survivors of infection and baseline (i.e. sampled before infection) individuals. We found many variants associated with stronger immunity in both sexes. Moreover, we also

identified candidates associated with a clear sex-specific effect along with a subset of variants associated with sexually antagonistic effects on survival, as evidenced by opposing slopes for allele frequency trajectories between baseline and survivors in the two sexes. I thus gathered evidence that indicates that the genetic architecture of immunity or resistance to pathogens is partially shared and partially distinct in the sexes.

In Chapter 4, I move beyond pathogens to answer another seminal prediction in sexual selection theory - does a male biased sex ratio lead to a stronger sexual selection? I test the prediction by manipulating sex ratios (male to female-biased) and quantifying how this changes selection against a homozygous mutation known to influence both sexual and non-sexual traits, thereby making individuals competitively inferior. By using a combination of mutants and wild type males for each sex ratio treatment, I was able to assess how relative paternity success of mutant males changes in each sex ratio treatment. I found robust evidence that the strength of sexual selection increases from female to male-biased sex ratios, and that it is the overall sex ratio and not the relative ratios of males and females that is associated with this. The results also indicate that stronger sexual selection against the deleterious *ebony* polymorphism in male-biased ratios is analogous to sexual selection promoting "good genes".

In the final chapter, I will summarize the major findings of each of the projects in my dissertation and connect my work to the broader sexual selection field.

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Chapter 2

Adaptation to a bacterial pathogen in *Drosophila melanogaster* is not aided by sexual selection

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Abstract

Theory predicts that sexual selection should aid adaptation to novel environments, but empirical support for this idea is limited. Pathogens are a major driver of host evolution and, unlike abiotic selection pressures, undergo epidemiological and co-evolutionary cycles with the host involving adaptation and counter-adaptation. Because of this, populations harbor ample genetic variation underlying immunity and the opportunity for sexual selection based on condition-dependent "good genes" is expected to be large. In this study, we evolved populations of *Drosophila melanogaster* in a 2-way factorial design manipulating sexual selection and pathogen presence, using a gram-negative insect pathogen *Pseudomonas entomophila*, for 14 generations. We then examined how the presence of sexual selection and the pathogen, as well as any potential interaction, affected the evolution of pathogen resistance. We found increased resistance to *P. entomophila* in populations that evolved under pathogen pressure, driven primarily by increased female survival after infection despite selection for resistance acting only on males over the course of experimental evolution. This result suggests that the genetic basis of resistance is in part shared between the sexes. We did not find any evidence of sexual selection aiding adaptation to pathogen, however, a finding contrary to the predictions of "good genes" theory. Our results therefore provide no support for a role for sexual selection in the evolution of immunity in this experimental system.

Keywords: sexual selection, good genes, adaptation, condition-dependence, Hamilton and Zuk hypothesis, pathogens.

Introduction

Darwin posited that sexual selection plays an important role in improving non-sexual fitness, writing that, "the strongest and most vigorous males, or those provided with the best weapons, have prevailed under nature, and have led to the improvement of the natural breed or species'' (Darwin, 1871). The modern version of this idea proposes that

sexually selected traits in males reflect "good genes" (Fisher, 1930; Zahavi, 1975; Iwasa *et al.*, 1991; Houle & Kondrashov, 2002), explaining potentially costly female choice by indirect benefits received in the form of increased offspring fitness. Theory suggests expression of sexually-selected traits should evolve to become dependent on overall condition—which would maintain signal fidelity—leading to accelerated rates of adaptation (Lorch *et al.*, 2003) and more efficient purging of deleterious mutations (Whitlock & Agrawal, 2009).

In line with predictions of positive effects of sexual selection (Cally *et al.*, 2019) on population performance, sexual selection has been found to diminish the likelihood of population extinction (Jarzebowska & Radwan, 2010; Lumley *et al.*, 2015). Experimental work in different insect taxa including *Drosophila* has also shown that the presence of sexual selection accelerates the purging of deleterious alleles in experimental populations (Radwan, 2004; Hollis *et al.*, 2009; Grieshop *et al.*, 2016). In several experiments, sexual selection facilitated adaptation to novel environmental challenges, including the evolution of desiccation resistance in *D. melanogaster* (Gibson Vega *et al.*, 2020), pesticide resistance in *Tribolium castaneum* (Jacomb *et al.*, 2016), and adaptation to a novel diet in *Callosobruchus maculatus* (Fricke & Arnqvist, 2007). However, an arguably larger body of experimental work has found no role for sexual selection in improving non-sexual fitness. Multiple experimental evolution studies failed to find population-level net benefits of sexual selection when examining larval competitive ability, net reproductive rate, or female fecundity (Promislow *et al.*, 1998; Holland & Rice, 1999; Long *et al.*, 2009 respectively). Moreover, a large body of work has also failed to demonstrate a role of sexual selection in adaptation in novel environments (e.g. to higher temperatures (Holland, 2002) or a novel diet (Rundle *et al.*, 2006)). There is also no evidence that overall mutation load from the genome is reduced under heightened sexual selection (Hollis & Houle, 2011; Arbuthnott & Rundle, 2012) (although in environments that are spatially complex, this is not true and the predicted beneficial effects of sexual selection on mutation load are seen (Singh *et al.*, 2017)). Thus, taken together, the literature is equivocal about the role of sexual selection in non-sexual fitness. This leaves an open

question about whether the "good genes" mechanism plays a role in adaptation in general or even in specific scenarios, like during adaptation to pathogens or parasites, where this role has been predicted to be most evident but remains largely untested.

One potential explanation for these mixed results is that the non-sexual fitness of populations is normally elevated by competition for mates—that is, sexual selection in the broad sense does have adaptive value—but these benefits are counterbalanced by the negative effects of sexual conflict and therefore invisible in many experimental designs. Sexual conflict arises because of an evolutionary conflict of interests between the sexes (Parker, 1979; Hosken *et al.*, 2019) which can manifest in two ways. The first, interlocus sexual conflict, is characterized by selection favoring traits that increase male competitive success even when these traits are accompanied by harm to females. Interlocus sexual conflict can lead to the evolution of female resistance and sexually antagonistic coevolution (Holland & Rice, 1999a; Chapman *et al.*, 2003; Rice *et al.*, 2006), reducing mean population fitness (Bonduriansky & Chenoweth, 2009; Long *et al.*, 2009, 2012). In *Drosophila*, interlocus sexual conflict acts through antagonistic effects on female fecundity and survival (Rice, 1996; Chapman, 2006), especially on the most fecund females (Long *et al.*, 2009). Intralocus conflict, on the other hand, involves sexually antagonistic pleiotropic effects of polymorphisms at the same locus in males and females (Bonduriansky & Chenoweth, 2009; Van Doorn, 2009; Innocenti & Morrow, 2010)) that constrain males and females from reaching sex-specific optima (Chippindale, 2001; Hollis *et al.*, 2014, 2019). Either form of sexual conflict leads to a burden on populations that might overwhelm any positive effects of sexual selection for mean population fitness (Bonduriansky & Chenoweth, 2009; Long *et al.*, 2009, 2012).

Male-male competition and female choice have been proposed to be particularly consequential for the evolution of pathogen resistance (Hamilton & Zuk, 1982; Folstad & Karter, 1992; Roberts *et al.*, 2004). Pathogens are a major evolutionary driver of the life histories of organisms (Price, 1980; Schmid-Hempel, 2005) due to their prevalence, diversity, and because they adapt to the host and represent a moving target for the

immune system. According to the Hamilton-Zuk hypothesis (1982), sexual ornaments indicate immunity towards prevalent pathogens or parasites (Hamilton & Zuk, 1982; Martin, 1990). A number of studies in birds have indeed demonstrated phenotypic correlations between male parasite or pathogen load and the quality of sexual ornaments (Hamilton & Zuk, 1982; Martin, 1990; Balenger & Zuk, 2014) or female preference towards the males (Blount *et al.*, 2003; Hund *et al.*, 2020). Yet, whether this phenotypic correlation should be positive or negative is not unequivocally predicted by mathematical models; either may be predicted depending on details of the model assumptions (Getty, 2002). These phenotypic correlations between sexual ornaments and parasite/pathogen resistance do not necessarily predict whether sexually attractive fathers will sire resistant offspring; rather, this key element of the "good genes" hypothesis is mediated by additive genetic correlations (Hamilton & Zuk, 1982). One way to test for this genetic correlation would be to track the evolution of resistance under controlled laboratory conditions (Kawecki *et al.*, 2012) where both the strength of sexual selection and pathogen pressure are manipulated. If there is an additive genetic correlation between sexually successful fathers and pathogen-resistant offspring, resistance should evolve more readily in populations where males also experience sexual selection.

Selection for improved immunity (including better physiological responses to immune challenges) in experimental populations has generally resulted in a robust and rapid response (Armitage & Siva-Jothy, 2005; Martins *et al.*, 2013; Joop *et al.*, 2014; Ferro *et al.*, 2019). Two studies that explored the effect of sexual selection on immunity by experimentally evolving populations with and without sexual selection have found that males and females diverge in their investment in innate immunity (measured as phenyloxidase activity; PO) (Hangartner *et al.*, 2015; Bagchi *et al.*, 2021). In both these studies (one on the flour beetle *Tribolium castaneum* and the other on the seed beetle *Callosobruchus maculatus* (Hangartner *et al.*, 2015; Bagchi *et al.*, 2021 respectively), females from polygamous populations had higher levels of PO than females from monogamous populations, with no effect on males from either of the two experimental regimes. The higher levels of PO in females from sexually selected populations did not

influence pathogen clearance in either study, although in one of the studies higher PO activity was correlated with lower survival in females upon bacterial infection (Bagchi *et al.*, 2021). These studies indicate how sexual selection and sexual conflict can drive sex-specific differences in male and female immunity. This pattern is not without exceptions; a study on the yellow dung fly, *Scathophaga stercoraria,* did not report sex differences in PO levels in populations evolved with or without sexual selection (Hosken 2001). Hosken (2001) also found that monogamous populations had higher PO levels than polygamous populations, although here also this difference did not translate into differences in bacterial clearance after infection (Hosken, 2001). The above studies manipulated the presence or absence of either a pathogen or sexual selection. In the work reported here, we manipulated both pathogen and sexual selection in order to test for effects of the presence of each, as well as any interaction, on the evolution of pathogen resistance.

We carried out a 2-way factorial evolutionary experiment manipulating sexual selection and exposure to a pathogen. We let replicate populations of *D. melanogaster* evolve for 14 generations either under controlled monogamy or random polygamy (i.e., with or without sexual selection (Hollis & Houle, 2011), in each generation exposing males to either an intestinal pathogen (a gram-negative bacterium *Pseudomonas entomophila*) or a sham treatment. In our experimental design, we only exposed males to the pathogen and allowed the males to interact with females beginning one day after exposure to the pathogen (we verified that males had cleared the bacteria from their gut at this timepoint, and thus did not infect females). With this design, we aimed to increase the opportunity for sexual selection to act via differential mating success of males differentially coping with infection. We aimed to address several interconnected questions.

First, and most simply, do *D. melanogaster* populations exposed to the pathogen as adults evolve resistance, measured as survival after infection, over a short timescale? Resistance to *P. entomophila* has been reported to evolve after only four generations of strong selection imposed by breeding from flies that survived a prior infection (Martins *et*

al., 2013). Second, if only one sex—in our design, males—experiences the pathogen, would evolved resistance to *P. entomophila* be detectable in the other sex? If evolved resistance is evident in both sexes, this would indicate a shared genetic basis. Third, would sexual selection lead to the evolution of differences in pathogen resistance even in the absence of the pathogen? This would be predicted if there were an additive genetic correlation between male sexual traits and resistance that were expressed irrespective of pathogen exposure (Joye & Kawecki, 2019). A result supporting this prediction has been reported in *Tribolium* (Hangartner *et al.*, 2015) and *Callosobruchus* (Bagchi *et al.*, 2021); however, the conclusion was based on quantifying an aspect of immune response rather than resistance to an actual pathogen. Fourth, does sexual selection accelerate the evolution of resistance in populations exposed to the pathogen, and does it do so to a greater degree than would be expected based on the sum of effects of sexual selection and pathogen exposure acting alone? This positive interaction between the effects of pathogen and sexual selection would be expected if heritable variation in pathogen resistance influenced infected males' sexual success.

The rationale of this study relied on the pathogen affecting the sexual success of males. Therefore, prior to the evolutionary experiment we tested if infection with *P. entomophila* affects competitive paternity share. Mortality in our laboratory population was much lower than is generally reported (Martins *et al.*, 2013; Faria *et al.*, 2015; Joye & Kawecki, 2019), but uninfected males had greater competitive paternity success than infected males. If genetic variation conferring resistance to *P. entomophila* has a similar positive effect on male competitive success after exposure to the pathogen, this scenario should provide an opportunity for female choice to amplify nonsexual selection and accelerate adaptation to pathogen.

Materials and Methods

Stock populations and experimental conditions

The experimental populations were established from a long-term laboratory population called Ives (IV) that was initiated from about 200 wild *D. melanogaster* of each sex collected in Massachusetts in 1975 (Charlesworth & Charlesworth, 1985). This population has been maintained in the lab at high density, with a census size in thousands, for more than 30 years and is adapted to the laboratory environment (Houle & Rowe, 2003). In the sexual competition experiment, we also used a reference population homozygous for a recessive *ebony* mutation previously backcrossed into the IV stock. To estimate pathogen virulence during experimental evolution, in each generation we ran a control using a line homozygous for a recessive *relish* mutation. The *relish* mutation blocks the Imd pathway that plays an important role in defense against gram negative bacterial pathogens (Hedengren *et al.*, 1999); *relish* mutants are therefore highly susceptible to *P. entomophila* (Vallet-Gely *et al.*, 2010).

All flies in the experiment were maintained on fly media composed of (for 1L water): 6.2g Agar powder (ACROS N. 400400050), 58.8g Farigel wheat (Westhove N. FMZH1), 58.8g yeast (Springaline BA10), 100ml grape juice; 4.9ml Propionic acid (Sigma N. P1386), 26.5 ml of Methyl 4-hydroxybenzoate (Nipagin M, VWR N. ALFAA14289.0) solution (400g/l) in 95% ethanol. Populations were kept at 25^0C with a 12L:12D cycle.

Sexual success of infected versus uninfected males

To determine whether infection has any effect on male sexual success, we compared the competitive paternity success of infected and sham-treated males (infection protocol described below). Because the infected and uninfected males came from the same population, we would not be able to distinguish paternity in direct competitions. We therefore competed each against males from a reference population homozygous for the *ebony* marker.

Each replicate consisted of five focal males (either infected (N = 38) or sham-treated (N = 39)) and five *ebony* males competing for five *ebony* females. These flies were allowed to interact for 48 hours before being discarded. The resulting offspring were scored upon

emergence as adults. The recessive *ebony* mutation enabled us to distinguish offspring sired by the focal males (which would have wild type cuticles) and those sired by the reference males (which would have dark cuticles). The proportion of wild-type offspring was then used as a measure of sexual success of the infected versus non-infected focal males. Even though the fraction of wild-type offspring may deviate from the actual fertilization success of focal males because of differences in egg-to-adult survival of wild-type and *ebony* offspring, this would affect the estimates for the two types of males in the same way.

Experimental regimes and selection protocol

To study the interplay between sexual selection (SS) and pathogen presence (P), we used a factorial design that manipulated the presence or absence of SS (polygamous versus monogamous mating systems) and the presence or absence of our pathogen, *P. entomophila,* resulting in 4 experimental regimes (+SS +P, +SS –P, -SS +P, -SS -P). Within each experimental regime, 3 replicate populations were established. To establish experimental populations, adults were obtained by amplifying flies from the IV base population stock, collecting virgin flies, and randomly assigning 80 males and 80 females to each of the 12 populations. At 5-6 days old, virgin males were orally infected with *P. entomophila* (protocol described in the following section) in +P treatments and sham-infected in –P treatments. Males were mated with virgin females for 72 hours after being exposed to infection for 24 hours (see below). Under the +SS experimental regimes, groups of 5 males and 5 age-matched virgin females were placed in interaction vials. Under the –SS regimes, groups of 1 male and 1 age-matched virgin female were placed in interaction vials. Flies were left in these interaction vials for 72 hours, after which mated females from each population were pooled and re-distributed in groups of 20 to new vials for egg laying. Females were allowed to lay eggs for 72 hours, after which they were discarded from the vials while larvae developed. The density of mated females was therefore controlled in the egg-laying vials but we did not further control for egg density, which appeared qualitatively the same across regimes and populations throughout the course of experimental evolution. We collected virgins from all experimental populations

on day 12 and 13 (and occasionally on day 14) from the start of egg laying. Although there was some adult emergence on the days before and after, these collections corresponded to the peak eclosion time and minimized the chance we inadvertently selected for faster or slower development. On emergence, virgins were collected and housed in groups of 20 in single-sex vials until they were 5-6 days old, at which point the experimental protocol was repeated. Populations were maintained under the experimental regimes for 14 generations at a population size of 160 individuals (80 males + 80 females).

Infections

The pathogen used in our experiments, *P. entomophila*, is a naturally-occurring gram-negative bacteria isolated from *D. melanogaster* in Guadeloupe (Vodovar *et al.*, 2005; Liehl *et al.*, 2006). It is acquired during feeding and at high doses kills about 60% of *D. melanogaster* adults within 72 hours and almost 70% of larvae in 48 hours (Liehl *et al.*, 2006). It has been found to elicit both local and systemic immune responses involving a range of host responses including the secretion of specific anti-microbial peptides, repair and regeneration of epithelial cells in the gut as a result of damage caused by the pathogen (Vodovar *et al.*, 2005; Liehl *et al.*, 2006), and leads to large scale changes in gene expression in response to this pathogen (Chakrabarti *et al.*, 2012). This system has been used to study the genetic basis of immunity (Chakrabarti *et al.*, 2012; Neyen *et al.*, 2014; Bou Sleiman *et al.*, 2015) as well as in an evolutionary context in work looking at life-history trade-offs (Vijendravarma *et al.*, 2015) and sexual selection (Joye & Kawecki, 2019).

We obtained an isolate of *P. entomophila* from Bruno Lemaitre (EPFL). Bacteria were plated from glycerol stocks 3 days prior to infection on standard LB-agar plates supplemented with 1% milk and grown for two days at room temperature. On the day before the infection, a single colony was transferred to a 50ml Erlenmeyer pre-culture flask with 12.5ml LB and incubated for 8 hours in a shaking incubator at 29°C and 180rpm. The pre-culture flask was then transferred to a 2L Erlenmeyer flask with 400ml LB

(or 1L Erlenmeyer with 200ml LB) and the culture was incubated overnight in the same shaking incubator at 29°C and 180rpm. On the next day, the bacterial culture was centrifuged at 2500 g at 4°C for 20 min. The pellet was re-suspended and mixed with sucrose and water to obtain a final infection cocktail with an OD of 300. The sham treatment was performed with a 2.5% sucrose solution.

Oral infection was performed as previously described (Neyen *et al.*, 2014). Flies were first starved for 4 hours and then transferred to a vial with a filter paper layered over food and soaked with 150µl of the bacterial cocktail. Males were left in these vials for 24-26 hours after which they were transferred to interaction vials with females. Dead flies were counted at 2, 4, 20 and 24 hours after pathogen exposure.

