Sexual selection and bacteria

Submitted by

Samuel Oyesiku-Blakemore

to the University of Exeter as a thesis for the degree of

Doctor of Philosophy in Biological Sciences

April 2020

I certify that all material in this thesis which is not my own work has been identified and that no material has previously been submitted and approved for the award of a degree by this or any other University.

Signature: ……..

Abstract

The role of symbiotic bacteria in determining their host's phenotype has become increasingly apparent in recent times. These bacterial communities can influence a range of host traits and fitness correlates. Symbiotic bacteria can alter their host's immune function, metabolism, reproductive fitness, sexual and social signals as well as behaviour. The amount of research into the host fitness effects of symbiotic bacterial is rapidly increasing, however; few studies are investigating how these effects vary across different host genotypes. This thesis investigates the relationship between host genetic background and bacterial symbionts across a range of sexually selected fitness measures in *Drosophila simulans*. We focused on two main types of bacterial symbionts; exosymbionts, that consisted of gut bacterial communities and surface bacteria that inhabit the fly cuticle, along with the bacterial endosymbiont W*olbachia pipentis. Wolbachia* is known to influence host fitness in a range of ways that vary from parasitic to mutualistic. The nature of these effects has previously been found to depend on both the host and the strain of *Wolbachia.* Previous work has attributed fitness effects found when curing *Wolbachia* infection with antibiotics to the change in the *Wolbachia* infection status. Antibiotic treatment is likely to change other bacterial components of the microbiota alongside removing *Wolbachia* infection.

In chapter 2 I found that antibiotic-caused male sexual-fitness rank changes across genotypes were caused by *Wolbachia* curing and not altering exosymbiotic bacterial communities. In Chapter 3 I found that the level of bidirectional cytoplasmic incompatibility suffered when mating with a standardised tester mate, was dependant on the genotype of the focal host. This effect was true for both focal males and females. In Chapter 4 I tested whether *D. simulans* populations evolving under elevated or relaxed natural and sexual selection for 38 generations differed in their gut bacterial communities. We found evolving under elevated sexual selection resulted in more diverse gut bacteria for males but not females. We also found sexual selection altered the gut bacterial community composition of both males and females. We found no effects of natural selection on gut microbial communities and no interaction between natural and sexual selection intensity on these communities. In Chapter 5 I found that altering exosymbiotic bacterial communities had fitness effects on both

males and females. In females these effects were only present when the bacterial communities were altered, not if the bacteria were simply removed. In Chapter 6 I found that *Wolbachia* infection affects female choosiness dependent on the females' genotype. Removing the exosymbiotic bacteria from females had no effect on their choosiness and genotype did not interact with this bacterial treatment. I also found that removing the symbiotic bacteria of females reduces their adult body size, however hosts infected with *Wolbachia* did not experience the same body size reduction with exosymbiont removal. Symbiotic bacteria are playing an important role in many sexually selected fitness traits. The direction and scale of these fitness effects depend on the host's genetic background. Sexual selection is also able to act on a host's gut bacteria. This means that a host's symbiotic bacteria are likely to play an important role in the evolutionary outcomes of sexual selection. This thesis increases our understanding of the role symbiotic bacteria play in sexual selection.

Acknowledgements

Firstly, I'm immensely grateful to both of my supervisors who have helped support and guide me though the entirety of my PhD. In the first year of my undergraduate degree, Dave Hosken managed to make evolutionary biology cooler than coral reefs and turtles. His enthusiasm for evolutionary biology is contagious and working in his lab has made me a better scientist. Beyond this, his support and encouragement has made this PhD possible, even when I didn't feel capable. Nina Wedell provided invaluable insight into the design and planning of this whole project. Beyond Nina's scientific insight, her attitude and optimism made even daunting tasks seem possible. I am so thankful to both my supervisors for their support, and for believing in me through this whole process.

Secondly, a huge shout out to the students, post docs and techs that made up fly lab and who would always be happy and willing to lend a hand. Mikey, Stefan, Matt, Will, Ali, Josie, MD, Andreas, Laura and Ruth, you guys are amazing. You were friends and my support throughout the insanity of fly work, so thank you. Without the friends I made within the department, completing a PhD would have been impossible. A special thank you to Ben, my radio co-host and partner in crime. When times were hard, you made things better.

To my family, who have provided me with endless support and inspiration that has shaped me in to the person I am today. My Grandmother is amazing, and I remember as a child her teaching me about her medical research, and thinking it was the coolest thing to 'invent' treatments! My parents, Sara and Andrew, who have always encouraged me to be the best I can, in whatever I set my mind to. They never fail to show their love or tell me how proud they are. There isn't the space to thank my family enough for all they have done, but I am eternally grateful. Finally, my partner Alicia, without you there would not be a PhD thesis here. You have provided love, support and guidance through it all (and lots of tea, too!). Thank you for being amazing, and staying up all night with me to help get this done!

Table of contents

List of tables and figures

Chapter 2

Chapter 3

Chapter 4

Chapter 5

Chapter 6

Author's declaration

Samuel Oyesiku-Blakemore (SOB) was funded by Natural Environmental Research Council, and all research presented within this thesis was conducted under the supervision of Prof. David J. Hosken (DJH) and Prof. Nina Wedell (NW). Each chapter was written by SOB in collaboration with DJH and NW. Additional support and contributions for each chapter are detailed below.

Chapter 2.The data were collected for assay one by A. Di Nisio and M.D. Sharma data for all other assays were collected by SOB, M.F. Hawkes (MFH), S. Store (SS), M. Davey (MD), Matthew Carey (MC), Josie Plachta (JP), Alicia Eveson (AE), M.D. Sharma (MDS) and CR Archer (CRA). The analyses were conducted by DJH and SOB.

Chapters 3, 5 and 6. Data were collected by SOB, MFH, SS, MC, JP, MDS and CRA. Data was analysed by SOB. Wing dissections were performed by SOB and Alison Skeats (AS).

Chapter 4. Eoin Duffy and Richa Joag maintained the selection lines. Gut dissections were performed by SOB and MFH. MDS ran the data clean up and pipeline through Qiime2. SOB, MFH and MDS ran analyses in the phyloseq package in RStudio.

Chapter 1

General introduction

Sexual selection can be thought of as variation in reproductive fitness (Hosken & House, 2011). Its mechanisms are mate choice, normally female choice, and mate competition, usually male-male. Male-male competition can occur both pre and post copulation and consists of males competing for access to females or male gametes competing for access to female gametes (sperm competition). Male-male competition causes selection to act on traits that make males better at monopolising access to females or their gametes. This leads to the evolution of exaggerated fighting weaponry (Berglund et al., 1996), male biased sexual size dimorphism (Fairbairn, 1997) and extreme sperm size or number (Gomendio & Roldan, 1991).

Females tend to be more choosey because on average they invest more in each reproductive event. Female choice can occur both pre and post copulation where females choose to mate with preferred males based on specific phenotypes or a combination of phenotypes (Andersson, 1994). Whilst understanding why male-male competition would evolve is fairly easy, understanding the evolution of female choice is more difficult. Female choice leads to the evolution of exaggerated male phenotypes for courtship or reproduction (Andersson, 1994) and females have evolved extreme female reproductive tracts that that allow for post-copulatory female choice (cryptic female choice) (Firman et al. 2017).

The evolution of exaggerated sexual traits can also be influenced by other organisms. For example, the presence of predators reduces the level conspicuous sexual colouration male guppies (*Poecilia reticulata*) (Endler, 1983). Increasingly we are becoming aware that the symbiotic bacterial communities that live on and in animals play an important part in determining their host's phenotype (Archie & Theis, 2011). For example, in humans the genes of the gut microbiome alone outnumber those in the human genome at least 100 to 1 (Gill et al., 2006). It is therefore highly likely that these microbial communities could be playing an important role in sexual selection, however these effects are rarely considered or explored.

Research into the influence of symbiotic bacteria on the sexually selected traits of their hosts is limited. The majority of research linking symbiotic bacteria to sexual selection so far has focused on the reproductive parasite *Wolbachia pipientis* (*Wolbachia*) (Werren et al. 2008). *Wolbachia* is an obligate bacterial endosymbiont found in the cytoplasm of arthropods. *Wolbachia* is maternally transmitted and incredibly abundant infecting between 20% and 76% of insect species (Stouthamer et al., 1999), as well as infecting mites, nematodes and crustaceans (Jeyaprakash & Hoy, 2000). This makes it one of the most prevalent bacterial symbionts in the animal kingdom. This alone would warrant the vast number of studies of *Wolbachia,* however it is the effects *Wolbachia* has as a reproductive parasite that receive the most investigation.