Bacterial load in infected males

To examine how fast *D. melanogaster* males clear the *P. entomophila* infection, we infected 1-2 day old virgin males in groups of 20 individuals as described above. We then measured bacterial load of individual flies at 4, 8 and 24 h from the onset of the infection treatment, randomly choosing 2 infection vials to sample at each timepoint. We carefully removed survivors by light anaesthesia and randomly selected 5 individuals.

Each individual fly was then placed in an Eppendorf tube containing small glass beads and 100µL of 70% ethanol to surface sterilize the fly cuticle. The tube was inverted a few times to ensure proper mixing after which the 70% ethanol was removed and replaced by 100µL of Luria broth (LB). We then placed the Eppendorf tubes on a Precellys bead ruptor for 30 seconds at 6000rpm in order to homogenize the flies. The homogenate was then serially diluted to obtain concentrations of 1:10, 1:100, 1:1000, 1:10,000 and 1:100,000. We plated 3 µL of each of these dilutions in 5 replicates on a single LB plate containing 1% milk. The plates were left for 50 hours at room temperature and colonies from each dilution and replicate were counted. For each dilution and time point combination, we calculated an average count of the number of colonies for the 5 technical replicates (from

each sample) followed by calculating the total colony forming units using the formula below:

$$Total\ Colony\ Forming\ Units = Number\ of\ colonies\ for\ a\ given\ dilution \times Dilution\ factor$$

Survival assays at generation 14

To assess adaptation to pathogen, two blocks of survival assays were done on males and females after 14 generations of experimental evolution. To avoid parental effects, we first reared individuals from all populations for one generation in a common garden. To establish the common garden, collected virgins were housed together in vials containing 20 males and 20 females. These individuals were allowed to mate for 72 hours, after which males were discarded. Females (N = 120 per block) from these mating vials were then collected and housed together for 72 hours in groups of 20 to lay eggs. After discarding the females, larvae were allowed to develop and emerging virgin males and females were collected and housed in single-sex groups of 20 each. Virgins (age at infection: Batch 1 – 5-7 days, Batch 2 – 4-5 days) were exposed to *P. entomophila* in the same manner as described above in single-sex groups of 20. After exposure to the pathogen for 24 hours (OD_{600nm} of infections: Batch 1 – 280, Batch 2 – 300) individuals from each vial were transferred to fresh vials and per vial deaths were scored at 2, 4, 20, 24, 28 (the first time point after transfer to new vials), 44, 52 and 72 hours after pathogen exposure. Alongside the infections, two vials were sham-treated for each of the populations to serve as controls. In each block, we again used flies with a *relish* mutation to ensure that the pathogen was virulent (Vallet-Gely *et al.*, 2010).

Statistical analysis

We performed all statistical analyses in R v3.4.3 with the package afex (Singmann *et al.*, 2015), a wrapper for lme4 (Bates *et al.*, 2011). We fit generalised linear mixed models (glmer) with the binomial family (logit link) where the response was the phenotype of each emerging fly (wild type or ebony, binary) in competitive mating success assays or the survival status of each fly (alive or dead; survival 72 hours post-infection) in the survival assays after 14 generations of experimental evolution. For the latter, we fit one

model that included all the data (both male and female survival) and included effects of sexual selection, pathogen presence, sex, and all interactions. We also fit simpler models on sex-specific subsets of the data that excluded an effect of sex. In all models, we included experimental block, population, and vial (nested within population) as random effects.

Results

To assess the potential for sexual selection to act on pathogen resistance, we first compared the paternity success of infected and sham-treated males in competition with males from a reference strain. We found that infected males had lower competitive mating success than uninfected males, as evidenced by a lower proportion of offspring sired by the focal males (treatment effect: $\chi^2_{df=1} = 4.45$; $p = 0.03$; **Figure 1**, **Table 1**). Infected males sired on average 59.2% of progeny in competition with the competitive standard, while uninfected males sired on average 68.5% of progeny in competition with the competitive standard. This result indicated that infection harms male paternity success and suggested that genetic variation contributing to infection resistance might be favored by sexual selection.

We also verified that the infected males had cleared the pathogen from their gut by the time they were placed with females. Although males harbored many live *P. entomophila* 4h after the onset of the infection treatment, no live bacteria were detected at 8 or 24 h (**Figure S1**), in agreement with earlier results (Bou Sleiman *et al.*, 2015). Thus, there was little opportunity for the males to transmit the infection to the females. Bacterial clearance from male guts does not preclude ongoing systemic and immune responses in males resulting from infection, however, making it plausible that males experience lasting effects of infection on sexual success.

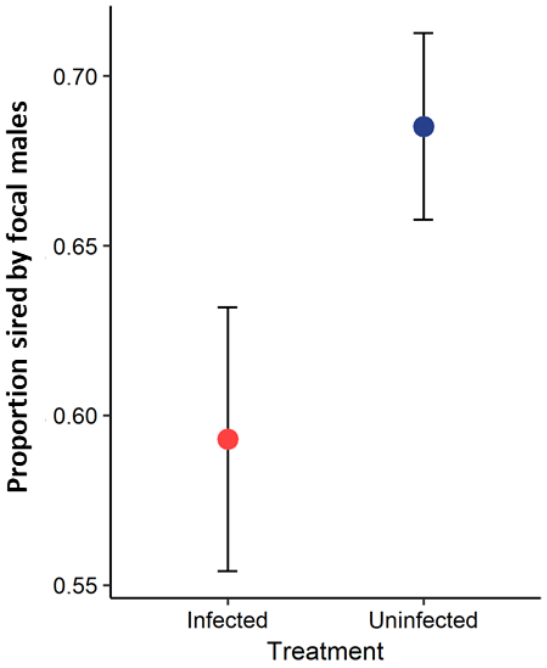

Figure 1 – Competitive reproductive success of infected versus uninfected wild type males in an assay including both pre- and post-copulatory effects. Paternity success is measured relative to a marked standard competitor.

We next evolved replicate populations with and without both sexual selection and pathogen for fourteen generations. Over the course of experimental evolution, *P. entomophila* virulence varied; the pathogen reliably killed a substantial fraction of the *relish* mutants (mean survival post infection 43% ± 10.7 (s.e.) in *relish* mutants, **Figure S2**). Survival at 24 hours was lower in experimental populations exposed to the pathogen (+P), averaging 92.4%, than it was in populations not exposed to the pathogen (-P), in which survival was 99.9%.

To compare resistance to *P. entomophila* in the populations subject to the different regimes, we measured their survival following infection after fourteen generations of experimental evolution and one generation of common garden rearing. In general, females survived less well after infection than males (**Figure 2**). Populations evolved

under pathogen pressure (+P evolutionary regimes) showed better post-infection survival than populations evolved without pathogen exposure (-P evolutionary regimes) (pathogen selection effect: $\chi^2_{df=1}$ = 8.89; p = 0.002; **Figure 2, Table 1**). A significant three-way interaction between sexual selection, pathogen, and sex (SS*Pathogen*Sex, $\chi^2_{df=1}$ = 5.91; p = 0.01) indicates a difference between males and females in how the interaction between sexual selection and pathogen presence affects post-infection survival, which we further explored in sex-specific analyses.

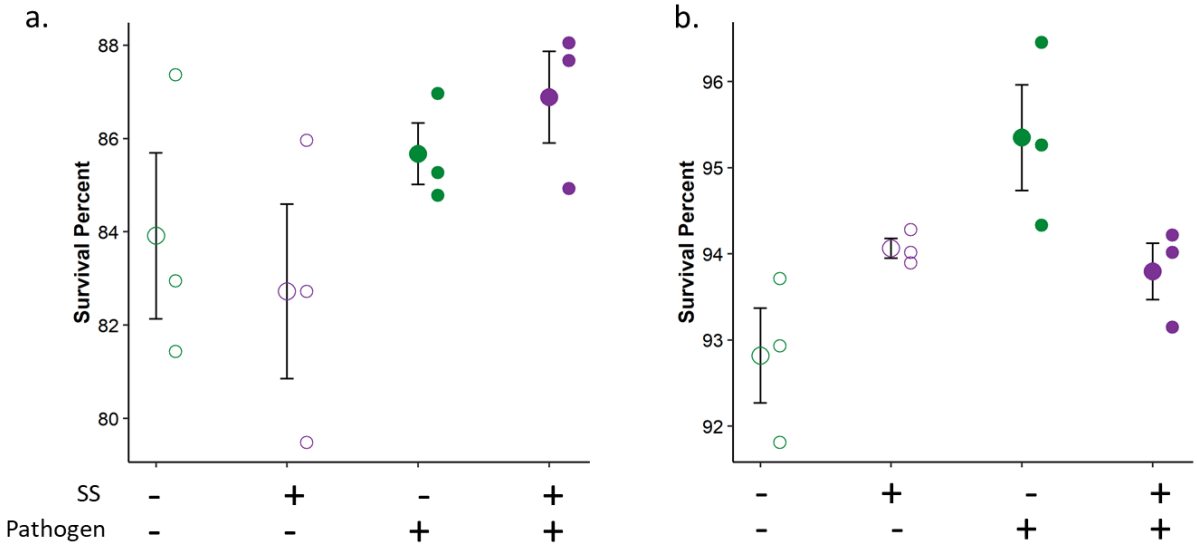

Figure 2 – Survival at 72 hours post infection with *P. entomophila*, for females (a) and males (b) pooled for both batches. Larger circles indicate the mean (± s.e.) of each evolutionary regime, while the smaller points represent the replicate populations within each regime.

In females, the sex-specific analyses showed that post-infection survival under +P regimes was better than that in the –P regimes (**Figure 2a**, pathogen selection effect: $\chi^2_{df=1}$ = 4.92; p = 0.026), but we detected no effect of sexual selection ($\chi^2_{df=1}$ = 0.04; p = 0.82) or any interaction between sexual selection and pathogen ($\chi^2_{df=1}$ = 0.93; p = 0.33). In

males, there was neither a significant effect of sexual selection (**Figure 2b**, $\chi^2_{df=1} = 0.094$; $p = 0.75$) nor pathogen ($\chi^2_{df=1} = 3.40$; $p = 0.06$). However, there was a significant interaction between sexual selection treatment and pathogen presence ($\chi^2_{df=1} = 4.71$; $p = 0.029$). For the good genes hypothesis to be true in our case, the +SS +P populations should have elevated survivorship compared to -SS +P regimes. However, in our study we see the opposite effect, with the -SS +P regimes surviving significantly better than +SS +P (**Figure 2b**, Tukey's post hoc comparison $p = 0.02$). At the same time, there is no difference between +SS populations evolved with and without pathogen. This difference in the effect of sexual selection that depended on whether the pathogen was present or not during the course of experimental evolution is what drives the significant interaction between sexual selection and pathogen.

Discussion

In our study, we aimed to address the interplay of sexual selection and pathogen presence on the evolution of resistance to a pathogen, *P. entomophila*. We found a signature of pathogen resistance in populations evolved under pathogen pressure for fourteen generations when compared to populations evolved without it. Surprisingly, despite only infecting males over the course of experimental evolution, resistance to pathogen was more prominent in females. We did not find any evidence that sexual selection can promote the evolution of resistance to the pathogen, contrary to the predictions of theory (Hamilton & Zuk, 1982; Westneat & Birkhead, 1998). We expected that the presence of sexual selection and pathogen pressure would act synergistically, resulting in a greater response to selection and therefore improved survival post-infection. We instead found an antagonistic interaction between the two in males, which could have possibly impeded the evolution of pathogen resistance.

Evolution of increased resistance of *D. melanogaster* to enteric infection and systemic infection has been seen in studies that have experimentally evolved fly populations with *P. entomophila* (Martins *et al.*, 2013; Gupta *et al.*, 2016). The study by Martins *et al.* (2013)

imposed very strong selection on both sexes, with pathogen-induced mortality up to 77% in the initial generations. In our experiment, pathogen selection was only applied on males and was associated with much lower mortality (5-25% depending on the generation). This lower virulence likely resulted from a difference in the bacterial genotype and/or the initial *Drosophila* gene pool; the IV population is generally robust and harbors high levels of genetic variation. It is likely that the overall strength of selection for resistance was therefore considerably lower in our experiment, but yet still sufficient to generate a detectable response. A stronger response to selection might have been obtained with a more virulent pathogen, or if both males and females had been infected each generation. Infecting females introduces a difficulty, however, in that reductions in female mating rate and fecundity make maintenance of experimental populations more challenging, and any reductions in female choosiness due to infection would be expected to diminish the importance of sexual selection. Lastly, it is also possible that effects of sexual selection and its interaction with the presence of pathogen, if present, would be detectable with a longer time scale as used in other studies (Rundle *et al.*, 2006; Fricke & Arnqvist, 2007). However, the time scale used in our experiment was sufficient to detect both evolved survival differences in females from different regimes as well as an interaction of sexual selection with pathogen resistance in males that indicated a negative effect of sexual selection on adaptation to pathogen.

The fact that females from populations under pathogen pressure evolved higher resistance despite not experiencing direct selection supports a shared genetic basis for immunity between the sexes. Indeed, in line with this idea (Collet *et al.*, 2016; Connallon & Hall, 2016), adaptation to desiccation resistance in experimentally evolved populations of *D. melanogaster* was observed both in males and females even when selection was imposed only on males (Gibson Vega *et al.*, 2020). Adaptation in our experiment may also be more evident in female post-infection survival simply because females show generally lower survival upon infection relative to males, which would make any evolved differences in survival easier to detect in females than males. Moreover, it is also plausible that alleles contributing to immunity that were favored in males under pathogen

pressure had a larger effect size on resistance in females, making female resistance towards the pathogen more detectable in this sex. We can exclude the possibility that selection did in fact act directly on females, for example by sexual or social transmission of the pathogen from males to females, because the pathogen was cleared by males by the time they encountered females. However, clearance of pathogen post infection from male guts does not preclude an ongoing immune response resulting from infection. This ability of a male to tackle the pathogen and mount a systemic or local immune response could have been a target of sexual selection.

In our study, we do not see any evidence that sexual selection aids the evolution of resistance to the pathogen. This however, does not preclude the possibility that there might have been benefits conferred by sexual selection in +SS regimes. Previous studies have attributed the lack of adaptation to novel environments to the negative effects of sexual conflict (Holland & Rice, 1999b; Rundle *et al.*, 2006). In a scenario where sexual conflict and sexual selection exert equal but opposing effects, both +SS –P and –SS +P regimes could show similar levels of adaptation. However, if sexual conflict negatively affected adaptation in our populations, we would have expected to find that populations exposed to the pathogen each generation but not experiencing sexual selection (-SS +P) would show a stronger signal of adaptation to pathogen than those exposed to pathogen and experiencing sexual selection (+SS +P). While our results on male survival after infection align with this idea, there is no signal of a cost to sexual selection in female survival after infection, leaving it difficult to attribute any importance to sexual conflict in our experiment.

In conclusion, our study found that populations of *D. melanogaster* evolved resistance to the insect pathogen *P. entomophila*, but this was either not facilitated (in females) or hindered (in males) by sexual selection. We expect that the low mortality in our study compared to previous work on this pathogen (Gupta *et al.*, 2013, 2016; Martins *et al.*, 2013; Joye & Kawecki, 2019), in which the majority of infected individuals die, provided a level of biological realism. The pathogen was still virulent enough to induce downstream

effects on male sexual success, suggesting that genetic variation conferring resistance to pathogen would provide a large target for sexual selection. In addition, because most males survived infection during the course of experimental evolution, this provided an opportunity for sexual selection to reinforce non-sexual selection by magnifying more subtle differences in pathogen resistance (e.g. differences in male condition or vigor that might emerge after weathering the infection). Despite a scenario that seems favorable for the detection of putative benefits of sexual selection—a relatively mild pathogen that might persist in natural host populations, that still yet influences mating success, in a host that harbors genetic variation for resistance—we found no such benefits.

Author contributions

S.S. and B.H designed experiments, S.S. performed experimental work. S.S. and B.H. analyzed the data. S.S., B.H. and T.J.K. wrote the manuscript and contributed to revisions.

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Data Accessibility

Data is available on dryad digital repository on this link - https://doi.org/10.5061/dryad.6djh9w12w

Competing interests

We declare that we have no competing interests.

Tables

Table 1. Models for survival assays at 72 hours post infection with *P. entomophila* after one generation of common garden rearing after generation 14.

Model	***Intercept***	***df***	***X^2***	***P***
Generation 14				
Survival (full model)				
SS	-2.24	1	0.01	0.91
Pathogen		1	8.89	***0.002***
Sex		1	245.32	$< 2.2e^{-16}$
SS:Pathogen		1	1.33	0.24
SS:Sex		1	0.14	0.70
Pathogen:Sex		1	0.0074	0.93
SS:Pathogen:Sex		1	5.97	***0.014***
Male Survival	-2.78			
Sexual selection		1	0.094	0.75
Pathogen		1	3.40	0.065
Sexual selection x Pathogen		1	4.71	***0.029***
Female survival	-1.72			
Sexual selection		1	0.04	0.82
Pathogen		1	4.92	***0.026***
Sexual selection x Pathogen		1	0.93	0.33

Supplemental Figures

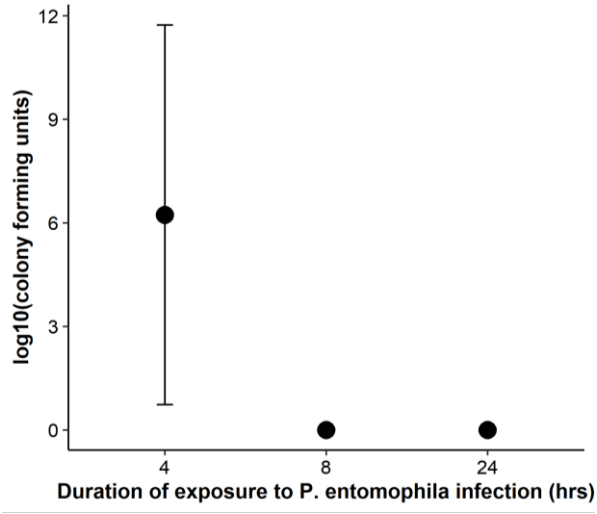

Figure S1. Internal bacterial load of virgin males exposed to *P. entomophila* following the infection protocol used in this study. The males harbor no detectable levels of live bacteria 8 hours from the onset of infection despite still being housed in the infection vials. Male survival in this experiment was similar to that seen during the course of the experimental evolution (survival at 24 hours: 94.5%).

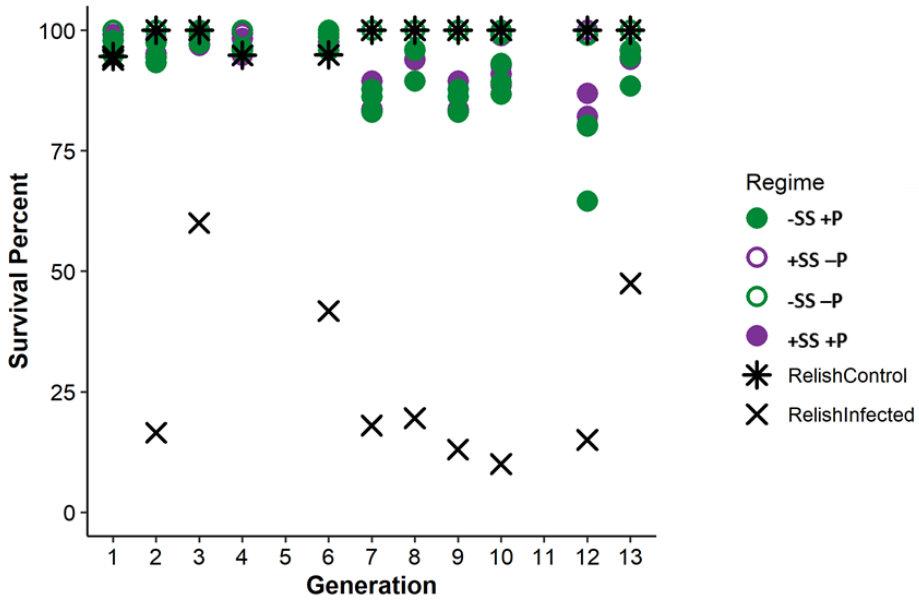

Figure S2. Survival of experimental evolution lines under *P. entomophila* infection (+SS +P and –SS +P) and sham infection (+SS –P and –SS –P). *relish* mutants were infected at every generation alongside experimentally evolved lines to estimate pathogen virulence
**relish* control refers to *relish* mutants exposed to sham infection to estimate background mortality in these lines.

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Chapter 3

The genomics of sex-specific and sexually antagonistic immunity in *Drosophila melanogaster*

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Introduction

Pathogens are rife in nature and immune performance is therefore important in determining survival (Punt *et al.* 2019). The diversity of pathogens requires multiple levels of responses from the immune system, at both the innate and adaptive (the two major arms of the immune system) levels. The genetic basis of the immune system involves multiple genes throughout the genome (Schmid-Hempel 2005; Lemaitre and Hoffmann 2007; Barreiro and Quintana-Murci 2010), many of which are pleiotropic influencing both immune responses and physiological and developmental processes. For instance, the reactive oxygen species provide an important mechanism in decimating pathogens, but also acts as signalling molecules (Forman *et al.* 2010; Lazzaro and Schneider 2014). This indicates that the genetic basis of immunity is broad.

While both sexes likely share some of the genetic basis of immunity through their shared genomes, there is also some degree of dimorphism between the sexes (Klein and Flanagan 2016; Duneau *et al.* 2017; Leech *et al.* 2019; Belmonte *et al.* 2020). For instance, males and females differ in major immune pathways, such as Toll and IMD (Klein and Flanagan 2016), leading to differences in how quickly each sex will clear a pathogen (Duneau *et al.* 2017; reviewed in Belmonte *et al.* 2020). Globally, the two sexes also show sexually dimorphic gene expression changes in response to pathogens, indicating broad intrinsic differences in how resistance functions in males and females (Duneau *et al.*, 2017).

Sexual dimorphism in immunity is driven by divergent fitness optima (Bonduriansky and Chenoweth 2009), as males and females face different selective pressures owing to different life histories (Zuk and McKean 1996; Rolff 2002; Zuk and Stoehr 2002; Nunn *et al.* 2008). For instance, optimal evolutionary strategies for males might involve greater relative investment into mating effort to maximise offspring quantity, while females might invest more towards maintenance and longevity. This gives rise to the prediction that the two sexes will invest in immunity differently to the extent that life-history optima differ (Zuk and McKean 1996; Rolff 2002; Zuk and Stoehr 2002; Nunn *et al.* 2008),

although which sex invests relatively more or less might be difficult to predict (Stoehr and Kokko 2006). Evidence for divergent evolutionary pressures on the two sexes is borne out by the fact that males and females achieve similar levels of immunocompetence using different mechanisms, and by different relative investment towards innate and adaptive arms of the immune system (Kelly *et al.* 2018). For example, in arthropods, females show higher bactericidal activity and melanisation, whilst males have higher interleukin responses to bacterial pathogens. These findings indicate that while immunity has a shared genetic basis, it also simultaneously has a sex-specific genetic architecture (Kelly *et al.* 2018).

The genetic architecture of immunity is relatively well-established in *Drosophila melanogaster* (Belmonte *et al.* 2020). Genome-wide association studies (GWAS) have highlighted the polygenic nature of immunity characterised by the additive effects of many variants, finding that a very small number of variants explain most of the variance in immunity levels (Tinsley *et al.* 2006; Magwire *et al.* 2012; Bou Sleiman *et al.* 2015). For example, chromosomal substitution line experiments have demonstrated that genetic polymorphisms underpinning pathogen recognition genes—rather than anti-microbial peptide expression—are significantly associated with variation in bacterial load (Lazzaro *et al.* 2006; Sackton *et al.* 2010). GWA studies have highlighted the diverse nature of variants associated with immunity in *D. melanogaster* towards different bacterial pathogens—e.g., *P. entomophila* (Bou Sleiman *et al.* 2015) and *P. aureginosa* (Wang *et al.* 2017)—involving pathways associated with stress response and developmental processes, morphogenesis, and tissue maintenance. Moreover, similar findings from GWAS in viral pathogens also bolster the polygenic basis of immunity (Magwire *et al.* 2012).

The genomic basis of sex-specific immunity, however, is not very well understood (Belmonte *et al.* 2020). Research has already demonstrated that variants on sex chromosomes can drive sex-specific effects on immunity and that polymorphisms on the X chromosome can lead to sex-specific gene expression and sexually antagonistic effects

on bacterial load (Hill-Burns and Clark 2009). Similarly, certain polymorphisms on the Y chromosome can drive differential autosomal and X gene expression associated with immunity (Kutch and Fedorka 2015). However, only a small subset of the GWA studies above has studied the sex-specific basis of immunity, finding relatively few candidate variants acting in a sex-specific manner and/or with sexually antagonistic effects on immunity (Wang *et al.* 2017; Shahrestani *et al.* 2021).

The genetic architecture of immunity is primarily known from studies on inbred lines (e.g. the *Drosophila melanogaster* genetic reference panel) (Bou Sleiman *et al.* 2015). Although sequenced inbred lines greatly facilitate GWA studies, high levels of homozygosity (DGRP lines have an inbreeding coefficient of 0.98; MacKay *et al.* 2012a) and the fixation of recessive alleles do not reflect a natural scenario. Moreover, the high degree of linkage disequilibrium between markers of the GWAS has limited our ability to determine which genetic variants are causative (MacKay *et al.* 2012b). To overcome the above issues, we used a lab-adapted, outbred population of *D. melanogaster* to identify the genomic architecture of sex–specific immunity. We performed a bulked segregant analysis and sequenced populations before (baseline) and after infection (survivors), measuring allele frequencies in pools of males and females independently. To perform the experiment, we split our batches into multiple independent blocks. This allowed us to both generate a massive sample size of well-estimated before/after allele frequencies, and we were able to see how repeatable allele frequency changes were from one replicate experiment to the next.

In our study, we used the host-pathogen system of *D. melanogaster* and *Pseudomonas entomophila*. The latter is a gram-negative generalist pathogen that leads to the activation of both local and systemic immune responses in the host including secretion of specific anti-microbial peptides (Vodovar *et al.* 2005; Liehl *et al.* 2006). A GWAS has identified 27 quantitative trait loci (QTL) associated with immunity against *P. entomophila*. While most of these QTLs were of "low effect", two of the most strongly associated variants explaining 15% of the variance in survival to pathogen were in the

neurospecific receptor kinase (*Nrk*) which is part of stress response. Another QTL was present in the *Gyc76C* gene, a modulator of the IMD pathway playing a role in response to salt stress. However, this study only examined females and did not identify candidates with high confidence (false discovery rate of 66% for the top candidate).

In this study, we set out to answer the following questions. First, what genetic variants underlie immunity in both sexes? Second, are there any sex-specific signatures of immunity in the genome (i.e. variants associated with immunity in one sex but not the other)? Here, we also asked if we could see any signal of sexually antagonistic effects on immunity, i.e. are there any alleles that are associated with greater resistance in one sex while having an opposite effect in the other sex. Our approach led to the identification of a strong signature of genetic variation throughout the genome involved in immunity in both sexes. Moreover, we identified many variants that differed between the sexes, with some candidates showing signatures of sexually antagonistic selection, i.e. the same allele associated with resistance in one sex and susceptibility in the other.

Materials and methods

Fly rearing

The experiment was carried out on a long-term laboratory population called Ives (IV) that was initiated from about 200 wild *D. melanogaster* of each sex collected in Massachusetts in 1975 (Charlesworth and Charlesworth 1985). This population has been bred under lab conditions for more than 30 years and has been adapting to the lab environment (Houle and Rowe 2003).

Flies were maintained on standard cornmeal medium, with the appropriate ethanol concentration added just prior to dispensing food into shell vials and bottles. We performed 8 batches of infection where we infected *D. melanogaster* with a naturally occurring intestinal pathogen *Pseudomonas entomophila*. For each batch, we first multiplied flies from the base IV population to a population size of approximately 2500

individuals established in vials with an adult density of 30 individuals per vial. They were allowed to mate for 3-4 days before being discarded from the vials. Emerging virgins ($N_{flies/sex/vial}$ = 20) from these vials were then collected and allowed to age for 3-4 days before using them in the experiment.

The fly medium composed of (for 1L water): 6.2g Agar powder (ACROS N. 400400050), 58.8g Farigel wheat (Westhove N. FMZH1), 58.8g yeast (Springaline BA10), 100ml grape juice; 4.9ml Propionic acid (Sigma N. P1386), 26.5 ml of Methyl 4-hydroxybenzoate (VWR N. ALFAA14289.0) solution (400g/l) in 95% ethanol. The temperature of the room was maintained at 25^0C with a 12L:12D cycle. All virgins were segregated using carbon dioxide as an anaesthetic.

On the day of the infection protocol, all vials were checked for the presence of larvae to confirm virginity. To obtain estimates of starting allele frequencies, serial numbers of 25 vials ($N_{total\ in\ 25\ vials}$ = 500) were chosen using a random number table specifying vial number, and all flies from these vials were collected in Eppendorf tubes and flash frozen at -80^0C for further DNA extraction. This experimental procedure was repeated for eight separate rearings that comprised our experimental blocks.

Infecting with P. entomophila

The pathogen used in this experiment, *P. entomophila*, is a naturally occurring gram-negative bacterium, isolated from *D. melanogaster* in Guadeloupe (Vodovar *et al.* 2005; Liehl *et al.* 2006); an isolate of *P. entomophila* extract was obtained from Bruno Lemaitre. To prepare the bacterial pellet for infection, bacteria were plated from glycerol stocks 3 days prior to infection on standard LB-agar plate supplemented with 1% milk and grown overnight for two days at room temperature. On the day before the infection, one single colony was transferred to a 50ml Erlenmeyer flask with 12.5ml LB and incubated for 8 hours in a shaking incubator at 29°C with 180rpm. Each pre-culture flask was then transferred to a 2L Erlenmeyer flask with 400ml LB (or 1L Erlenmeyer with 200ml LB) and the culture was incubated overnight in the same shaking incubator as the pre-culture.

The following day, flies were infected with the pathogen cocktail which was prepared by first centrifuging the bacterial culture at 2500 g at 4°C for 20 min. The pellet was re-suspended and mixed with sucrose and water to obtain a final infection cocktail with an OD of 200 (measured at 600nm) and 2.5% sucrose (the control contained 2.5% sucrose solution).

Virgin flies ($N_{infected\ males\ or\ females}$ = 1000; $N_{control\ males\ or\ females}$ = 100) were exposed to pathogen (or control) in single sex vials of 20 individuals each through an oral infection route when they were 3-4 days old. Oral infection was performed as previously described (Neyen *et al.* 2014). The flies were first starved for 4 hours and then transferred to a tube with a filter paper layered over the food with 150µl of bacterial cocktail. The flies were left on these vials for 24-26 hours after which they were transferred to new vials with fresh food. Survivor counts were performed at 1, 4, 28, 42, 48 and 72 hours after the flies were transferred to infection vials. After the last count at 72 hours, all survivors were anaesthetized on a tray under the microscope and groups of 100 flies from this pool were transferred to Eppendorfs and flash frozen in liquid nitrogen. From the frozen survivor pools of each sex, 3 Eppendorfs containing 100 flies each were randomly chosen for sex-wise DNA extraction. Before proceeding for the extraction, flies from the chosen tubes were pooled together on ice and re-divided into pools of 25 flies in fresh Eppendorf tubes.

DNA extraction

To capture population-level genomic variation resulting from infection, we contrasted allele frequencies from baseline and survivors that we obtained by pooled sequencing. We collected males and females from the base population and their surviving counterparts from each infection batch, making up to a total of four of samples per batch. To make up each sample, we pooled 300 individuals, 600 chromosomes in each pool per status (baseline or survivor) that resulted in the generation of one sequencing library. We followed the recommendations from Schlötterer *et al.* (2014), where they say that while it is optimal to have at least a pool of 40 individuals, a larger pool size of

individuals results in higher accuracy of population level estimates resulting from pooled sequencing. To yield a fair representation of each individual in the pool, the pool was subdivided, DNA was extracted and concentration was measured from these sub-pools. These were then re-pooled in one sample by combining equal quantities of homogenates from each sub-pool (Lynch *et al.* 2014). From this master pool, 180µl was taken as a starting material for DNA extraction.

DNA extraction from preserved samples was done using the Qiagen DNeasy Blood and Tissue kit (Qiagen, Hilden, Germany) following the manufacturer's protocol. Individuals in each tube were first homogenised using the homogenisation buffer (provided with the kit) using small glass beads on the Precellys 24 Tissue homogeniser. This was followed by a proteinase K treatment (Qiagen ID 19133; 20mg/ml) at 56 degrees for 3 hours. The homogenate was pipetted out into fresh tubes and spun for 30sec to ensure that the supernatant was clear and then stored at -20^{0}C for a week before proceeding with the extraction.

DNA Sequencing

Paired end libraries were prepared using the TruSeq Nano DNA sample preparation kit (Illumina, San Diego, CA, USA) followed by a gel size selection performed on Pippin Prep (Sage Science, Beverly, MA, USA) to select fragments in the range of 450-490bp (350bp of library + Illumina adaptor). Paired-end reads were sequenced on the Illumina HiSeq 3000/4000 and FASTQ files were obtained with Illumina base calling pipeline bcl2fastq2 v2.17.1.14.

Before mapping, adaptors were removed using CUTADAPT (v1.18), followed by low quality reads (>30). Mapping was done with BWA v0.7.17 against the *D. melanogaster* reference genome (Release 6 plus ISO1 MT; dm6). We used the default mapping parameters of the software except for specifying -M (to mark the shorter split hits as secondary alignments). The alignment output files was specified in the SAM format. These SAM files were then filtered for reads mapped in proper pairs with a minimum

(and stringent) mapping quality of 30 and with no secondary alignments (-q = 30 and -F 0x100). This was done using SAMtools v1.4 (Li *et al.* 2009); the reads were then converted to a BAM format also using SAMtools v1.4. The mean mapped reads per sample was 42.6 million with 83.0% of them mapped. The BAM files were filtered for PCR duplicates with PICARD v2.17.8 (MarkDuplicates function). These files were then aligned around InDels using GATK 3.8.0. The filtered SAM files were combined into one file using SAMtools mpileup. From here on, we used the Popoolation2 software (Kofler *et al.* 2011) v1.2.01 and performed heuristic SNP calling based the following parameters: 1) minimum coverage from all samples 20, 2) maximum coverage from all samples 1000, 3) minimum read count for a given allele 5, across all samples pooled. We then processed the allele counts in R v3.4.3. First, we filtered all mono and tri-allelic variants, followed by the exclusion of variants with a coverage less than 20 or greater than 1000. Then, mean allele frequencies for males and females were calculated separately and only variants having allele frequencies between 0.05 and 0.95. The final number of variants surviving after these filters were 257,669. For the statistical models, we only considered data from chromosomes 2L, 2R, 3L, 3R, 4 and X.

We did not include variants on the Y chromosome for our analysis, even though polymorphisms on the Y chromosome are known to influence sex-specific immunity (Kutch and Fedorka 2015). This is because mapping of the Y chromosome results in weakly mapped and mis-mapped reads on autosomes, owing to highly repetitive and homologous sequences to autosomes (Chang *et al.* 2022), leading to erroneous allele frequency estimates between the two sexes.

Statistical analyses

We used R v3.4.3 with the package afex (Singmann *et al.* 2015) a wrapper for lme4 (Bates *et al.* 2011) for all statistical analyses. We fit generalized linear mixed models (glmer) with the binomial family (logit link) to assess differences in male and female survival towards the pathogen. In this model, the response variable was the count of the dead flies versus

counts of flies surviving from each replicate vial post infection with *P. entomophila* predicted by sex and a random effect of experimental block and vial.

We ran linear mixed effects models (lmer with a normally distributed error term) on allele frequencies at each locus to identify potential candidate variants underlying immunity in both sexes and to resolve candidate variants associated with an interaction of sex and immunity.

Allele frequency ~ C + S + C*S,
Where C refers to status – baseline (prior to infection) and survivor (post-infection) and S refers to sex; "experimental block" was taken as a random factor. Allele frequencies were transformed using the arcsine square root transformation commonly used to transform proportion data (Shahrestani *et al.* 2021). Linear mixed models, with even non-transformed response variables performed almost as well as the quasibinomial and better than regular binomial models (Wiberg *et al.* 2017).

We then calculated the false discovery rate (FDR) using the method by Storey and Tibshirani (2003) using the "qvalue" R package (version2.16.0; Storey and Tibshirani 2003). We chose a threshold of 0.2 to define top candidate variants that change consistently across all or most of the replicates.

Annotation of variants
All variants that crossed the threshold of q value<0.2 were run on variant effect predictor (McLaren *et al.* 2016) using the default parameters. The resulting gene names were matched to gene functions upon search on the Flybase database.

Functional analysis
We used the GOwinda software (Kofler and Schlötterer 2012) to identify enriched GO terms based on our candidate sites for those a. underlying immunity in both the sexes, b. showing sex-specific and sexually antagonistic immunity. For each individual analysis, we

plugged in the candidate list of variants found significant from the model outputs above. A gene annotation file for the *D. melanogaster* reference genome (6.14) was obtained from FlyBase, and a gene set file for relevant GO terms was obtained from FuncAssociate3.0 (Berriz *et al.* 2009). We then set the software to run for 10^5 simulations using the "gene definition" gene and mode parameters set to "gene" (i.e. we did not consider upstream or downstream regions in our analysis).

Results

Survival against P. entomophila

We found that on average 77±3% females and 73±3% males survived 72 hours post infection with *P. entomophila* (**Figure 1**). There was no survival difference between the two sexes ($\chi^2_{df=1}$ = 0.29, p = 0.53; **Supplementary Table 3**) and the average survival for controls, run for every block was 96.9±0.9% and 98.0±0.7% for females and males respectively. The variance explained by "block" as a random factor was 0.08± 0.28.