Wolbachia is maternally transmitted meaning males are evolutionary dead ends and so its evolutionary optimum is not always aligned with that of its host. This means there has been selection on *Wolbachia* to alter its host's reproductive biology, which can have fitness effects ranging from beneficial to costly. In some nematode worms and parasitic wasps *Wolbachia* is essential for normal reproduction (Bandi et al., 1999; Dedeine et al., 2001). *Wolbachia* alters host reproduction in many other ways even causing extreme reproductive phenotypes. These reproductive phenotypes include cytoplasmic incompatibility, parthenogenesis, male killing and feminisation of genetic males (see box 1 for description).

These significant *Wolbachia* induced changes in reproductive phenotype can be costly, and so there is selection on the host to overcome these costs. This can lead to host parasite coevolution. Experiments using the two closely related Drosophila species, *D. melanogaster* and *D. simulans,* have transinfected *D. simulans* with a strain of *Wolbachia* from *D. melanogaster*. They found that in *D. simulans* the strain causes 98% CI induced embryonic mortality compared to 18-32% in *D. melanogaster (*Poinsot et al. 1998*)*. This suggests that the *D. melanogaster* has coevolved with the *Wolbachia* to reduce the level of CI. They can also have important effects on sexual selection, for example where *Wolbachia* induces male killing in the butterfly *Acraea encedon*, the sex ratio is extremely female biased and has led to sex role reversal (Jiggins et al. 2000).

In the woodlouse *Armadillidium vulgare,* feminising *Wolbachia* has caused the

evolution of a new mechanism of sex determination in some populations. Females are ancestrally and in some populations the heterogametic sex with males being ZZ and females ZW. In some populations with the feminising *Wolbachia* strain the female determining W chromosome has been lost so all individuals are ZZ. This means that *Wolbachia* infection is the sex-determining factor and there has been selection on the reduction of *Wolbachia* transmission efficiency (Rigaud & Juchault, 1993). Further work on *A. vulgare* has found that as populations become female biased and sex roles reverse males prefer to mate with 'real' females than feminised males (Moreau et al. 2001). This then leads to the question of how in some populations the female determining W chromosome has been lost. If males are more likely to choose to mate females carrying the W chromosome, then there should be selection on its maintenance in the population.

Wolbachia can also have effects on host reproduction beyond these large phenotype changes, which are diverse and seem to depend on the host and the strain of *Wolbachia*. These effects have important implications for sexual selection in their host. In male *D. simulans Wolbachia* has been associated with a decrease in fertility, sperm production and sperm competitive ability (Snook et al., 2000; Champion de Crespigny & Wedell, 2006). This is in contrast with *Wolbachia*'s effect in the flour beetle *Tribolium confusum* where infected males have an increased fertility (Wade & Chang, 1995). Finding conflicting examples of the effect of *Wolbachia* infection is not rare which further highlights that the host and strain interact in a dynamic way. In both *D. melanogaster* and *D. simulans, Wolbachia* infection increases male mating rate (Champion de Crespigny et al., 2006). This may have evolved as a host response because CI rates are reduced with multiple matings (Karr et al., 1998), or it may be caused by the *Wolbachia* to increase the males' chance of mating with uninfected females.

The female effects of *Wolbachia* infection are also well studied and are equally variable. Female *T. confusum* infected with *Wolbachia* have fewer offspring than uninfected females (Wade & Chang, 1995). This is the opposite of what is found in males suggesting there may be sexual conflict over *Wolbachia* infection. Female *D. simulans* from California show the incredible speed at which the fitness effects of *Wolbachia* can evolve, where infection has changed from causing a reduction in

fecundity of 20% to a benefit of 10% over 20 years (Weeks et al., 2007). This also displays how hosts and *Wolbachia* coevolve and why the fitness effects are so varied. For example, in different strains of *D. melanogaster* infected with the same strain of *Wolbachia* there were variable fitness effects across fecundity and survival (Fry et al., 2004). Some *D. melanogaster* strains showing enhanced survival or fecundity in infected females and others showing no effect or one even incurring a cost.

There are clearly many effects that *Wolbachia* has on its host, however the majority of studies that compare *Wolbachia* infected and uninfected individuals use antibiotics to remove the infection. This antibiotic treatment is not a targeted approach and will remove all non-resistant symbiotic bacteria. Only since recent advances in molecular techniques have the importance of these other bacterial symbionts started to be realised. A variety of symbiotic bacteria have been shown to influence signalling, male attractiveness, kin recognition, and female choice while also having wider behavioural effects.

When courting, males use a variety of signals in attempt to attract a mate, bacteria play an important role in many of these signals in a number of ways. Sexual signals can be incredibly diverse ranging from olfactory pheromones to colourful visual displays, or behaviours such as dancing. Hawaiian bobtail squid (*Euprymna scolopes)* have a light organ within their mantle that contains the bioluminescent bacteria, *Vibrio fischeri,* that is used for camouflage. The Hawaiian bobtail squid is infact able to control the level of bioluminescence emitted by bacteria (Boettcher et al., 1996). It is also possible these bioluminescent bacteria could be involved in courtship, however little is known about the reproductive behaviour of bobtail squid. Under laboratory conditions the closely related Atlantic bobtail squid (*Sepiola atlantica*) was observed mating and there appeared to be no courtship (Rodrigues et al., 2009), however, this was with only 5 matings and it is unlikely to be representative of what happens in the wild. As these squid are active at night, it is definitely possible that the bioluminescence is involved in courtship, however this needs further investigation.

Many birds have extravagant and colourful plumages that have evolved as a result of sexual selection. Females have frequently been shown to choose the showiest male, however explaining why females choose these more conspicuous males is more

difficult. One explanation, the handicap principle, is that the traits are costly to carry and so only the best condition males can afford to carry them (Zahavi, 1975). The cost of carrying these signals appears to be mediated by symbiotic bacteria in some cases. Bird's feathers are home to bacteria communities a subset of which are known as feather degrading bacteria (FDB) because of their ability to break down feathers. These FDB are phylogenetically diverse (Onifade et al. 1998) and relatively prevalent across bird species (Burtt & Ichida, 1999).

In spotless starlings (*Sturnus unicolor*), feathers responsible for male sexual signaling are more susceptible to degradation and habour more bacteria than feathers not used for signalling (Ruiz-Rodríguez et al., 2015). There are further examples of feathers involved in sexual signalling being degraded faster than normal feathers. Unmelanised areas of feathers are degraded by FDB faster than melanised ones (Ruiz-De-Castañeda et al., 2012), and often white feathers or patches are involved in sexual signalling. In the European flycatcher (*Ficedula hypoleuca*) males have larger and brighter white patches on their wings than females and these patches are sexually selected. The white patches of the feathers are degraded faster than the dark areas of the same feathers (Ruiz-De-Castañeda et al., 2012). With these wing feathers not only involved in signalling but also flight having more degradable feathers will be costly and symbiotic bacteria are responsible for some of these costs (Ruiz-De-Castañeda et al., 2012). Symbiotic bacteria do not only impose some of the costs of sexual displays in birds they also help to protect against them. The uropygial glands of birds produce oily secretions that are used to protect the feathers while improving their flexibility and waterproofness (Moreno-Rueda, 2017). The secretion also reduces the growth of feather degrading bacteria, and the size of the gland inversely correlates with feather degradation (Moreno-Rueda, 2010). Symbiotic bacteria that live within the uropygial gland produce antibiotic peptides that protect against the feather degrading bacteria (Martín-Vivaldi et al., 2009).

The symbiotic bacteria that live within animals' guts have become the focus of many studies recently, and their impact on host fitness is hugely diverse and stretches beyond the obvious digestive and immune functions. In *D. melanogaster* alterations to the composition of the gut microbiota caused changes in male cuticular hydrocarbon (CHC) profile, which are pheromones used in courtship (Sharon et al. 2010; Inglby,

2015). Females also preferred to mate with males that had similar gut microbiota to them (Sharon et al. 2010). The effects found in this study could not be replicated in when the work was repeated by Leftwich et al. (2017) although this study failed to find any diet based assortative mating they did find *D. melanogaster* raised on different diets had different gut bacterial communities. The diet based assortative mating effect has been by Najarro et al. (2015). Further work has suggested that variation in the presence and amount of fungicide in the diets used in these experiments may explain the difficulty in replicating these result (Obadia et al. 2018). Fungicide present in the diets used has been shown to potentially alter the gut bacterial profiles of *D. melanogaster* (Obadia et al. 2018). Despite these various studies the microbiota associated assortative mating appear to be present when the diet mediated assortative matings are also present. In Mediterranean fruit flies (*Ceratitis capitata*) changes to the symbiotic gut bacteria alters male reproductive success (Ami et al., 2009). When sterilising males for use in pest control the gut microbiota was altered this caused the males to be less successful at securing matings. This effect was removed after reinfecting the flies with gut bacteria they had lost (Ami et al., 2009). Gut bacteria inhibits kin recognition in *D. melanogaster* where removing the gut microbiota caused males to invest significantly less when copulating with siblings (Lize et al., 2014). It is possible that there is conflict between the gut bacteria and host over male reproductive investment. The gut bacteria benefit from males mating with siblings because a large portion of the gut microbiota is maternally transmitted (Wade, 2014). Although males benefit by limiting their investment when mating with siblings as offspring may suffer from inbreeding costs (Okada et al., 2011). The possibility of host-gut conflict has yet to be explored and warrants further testing.

Gut microbiota have even been shown to have important effects on host behaviour. The most notable of these is in mice, where changes in gut microbiota caused increased anxiety and neurochemical changes (Neufeld et al., 2011). Behavioural changes are likely to have important implications in terms of sexual selection however so far no work has looked at the gut-brain axis from this angle. In the most extreme case, gut bacteria can cause reproductive isolation between two closely related species from the genus *Nasonia*. The hybrid lethality between *Narsonia vitripennis* and *Narsonia giraulti* is almost completely removed by the curing of their gut bacteria and can be brought back by reinfection (Brucker & Bordenstein, 2013). This highlights how

the effects of animal's gut bacteria can be very similar to the effects of *Wolbachia* where this hybrid lethality is similar to cytoplasmic incompatibility.

Clearly, *Wolbachia* amongst other reproductive parasites, play an important role in host reproduction, and therefore in sexual selection, also. The effects reproductive parasites have on their host are becoming increasingly important to understand as their potential for pest control is explored. If *Wolbachia* infection causes males to be less able to secure matings or to produce fewer and or less competitive sperm, then releasing them into the population may not be a cost effective way to reduce pest numbers. There is still potential to explore how *Wolbachia* infection interacts with host genotype. With most studies of *Wolbachia,* ignoring the potential impact of other symbiotic bacteria. The varied effects of the gut microbiota alone show how important they can be in terms of host reproduction. It is therefore important to control for the effects of the gut microbiota when testing the reproductive consequences of *Wolbachia* infection. Despite the wider fitness effects of symbiotic gut bacteria becoming increasingly well studied, its impact on sexual selection is still poorly understood. It will be important to control for symbiotic bacteria when studying all aspects of sexual selection. For example *Wolbachia* can bias estimates of sexual conflict in *D. simulans* (Duffy et al. 2019). The classic view of sexual selection may need to be adjusted as we become increasingly aware that symbiotic bacteria plays an important role in shaping both male and female reproductive fitness.

This thesis will explore how the gut microbiota and host genotype interact. We use the model organism *D. simulans* to investigate these interactions. *Drosophila simulans* separated from its closely related sister species *D. melanogaster* around 2 million years ago (Powell, 1997). Although *D*. *melanogaster* is the more frequently studied model species there are substantial differences between the two species in relation to both natural (Chakir et al. 2002) and sexual selection (Taylor et al. 2009). Using a variety of model species provides us with a more complete picture of the natural world. *Drosophila simulans* are an ideal model to study the effects of the microbiota on several aspects of sexual selection. Females control mating decisions in *D. simulans,* so there is no forced copulation once females are sexually mature (Spieth, 1974; Markow, 2000). This allows us to study female choosiness and preference, as well as male attractiveness and competitive ability. *Drosophila spp.* also have relatively simple

symbiotic microbial communities (Wong et al. 2013). *Wolbachia* infection has also been found to impact range of sexually and naturally selected traits in *D. simulans* (Champion de Crespigny & Wedell, 2006; Weeks et al. 2007; Champion de Crespigny & Wedell, 2007; Bi et al. 2019). We are also able to use iso-genetic strains (isofemale lines, hereafter genotypes), as they are powerful way to assess naturally occurring genetic variation in a population and enable us to repeatably measure a range of fixed genotypes (Hoffmann & Parsons, 1988; David et al. 2005). Therefore, we are able to test the fitness effects of the microbiota across a range of genotypes.

In Chapter 2, we investigate effects of *Wolbachia* infection and microbiota changes on male sexual-fitness across different genotypes. We used antibiotics to cure *Wolbachia* infection and then manipulated the microbiota in different ways to test if Wolbachia or other aspects of the microbiota caused the fitness rank changes with antibiotic treatment. In Chapter 3 we explore the possibility of bidirectional cytoplasmic incompatibility between the strain of *Wolbachia* our focal isofemale lines are infected with, and the strain our tester *ebony* flies are infected with. We also test if the level of CI depends on the host's genotype when all focal flies were infected with the same *Wolbachia* strain. In Chapter 4 we evolve populations under either elevated or relaxed, natural and sexual selection in a fully-factorial manner. We then sequence the gut microbial communities of each population replicate for males and females using 16s rRNA amplicon sequencing. We test the gut microbiome response to natural and sexual selection in both males and females and the interactions between natural and sexual selection. In Chapter 5 we manipulate the symbiotic microbial communities of males and females and include a third microbial treatment of a novel microbiota collected from *Drosophila pseudoobscura* raised on a different diet. This allows us to compare the fitness effects of microbiota removal to the alteration of the microbiota to a community that is less adaptive. We manipulated the microbiota across genotypes to test if the response depends on the host's genetic background. We test the fitness effects of this microbiota manipulation across a range of fitness measures in both males and females. In Chapter 6 we investigate how *Wolbachia* infection and other aspects of the microbiota affect female choosiness and body size across different genotypes.

Box 1.

Cytoplasmic incompatibility (CI) is the most widespread and studied *Wolbachia*-induced phenotype (reviewed in Hoffmann & Turelli, 1997). CI occurs when males infected with a strain of *Wolbachia* mate with a female that is either uninfected or infected with a different strain of *Wolbachia* resulting in zygote mortality. All other crosses result in no zygote mortality meaning that females without that strain of *Wolbachia* have a lower reproductive output and that strain spreads throughout the population. One reason this phenotype has received so much attention is its possible use in pest control, by releasing CI inducing *Wolbachia* infected males into the population. If these males mate with uninfected wild females they will not produce offspring and so pest numbers will decrease (LePage & Bordenstein, 2013).

Parthenogenesis inducing *Wolbachia* strains cause females to produce *Wolbachia* infected daughters without fertilisation from a male. Unfertilised eggs that normally would develop into a haploid male, which are an evolutionary dead end for the infecting *Wolbachia*, actually develop into diploid females. This means that the *Wolbachia* strain spreads throughout the population, as infected females can produce double the number of daughters as uninfected females. As a result this changes in the sex ratio with the population becoming female biased which can have further effects on sexual selection. *Wolbachia* induced parthenogenesis has been found in thrips, wasps and mites (Arakaki et al., 2001; Huigens et al., 2004; Weeks & Breeuwer, 2001).

Male killing strains of *Wolbachia* act by killing any genetic male offspring. This phenotype only evolves when there is an effect of sibling competition on offspring fitness/survival. Male killing strains can spread through a population as females do not waste resources caring for sons or there is reduced competition for their daughters. Similar to parthenogenesis male killing also causes the population to become females biased. *Wolbachia* induced male killing can be found in Coleoptera, Lepidoptera and Diptera (Hurst et al., 1999; Hurst et al., 2000).

Wolbachia induced feminisation of genetic males is a process where genetically male offspring of *Wolbachia* infected females develop as functional females. *Wolbachia* induced feminisation occurs in some Crustacea, Hemiptera, and Lepidoptera (Rousset et al., 1992; Kageyama et al., 2002; Negri et al., 2006). Inducing feminisation evolves as it benefits the *Wolbachia* in a similar way to inducing parthenogenesis it means that all offspring females produce are daughters and so are able to transmit *Wolbachia*.