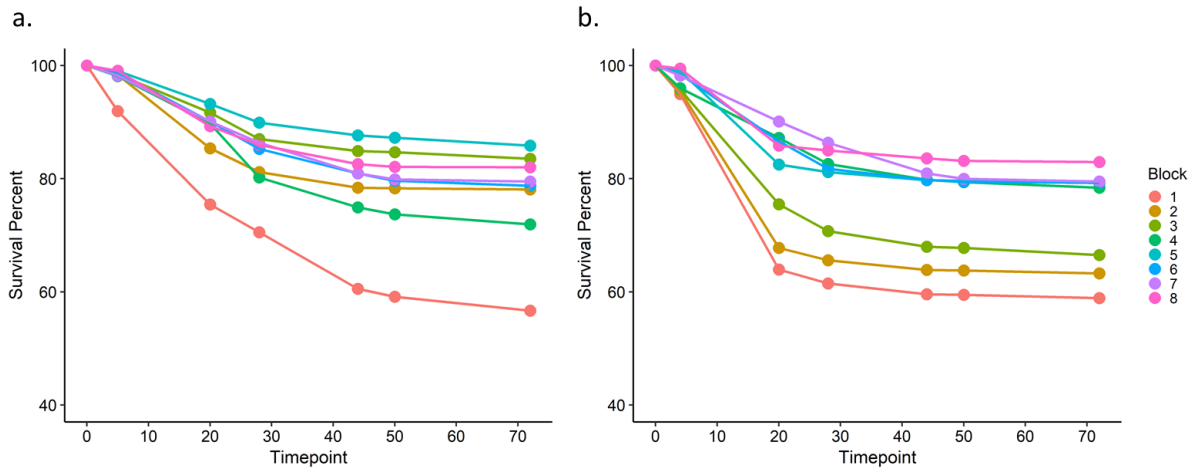

Figure 1. Post infection survival of females (a.) and males (b.) with *P. entomophila*.

Global patterns of genomic differentiation

We sequenced pools of 300 males and females from baseline and survivors for each batch of infection with *P. entomophila*. We identified 257,669 SNPs that satisfied the stringent SNP calling and filtering criteria. These SNPs were spread across the five major chromosomal arms (0.81% on X, 27.5% on 2L, 20.6% on 2R, 22.2% on 3L, 28.6% 3R), with a few on the dot chromosome 4 (0.069%). We did not find many variants on the X chromosome even though the total percentage of mapped reads was high. We used a stringent map quality filter and it is possible that many variants on the X chromosome were filtered out because of this, including those with a valid secondary alignment (e.g. to the Y chromosome). Genes on the X chromosome are often weakly mapped because they are homologous to genes on the autosomes (Webster *et al.* 2019).

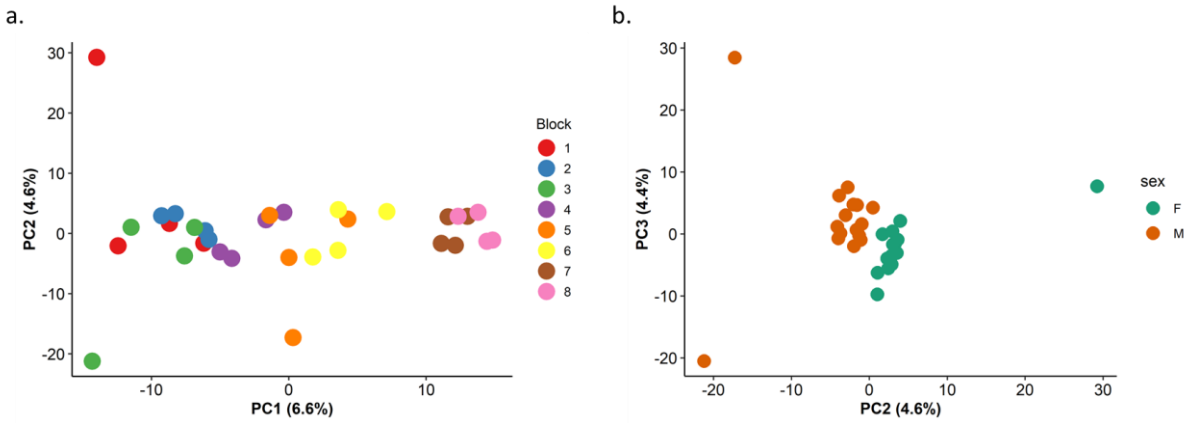

Figure 2. Principal component analysis on allele frequencies from 257,669 SNPs that satisfied the stringent SNP calling and filtering criteria, a. SNPs are separated by blocks indicating genetic drift in the founder population over time through the experiment. b. separation by sex in seen in the second and third and principal components.

Principal component analysis on allele frequencies of all SNPs distinguished the 8 temporally-ordered blocks on PC1 and PC2 (**Figure 2**; PC1: f = 63.0, P<0.001 and PC2: f = 0.94, P<0.001). This indicates that samples obtained within an experimental block,

whether male or female, or baseline or infection survivors, were more similar to one another than they were to samples from other experimental blocks. This observation likely reflects some genome-wide genetic drift in the founder/base population between experimental blocks over time. The following PCAs, from second to fourth, separated the sexes (**Figure 2**, PC1: t = -0.69, P = 0.49; PC2: t = -4.24, P <0.001; PC3: t = 2.18, P = 0.045; PC4: t = -4.93, P = <0.001; t = -0.59, P = 0.56), indicating that sex is a strong driver of genome-wide differences. It was only on the fifth PC that baseline and survivors were distinguishable (**Figure 2** and **Figure S1**, Welch's t-test - PC 1 scores t = 0.72, P = 0.47 (shows no separation between baseline and survivors); PC 5 scores t = -2.27, P = 0.037).

Variants underlying immunity

In our dataset, we detected 93 variants underlying immunity in both sexes at q <0.20 (**Supplementary Table 1**). These variants were primarily located across the different chromosomal arms of the autosomes (**Figure 3a**.); we found only one variant on the X chromosome.

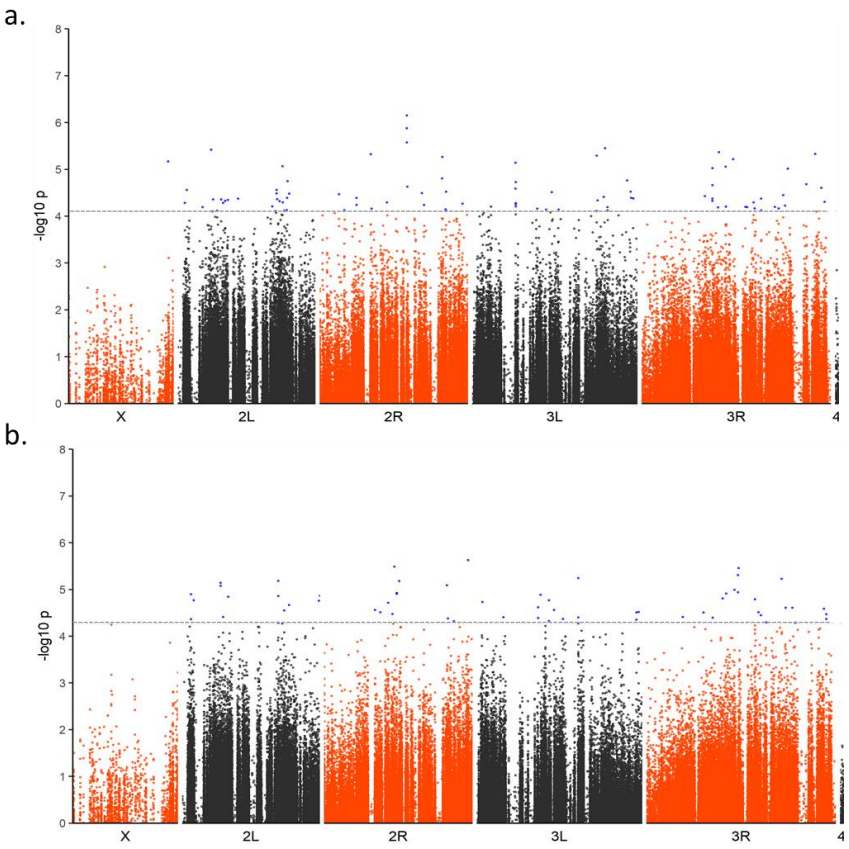

Figure 3. Differentiation of allele frequencies between baseline and survivor populations underpinning a. immunity in both sexes and b. interaction of immunity with sex. The grey dashed line refers to the –log(pvalue) corresponding to the storey's q value threshold of 0.2.

The estimate of pi0 (the proportion of true nulls across all tests) was 88%, indicating a strong signal (~12% of all variants tested) of marker association with survival to infection, indicating a broad genomic basis of immunity. **Figure 4** shows some representative SNPs underlying immunity and their allele frequency trajectories from baseline to survivors. On qualitative assessment of these candidates, we found that 23% of these were separated by less than a 100kb (a window chosen by Kawecki *et al.* 2021 to identify loci that might have been in linkage disequilibrium). It is possible that candidates within these 13 clusters were under linkage disequilibrium and might represent false positives, as they are not independent from neighbouring candidates (**Supplementary Table 1**).

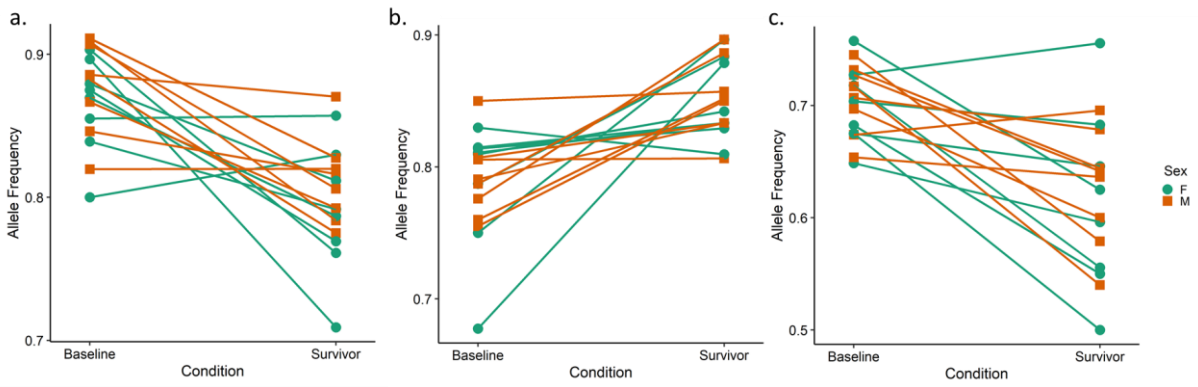

Figure 4. Representative candidate variants underlying immunity in both sexes. a. missense variant on locus 2R:14823520 in *pgm2b* gene (p = 7.11E-07; q value = 0.12) predicted to enable intramolecular transferase activity, phosphotransferases, involved in carbohydrate metabolic process and is expressed in spermatozoon. b. missense variant on locus 3L:7359780 in *pst* gene (p = 7.25E-06; q value = 0.13) annotated for function associated with long term memory and protein secretion. c. missense variant on locus 2L:7710491 in *tep3* coding for Thioester-containing protein 3 (p = 5.22e-05; q value – 0.19) - involved in the defence response to Gram-positive bacteria.

We found 63 variants underlying an interaction between infection status (baseline and survivor) and sex (**Supplementary Table 2**) across the different chromosomes (**Figure 3b.**). Amongst these candidates, 20 variants were associated with sexually antagonistic effects. These candidates were chosen on the basis that the gradient of the regression slope for these candidates was opposite with the similar magnitude. **Figure 5** shows representative candidate variants with sex-specific and sexually antagonistic associations.

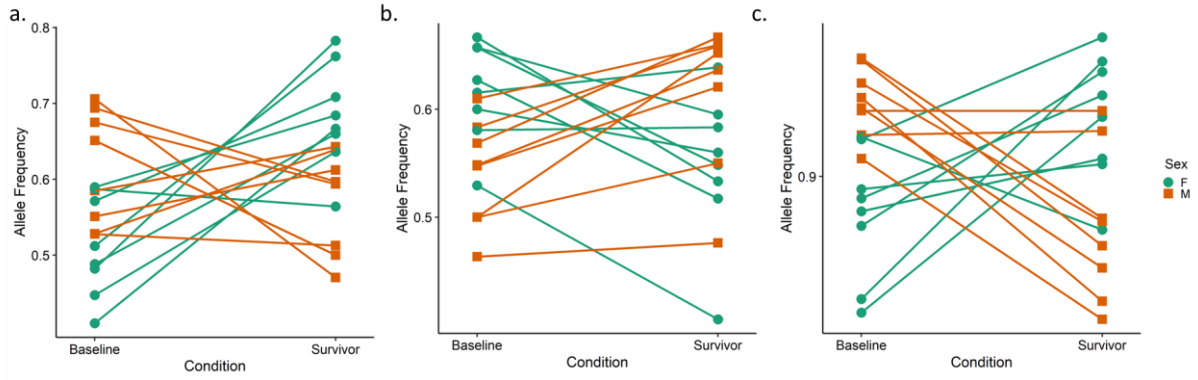

Figure 5 – Representative candidate variants underlying interaction between immunity and sex. a. missense variant on locus 3L:14633220 in the *dlp* gene (dally like; p = 4.28E-05, q value = 0.18), coding for a glypican that regulates the signaling strength and range of morphogens encoded by *hedgehog* and *wingless* genes. b. intronic variant on the locus 2R:24499737 in the *ssl* gene (Suppressor of Stellate-like; p = 2.36E-06 , q value = 0.15) showing sexually antagonistic selection. This gene enables protein kinase regulator activity and predicted to be involved in protein phosphorylation and regulation of protein serine/threonine phosphatase activity and to be part of protein kinase CK2 complex. c. synonymous variant on the locus 2R:11956089 in the *exp* gene (expansion; p = 3.25E-06 , q value = 0.15) showing sexually antagonistic selection, and encoding an atypical Smad-like protein which regulates tube size in the trachea through receptor tyrosine kinase (RTK) pathways, as well as apical secretion pathways.

The pi0 estimate of true null p values for sex-specific candidates was 89%, again indicating a broad genomic target (11% of the total variants) for sex-specific immunity. We found that 20.6% of these candidates were less than 100kb distance from each other, indicating a high likelihood of linkage disequilibrium between them. This corresponded to 10 clusters of two or more candidates (**Supplementary Table 2**).

We carried out a further examination of sex-specific variants underlying immunity, given that our PC analysis revealed that variants separated on the basis of sex. Post-hoc comparisons between baseline and survivors, in both males and females, revealed 43

candidates underlying female-specific immunity under FDR threshold of 20%; we retrieved no male specific candidates here.

To confirm the sex-specificity of candidates, we verified if top candidates for each sex brought about a multivariate separation (in the PCA) of the opposite sex. Since we found no male-specific candidates at the FDR threshold of 0.2, we chose an arbitrary threshold of p < 1E-04 and found 98 candidate variants for each sex. This allowed us to see if there were global differences in the genetic architecture of immunity of males and females. We plotted the top sex-specific candidates and found that the top candidates for a given sex were able to separate baseline from survivors of that sex (as expected). However, the same candidate list applied to the opposite sex failed to differentiate baseline from survivors (**Figure 6**; Welch's t test on the PC1 scores of top female candidates: Female t = -18.90, p <0.001; Male t = -0.12, p = 0.9; top candidates male candidates: sex - Female t = -0.14, p = 0.8; Male t = -26.3, p <0.001). We found no evidence that the top candidates associated with infection survival in one sex were associated with infection survival in the other sex, suggesting a partially distinct genetic basis of immunity.

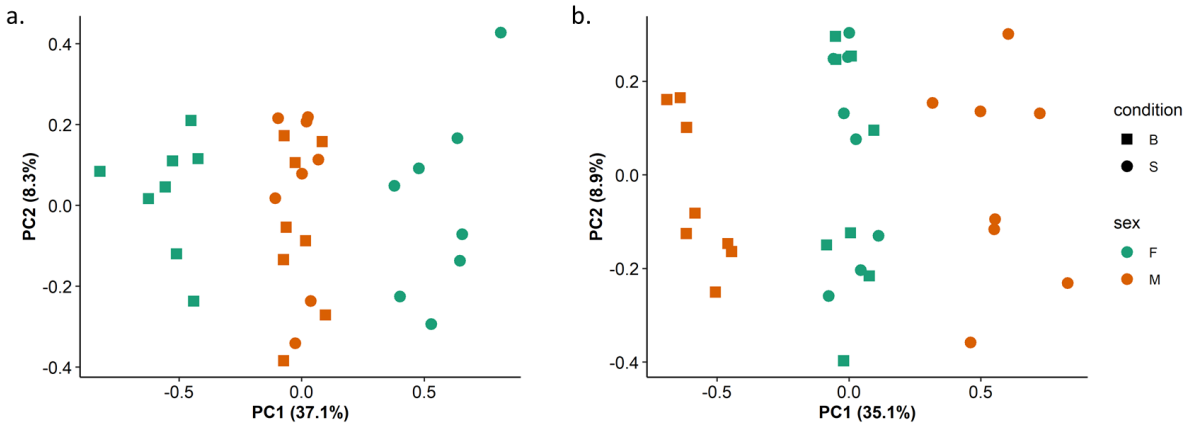

Figure 6. PCA of top 98 female (panel a) and male candidates (panel b) obtained under the threshold 1E-04. The multivariate separation between baseline (B, solid squares) and survivor (S, solid circles) females and males respectively for the top candidates obtained for each sex, and the lack of separation for the other sex indicates some degree of sex-specific architecture of immunity.

Functional characterisation of candidates

Our variants fell in many categories, the top three being intronic (31%), upstream (28%) and downstream (28%) variants (**Supplementary Table 1**). We found more candidate variants underlying immunity in the following categories than expected by chance - intronic variants ($\chi^2_{df = 1}$= 10.8, p <0.001), upstream variants ($\chi^2_{df = 1}$= 3.6, p = 0.06), downstream variants ($\chi^2_{df = 1}$= 6.8, p = 0.008), non-coding transcripts ($\chi^2_{df = 1}$= 18.8, p <0.001), 5′ UTR variants ($\chi^2_{df = 1}$= 7.47, p = 0.006). Although, none of the variant categories was enriched in the candidate variants underlying an interaction between infection survival and sex.

The 93 variants underlying immunity fell in 177 candidate genes, while the 63 variants underlying interaction of infection status with sex were annotated to 94 genes (more number of annotated genes than variants indicate that a bulk of detected variations might have been in the coding regions of genes). We found 34 GO terms in our candidate list underlying immunity in both sexes, 2 GO terms for the candidate list underlying interaction of status with sex and 7 GO terms underlying female-specific immunity, with p value <0.05. However, none of these survived the FDR threshold **(see Supplementary Table 4).**

Overlap of genes underlying variants from previous work

We found no overlaps between candidate genes identified in our study with a previous study by Bou Sleiman et al. (2015). In this study, the authors established a list of 27 significant QTLs (under p value threshold 10^{-5}, corresponding to an FDR of 0.66) contained within 30 genes, implicated in variation in immunity in the DGRP lines with P. entomophila as a pathogen.

Then we compared gene expression data from the same study as above that contrasted resistant versus susceptible DGRP lines. To perform the GWAS, the authors chose 4

susceptible and 4 resistant lines from the bottom and top 10% surviving under P. entomophila infection. On comparing these 8 lines under two phenotypic categories, they found 35 (FDR < 0.2 and logFC > 2) and 65 (FDR < 0.2 and logFC > 1) differentially expressed genes at 4 hours post infection. We did not find any overlaps between our candidate gene list with the list of Bou Sleiman *et al.* (2015).

Discussion

In this study, we found that immunity is a complex trait determined by multiple loci across the genome and that sexual dimorphism in immunity is underpinned by various variants with sex-specific and sexually antagonistic effects. We also found variants associated with immunity that were unique to each sex, pointing towards a partially independent genetic architecture. Put together, our evidence suggests that sexual dimorphism in immunity can still exist despite the two sexes surviving similarly in the face of the pathogen.

Our results indicate that a large amount of the genome is associated with sex-specific immunity. Sex-specific alleles, like those detected in our study, could lead to sex-biased gene expression. This could happen if candidate variants are preferentially located upstream or near genes, potentially leading to differential gene expression in the sexes. While we did not find sex-specific candidate variants enriched in coding regions, the possibility of sex-biased gene expression is interesting with broad consequences for selection. For instance, sex-biased genes show increased levels of genetic diversity and a footprint of relaxed selection (Sayadi *et al.* 2019), given that selection on sex-bias genes acts primarily in one sex and weakly or not at all in the other sex. However, despite the overall pattern of relaxed selection on sex-biased genes, Sayadi *et al.* (2019) found that weakly female biased genes showed balancing selection, while male-biased genes showed signs of overall purifying selection. These findings might then indicate that different selection pressures might operate in male and female biased genes potentially leading to varying levels of genetic diversity on

loci on these genes. Alternatively, alleles with sex-specific effects on immunity might be associated with similar expression pattern in the sexes, although having a positive effect in one sex and negative in the other.

Genetic diversity of variants underpinning major life-history traits is known to be maintained by sexually antagonistic selection or sex-specific variation (Connallon and Chenoweth 2019; Sayadi *et al.* 2019). Because sexual dimorphism in immunity is predicted to be underscored by divergent fitness optima supported by divergent life-history trade-offs, one could expect that at least some variants associated with sex-specific effects on immunity could also be associated with life-history traits. Finding variants with sex-specific effects on immunity is then of broad significance as immunity to biologically realistic pathogens can potentially underpin the broad non-alignment of the interests of the sexes.

We did not find any variants associated with sex-specific immunity on the X chromosome. It is possible that given the low marker density on this chromosome, detecting any signal was difficult. The X chromosome is predicted to harbour sexually antagonistic variants, given that genes on this chromosome face stronger selection in females than males (Rice 1984; Gibson *et al.* 2002). Moreover, X-linked variants underlying sexually antagonistic effects on immunity have been found in *D. melanogaster* (Hill-Burns and Clark 2009). However, there is also evidence of little enrichment or no enrichment for variants with sex-specific or sexually antagonistic variation on the X chromosome compared to autosomes (Ruzicka *et al.* 2019; Sayadi *et al.* 2019). This might be explained by dominance reversal of alleles on the X chromosome, in which each allele is partially or completely dominant in selective contexts in which it is favoured and recessive in contexts in which it is harmful, leading to sex biased gene expression, thereby liberating these loci from sexually antagonistic selection (Fry 2010, Ruzicka et al 2010).

Our finding of certain candidates with sexually antagonistic association with pathogen survival comes with a caveat. Some of the antagonistic candidates have different starting baseline allele frequencies - differences between baseline allele frequencies of sexes might be real owing to sexually antagonistic selection on developmental genes (Chippindale 2001). A novelty of our work though, is that we identify sexually antagonistic variants underlying immunity on the autosomes, as predicted by Geeta Arun *et al.* (2021). Sexual dimorphism in survival towards certain pathogens is known, and females generally survive better than males against certain bacterial and fungal pathogens (Duneau *et al.* 2017; Shahrestani *et al.* 2018). However, the lack of dimorphism in survival, like in our study, might not be entirely surprising, as the two sexes have a mutual interest in resisting the pathogen. This result also does not preclude the possibility that the sexes resist the infection via different mechanisms (Kelly *et al.* 2009), an idea that is also supported by our work. Our post-hoc analysis indeed indicates that a part of the genetic architecture underlying immunity is different between the sexes, suggesting that the sexes might deal with immunity via different mechanisms although converging on a similar immunocompetence, as evidenced by our survival results.

We found a broad genomic signature associated with immunity in both sexes. This signature was in part underpinned by an enrichment of candidates in the upstream regions. This suggests that some degree of difference in resistance between baseline and survivor individuals might be due to differential gene expression, potentially mediated by differential regulation of transcription. Further functional work by analysing gene expression differences between baseline and survivors might be able to validate this prediction.

Our results are interesting because, unlike previous work on this pathogen, our study was on genetically diverse populations, making our findings more likely to generalize when compared to studies on inbred lines (Bou Sleiman *et al.* 2015). Additionally, we can pinpoint with high confidence the candidate variants associated with immunity at stringent FDR threshold compared to previous work. We also found evidence that a

considerable proportion of the genome (>10%), as evidenced by the pi0 values, is associated with immunity. Most importantly, we used both sexes in our study, enabling us to elucidate candidates (those with sex-specific effects) that would normally not be detected in a single-sex study.

There are certain shortcomings of our study though. One, we lack functional validation of our candidates and as a result we cannot determine whether the variants we detected bring about real effects on immunity, and the mechanisms by which these effects could be realised. For instance, it would be interesting to follow the course of Duneau *et al.* (2017) and Bou Sleiman *et al.* (2015), by using RNAi lines to validate certain candidate genes such as *tep3*, known to influence defence against gram-positive bacteria, found in our study. Moreover, certain genes in the Toll pathway lead to sexual dimorphism towards gram positive and negative bacteria (Duneau *et al.* 2017), and a functional analysis would also help identify downstream targets of our candidate variants to verify if this is true in our study. Two, we lack a formal analysis of linkage disequilibrium to be able to pinpoint if contiguous loci within certain clusters are independently associated in response to infection. Third, we might be limited by the generalisability of the candidates found in our study, as these might not reflect selection on populations, given that some of these variants might be selected against if they are too harmful in one sex or have negative epistatic interactions with important life-history traits.

In conclusion, our study highlights that sexual dimorphism in immunity is maintained by genetic variation on large portions of the genome. Different selective pressures – shared, sex-specific and sexually antagonistic selection, might maintain this variation. Our results suggest that immunity, an important fitness-determining trait, that is predicted to be sexually selected could indeed signal overall genetic variation and thereby genetic quality.

Author contributions

S.S. and B.H. and designed experiments, S.S. performed experimental work. S.S., B.H. and T.J.K. analyzed the data. S.S., B.H. and T.J.K wrote the manuscript and contributed to revisions.

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Data Accessibility

Data will be made available on dryad digital repository on acceptance.

Competing interests

We declare that we have no competing interests.

Supplemental Figures

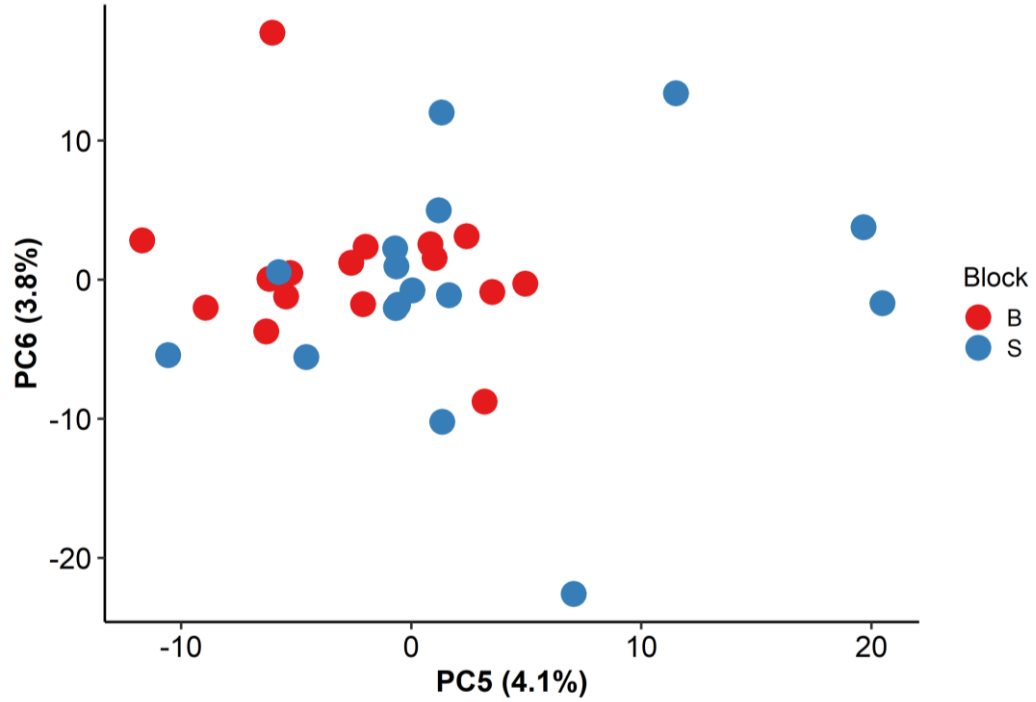

Figure S1 – Multivariate separation of baseline individuals (B in red solid circles) from survivors (S in blue solid circles) for all the 257,669 SNPs satisfying stringent filtering, and those included in models.

Supplemental Tables

Supplementary Table 1 – Variant consequences for 256,669 variants that passed the stringent filtering, and those included in models.

	Variant consequence	Percentage found
1	Intronic	31
2	Upstream	28
3	Downstream	28
4	Intergenic	2
5	Synonymous	4
6	3′ UTR	2
7	Non-coding transcript	1
8	Missense	1
9	5′ UTR	1
10	Non-coding exonic transcripts	1
11	Gain of stop codon (0.00001%), retention of stop codon (0.00001%), loss of stop codon (0.00001%), loss of start codon (0.00001%), coding sequence variants (0.00001%) and other categories	1

Supplementary Table 2 - Candidate variants underlying immunity in both sexes. Distance refers to distance in kb between contiguous variants.

Location	Consequence	Distance (kb)	p value	q value
2L:1184136	downstream_gene_variant;CG4896 5_prime_UTR_variant;Tgt upstream_gene_variant;CG5001 upstream_gene_variant;CG5126	332.04	5.20E-05	0.19
2L:1516182	upstream_gene_variant;Or22a downstream_gene_variant;CG18132 downstream_gene_variant;halo	2701.62	2.75E-05	0.19
2L:4217808	Synonymous_gene_variant; -	1460.64	6.43E-05	0.195387
2L:5678448	upstream_gene_variant;CG12511	184.30	3.81E-06	0.12
2L:5862756	intron_variant;rau	123.52	7.91E-05	0.19
2L:5986278	downstream_gene_variant;eIF4A upstream_gene_variant;ifc	689.17	4.39E-05	0.19
2L:6675451	downstream_gene_variant;lid downstream_gene_variant;Nse1 upstream_gene_variant;Spn27A downstream_gene_variant;CG34310 downstream_gene_variant;Nhe3 downstream_gene_variant;cup	727.46	7.60E-05	0.19
2L:7402920	downstream_gene_variant;CG34310 5_prime_UTR_variant;CG5171 upstream_gene_variant;CG5177 upstream_gene_variant;CG5160 intron_variant;CG5171 upstream_gene_variant;CG5177 downstream_gene_variant;CR44079 upstream_gene_variant;CG5171 upstream_gene_variant;lncRNA:CR46205	307.57	4.38E-05	0.19
2L:7710491	missense_variant;Tep3 upstream_gene_variant;Ntl upstream_gene_variant;tRNA:Tyr-GTA-1-9 downstream_gene_variant;CG33296	337.07	5.22E-05	0.19
2L:8047567	upstream_gene_variant;asRNA:CR44990 upstream_gene_variant;RpL36A downstream_gene_variant;PIG-U upstream_gene_variant;Uba4 upstream_gene_variant;Sgp upstream_gene_variant;Acer	469.76	4.75E-05	0.19
2L:8517330	5_prime_UTR_variant;mRpL51 upstream_gene_variant;CG13097 upstream_gene_variant;Uba4 upstream_gene_variant;Sgp upstream_gene_variant;Acer	1779.08	4.51E-05	0.19

2L:10296411	downstream_gene_variant;CG13138 intron_variant;CYLD upstream_gene_variant;Hand upstream_gene_variant;Ufd4 upstream_gene_variant;asRNA:CR44968	5795.75	4.22E-05	0.19
2L:16092163	upstream_gene_variant;CG4891	576.87	6.21E-05	0.19
2L:16669035	intron_variant;Trpgamma	177.01	7.82E-05	0.19
2L:16846050	downstream_gene_variant;CG31810 intron_variant;CG13284 downstream_gene_variant;SclB downstream_gene_variant;SclA	0.009	3.27E-05	0.19
2L:16846059	downstream_gene_variant;CG31810 intron_variant;CG13284	108.50	2.74E-05	0.19
2L:16954563	upstream_gene_variant;lncRNA:CR44393	406.87	4.37E-05	0.19
2L:17361442	upstream_gene_variant;CG31804 non_coding_transcript_exon_variant;lncRNA:CR44346	509.43	4.81E-05	0.19
2L:17870881	intron_variant;CadN2	13.94	8.59E-06	0.13
2L:17884829	intergenic_variant;-	304.78	5.07E-05	0.19
2L:18189610	upstream_gene_variant;Ptp36E intron_variant;CG42750 upstream_gene_variant;lncRNA:CR44900 upstream_gene_variant;Ptp36E	379.59	7.65E-05	0.19
2L:18569208	intron_variant;Pde11	63.67	7.43E-05	0.19
2L:18632878	intron_variant;MESR3	93.97	4.06E-05	0.19
2L:18726849	intron_variant;CG10348	286.62	1.80E-05	0.19
2L:19013470	downstream_gene_variant;robl37BC upstream_gene_variant;RpL30 upstream_gene_variant;snoRNA:Me28S-A2113 downstream_gene_variant;lncRNA:CR45292	19013.47	3.33E-05	0.19
2R:3290250	intergenic_variant;-	882.93	3.40E-05	0.19
2R:4173184	intergenic_variant;-	2081.59	7.46E-05	0.19
2R:6254783	intron_variant;Pld	0.016	4.07E-05	0.19
2R:6254799	intron_variant;Pld	2421.35	5.75E-05	0.19
2R:8676158	intron_variant;Acsl 5_prime_UTR_variant;Acsl upstream_gene_variant;UQCR-11L downstream_gene_variant;CG30355 upstream_gene_variant;Acsl	142.007	4.76E-06	0.12
2R:8818165	intron_variant;sns	2611.20	6.95E-05	0.19
2R:11429374	5_prime_UTR_variant;Drip intron_variant;Drip	3394.13	5.12E-05	0.19

	upstream_gene_variant;Drip downstream_gene_variant;lncRNA:CR43771			
2R:14823509	upstream_gene_variant;Pgm2b downstream_gene_variant;lncRNA:CR45329	0.003	1.32E-06	0.12
2R:14823512	upstream_gene_variant;Pgm2b downstream_gene_variant;lncRNA:CR45329	0.008	2.66E-06	0.12
2R:14823520	upstream_gene_variant;Pgm2b downstream_gene_variant;lncRNA:CR45329	119.84	7.11E-07	0.12
2R:14943362	downstream_gene_variant;Lap1 intron_variant;CG10257 downstream_gene_variant;aPKC	2463.18	2.36E-05	0.19
2R:17406549	downstream_gene_variant;CG10939 downstream_gene_variant;Vajk4 missense_variant;Camp	355.14	3.24E-05	0.19
2R:17761690	downstream_gene_variant;HLH54F intron_variant;CG5009 downstream_gene_variant;OstDelta	3087.64	5.79E-05	0.19
2R:20849330	downstream_gene_variant;mir-3643 downstream_gene_variant;CG9993 missense_variant;CG17999	50.64	1.56E-05	0.19
2R:20899970	upstream_gene_variant;otp intron_variant;otp downstream_gene_variant;asRNA:CR43160	531.34	5.46E-06	0.12
2R:21431319	intron_variant;Sdc	78.15	7.18E-05	0.19
2R:21509469	intron_variant;MESK2	0.005	3.02E-05	0.19
2R:21509474	intron_variant;MESK2	2790.28	7.66E-05	0.19
2R:24299763	downstream_gene_variant;Fatp3	24299.76	5.43E-05	0.19
3L:3183452	intron_variant;Girdin upstream_gene_variant;CG32284 upstream_gene_variant;CG14957 upstream_gene_variant;CG42494 downstream_gene_variant;CG42525 upstream_gene_variant;CG14964	4176.30	6.31E-05	0.19
3L:7359760	upstream_gene_variant;Sec63 upstream_gene_variant;CTCF downstream_gene_variant;Sh3beta downstream_gene_variant;asRNA:CR45822 downstream_gene_variant;asRNA:CR45835	0.02	2.60E-05	0.19
3L:7359780	upstream_gene_variant;Sec63 upstream_gene_variant;CTCF missense_variant;pst downstream_gene_variant;Sh3beta downstream_gene_variant;asRNA:CR45822 downstream_gene_variant;asRNA:CR45835	25.50	7.25E-06	0.12

	intron_variant;smid			
3L:7385289	downstream_gene_variant;CG8564	34.92	5.51E-05	0.19
	upstream_gene_variant;SMSr			
3L:7420210	upstream_gene_variant;CG42458	2.93	1.87E-05	0.19
3L:7423148	upstream_gene_variant;CG42458	0.006	6.16E-05	0.19
3L:7423154	upstream_gene_variant;CG42458	3635.56	5.31E-05	0.19
3L:11058719	upstream_gene_variant;Blos2 downstream_gene_variant;CG32075 downstream_gene_variant;CG32069 downstream_gene_variant;CG6321	1516.28	6.90E-05	0.19
3L:12575007	upstream_gene_variant;sowah	942.27	7.28E-05	0.19
3L:13517285	downstream_gene_variant;Liprin-beta upstream_gene_variant;Vps36	1142.53	3.08E-05	0.19
3L:14659824	downstream_gene_variant;bru3 intron_variant;dlp downstream_gene_variant;lncRNA:CR45888	6472.58	7.24E-05	0.19
3L:21132404	intron_variant;lCA69 downstream_gene_variant;CG10565 upstream_gene_variant;ko downstream_gene_variant;Ac78C	25.61	7.71E-05	0.19
3L:21158023	downstream_gene_variant;CG10565 downstream_gene_variant;Ac78C intron_variant;Ac78C	121.09	5.10E-06	0.12
3L:21279113	downstream_gene_variant;CG12974 downstream_gene_variant;ppl intron_variant;AcCoAS upstream_gene_variant;AcCoAS	1071.44	4.63E-05	0.19
3L:22350554	downstream_gene_variant;CG12974 intron_variant;Ten-m downstream_gene_variant;lncRNA:CR45963 upstream_gene_variant;lncRNA:CR45964 intron_variant,non_coding_transcript_variant;lncRNA:CR45962	237.26	3.89E-05	0.19
3L:22587815	intron_variant;CG14459	410.18	3.52E-06	0.12
3L:22997998	upstream_gene_variant;nrm	0.003	6.42E-05	0.19
3L:22998001	upstream_gene_variant;nrm	3351.50	7.53E-05	0.19
3L:26349507	downstream_gene_variant;CG42598 intergenic_variant;-	591.8	1.73E-05	0.19
3L:26941307	intergenic_variant	66.46	3.01E-05	0.195387
3L:27007774	intergenic_variant;-	366.87	4.11E-05	0.19
3L:27374644	intron_variant;Dbp80	27374.6	4.16E-05	0.19
	upstream_gene_variant;pug	1305.73	3.74E-05	0.19