References

Ami, E.B., Yuval, B. & Jurkevitch, E., 2009. Manipulation of the microbiota of mass-reared Mediterranean fruit flies *Ceratitis capitata* (*Diptera*: *Tephritidae*) improves sterile male sexual performance. *ISME J*, 4(1), pp.28–37.

Andersson, M., 1994. Sexual selection. Princeton University Press; Princeton, NJ.

Arakaki, N., Miyoshi, T. & Noda, H., 2001. *Wolbachia*–mediated parthenogenesis in the predatory thrips *Franklinothrips vespiformis* (*Thysanoptera*: *Insecta*). *Proceedings of the Royal Society of London B: Biological Sciences*, 268(1471), pp.1011–1016.

Archie, E.A. & Theis, K.R., 2011. Animal behaviour meets microbial ecology. *Animal Behaviour*, 82(3), pp.425–436.

Bandi, C. et al., 1999. Effects of tetracycline on the filarial worms *Brugia pahangi* and *Dirofilaria immitis* and their bacterial endosymbionts *Wolbachia*. *International Journal for Parasitology*, 29(2), pp.357–364.

Berglund, A., Bisazza, A. & Pilastro, A., 1996. Armaments and ornaments: an evolutionary explanation of traits of dual utility. *Biological Journal of the Linnean Society*, 58(4), pp.385–399.

Bi, J. et al., 2019. *Wolbachia* infection may improve learning and memory capacity of *Drosophila* by altering host gene expression through microRNA. *Insect Biochemistry and Molecular Biology*, 106, pp.47–54.

Boettcher, K.J., Ruby, E.G. & McFall-Ngai, M.J., 1996. Bioluminescence in the symbiotic squid *Euprymna scolopes* is controlled by a daily biological rhythm. *Journal of Comparative Physiology A*, 179(1), pp.65–73.

Brucker, R.M. & Bordenstein, S.R., 2013. The hologenomic basis of speciation: Gut bacteria cause hybrid lethality in the genus *Nasonia*. *Science,* 341 (6146), pp.667–669.

Burtt Jr., E.H. & Ichida, J.M., 1999. Occurrence of feather-degrading *Bacilli* in the

plumage of birds. *The Auk*, 116(2), pp.364–372.

Chakir, M. et al., 2002. Male sterility thermal thresholds in *Drosophila*: *D. simulans* appears more cold-adapted than its sibling *D. melanogaster*. *Genetica*, 114(2), pp.195–205.

Champion de Crespigny, F.E., Pitt, T.D. & Wedell, N., 2006. Increased male mating rate in *Drosophila* is associated with *Wolbachia* infection. *Journal of Evolutionary Biology*, 19(6), pp.1964–1972.

Champion de Crespigny, F.E. & Wedell, N., 2006. *Wolbachia* infection reduces sperm competitive ability in an insect. *Proceedings of the Royal Society B: Biological Sciences*, 273(1593), pp.1455–1458.

Champion de Crespigny, F.E. & Wedell, N., 2007. Mate preferences in *Drosophila* infected with *Wolbachia*? *Behavioral Ecology and Sociobiology*, 61(8), pp.1229–1235.

David, J.R. et al., 2005. Isofemale lines in *Drosophila*: An empirical approach to quantitative trait analysis in natural populations. *Heredity*, 94(1), pp.3–12.

Dedeine, F. et al., 2001. Removing symbiotic *Wolbachia* bacteria specifically inhibits oogenesis in a parasitic wasp. *Proceedings of the National Academy of Sciences,* 98 (11), pp.6247–6252.

Duffy, E., Archer, C.R., Sharma, M.D., et al., 2019. *Wolbachia* infection can bias estimates of intralocus sexual conflict. *Ecol Evol*, 9, pp.328– 338.

Endler, J.A., 1983. Natural and sexual selection on color patterns in *poeciliid* fishes. *Environmetal Biology of Fishes* 9, pp.173–190.

Fairbairn, D.J., 1997. Allometry for sexual size dimorphism: Pattern and process in the coevolution of body size in males and females. *Annual Review of Ecology and Systematics*, 28, pp.659–687.

Firman, R.C., Gasparini, C., Manier, M.K. & Pizzari, T., 2017. Postmating female control: 20 years of cryptic female choice. *Trends in Ecology & Evolution*, 32(5), pp.368-382.

Fry, A.J., Palmer, M.R. & Rand, D.M., 2004. Variable fitness effects of *Wolbachia* infection in *Drosophila melanogaster*. *Heredity*, 93(4), pp.379– 389.

Gill, S. et al., 2006. Metagenomic analysis of the human distal gut microbiome. *Science*, 312(5778), pp.1355–1359.

Gomendio, M. & Roldan, E.R.S., 1991. Sperm competition influences sperm size in mammals. *Proceedings of the Royal Society B: Biological Sciences*, 243(1308), pp.181–185.

Hoffmann, A. & Parsons, P., 1988. The analysis of quantitative variation in natural populations with isofemale strains. *Genetics Selection Evolution*, 20(1), p.87.

Hoffmann, A.A. & Turelli, M., 1997. Cytoplasmic incompatibility in insects. In: O'Neill S.L., Hoffman, A.A. & Werren, J.H., (eds). *Influential Passengers: Inherited Microorganisms and Arthropod Reproduction*. Oxford University Press, Oxford, UK, pp.42–80.

Hosken, D.J. & House, C.M., 2011. Sexual selection. *Current Biology*, 21(2), pp.62-65.

Huigens, M.E. et al., 2004. Natural interspecific and intraspecific horizontal transfer of parthenogenesis–inducing *Wolbachia* in *Trichogramma* wasps. *Proceedings of the Royal Society B: Biological Sciences*, 271(1538), pp.509–515.

Hurst, G.D.D. et al., 2000. Male-killing *Wolbachia* in *Drosophila*: A temperature-sensitive trait with a threshold bacterial density. *Genetics*, 156(2), pp.699–709.

Hurst, G.D.D. et al., 1999. Male–killing *Wolbachia* in two species of insect. *Proceedings of the Royal Society B: Biological Sciences*, 266(1420), pp.735–740.

Ingleby, F.C., 2015. Insect Cuticular Hydrocarbons as Dynamic Traits in Sexual Communication. *Insects*, 6, pp.732-742.

Jiggins, F.M., Hurst, G.D. & Majerus, M.E., 2000. Sex-ratio-distorting *Wolbachia* causes sex-role reversal in its butterfly host. *Proceedings of the Royal Society B: Biological Sciences.*, 267(August 1999), pp.69–73.

Kageyama, D. et al., 2002. Feminizing *Wolbachia* in an insect, *Ostrinia furnacalis* (*Lepidoptera: Crambidae*). *Heredity*, 88(6), pp.444–449.

Karr, T.L., Yang, W. & Feder, M.E., 1998. Overcoming cytoplasmic incompatibility in *Drosophila*. *Proceedings of the Royal Society B: Biological Sciences*, 265(1394), pp.391–395.

Kokko, H. & Rankin, D.J., 2006. Lonely hearts or sex in the city? Density- dependent effects in mating systems. *Philosophical Transactions of the Royal Society B: Biological Sciences*, 361(1466), pp.319–334.

Leftwich, PT. et al., 2017. Gut microbiomes and reproductive isolation in *Drosophila*. *PNAS,* 114, pp.12767–12772.

LePage, D. & Bordenstein, S.R., 2013. *Wolbachia*: Can we save lives with a great pandemic? *Trends in parasitology*, 29(8), pp.385–93.

Lize, A., McKay, R. & Lewis, Z., 2014. Kin recognition in *Drosophila*: the importance of ecology and gut microbiota. *ISME J*, 8(2), pp.469–477.

Markow, T.A., 2000. Forced matings in natural populations of *Drosophila*. *American Naturalist*, 156(1), pp.100–103.

Martín-Vivaldi, M. et al., 2009. Antimicrobial chemicals in hoopoe preen secretions are produced by symbiotic bacteria. *Proceedings of the Royal Society B: Biological Sciences*, 277(1678), pp.123–130.

Moreau, J. et al., 2001. Sexual selection in an isopod with *Wolbachia*-induced sex reversal: males prefer real females. *Journal of Evolutionary Biology*, 14(3), pp.388–394.