3R:10693028	upstream_gene_variant;CG14683 downstream_gene_variant;Skeletor 5_prime_UTR_variant;Skeletor			
3R:11998758	upstream_gene_variant;Ect3 intron_variant;Tk	0.02	4.26E-05	0.19
3R:11998778	upstream_gene_variant;Ect3 intron_variant;Tk	0.039	4.75E-05	0.19
3R:11998817	upstream_gene_variant;Ect3 intron_variant;Tk	0.043	2.18E-05	0.19
3R:11998860	upstream_gene_variant;Ect3 intron_variant;Tk	953.21	9.44E-06	0.13
3R:12952070	intron_variant;grsm	115.26	6.50E-05	0.19
3R:13067333	intron_variant;sim	1163.67	4.30E-06	0.12
3R:14231011	upstream_gene_variant;CG9624 upstream_gene_variant;CG9631 downstream_gene_variant;CG43291	23.43	8.83E-06	0.13
3R:14254445	intron_variant;MetRS-m upstream_gene_variant;CG43179	1247.49	6.29E-05	0.19
3R:15501939	intergenic_variant;-	2061.87	6.06E-06	0.12
3R:17563809	intron_variant;Hmx	316.95	6.32E-05	0.19
3R:17880765	intron_variant;Rim	1082.03	6.40E-05	0.19
3R:18962797	intron_variant;CG42613 intron_variant;CG43732 downstream_gene_variant;CG43732	191.99	5.10E-05	0.19
3R:19154787	upstream_gene_variant;nos 3_prime_UTR_variant;CG11779 upstream_gene_variant;CG5835	1070.83	6.81E-05	0.19
3R:20225618	upstream_gene_variant;nos intron_variant;Nlg4	0.043	4.22E-05	0.19
3R:20225661	intron_variant;Nlg4	2308.96	7.58E-05	0.19
3R:22534628	intron_variant;CG5326 upstream_gene_variant;CG33093 upstream_gene_variant;CG33099 intron_variant;cnc upstream_gene_variant;cnc	668.8	6.38E-05	0.19
3R:23203428	synonymous_gene_variant; fzz	747.74	6.84E-05	0.19
3R:23951177	downstream_gene_variant;GluProRS upstream_gene_variant;CG12268	320.023	3.57E-05	0.19
3R:24271200	upstream_gene_variant;Orct2 5_prime_UTR_variant;jar	452.61	5.97E-05	0.19
	intron_variant;tok	3145.52	9.71E-06	0.13

3R:24723817	upstream_gene_variant;CG13630 upstream_gene_variant;snoRNA:Me18S-C1280			
3R:27869345	intron_variant;CG34353 downstream_gene_variant;CG12426	1510.85	2.07E-05 2.07E-05	0.19 0.19
3R:29380195	intron_variant;Ptp99A	221.10	4.72E-06	0.12
3R:29601299	downstream_gene_variant;alph intron_variant;alph upstream_gene_variant;CG7568	821.05	8.04E-05	0.19
3R:30422352	upstream_gene_variant;mRpS18C downstream_gene_variant;CG2218 downstream_gene_variant;CG15536 downstream_gene_variant;CG15535 5_prime_UTR_variant;CG42740	576.19	2.49E-05	0.19
3R:30998544	intron_variant;5-HT7 upstream_gene_variant;lncRNA:CR46117	30998.54	4.96E-05	0.19
X:22280229	intergenic_variant;-		6.78E-06	0.12

Supplementary Table 3 – Candidate variants underlying sex-specific immunity.

Location	Consequence	Distance (kb)	p value	q value
2L:1505680	upstream_gene_variant;CG7420 downstream_gene_variant;asRNA:CR44064	0.005	4.31E-05	0.18
2L:1505685	upstream_gene_variant;CG7420 upstream_gene_variant;CG7420 downstream_gene_variant;asRNA:CR44064	464.5	1.28E-05	0.15
2L:1970248	upstream_gene_variant;CG7420 downstream_gene_variant;tRNA:Arg upstream_gene_variant;Der	4568.4	1.70E-05	0.15
2L:6538674	5_prime_UTR_variant;erm intron_variant;eya	0.002	7.26E-06	0.15
2L:6538676	intron_variant;eya upstream_gene_variant;eya	451.06	8.41E-06	0.15
2L:6989738	intron_variant;uif	857.06	3.90E-05	0.18
2L:7846800	3_prime_UTR_variant;CG14535	8527.9	1.44E-05	0.15
2L:16374784	intergenic_variant; -	0.001	5.20E-05	0.19
2L:16374785	intergenic_variant; -	0.006	1.39E-05	0.15
2L:16374791	intergenic_variant; -	685.7	6.57E-06	0.15
2L:17060558	intergenic_variant; -	313.1	5.32E-05	0.19
2L:17373680	synonymous_variant;Lrch	837.8	2.81E-05	0.17
2L:18211572	upstream_gene_variant;CG31802 intron_variant;CG42750	5110.2	2.17E-05	0.17
2L:23321815	intron_variant,non_coding_transcript_variant;CR42530	191.7	1.75E-05	0.15
2L:23513577	intergenic_variant; -	23513.5	1.37E-05	0.15
2R:8638729	intergenic_variant; -	916.9	2.77E-05	0.17
2R:9555688	upstream_gene_variant;Or45b downstream_gene_variant;mir14 downstream_gene_variant;lncRNA:CR43651	1311.1	3.08E-05	0.17
2R:10866840	downstream_gene_variant;asRNA:CR45281 intron_variant;CG30015	759.02	1.94E-05	0.15
2R:11625861	intron_variant MODIFIER CG9005	125.2	3.36E-05	0.18
2R:11751115	intergenic_variant; -	204.9	5.41E-05	0.19
2R:11956089	intron_variant;exp	409.5	3.25E-06	0.15
2R:12365645	downstream_gene_variant;CG30043 upstream_gene_variant;CG33012 intron_variant;CR33013	0.003	1.24E-05	0.15
2R:12365648	downstream_gene_variant;CG30043 upstream_gene_variant;CG33012 intron_variant;CR33013	377.9	1.19E-05	0.15
2R:12743605	intron_variant;CG42663	8113.7	6.66E-06	0.15
2R:20857370	intergenic_variant; -	191.2	8.13E-06	0.15
2R:21048651	downstream_gene_variant;shg	1035.04	4.20E-05	0.18
2R:22083694	downstream_gene_variant;GlcT	2416.04	4.78E-05	0.19

	synonymous_variant;Synj			
	downstream_gene_variant;CG13502			
	downstream_gene_variant;Crtp			
2R:24499737	downstream_gene_variant;Prosalpha4T2	24499.7	2.36E-06	0.15
	synonymous_variant;Ssl			
	downstream_gene_variant;lncRNA:Yu			
3L:968797	intron_variant;Glut1	3568.1	1.86E-05	0.15
	synonymous_variant;CG11353			
3L:4536930	downstream_gene_variant;CG32243	5881.02	3.93E-05	0.18
	upstream_gene_variant;Tie			
3L:10417955	upstream_gene_variant;-	0.007	2.40E-05	0.17
	downstream_gene_variant;CG12362			
3L:10417962	upstream_gene_variant; -	415.3	4.06E-05	0.18
	downstream_gene_variant;CG12362			
3L:10833323	upstream_gene_variant;tna	1437.4	1.29E-05	0.15
	downstream_gene_variant;CG32100			
3L:12270817	downstream_gene_variant;Sms	0.004	1.70E-05	0.15
	synonymous_variant;Pbgs			
	upstream_gene_variant;app			
	downstream_gene_variant;Sms			
3L:12270821	downstream_gene_variant;CG32100	828.08	4.71E-05	0.19
	missense_variant; Pbgs			
	upstream_gene_variant; app			
3L:13098910	;	1534.3	2.76E-05	0.17
	upstream_gene_variant;			
3L:14633220	missense_variant;dlp	2622.3	4.28E-05	0.18
	upstream_gene_variant;RecQ5			
	upstream_gene_variant;CG6497			
3L:17255545	intron_variant,non_coding_transcript_variant	0.004	5.74E-06	0.15
	upstream_gene_variant;lncRNA:CR43874			
	upstream_gene_variant;CG6497			
3L:17255549	intron_variant,non_coding_transcript_variant; lncRNA:CR43870	0.002	4.00E-05	0.18
	upstream_gene_variant; lncRNA:CR43874			
	upstream_gene_variant; CG6497			
3L:17255551	intron_variant,non_coding_transcript_variant	9871.041	5.32E-05	0.19
	upstream_gene_variant MODIFIER lncRNA:CR43874			
3L:27126592	intergenic_variant; -	0.015	4.44E-05	0.18
3L:27126607	intergenic_variant; -	329.3	3.12E-05	0.17
3L:27455947	intergenic_variant; -	27455.9	3.04E-05	0.17
3R:6144044	intergenic_variant; -	3507.3	3.89E-05	0.18
	downstream_gene_variant;Dh44			
3R:9651410	3_prime_UTR_variant; CG9492	1544.5	3.12E-05	0.17
	downstream_gene_variant;CR43441			
3R:11195939	upstream_gene_variant;CG18577	1696.3	4.03E-05	0.18
	upstream_gene_variant;cu			

3R:12892303	downstream_gene_variant synonymous_variant LOW d-cup	607.053	1.56E-05	0.15
3R:13499356	downstream_gene_variant MODIFIER CR33929 intergenic_variant; -	1418.7	1.23E-05	0.15
3R:14918148	upstream_gene_variant;Sdr	592.9	1.02E-05	0.15
3R:15511070	upstream_gene_variant;CG44013 upstream_gene_variant;CG44014	0.001	4.92E-06	0.15
3R:15511071	upstream_gene_variant;CG44013 upstream_gene_variant;CG44014	60.3	1.15E-05	0.15
3R:15571414	intergenic_variant; -	2769.4	3.48E-06	0.15
3R:18340827	intron_variant;CG15803	633.2	1.62E-05	0.15
3R:18974103	downstream_gene_variant;Smu1 downstream_gene_variant;euc	396.3	3.08E-05	0.17
3R:19370466	intron_variant;Ino80	881.5	3.58E-05	0.18
3R:20252005	upstream_gene_variant; upstream_gene_variant;cic	2623.5	5.11E-05	0.19
3R:22875555	intergenic_variant; -	657.6	5.91E-06	0.15
3R:23533242	downstream_gene_variant;Ugt303B2 downstream_gene_variant;Ugt303B1 missense_variant;Ugt303B3	1148.3	2.47E-05	0.17
3R:24681636	upstream_gene_variant;CG10175 intron_variant;slo	527.2	2.47E-05	0.17
3R:25208869	intron_variant;CG13654 upstream_gene_variant;Cad96Ca	4832.7	5.13E-05	0.19
3R:30041576	upstream_gene_variant;CG13654 downstream_gene_variant;Sry synonymous_variant;Sry upstream_gene_variant;Sry upstream_gene_variant;CG7943 downstream_gene_variant;RpL32 upstream_gene_variant;janA upstream_gene_variant;janB upstream_gene_variant;ocn upstream_gene_variant;ZIPIC 3_prime_UTR_variant;Sry synonymous_variant;Sry	376.001	2.60E-05	0.17
3R:30417577	downstream_gene_variant MODIFIER CG2217 downstream_gene_variant MODIFIER CG42740	0.014	3.40E-05	0.18
3R:30417591	downstream_gene_variant MODIFIER CG2217 downstream_gene_variant MODIFIER CG42740	30417.5	4.28E-05	0.18

Supplementary Table 4 – GO terms passing the threshold of p < 0.05 (but not FDR cut-off of 0.2) associated with candidate SNPs underlying immunity and interaction of infection status with sex.

GO term	Average number of genes found in GO term	Genes found when every gene is counted once	p value (uncorrected)	FDR corrected p value	Number of unique genes per GO term	Total genes for given GO category	Description of GO term	Gene associated with GO term
				GO terms associated with immunity				
GO:0016401	0.006	1	0.00592	0.311112	1	3	palmitoyl-CoA oxidase activity	cg5009
GO:0042579	0.018	1	0.01779	0.311112	1	33	microbody	cg5009
GO:0006635	0.018	1	0.01779	0.311112	1	11	fatty acid beta-oxidation	cg5009
GO:0016634	0.018	1	0.01779	0.311112	1	11	oxidoreductase activity, acting on the CH-CH group of donors, oxygen as acceptor	cg5009
GO:0019395	0.018	1	0.01779	0.311112	1	12	fatty acid oxidation	cg5009
GO:0030258	0.018	1	0.01779	0.311112	1	24	lipid modification	cg5009
GO:0072329	0.018	1	0.01779	0.311112	1	13	monocarboxylic acid catabolic process	cg5009
GO:0009062	0.018	1	0.01779	0.311112	1	12	fatty acid catabolic process	cg5009
GO:0034440	0.018	1	0.01779	0.311112	1	13	lipid oxidation	cg5009
GO:0005777	0.018	1	0.01779	0.311112	1	22	peroxisome	cg5009
GO:0003997	0.018	1	0.01779	0.311112	1	6	acyl-CoA oxidase activity	cg5009
GO:0044242	0.018	1	0.01801	0.311112	1	25	cellular lipid catabolic process	cg5009
GO:0016042	0.019	1	0.01864	0.311112	1	39	lipid catabolic process	cg5009
GO:0003995	0.022	1	0.02203	0.311112	1	15	acyl-CoA dehydrogenase activity	cg5009
GO:0006631	0.022	1	0.02223	0.311112	1	47	fatty acid metabolic process	cg5009
GO:0032787	0.023	1	0.02265	0.311112	1	76	monocarboxylic acid metabolic process	cg5009
GO:0050660	0.026	1	0.02631	0.311112	1	61	flavin adenine dinucleotide binding	cg5009
GO:0016627	0.028	1	0.02755	0.311112	1	39	oxidoreductase activity, acting on the CH-CH group of donors	cg5009
GO:0016054	0.029	1	0.02921	0.311112	1	38	organic acid catabolic process	cg5009
GO:0046395	0.029	1	0.02921	0.311112	1	38	carboxylic acid catabolic process	cg5009

GO:0003333	0.03	1	0.02996	0.311112	1	30	amino acid transmembrane transport	cg7888
GO:0015837	0.033	1	0.03254	0.311112	1	42	amine transport	cg7888
GO:0006865	0.033	1	0.03254	0.311112	1	38	amino acid transport	cg7888
GO:0071705	0.033	1	0.03254	0.311112	1	50	nitrogen compound transport	cg7888
GO:0044255	0.038	1	0.03743	0.311112	1	175	cellular lipid metabolic process	cg5009
GO:0006082	0.038	1	0.03757	0.311112	1	244	organic acid metabolic process	cg5009
GO:0042180	0.038	1	0.03757	0.311112	1	266	cellular ketone metabolic process	cg5009
GO:0043436	0.038	1	0.03757	0.311112	1	244	oxoacid metabolic process	cg5009
GO:0019752	0.038	1	0.03757	0.311112	1	244	carboxylic acid metabolic process	cg5009
GO:0046942	0.041	1	0.04022	0.313605	1	52	carboxylic acid transport	cg7888
GO:0015849	0.041	1	0.04022	0.313605	1	52	organic acid transport	cg7888
GO:0044282	0.048	1	0.04705	0.343998	1	134	small molecule catabolic process	cg5009
GO:0005275	0.05	1	0.04924	0.343998	1	57	amine transmembrane transporter activity	cg7888
GO:0015171	0.05	1	0.04924	0.343998	1	52	amino acid transmembrane transporter activity	cg7888

GO terms associated with interaction between infection status and sex

GO:0016746	0.019	1	0.01873	1	1	155	transferase activity, transferring acyl groups	cg11353
GO:0016747	0.019	1	0.01873	1	1	134	transferase activity, transferring acyl groups other than amino-acyl groups	cg11353

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Chapter 4

Male-biased sex ratios increase sexual selection against a genetic polymorphism in *Drosophila melanogaster*

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Abstract

Sexual selection explains the enormous diversity and display of exaggerated traits in nature. A major factor hypothesized to influence the strength of sexual selection is the operational sex ratio (OSR). However, the rationale for the relationship between OSR and sexual selection is debatable. Moreover, various metrics used to study the relationship between OSR and sexual selection have led to mixed results. In this study, we investigated how the operational sex ratio influences the strength of selection on a Mendelian trait in *Drosophila melanogaster*. To this end, we measured competitive mating success of two genotypes, a homozygous mutant of the *ebony* gene and the wild type, under different sex ratio treatment. We also manipulated the mating pool density, as it is an important covariate of OSR. The strength of sexual selection increased as sex ratio became increasingly male-biased. Moreover, our results indicated that the sex ratio and not the absolute densities of males and females influenced the strength of sexual selection. We also found that the strength of sexual selection waned over consecutive days, highlighting a change in the relative selection on pre- and post-copulatory mate competition. Our study bolsters the idea that heightened sexual selection in male-biased sex ratios can lead to overall population fitness benefits by selecting against "bad genes".

Keywords: operational sex ratio, sexual selection, male-male competition.

Introduction

Sexual selection explains the enormous diversity of exaggerated traits in nature. Darwin (1871) noted that these traits might increase the sexual success of the bearer despite their apparent non-sexual costs, which was at odds with natural selection. Quantifying the strength of sexual selection can aid in understanding its evolutionary consequences, such as trade-offs associated with the development and maintenance of sexual traits (Rowe & Houle, 1996; Bonduriansky & Chenoweth, 2009), the degree of sexual dimorphism (Darwin, 1871; Andersson, 1994; Kelly, 2008; Sword & Simpson, 2008; Vanpé

et al., 2008), or sexual conflict (Parker, 1979; Chapman *et al.*, 2003; Bonduriansky & Chenoweth, 2009).

A major factor hypothesized to influence the strength of sexual selection is the operational sex ratio (OSR), i.e., the relative numbers of potentially interacting males and females capable of mating at a given time (Emlen & Oring, 1977; Clutton-Brock & Parker, 1992; Kvarnemo & Ahnesjö, 1996; although see Shuster, 2009). Specifically, the strength of sexual selection on males and females is expected to increase as the OSR becomes more male- and female-biased, respectively (Emlen & Oring, 1977). This is based on the assumption that stronger competition for mates between members of the more abundant sex leads to higher variance in mating success, and that higher variance results in stronger sexual selection (Emlen & Oring, 1977; Shuster, 2009; Klug *et al.*, 2010; Jennions *et al.*, 2012). However, these assumptions are debatable (Arnold & Duvall, 1994; Shuster & Wade, 2003; Shuster, 2009). For instance, the assumption that sex ratio skew increases variance in mating success is not a logical necessity (Jennions *et al.*, 2012). But more importantly, even if it does, an increase in mating success could also be partially or completely stochastic. This warrants empirical studies on the relationship between OSR and the strength of sexual selection.