Moreno-Rueda, G., 2010. Uropygial gland size correlates with feather holes, body condition and wingbar size in the house sparrow *Passer domesticus*. *Journal of Avian Biology*, 41(3), pp.229–236.

Moreno-Rueda, G., 2017. Preen oil and bird fitness: a critical review of the evidence. Biol Rev Camb Philos Soc, 92(4), pp.2131-2143.

Najarro, M.A. et al., 2015. Choosing mates based on the diet of your ancestors: replication of non-genetic assortative mating in Drosophila melanogaster. *PeerJ*, *3*, e1173.

Negri, I. et al., 2006. Feminizing *Wolbachia* in *Zyginidia pullula* (*Insecta, Hemiptera*), a leafhopper with an XX/X0 sex-determination system. *Proceedings of the Royal Society B: Biological Sciences*, 273(1599), pp.2409–2416.

Neufeld, K.M. et al., 2011. Reduced anxiety-like behavior and central neurochemical change in germ-free mice. *Neurogastroenterology & Motility*, 23(3), pp.255–e119.

Odabia, B. et al., 2018. Diet influences host–microbiota associations in *Drosophila. PNAS*, 115 (20), E4547-E4548.

Okada, K. et al., 2011. Male attractiveness, fertility and susceptibility to oxidative stress are influenced by inbreeding in *Drosophila simulans*. *Journal of evolutionary biology*, 24(2), pp.363–371.

Onifade, A.A. et al., 1998. A review: Potentials for biotechnological applications of keratin-degrading microorganisms and their enzymes for nutritional improvement of feathers and other keratins as livestock feed resources. *Bioresource Technology*, 66(1), pp.1–11.

Poinsot, D., Bourtzis, K., Markakis, G., Savakis, C. & Merçot, H., 1998. *Wolbachia* transfer from *Drosophila melanogaster* into *D. simulans*: Host effect and cytoplasmic incompatibility relationships. *Genetics*, *150*(1), pp.227–237.

Powell, J.R., 1997. *Progress and Prospects in Evolutionary Biology: The Drosophila Model*. Oxford University Press, Oxford, UK.

Rigaud, T. & Juchault, P., 1993. Conflict between feminizing sex ratio distorters and an autosomal masculinizing gene in the terrestrial isopod *Armadillidium vulgare*. *Genetics*, 133(2), pp.247–252.

Rodrigues, M. et al., 2009. Mating behavior of the Atlantic bobtail squid *Sepiola atlantica* (*Cephalopoda: Sepiolidae*). *Vie et Milieu*, 59(3-4), pp.271–275.

Rousset, F. et al., 1992. *Wolbachia* endosymbionts responsible for various alterations of sexuality in arthropods. *Proceedings of the Royal Society B: Biological Sciences*, 250(1328), pp.91–98.

Ruiz-De-Castañeda, R. et al., 2012. Bacterial degradability of an intrafeather unmelanized ornament: A role for feather-degrading bacteria in sexual selection? *Biological Journal of the Linnean Society*, 105(2), pp.409–419.

Ruiz-Rodríguez, M. et al., 2015. Bacteria and the evolution of honest signals. The case of ornamental throat feathers in spotless starlings. *Functional Ecology*, 29(5), pp.701–709.

Sharon, G. et al., 2010. Commensal bacteria play a role in mating preference of *Drosophila melanogaster*. *Proceedings of the National Academy of Sciences*, 107(46), pp.20051–20056.

Snook, R.R. et al., 2000. Offsetting effects of *Wolbachia* infection and heat shock on sperm production in *Drosophila simulans*: analyses of fecundity, fertility and accessory gland proteins. *Genetics*, 155(1), pp.167–178.

Spieth, H.T., 1974. Courtship behavior in *Drosophila*. *Annual Review of Entomology*, 19(1), pp.385–405.

Taylor, M.L., Sharma, M.D. & Hosken, D.J., 2009. Sexual selection in flies: A comparison of *Drosophila simulans* and *D. melanogaster*. *Animal Biology*, 59(4), pp.391–402.

Wade, M.J. & Chang, N.W., 1995. Increased male fertility in *Tribolium confusum* beetles after infection with the intracellular parasite *Wolbachia. Nature*, 373(6509), pp.72–74.

Wade, M.J., 2014. Paradox of mother's curse and the maternally provisioned offspring microbiome. *Cold Spring Harbor perspectives in biology*, 6(10).

Weeks, A.R. et al., 2007. From parasite to mutualist: Rapid evolution of *Wolbachia* in natural populations of *Drosophila*. *PLoS Biology*, 5(5), pp.0997–1005.

Weeks, A.R. & Breeuwer, J.A.J., 2001. *Wolbachia*–induced parthenogenesis in a

genus of phytophagous mites. *Proceedings of the Royal Society B: Biological Sciences*, 268(1482), pp.2245–2251.

Werren, J., Baldo, L. & Clark, M., 2008. Wolbachia: master manipulators of invertebrate biology. *Nat Rev Microbiol*, 6, pp.741–751.

Wong, A.C.-N., Chaston, J.M. & Douglas, A.E., 2013. The inconstant gut microbiota of *Drosophila* species revealed by 16S rRNA gene analysis. *The ISME Journal*, 7(10), pp.1922–1932.

Zahavi, a., 1975. Mate selection-a selection for a handicap. *Journal of theoretical biology*, 53, pp.205–214.

Chapter 2

The gut microbiome, *Wolbachia* and intergenomic epistasis for sexual fitness.

Abstract

A number of recent studies have documented apparent effects of gut bacteria on host fitness. However, many of these have not satisfactorily accounted for other curable symbionts that could also alter host phenotypes. One of these is the near ubiquitous endosymbiont of insects, *Wolbachia*. *Wolbachia* greatly affect host fitness, and treating hosts with antibiotics to investigate gut bacteria also affects *Wolbachia*, the relative contribution of the two to documented host-fitness impacts remains unclear. Here we simultaneously assess the impact of gut bacteria and *Wolbachia* on the male sexual-fitness of *Drosophila simulans* genotypes. We show that antibiotic treatment has major impacts on male fitness ranks. This is entirely driven by host genotype interactions with *Wolbachia* infection status, with no detectable effects of changes to the gut microbiome. These results show that to ascribe gut bacterial effects with certainty, accounting for the effects of other host commensals is critical. Furthermore, they suggest that *Wolbachia* may be a cryptic but important source of intergenome epistatic fitness-variation.

Introduction

The role of commensal bacteria in determining host phenotypes has only recently begun to be explored in detail (Archie & Theis, 2011). While we have long known that some “infections” have large fitness effects (Burnet & White, 1972), the acceptance that the total host microbiome may be important in host phenotype determination is new. Diverse bacterial communities live on and in animals, and these communities can influence a range of host traits and fitness correlates (Coyte et al. 2015). For example, bacteria can be key players in animal recognition, with communities inhabiting hyena scent-glands seemingly responsible for chemical cues used in social interactions (Theis et al. 2013). Equally, within-group similarity in the bacterial

communities inhabiting the anal scent-secretion of meetkats appears to be important in social interactions (Leclaire et al. 2014). Gut bacterial commensals have also been linked to obesity and human health (Turnbaugh et al. 2009; Coyte et al. 2015).

It has also been suggested that gut bacterial communities have important effects on traits more closely linked to fitness, and in *Drosophila melanogaster,* treatment with antibiotics alters the fly gut microbiota and decreases longevity (Brummel et al. 2004), and also influences mate choice (Sharon et al. 2010). Changes in mate preference resulting from antibiotic exposure (e.g. Sharon et al. 2010) are potentially extremely important as this could shed light on the assortative mating seen in iconic studies of speciation that used flies experimentally evolving on different diets (Dodd, 1989). Diet can also alter gut microbiota and the gut microbiota may influence fly cuticular hydrocarbons (CHCs), which are key determinants of male attractiveness (Ingleby et al. 2014). Thus bacteria might underpin gene-flow disruption across diets caused by reduced mating rates between flies developing of different food. This has clear implications for speciation and our understanding of the mechanisms generating it. Unfortunately a common means of disrupting the gut biota of *Drosophila* involves treatment with antibiotics, but this has effects beyond the gut as antibiotics also kill other curable symbionts, including *Wolbachia* a widespread intracellular parasite that infects every insect order (Serbus et al. 2008). The loss of *Wolbachia* is of particular importance as they can profoundly influence host phenotype (Werren, 1997), and have been implicated in male fitness and mate choice previously (Koukou et al. 2006; De Crespigny & Wedell, 2006).

Wolbachia are cytoplasmically inherited rickettsiae (Werren, 1997) and cause a range of phenotypes that vary in their effects from mutualistic, to commensal, to parasitic (Werren et al. 2008). Many of the effects on hosts are directly linked to their transmission mode, which explains why they kill or feminize males, and induce parthenogenesis and cytoplasmic incompatibility (Werren et al. 2008). In their more mutualistic interactions with hosts, *Wolbachia* can salvage ovarian defects and protect against viruses (Starr & Cline, 2002; Martinez et al. 2014). Additionally, mate choice effects have been attributed to them, with antibiotic treatment altering male attractiveness and fitness ranks (Koukou et al. 2006; Miller et al. 2010). However as noted above, curing flies of *Wolbachia* also cures them of their gut biota, and although

it was thought that the crossing regime used in a previous study assessing the mate-choice effects of *Wolbachia* infection controlled for gut biota (Koukou et al. 2006), this was not directly assessed. As a result, it is currently not clear if the strong fitness effects of antibiotic treatment in *Drosophila* are due to loss of *Wolbachia* infection or altered gut biota.

Drosophila simulans is a close relative of *D. melanogaster*. As with the latter, CHCs are important determinants of male attractiveness (Ingleby et al. 2013a) and they are influenced by diet (Ingleby et al. 2013b). Thus it is possible that gut bacteria which are also influenced by diet, alter CHCs and male fitness ranks in *D. simulans*. Additionally, *Wolbachia* infection alters one male fitness component, sperm competitiveness (Champion de Crespigny & Wedell, 2006), so there is potential for both the gut bacterial community and *Wolbachia* to affect male fitness. Here we tested for the effects of gut biota and *Wolbachia* on relative male fitness. We used *D. simulans* iso-genetic strains (isofemale lines, hereafter genotypes or isolines), as they are powerful way to assess naturally occurring genetic variation in a population and enable researchers to repeatably measure a range of fixed genotypes (Hoffmann & Parsons, 1988; David et al. 2005). We first assessed the effects of antibiotic treatment on a range of sexual fitness measures of host genotypes and then subsequently tested to see whether gut microbiota or *Wolbachia* were responsible for the changes in fitness we documented.

Materials and Methods

Drosophila simulans isolines used in this experiment were originally collected from Greece (Ingleby et. al., 2013a) and were maintained for > 45 generations with full-sib matings (n= 25 brothers and 25 sisters/isoline). Thus, each isoline could be considered as being distinct genotypes (David et al., 2004). All stocks were reared on a standard cornmeal-based Jazzmix diet (hereafter Jazzmix) (supplied by Applied Scientific, UK) at 25°C on a 12:12 hour light:dark cycle (unless stated otherwise).

Assay 1. *Antibiotics and Fitness Ranks*

Thirty of these isolines were randomly selected and assessed for *Wolbachia* infection prior to the start of our investigation. Two were found to be naturally uninfected and excluded from further use due to design considerations. The remaining 28 isolines were split into two sets, one was subject to antibiotic treatment (see *Wolbachia* curing section below; called *cured isolines* hereafter) and the other was maintained as per the standard protocol described above (called *infected isolines* hereafter). Two *ebony* (a recessive, phenotypic body-colour mutant) stock populations were also established (stocks from Tucson stock centre) and maintained at the standard conditions described above (*ca*. 800 flies/cage) – one population was cured of *Wolbachia,* the other maintained with its natural infection.

Wolbachia curing: To cure flies they were reared on '*Drosophila* quick mix medium' (Blue media: Blades Biological, Edenbridge, Kent, U.K.) at 25°C and a 12:12 h light:dark during the curing and recovery process. Briefly, 25 males and 25 females from each isoline (and an *ebony* stock subset) were allowed to oviposit for three days on food supplemented with 0.03% of the antibiotic Tetracycline HCL (Sigma Aldrich). Offspring collected from these vials were used to start the next generation. This process was repeated for three generations, after which the presence or absence of *Wolbachia* was confirmed via PCR analysis (see *Wolbachia* screening section below) of 20 males and females per isoline (and *ebony* stock). Following confirmation of *Wolbachia* absence from the *cured* isolines (and *ebony* stock), we allowed them to recover for three generations on non-tetracycline blue media before any experiments were performed.

Wolbachia screening: To determine the infection status of individuals, PCR amplification of *Wolbachia*-specific genes was conducted on DNA extracts of adult flies. Flies were squashed in 48 µl of STE buffer (Fisher Scientific; 25 mM NaCl, 10 mM Tris-HCl pH=8.0, 1 mM EDTA), and incubated with 2µl Proteinase K (0.5 mg ml^{-1}) for 30 min at 56°C. The homogenate was heated at 95°C for 2 min to deactivate the Proteinase K, diluted 1:30 with DNase free water and was then used for PCR amplification. *wsp* primers used were *wsp* 81F (5′-TGGTCCAATAAGTGATGAAGAAAC-3′) and *wsp* 691R (5′-

AAAAATTAAACGCTACTCCA-3′) (Zhou et al. 1998). Cytoplasmic DNA extracts from known positive samples were used as positive controls and sterile water was used instead of DNA in the negative controls. The PCR program used was: 94 °C for 4 min; 35 cycles of 95 °C for 30 sec, 52 °C for 30 sec, and 72 °C for 1 min; 72 °C for 4 min. PCR products were run on a 1% agarose gel, stained with RedSafe™ and visualized under an UV transilluminator.

Assay 1: Fitness of focal male versus ebony males.

We used competitive male reproductive output (the number of offspring sired by focal males competing against two *ebony* males for access to two *ebony* females) as a measure of male fitness. This was scored as the proportion of offspring that were sired by the focal male's (*wild-type*) averaged to produce a mean isoline score. We used 4 mating combinations: CxC (cured $_{focal}$♂ + 2 cured $_{ebony}$♂ + 2 cured $_{ebony}$♀; *n*= 139), CxI (cured $_{focal}$♂ + 2 infected $_{ebony}$♂ + 2 infected $_{ebony}$♀; *n*=122); IxI (infected $_{focal}$♂ + 2 infected $_{ebony}$♂ + 2 infected $_{ebony}$♀; *n*=128) and IxC (infected $_{focal}$♂ + 2 cured $_{ebony}$♂ + 2 cured $_{ebony}$♀; *n*=95) and tested 5 males from each isoline per mating combination. Briefly, each focal male was housed with two *ebony* males and two *ebony* females for 48 hours, then males were removed and females were moved into fresh egg-laying vials for 48 hours and then again for 72 hours. All fly transfers were performed without anaesthesia (Champion De Crespigny & Wedell, 2008). Offspring from each vial were counted on the 8th day after the first eclosions. This measure has been shown to be a good proxy for lifetime productivity from a single copulation (Taylor et al. 2008; Nguyen & Moehring 2015). All parental flies were subsequently screened for *Wolbachia* to verify mating combinations. Any individuals not matching purported treatments were excluded from further analysis.

Analyses 1: Monte Carlo simulations were used to test if isoline ranking in one treatment was predictive of its ranking in another treatment. We tested the following combinations (focal males x ebony female): CxC vs IxC and CxI vs IxI. Briefly, an actual correlation value was calculated and then ranks in each treatment were shuffled (within treatments) without replacements. Ranks were shuffled 10,000 times and used to calculate a two-tailed *P*-value. *P*-values > 0.05 mean that there was no significant correlation between the ranking across treatment pairs (i.e. ranking in one treatment

does not predict ranking in another treatment). These results were additionally verified using GLMM (using the arcsine square root transformed offspring proportion) with Treatment as a fixed effect and Isoline as a random effect. For robustness, we then used the raw proportional data in a generalised linear mixed effect model, taking into account the quasibinomial distribution of the dispersion parameter and treated Treatment and isoline IDs as fixed effects against the offspring count. Model simplification was used to test the significance of the interaction term. All analyses were concordant, so we only present the randomization outcomes here.