One approach to test the above hypothesis explored the relationship between OSR and the opportunity for sexual selection (I_S) (Wade, 1979; Arnold & Wade, 1984; Shuster & Wade, 2003). I_S is defined as the square of the coefficient of variation in mating success for a given sex and measures the maximum slope of regression of relative fitness on a quantitative trait; it thus has merits as a measure of sexual selection (Krakauer *et al.*, 2011). However, I_S might not be the most reliable metric as it can also be dictated by stochastic factors (Klug *et al.*, 2010; Jennions *et al.*, 2012; Rios Moura & Peixoto, 2013), and it is not surprising that numerous independent studies using I_S to measure selection in varying operational sex ratios have found mixed results. For instance, some empirical studies report a positive relationship between OSR and I_S (Jones *et al.*, 2004; Mills *et al.*, 2006; DuVal & Kempenaers, 2008; Vanpé *et al.*, 2008; Croshaw, 2010; Wacker *et al.*, 2013),

while others report a negative relationship (Clutton-Brock *et al.*, 1997; Fitze & Le Galliard, 2008).

Similar to I_S, other metrics based on individual variance in mating success have also been used to measure the strength of sexual selection (reviewed in Fairbairn & Wilby, 2001; Jones *et al.*, 2004; Mills *et al.*, 2006; Croshaw, 2010; Klug *et al.*, 2010), but often do not agree with each other. In general, individual variance-based metrics of sexual selection have been questioned because these variances cannot separate stochastic variation in mating success and actual selection on sexual traits (Klug *et al.*, 2010; Jennions *et al.*, 2012).

Selection (sexual or otherwise) is about the relationship between fitness and trait(s) - the slope of regression signifies the relationship (the covariance) between a trait and its association with relative fitness. Then, the product of the regression slope, the selection gradient, and additive genetic variance predict the evolutionary change in quantitative genetic traits (Lande & Arnold, 1983), a more direct approach to study evolution. Such trait-based approaches (e.g. selection differential, selection gradient) have been used to quantify sexual selection, however here too, results have been equivocal in ascertaining the link between operational sex ratios and the strength of selection. For instance, while stronger sexual selection on certain traits was observed in male-biased compared to equal (Jones *et al.*, 2004) or female-biased sex ratios (Wacker *et al.*, 2013), the reverse was observed in other studies (Klemme *et al.*, 2007; Fitze & Le Galliard, 2008; Head *et al.*, 2008).

Here, we use an experimental approach to study the effect of operational sex ratio on the strength of sexual selection on a Mendelian trait in *Drosophila melanogaster*. Focusing on a Mendelian rather than a quantitative trait provides a direct link to the underlying genetic basis as well as might result in greater statistical power, given that Mendelian traits produce distinct trait values compared to continuous values of quantitative traits. Specifically, we let males homozygous for a mutant *ebony* allele compete for females

with wild-type males. The *ebony* males are less competitive compared to wild-type males (Kyriacou *et al.*, 1978) and so we asked how the sexual selection coefficient (estimated from competitive paternity success) against *ebony* is affected by different OSR treatments. With this approach, we directly measure the totality of sexual selection, including pre- and post-copulatory components, in different sex ratio scenarios.
Along with OSR, we manipulated the total number of interacting individuals (i.e., mating pool density). Differences in OSRs inherently imply a change in the number of at least one sex. While the rationale for the proposed effect of OSR on the strength of sexual selection is based on the numbers of the two sexes relative to each other, an apparent effect of OSR could be driven by the absolute density of one sex in the mating pool (Fairbairn & Wilby, 2001; Head *et al.*, 2008; Wacker *et al.*, 2013). Experimental evidence suggests that mating pool density can also act independently of OSR to negatively influence pre-copulatory mate competition (McLain, 1992; Jirotkul, 1999). Density and male-biased sex ratios can also synergistically influence the strength of sexual selection; a relatively greater reduction in I_s was observed in low density male-biased treatments compared to equal sex ratios at similar density strong in male-biased high density treatments, indicating an interaction between the two variables (House *et al.*, 2019). Although neither sex ratio or density nor their interaction influenced I_s (Head *et al.*, 2008). Simultaneously manipulating OSR and density allows us to disentangle to some extent the effects of these two factors.

We tested the effects of OSR and density on the strength of sexual selection against the *ebony* males in two experiments with somewhat different designs. In experiment 1, we varied male and female densities in a fully factorial design; this resulted in various combinations of OSR and total density. In experiment 2, we factorially combined three different values of OSR and three total densities (males + females). We analysed both experiments in two alternative frames of reference, one using male and female densities as predictor variables of the relative success of *ebony* males, the other using OSR and total density of both sexes as predictors. While the mating interactions in experiment 1 were limited to 24 h, experiment 2 was performed over three consecutive days, a period

during which the focus of sexual selection might transition from mate competition / mate choice by initially virgin females to sperm competition and its avoidance, mediated in part by seminal fluid proteins. We aimed to address three interrelated questions. First, does the strength of selection increase with increasing male-biased sex ratios, given that more skewed sex ratios are predicted to have stronger competition amongst males for mates? Second, does density or its interaction with sex ratio influence the strength of sexual selection? Third, do any effects of density and OSR on the strength of sexual selection change over time after the onset of sexual interactions?

Materials and methods

Fly rearing and maintenance

The experimental populations used here were derived from a long-term laboratory population called IV that was initiated from about 200 wild D. melanogaster of each sex collected in Massachusetts in 1975 (Charlesworth & Charlesworth, 1985). This population has been maintained in the lab at high density, with a census size in thousands, and is adapted to the laboratory environment (Houle & Rowe, 2003). For fitness assays involving a competitor, we used a second laboratory population derived from the IV population that carries a recessive ebony mutation (IVe).

The IVe population was established in 1992 after a spontaneous recessive ebony mutation was repeatedly backcrossed with the IV population. The IVe population has been maintained in the laboratory for many generations and has shown to be weaker at non-sexual traits (e.g. larval competition; (Houle & Rowe, 2003)) and sexual competition (Hollis & Kawecki, 2014) and because of their darker cuticle, provide a competitive standard easily distinguished from wild type flies used in our experiments.

All flies were reared on 2% yeast media (water, agar [Milian CH], brewer's yeast [Migros CH], cornmeal, sucrose, and Nipagin [Sigma-Aldrich CH]) and maintained on a 12L:12D photoperiod at 25^{0}C and a relative humidity of 55%.

Measuring competitive paternity success

To determine the strength of sexual selection against the ebony mutation, we compared competitive paternity success of wild type IV males against IVe males in different sex ratio and density treatments. The two types of males were allowed to compete for females from the same ebony population and the resulting offspring were scored upon emergence as adults. This approach enabled us to distinguish offspring sired by the wild-type males, which would have typical dark orange cuticles, and those sired by the reference males, which would have dark cuticles. We then used the proportion of wild type to ebony offspring as a measure of sexual success of the ebony relative to wild type males. In our competitive mating assays, we used twice the number of ebony to wild type males to maximise statistical power. Ebony males are weaker at sexual competition compared to wild types, so by using more of the former compared to the latter, we wanted to ensure the proportion of offspring sired by the two types of males was centred around 0.5, giving us scope to assess changes in competitiveness of our focal ebony males.

Experiment 1

In experiment 1, we simultaneously manipulated male and female densities in a factorial design. For this, we put together 6, 9, or 12 males (M) in all possible combinations with 6, 9, or 12 females (F) to yield a range of sex ratios from 1:2 female-biased to 2:1 male-biased sex ratios with a total density range of 12-24 individuals in a bottle of 175ml with fresh food. For this, we anesthetised 5-6 day old virgin males and females and transferred them into bottles (volume: 175ml) containing fresh food. These flies were then allowed to interact for 24 hours before being discarded, after which the eggs laid in these vials were allowed to develop. In a pilot experiment, we determined that even at egg densities as high as 300 (an expected number of eggs from a maximum of 12 females used in this experiment), there was no difference in relative survival of larvae from the two phenotypes (**Figure S1**). Upon emergence after 11-12 days, all offspring were collected, frozen at -20^{0}C, and scored to estimate paternity. We performed 2 independent blocks of

the above-mentioned competition trials over 4 weeks with 33 and 45 replicates respectively.

Experiment 2

In this experiment, we set out to quantify temporal changes in the effects of sex ratio and density. With this aim, we measured competitive paternity success of mutant ebony males for three subsequent days with some modifications from experiment 1. First, we used three total densities and three sex ratios (density = 18, 36, and 54 individuals and sex ratio = 1:2 male-biased, 1:1 equal, and 2:1 female-biased ratios). Second, we used a greater number of individuals to reduce noise from individual variation and larger (internal volume: 391ml) and somewhat more complex containers to avoid crowding compared to bottles used in the previous experiment. Therefore, large arenas with two independent food patches (further divided by partitions), allowed for more space for females to potentially escape male harassment (Yun et al., 2017). Third, to quantify paternity of ebony versus wild type males, we randomly sampled a pre-determined number of eggs (n = 50) from the total number of eggs laid in a treatment and reared them separately. We did this in order to avoid differential effects of crowding on larval viability from the two different genotypes, given that we expected a greater total number of eggs in this experiment resulting from a larger number of individuals per treatment compared to experiment 1.

We set up the different sex ratio and density treatments as before. The container/arena (**Figure S2**) had two replaceable circular inserts (petri-dishes) that were filled with food or medium for collecting eggs (agar+orange juice sprinked with Baker's yeast). Fresh food was replenished every day, for three consecutive days of the experiment, once in the morning and it was replaced in the evening by egg laying medium. We then returned the next morning and randomly sampled 45-50 eggs (to control for density) and placed them in vials of fresh fly food to develop. Adults were scored on emergence, and competitive paternity success of the mutants was estimated as in experiment 1. We performed four

blocks of the experiment spread over 8 weeks, with 44, 89, 79 and 78 total replicates each.

Statistical analysis

We performed all statistical analyses in R v3.4.3 (R Core Development Team, 2021) with the package afex (Singmann et al., 2015), a wrapper for lme4 (Bates et al., 2011). For both experiments, we fit generalized linear mixed models (glmer) with the binomial family (logit link), where the response was the count of the ebony versus wild type offspring emerging from each replicate mating trial. Note that the (sexual) fitness of ebony males relative to wild type males is

$$Relative\ fitness\ of\ ebony\ males = \frac{1}{2}\frac{Number\ of\ ebony\ offspring}{Number\ of\ wild\ type\ offspring}$$

(the division by 2 accounts for the 2:1 ratio of ebony to wild type males). Thus, logit of the proportion of ebony offspring equals ln(relative fitness of ebony males) – ln(2). Thus, the parameter estimates from our models can be directly interpreted in terms of the relative sexual fitness of ebony.

We analysed both experiments in two alternative reference frames: (1) using male and female densities as the main continuous explanatory variables of interest and (2) using the $\log_2$ of the operational sex ratio and total density of males + females as the explanatory variables. For experiment 2, we also included the day of egg collection as the third continuous explanatory variable (expressed as 0, 1 and 2; this way the estimates of other explanatory variables can be interpreted as applying to the first day of the sexual competition); we also fit a separate model for each day. All models included the experimental block and the replicate as random effect. All interactions (including three-way interactions) between explanatory variables were initially included; those with $p > 0.10$ were dropped from final models. Significance was assessed with likelihood ratio tests.

Results

As expected, ebony males sired, in general, fewer offspring than would be expected under random mating, consistent with sexual selection acting against the ebony mutation (**Figures 1** and **2**). We analysed both experiments in two alternative reference frames: treating the female and male densities as predictor variables, and treating the (log2-transformed) sex ratio and total density (the number of males + females) as predictors. In experiment 1, paternity success of ebony males increased with female density (slope estimate = 0.096 ± 0.037, $\chi^2_{df=1}$ = 6.15, p = 0.013); no effect of male density could be detected even though the associated slope estimate was negative (-0.040 ± 0.037). We did not find a significant interaction between male and female density (**Supplementary Table 1**). In the alternative framework, with overall sex ratio and total density as the predictors, we found a significant negative effect of sex ratio ($\chi^2_{df=1}$ = 6.76, p = 0.0092; **Figure 1**): competitive paternity success of ebony males decreased with increasingly male-biased sex ratios. We did not detect any effect however of total density ($\chi^2_{df=1}$ = 1.23, p = 0.26) or sex ratio × density interaction ($\chi^2_{df=1}$ = 2.25, p = 0.13, excluded from the final model; **Supplementary Table 1**).

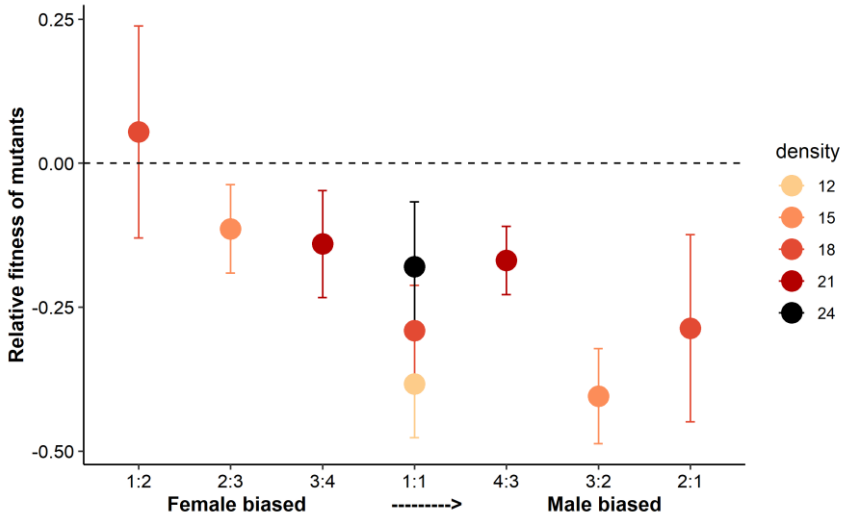

Figure 1. Data from experiment 1 indicates that higher relative fitness, which is indicative of lower competition from wild-type males gradually reduces over the continuum from female to male-biased sex ratios.

In experiment 2, in our joint model (with all collection days) we found that both male and female densities had a significant effect, however with opposite signs for slope estimates (**Table 1**; slope estimate: Females = 0.50 ± 0.089, Males = -0.54 ± 0.095). In models with overall sex ratio, we found that while sex ratio had a significant negative effect ($\chi^2_{df=1}$ = 11.40, $p<0.001$) on the relative success of *ebony* males, density did not ($\chi^2_{df=1}$ = 0.092, p = 0.76; **Supplementary Table 1**). In this model, although the day of collection did not have a significant effect ($\chi^2_{df=1}$ = 0.16, p = 0.68), the interaction of collection day with sex ratio was significant ($\chi^2_{df=1}$ = 9.95, p = 0.001), indicating a temporal pattern of the effect of sex ratio on competitive paternity success of mutants. In a subsequent simpler model (on removal of interaction of sex ratio with density; **Table 1**), we confirmed that sex ratio significantly influences the competitive paternity success of *ebony* males and that indeed there is a temporal pattern ($\chi^2_{df=1}$ = 42.87, $p<0.001$ and $\chi^2_{df=1}$ = 9.99, p = 0.001 respectively). Moreover, the day-wise models corroborate our finding about the interaction of sex ratio with collection day, showing that the effect of sex ratio significantly wears off over the experimental collection days from 1 to 3, even though it was significant on each day (**Table 1**; slope estimates: Day 1 = -0.57 ± 0.08, Day 2 = -0.22 ± 0.07, Day 3 = -0.21 ± 0.08, **Figure 2**).

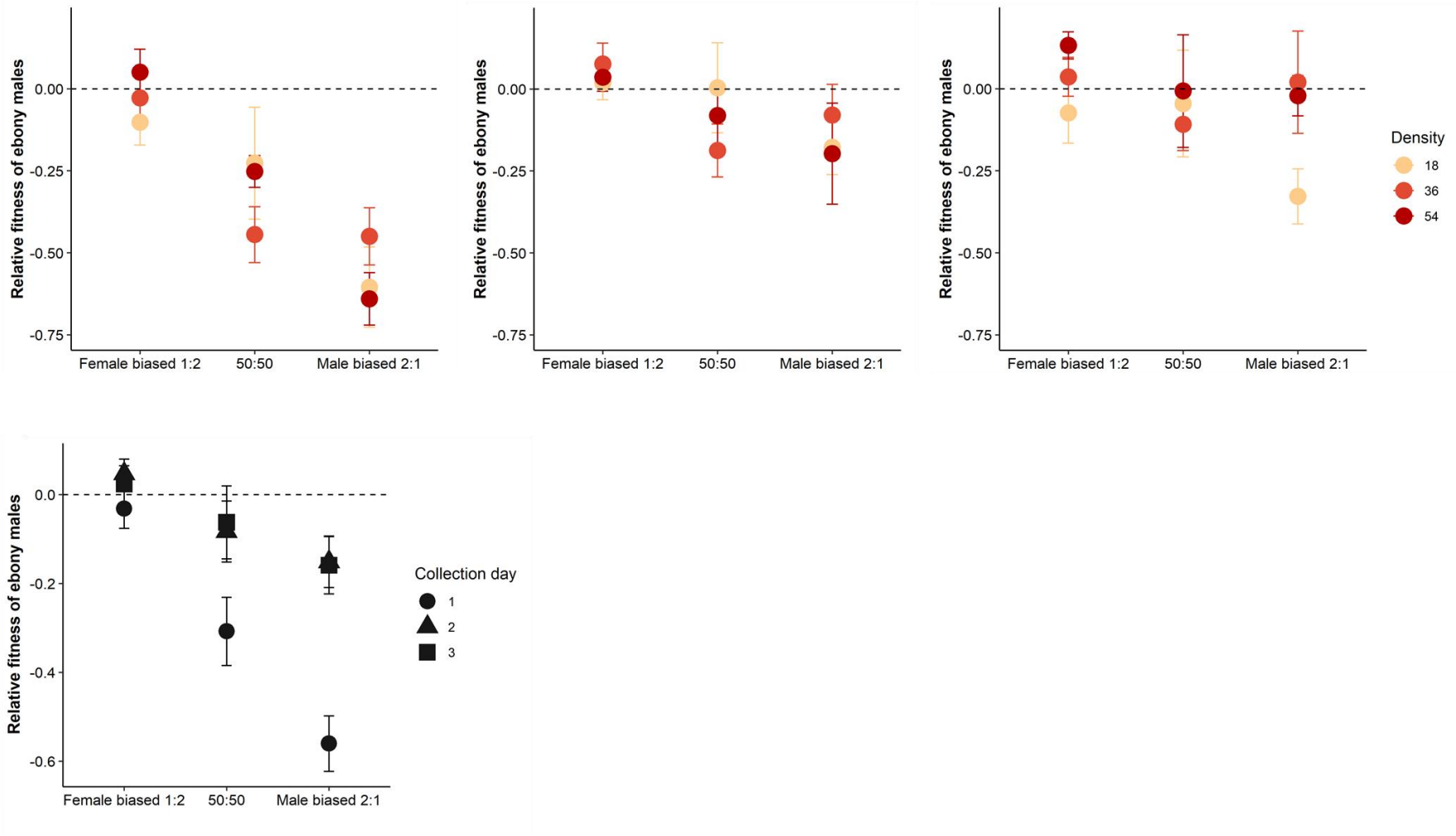

Figure 2. Experiment 2: a, b and c represent relative fitness on experimental days 1 (where all individuals were virgins), 2 and 3 respectively, while d. shows a day-wise change in relative fitness over different sex ratios. These plots indicate that effects of sex ratio weaken temporally over the experiment.

Discussion

In this study, we performed two independent experiments to study how sex ratio influences the strength of sexual selection against a genetic polymorphism. We found that the strength of selection against the *ebony* polymorphism increased through the gradient from female-biased to male-biased ratios. Our results indicate that the overall sex ratio, and not the relative densities of males and females, influences the strength of sexual selection. Interestingly, the effect of sex ratio slowly diminished as the experiment progressed to its third and final day indicating a temporal pattern of sex ratio on sexual selection.

Sex ratio is a debated indicator of the strength of sexual selection (Klug *et al.*, 2010; Kokko & Jennions, 2014). While biased sex ratios can lead to stronger mate competition and mate monopolization by the less abundant sex, resulting in stronger sexual selection (Emlen & Oring, 1977; Shuster, 2009; Klug *et al.*, 2010; Jennions *et al.*, 2012), this is not a given. We find that the strength of sexual selection against the mutant *ebony* allele increases from female to male-biased ratios. Polymorphisms on the *ebony* gene have pleiotropic effects such as dark brown cuticle colouration and partial visual impairment (Kyriacou *et al.*, 1978). They also influence mating traits such as wing vibration frequencies and locomotor activity (Jacobs, 1960; Kyriacou, 1981). Our findings therefore indicate that sexual selection acts on a pleiotropic locus that influences both sexual and non-sexual traits. This evidence is complementary to previous work that reported selection under biased sex ratios on certain sexual traits. For instance, in two-spotted gobies, two out of the four sexual traits examined were under stronger selection in equal-sex ratio compared to female-biased sex ratios (Wacker *et al.*, 2013). Similarly, in rough-skinned newts, a higher selection gradient for longer tail length, a sexually-selected trait, was seen in male-biased ratios compared to equal sex ratios (Jones *et al.*, 2004).

Our experiments assay the joint contributions of pre- and post-copulatory mate competition on the relative paternity success of *ebony* versus wild-type males. However,

our observation that the strength of sexual selection wanes temporally over the experiment, particularly from day 1 to 2 when most females would have mated, might indicate that the relative balance between pre- and post-copulatory sexual selection changes over time. For instance, it is plausible that higher relative selection on pre-copulatory versus post-copulatory competition operates at the start of the experiment, where all individuals are virgins and compete for first mating. However, as the experiment progresses, and as more females are inseminated, the focus of selection likely shifts from purely pre-copulatory to a mix of pre- and post-copulatory selection. The *ebony* males are visually impaired and lack appropriate courtship behaviours compared to wild-type males, making *ebony* males weaker at pre-copulatory competition compared to wild-types (Jacobs, 1960; Kyriacou *et al.*, 1978; Kyriacou, 1981). This possibly leads to stronger pre-copulatory selection than post-copulatory selection against *ebony* (relative to wild type), explaining the decreasing strength of selection over time.

We found that in both experiments, sexual selection reduced at female-biased compared to male-biased ratios. These results are robust to the different settings used in the two experiments. First, even though the overall sex ratios in a population of Drosophila is 50:50, observations in the wild indicate that male-biased mating aggregations are commonplace where a single female is chased and surrounded by multiple males (Atkinson & Shorrocks, 1977; Soto-Yéber *et al.*, 2018). Second, large and relatively spatially structured arenas (with clustering of food resources) used in experiment 2 might allow females to escape male harassment, as in the wild, thereby reflecting closer to real selection pressures as in nature.

In conclusion, our work shows that OSR is an important predictor of sexual selection with male-biased ratios leading to stronger sexual selection. Theory predicts that sexual selection can lead to the betterment of overall population fitness via selection on "good genes" (Rowe & Houle, 1996; Lorch *et al.*, 2003), which is supported by experimental work (Cally *et al.*, 2019). These good genes are predicted to capture overall genomic variation for not only sexual but also non-sexual traits – thereby linking sexual with non-

sexual selection (Rowe & Houle, 1996; Lorch *et al.*, 2003; Tomkins *et al.*, 2004). Our findings that male-biased sex ratios lead to stronger selection against a pleiotropic locus, implicated in non-sexual and sexual traits, is in line with this prediction. Our results indicate that male-biased sex ratios strengthen "good genes" effects and promote population-level effects.

Author contributions

S.S., T.J.K. and B.H. and designed experiments, S.S. performed experimental work. S.S., T.J.K. and B.H analyzed the data. S.S. and T.J.K. wrote the manuscript and S.S., T.J.K. and B.H. contributed to revisions.

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Data Accessibility

Data will be made available on dryad digital repository on acceptance.

Competing interests

We declare that we have no competing interests.

Tables

Table 1. Competitive paternity success of mutant *ebony* males recorded as the phenotypes of offspring obtained from mutant and wild type males across different sex ratio and density treatments.

Model	Parameter	d.f.	X^2	p
Experiment 1				
Intercept	-0.27 ± 0.46			
Female Density	0.096 ± 0.037	1	6.15	*0.013*
Male Density	-0.040 ± 0.037	1	1.20	0.27
Intercept	-0.26 ± 0.46			0.57
Sex ratio	-0.45 ± 0.17	1	6.76	*0.0092*
Total Density	0.027 ± 0.024	1	1.23	0.26
Experiment 2				
Joint model for all days with male and female density				
Intercept	0.23 ± 0.38			
Female density	0.50 ± 0.089	1	30.90	*<0.001*
Male density	-0.54 ± 0.095	1	31.45	*<0.001*
Day of collection	-0.29 ± 0.30	1	0.94	0.32
Female density × Day of collection	-0.12 ± 0.069	1	3.09	0.07
Male density × Day of collection	0.26 ± 0.074	1	12.62	*<0.001*
Reduced joint model for all days with overall sex ratio				
Intercept	0.13 ± 0.16	1		
Sex ratio	-0.52 ± 0.077	1	42.87	*<0.001*
Day of collection	0.049 ± 0.12	1	0.15	0.69
Density	-0.0015 ± 0.0045	1	0.10	0.74
Sex ratio × Day of collection	0.19 ± 0.06	1	9.99	*0.001*
Density × Day of collection	0.0064 ± 0.0035	1	3.25	0.071

Collection day 1				
Intercept	0.002 ± 0.07	1		
Sex ratio	-0.57 ± 0.08	1	35.34	*<0.001*
Collection day 2				
Intercept	0.52 ± 0.07	1		
Sex ratio	-0.22 ± 0.07	1	8.56	*0.003*
Collection day 3				
Intercept	0.51 ± 0.07	1		*<0.001*
Sex ratio	-0.21 ± 0.08	1	5.58	*0.018*

Supplemental Figures

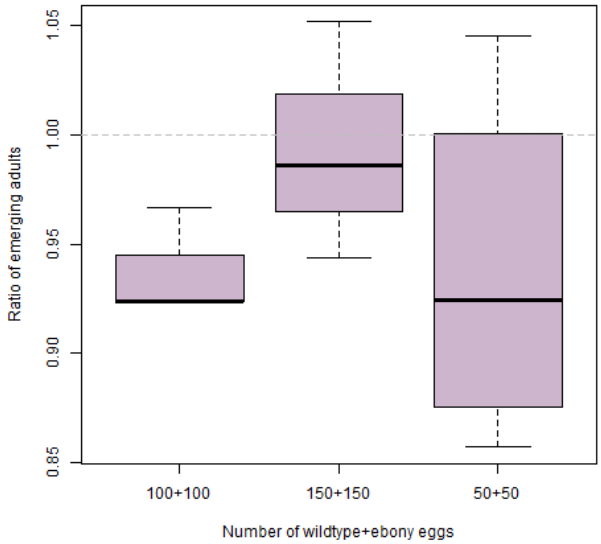

Figure S1. Relative larval survival of *ebony* and wild type eggs. Eggs from the two genotypes were taken in equal proportion (i.e. 1:1) and reared at three chosen densities (N = 100, 200 and 300). There is no difference in adult emergence between the two phenotypes.

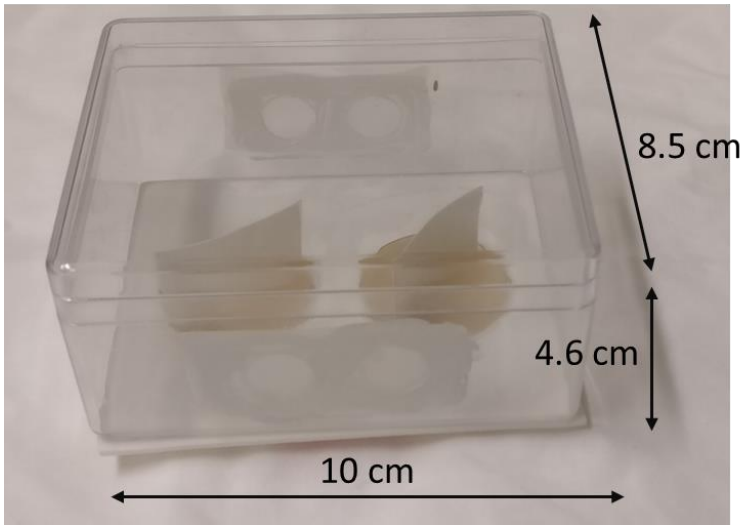

Figure S2. Arenas used for experiment 2. The arena has two replaceable circular inserts (petri-dishes) that were filled with food or medium for collecting eggs (agar + orange juice sprinkled with Baker's yeast). The partition on the food petri-dishes adds to the spatial complexity and large volume (391ml) of the arena potentially also allows females enough room to escape from male harassment.

Supplemental Tables

Supplementary Table 1. Competitive paternity success of mutant *ebony* males recorded as the phenotypes of offspring obtained from mutant and wild type males across different sex ratio and density treatments.

Model	Parameter	d.f.	X^2	p
Experiment 1				
Intercept	-3.50 ± 5.35			
Female Density	2.15 ± 2.51	1	0.72	*0.39*
Male Density	0.86 ± 2.44	1	0.12	0.72
Female Density × Male density	-0.60± 1.14	1	0.27	0.60
Intercept	-0.25 ± 0.460			0.58
Sex ratio	-2.99 ± 1.69	1	3.093	0.078
Total Density	0.026 ± 0.024	1	1.16	0.28
Sex ratio × Total Density	0.14 ± 0.096	1	2.25	0.13
Experiment 2				
Joint model for all days with overall sex ratio				
Intercept	0.12 ± 0.16			
Sex ratio	-0.48 ± 0.14	1	11.40	*<0.001*
Density	-0.0013 ± 0.0045	1	0.092	0.76
Day of collection	0.051 ± 0.12	1	0.16	0.68
Sex ratio × Density	-0.0013± 0.0034	1	0.14	0.80
Sex ratio × Day of collection	0.19 ± 0.06	1	9.95	*0.001*
Density × Day of collection	0.0064 ± 0.0035	1	3.22	0.072

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Chapter 5

General discussion

Major findings and future directions

This thesis brings together three important themes in sexual selection. First, I experimentally tested the predictions of the "good genes" hypothesis, namely that traits under sexual selection are honest signals of non-sexual fitness, specifically resistance to parasites. I built upon this work by investigating the genetic basis of sexual dimorphism in immunity, to understand pathogen-driven sexual selection. Third, I tested the relationship of an important demographical variable, sex ratio with sexual selection.

In Chapter 2, contrary to predictions of the "good genes" hypothesis, I found no evidence that sexual selection can promote the evolution of resistance to a pathogen (i.e., adaptation). Furthermore, I could not ascertain if sexual conflict, often attributed to counterbalancing the beneficial effects of sexual selection in populations (Chapman *et al.* 2003; Hosken *et al.* 2019), was one of the reasons behind the lack of adaptation in regimes under sexual selection. Intriguingly, in evolutionary regimes under pathogen pressure and sexual selection, male survival was depressed – in line with predicted negative effects of sexual conflict – but not females. It was therefore not possible to conclusively attribute lack of adaptation to sexual conflict.

The preliminary finding that the sexual success of infected males was lower than uninfected males indicated a potential for sexual selection to act on pathogen resistance. If genetic variation conferring resistance to *P. entomophila* had a similar positive effect on male competitive success after exposure to the pathogen, this scenario should have provided an opportunity for female choice to amplify non-sexual selection and accelerate adaptation to pathogen. We were not able to detect adaptation to pathogen in regimes under sexual selection and pathogen due to low mortality. However, it does not preclude

that sexual selection and pathogen presence did not act towards increasing male-competitiveness and female choice for resistant and "fitter" males. For instance, did males from sexual selection regimes have better reproductive success than males from regimes without sexual selection, and did this improve under pathogen pressure? In addition, were females under the sexual selection and pathogen evolutionary regimes able to discriminate between resistant and non-resistant males both in the presence and absence of pathogen? In fact, experiments to test competitive success of males from all regimes was one of the directions I wanted to take after the course of experimental evolution. However, I could not phenotype these populations further due to limitations on lab access brought on by COVID-19.

Interestingly, I observed sexually-dimorphic responses to selection. Counter to my expectation, when pathogen pressure was applied to males, only females evolved improved resistance. This observation implied a shared genetic basis of immunity, a prediction that I confirmed in Chapter 3, wherein I identified various loci on the genome associated with immunity in both sexes. I found that this broad genomic signature was, in part, associated to an enrichment of candidates in the upstream regions of genes, which might contribute to differential transcriptional regulation. This could suggest that at least some degree of difference between baseline and survivor individuals could potentially be attributed to differential gene expression of important immunity related genes. Further experiments assaying gene expression differences in baseline and survivors might help understand the mechanisms behind the action of alleles associated with immunity.

In Chapter 3, I highlight that the response to a pathogen pulls together a large chunk of the genome. The broad genomic signature might then be maintained via different selective pressures - shared, sex-specific, and sexually antagonistic selection. A possible interpretation of this is that if immunity is a basis for female choice, the trait might signal overall genetic variation and thereby, the genetic quality of a male.

The findings of sex-specific loci associated with immunity could have broad implications for sexual selection. It is likely that the effects of sexual selection on "good genes" related to immunity might be weakened by potential sex-specific effects of alleles. Sex-specific alleles might be associated with negative effects on immunity in one sex, but not the other.

I found a broad genomic signature of immunity, with the caveat that these results might not be generalisable to other populations. It is very plausible that candidate genes found in the single generation study of Chapter 3 might not reflect genes associated with immunity in the experimental evolution regimes of Chapter 2. It is possible that some candidate variants might be selected against in populations if they are too harmful in one sex or have negative epistatic interactions with important life-history traits. It thus, remains untested if candidate variants I found in Chapter 3 are the "fuel of evolution".

The choice of pathogen in Chapters 2 and 3 may have consequences for the interpretation of results. My choice of *P. entomophila* as a model had three major advantages. First, it caused relatively low rates of mortality (5-25%), making it biologically realistic. Second, I found that the competitive paternity success of infected males was ~10% lower than in uninfected males, therefore providing an opportunity for sexual selection to act on pathogen resistance. Third, infection by this pathogen was able to generate a broad signal on the genome with loci associated with immunity. However, despite the advantages, the choice of pathogen presented some corresponding challenges. The comparatively low mortality rate in my experiments (5-25%, versus 75% in other studies (Martins *et al.* 2013)) meant that the pathogen imposed weak selection. This combined with the inconsistency in virulence (personal communication with Bruno Lemaitre and Tadeusz Kawecki) across infection batches led to fluctuating selection pressure over generations thereby resulting in an unclear signal of adaptation after the course of experimental evolution. Taking stock of the advantages and disadvantages of this pathogen, in Chapter 4 I decided to study sexual selection without pathogens.

In Chapter 4, I found that the strength of sexual selection increased with the ratio of males to females (i.e. sexual selection is comparatively stronger in male-biased populations). This result might indicate the "purging" of "bad genes" under male-biased sex ratios characterised by heightened sexual selection. In the light of this result, in hindsight, I could have used male-biased sex ratios (as opposed to equal sex ratios for sexual selection regimes) to increase the strength of sexual selection in experimental regimes of Chapter 2. However, it is likely that we might not have observed the predicted benefits of increased sexual selection in a multi-generation experiment. Indeed, male-biased sex ratios might be characterised by higher sexual conflict (Wigby and Chapman 2004), thereby overshadowing the more subtle positive effects of sexual selection on net population fitness. Another concern that we would have had to consider when manipulating the mating system would have been that of effective population size (Snook 2001; Reuter *et al.* 2008). A bias in sex ratio entails a reduction in effective population size, which in turn increases the effect of inbreeding, making it more challenging to quantify the effect of sexual selection alone.

Temporal patterns of sexual selection indicate that while some genotypes might be poorer at pre-copulatory mate competition, they might subsequently be able to regain, to some extent, the lost mating opportunities and gain a share in paternity against another "more superior" genotype. My results provide evidence of this idea. Competitive paternity success gradually increased in favour of *ebony* males, albeit without totally out-competing wild type males. The *ebony* males are visually impaired and are deficient in appropriate courtship behaviours (such as wing vibration stimulation) compared to wild type males, making the former weaker at pre-copulatory competition compared to wild types (Jacobs 1960; Kyriacou *et al.* 1978; Kyriacou 1981). This possibly led to stronger pre-copulatory than post-copulatory selection against *ebony* (relative to wild type), explaining the decreasing strength of selection over time. Future work could test if these temporal patterns hold true with other genotypes – for instance especially with genotypes that are known to be poorer at pre-copulatory mate competition and post-copulatory sperm competition.

In conclusion, from the findings presented in this thesis, I challenge the "good genes" hypothesis about pathogens, positing that choosy females are favoured by selection because chosen males carry genetic variants for immunity that confer higher fitness. This finding has important implications on sexual selection as it highlights that the quest for deciphering the basis of female choice is far from over. Future work could use a wide range of pathogens to clarify the contexts (if any) under which the predictions of the "good genes" hypothesis by Hamilton and Zuk (1982) are accurate for populations. Moreover, understanding the role of pathogens in sexual selection also requires a better understanding of how immunity works. Much remains to be discovered regarding the mechanisms underlying immunity, its trade-offs with other life-history functions, and the sex-specific and sexually antagonistic components of immunity. Lastly, male-biased sex ratios seem to be an important demographic context in which the positive effects of sexual selection, i.e., the purging of "bad genes", might be visible. Future work should address the findings are generalizable over other genetic polymorphisms and other animal taxa with different mating systems.

My work with *D. melanogaster* has shed light on three outstanding questions in sexual selection. I highlight that the issue of non-alignment of sexual and natural selection is still unresolved putting into question the consequences of sexual selection for populations.

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