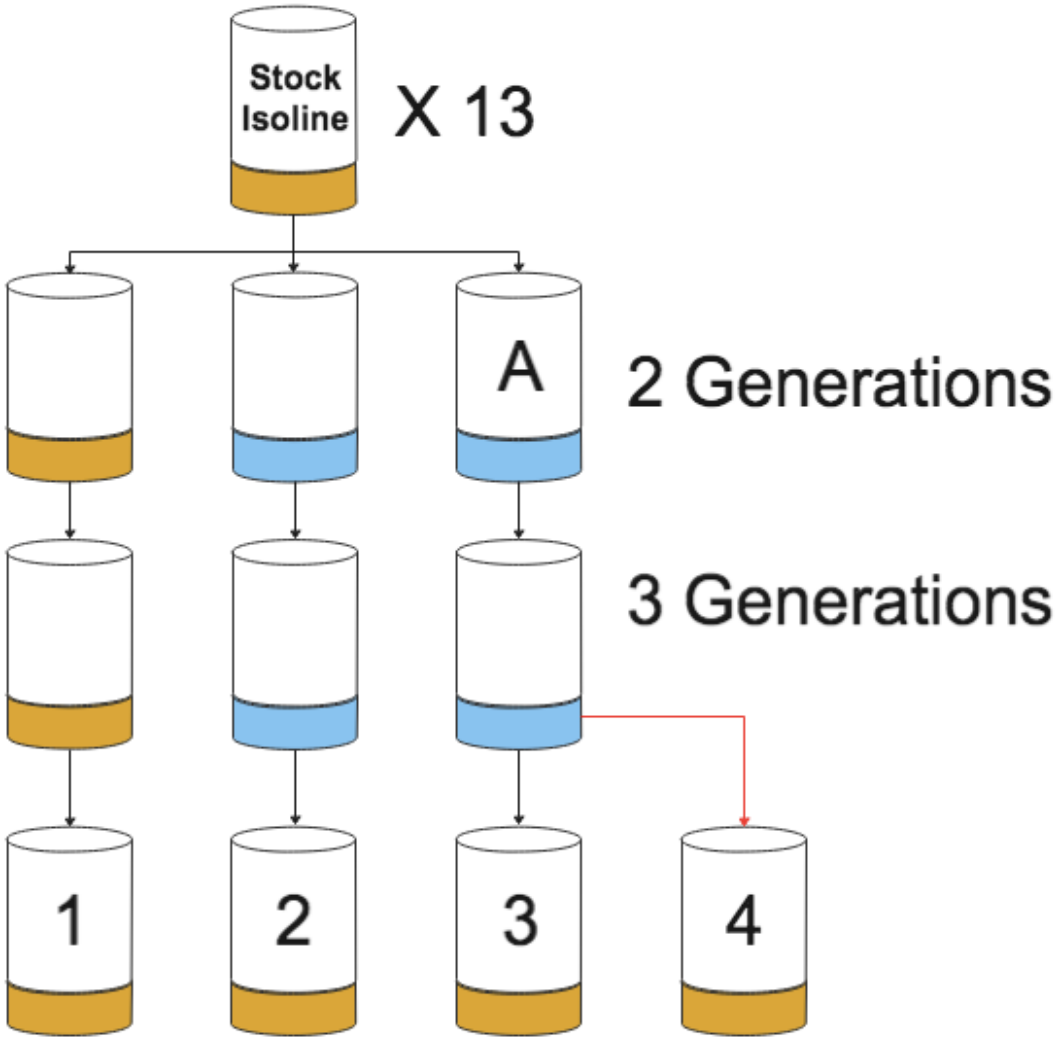

Figure 1. Experimental treatment for each isoline (N=13). Vials with brown food are raised on Jazzmix blue vials are raised on blue food. A represents antibiotic treatment with 0.03% tetracycline. The red arrow represents where the food was inoculated with the gut bacteria collected from flies in treatment 1 using the inoculation method described in assay 2. Treatment 1 is the standard control, treatment 2 is the food control, treatment 3 is the cured and 4 is the reinfected treatment.

Assay 2. Wolbachia*, Gut Biota and Male Reproductive Success*

To establish whether changes in fitness ranks were caused by gut bacteria or *Wolbachia*, we haphazardly selected 13 uncured isolines and established sub-lines that were exposed to one of 4 experimental treatments. Flies were *1*) untreated and left on their standard Jazzmix food (+W+G), *2)* untreated but reared on the curing food (+WΔG), *3*) treated with antibiotics (–WΔG) or *4*) treated with antibiotics and re-infected with previous gut bacteria (–W+G) – where W = Wolbachia (+ or -) and G is gut bacteria, altered (Δ) or not (+) (Figure 1).
As above, curing involved rearing flies on Drosophila Quick Mix Media Blue (Blades Biological) treated with 0.03% tetracycline hydrochloride but here this was done for two generations. The flies were then allowed to recover on the blue media without tetracycline for 3 generations before being moved back onto Jazzmix.

To re-infect flies (–W+G), the gut bacteria from their matching untreated sub-line was used. To collect the gut bacteria 25 males and 25 females from each sub-line were allowed to live and interact as normal on Jazzmix in 50ml vials for 3 days. The flies were then removed and 2ml of PBS was added to each vial on top of the food. The vial was then vortexed so the top layer of food mixed with the PBS and the liquid was pipetted out the vial into sterile 2ml Eppendorfs. The Eppendorfs were centrifuged at 10000rmp for 3 minutes to pellet the bacterial cells and the supernatant discarded. The pellet was re-suspended in 500ul of PBS and stored at 4^{o}C. Twenty-five females from each of the treatment 2 sub-lines (antibiotic treated but re-infected) were allowed to egg lay for 24 hours. The eggs were then dechorionated using 50% bleach, this removes any maternally transferred bacteria from the eggs, then rinsed in PBS. Eggs were then placed on fresh Jazzmix in 50ml vials and inoculated with 100ul of the line-appropriate gut-bacteria solution. The eggs were then allowed to develop and the adult flies were moved onto new Jazzmix.

In treatment 3 (reared on the curing food: (+WΔG)) flies from the sub-lines were reared on the same blue food as the antibiotic treated lines *without* the addition of tetracycline for 5 generation and then moved back onto Jazzmix. While for treatment 4 (+W+G), sub-lines were maintained as normal on Jazzmix and were not exposed to antibiotics.

Gut bacterial assay: To test if experimental treatments (–WΔG; –W+G; +WΔG; +W+G) altered bacteria as predicted, we sampled 10 of the 13 sub-lines from each treatment (this subsampling was purely for logistical reasons). Ten male and female guts/line were sterilely dissected out and pooled by sex in 100ul of PBS. The guts were crushed by hand using a sterile pestle. DNA was extracted using the DNeasy Blood & Tissue Kit (qiagen). *PCR of the DNA extracted from the guts* was used to test for the presence or absence of the 5 most common bacterial species in *Drosophila* guts (*Lactobacillus brevis, Lactobacillus fructivorans, Lactobacillus plantarum, Acetobacter pomorum* and *Acetobacter tropicalis*), ascertained using taxon specific primers (Wong et al. 2013). The PCR protocol was an initial denaturing step of 95^{O}C for 3 minutes. Followed by 30 cycles of 95^{O}C for 35 seconds, 66^{O}C for 35 seconds (56^{O}C for 16S8F + 16S1492R, *A. tropicalis and L. brevis*), 72^{O}C for 1 minute and a final extension at 72^{O}C for 10 minutes. Products were run on a 1% agarose gel to test band quality and size. Primers 16S8F and 16S1492R (Lane, 1991) were used to amplify the 16S rRNA genes.

Assay 2: Fitness post manipulation of Wolbachia and gut biota.

To test for effects of *Wolbachia* and the gut microbiota on male fitness we compared the 4 treatments, –WΔG, –W+G, +WΔG and +W+G. 20 males and females from each subline were allowed to egg lay on 30ml of Jazzmix for 3 days and virgin males were collected from these vials. These males were allowed to sexually mature (for 4 days) and were then competed against 2 virgin *ebony* competitor males for access to 2 virgin *ebony* females (as described for *Fitness assays 1* above) all from the *Wolbachia* infected ebony population. We used infected testers because this best avoids any potential cytoplasmic incompatibility although leaves the possibility of birectional cytoplasmic incompatibility (O'Neill & Karr, 1990) or male killing.

Analyses 2: To assess whether fitness effects were likely to be due to changes in gut biota or *Wolbachia*, we first had to establish that after curing flies we could re-establish the initial gut microbiota and that our antibiotic and diet treatments affected gut microbiota as expected. To test for the effects of experimental treatments (antibiotic and diet manipulations) on gut biota we used meta-analytic techniques (Rosenthal, 1991). First, contingency table tests were used to compare infection rates of the major bacterial group across treatments, and then *P*-values for each comparison were converted to *z*-scores that were subsequently combined using Stouffer's method (Rosenthal, 1991). We then assessed the statistical significance of these scores using

standard probabilities of the Normal Distribution. We then used GLMMs to assess the impact of *Wolbachia* and gut bacteria on male fitness while controlling for genetic background (founding isoline), before finally conducting rank correlations when using either gut treatment (ΔG vs. +G) or *Wolbachia* infection status (-W vs. + W) to split the data.

Assay 3. Wolbachia *and gut microbiota manipulation without diet manipulation*

We randomly selected 6 uncured isolines and established sub-lines that were exposed to one of 4 experimental treatments. In all treatments the experimental flies had their gut bacteria removed by dechorionating their eggs and then were either re-infected with their original bacteria from the matching isoline (re-infected) or inoculated with sterile PBS as a control (removed) to provide the gut bacteria +/- treatments. Flies were *1*) treated with antibiotics and had their gut bacteria removed (–W-G), *2*) treated with antibiotics and re-infected (–W+G), *3*) untreated but had their gut bacteria removed (+W-G) or *4*) untreated and re-infected (+W+G) – where W = Wolbachia (+ or -) and G is gut bacteria, removed (-) or removed and then re-infected (+). As above, curing involved rearing flies on Drosophila Quick Mix Media Blue (Blades Biological) treated with 0.03% tetracycline hydrochloride but here this was done for two generations. The flies were then allowed to recover on the blue media without tetracycline for 3 generations before being moved back onto Jazzmix.

The experimental flies from both sub treatments of each isoline (W+/W-) had their gut bacteria removed by dechorionating their eggs as in assay 2. Then 20 eggs were distributed into each small vial containing 7ml Jazzmix food. Then half of these vials were inoculated with 100ul of the gut bacteria collected from the matching isoline as the + gut bacteria treatment. The other half were inoculated with 100ul sterile PBS as the – gut bacteria treatment. Gut bacteria for inoculation was collected in the same way as for fitness assay 2*.*

Assay 3: Male attractiveness post Wolbachia *and gut biota manipulation.*

To test for effects of *Wolbachia* and the gut microbiota on male fitness we compared the 4 treatments, –W-G, –W+G, +W-G and +W+G. We used male attractiveness (latency to mate with a tester female) as a measure of fitness. This is a standard

measure of male attractiveness (Speith, 1974; Ritchie et al. 1999; Taylor et al. 2008; Ingleby et al. 2013c: discussed in Narraway et al. 2010) and mating success is a key measure of *Drosophila* fitness (Powell, 1997). To collect focal males of each treatment the eggs from the gut treatment process above were allowed to develop and males collected as virgins every 6 hours. These virgin males were stored in vials of 5-10 individuals then allowed to mature for 3-6 days. To collect tester females 25 male and 25 female ebony flies were allowed to egg lay for 2 days in each large vial containing 30ml Jazzmix. The eggs were left to develop until female virgins were collected as every 6 hours and stored in groups of 10. These flies aged for 3-5 days and used as tester mates for both males and females. All tester females were moved into individual vials ~12 hours before being introduced to a male. Individual males were introduced to the female 1 hour after incubator lights came on and the time from introduction until mating (mating latency) and mating duration were recorded by observers blind to the treatment of each pair. The focal flies were then removed and stored at -20 until they were tested for size with wing measurements.

Analyses 3: All analyses were performed in RStudio version 1.1.383 (RStudio Team, 2016) using R version 3.6.2 (R Core Team, 2019). To assess whether fitness effects were likely to be due to changes in gut biota or *Wolbachia,* we used Kaplan-Meier curves to visualize the data and analysed differences in male attractiveness using a cox proportional hazard model (Kaplan & Meier 1958; Cox 1972) with isoline, gut bacteria treatment, and *Wolbachia* infection status as co-variates and mating being the hazard. This allows us to tests which factors impact the time to the event and also include individuals that did not mate during the observation period. We then tested the significance and interactions of all the risk factors using the ANOVA function in the car package (Fox & Weisberg, 2018).

Results

Antibiotic treatment (Methods: Assay 1) altered the relative male-fitness ranks of isolines. Monte Carlo simulations showed there was no correlation between the fitness ranks of isolines when males were treated (cured) with antibiotics or not (infected) (Figure 2). This was true whether the tester females were cured or infected (correlations in cured tester females: mean Spearman's rho < 0.001; p = 0.77.

correlations in infected tester females: mean Spearman's rho = 0.002; p = 0.25). Thus treating isolines with antibiotics fundamentally changed the relative fitness-ranks of male genotypes although these changes cannot be deemed statistically significant due to the nature of our analysis.

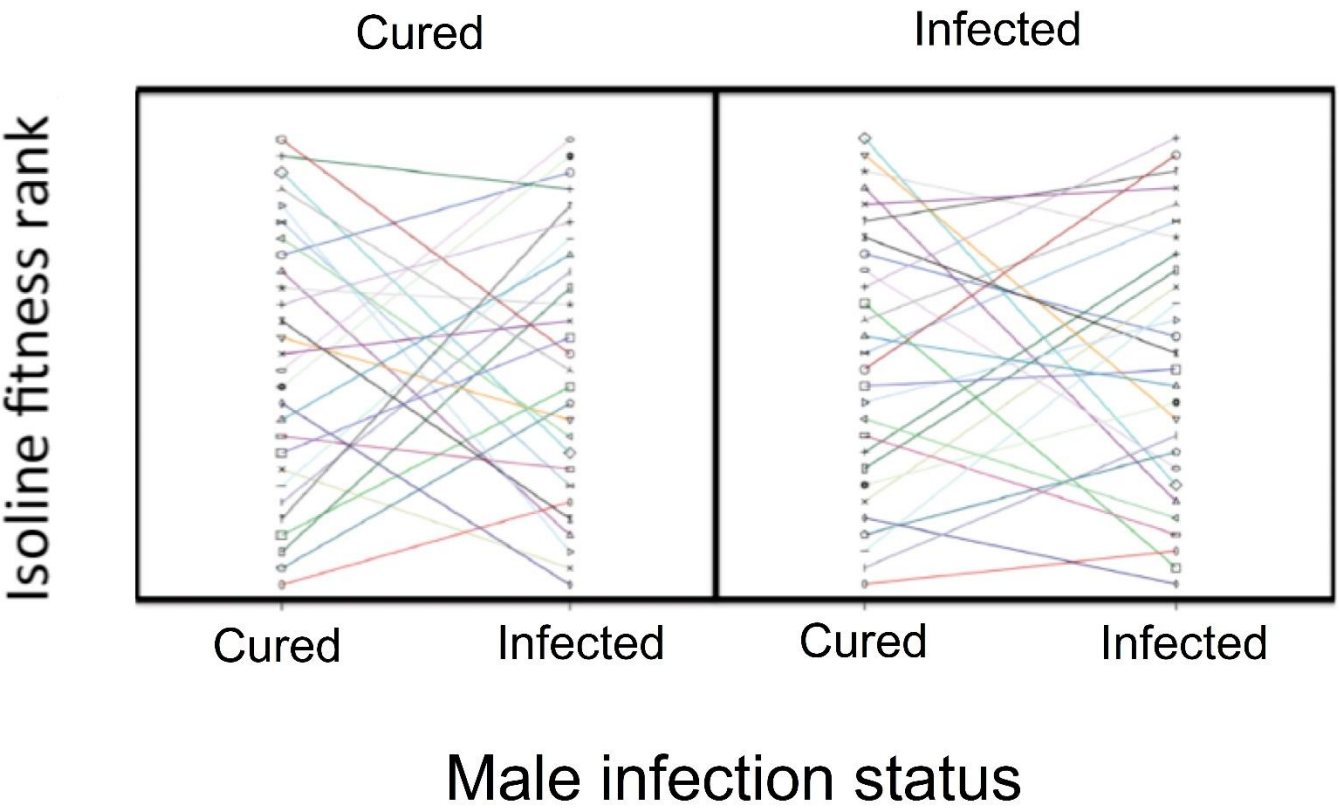

Figure 2. The relative competitive male-fitness ranks of genotypes (isolines) either untreated (infected) or treated (cured) with antibiotics when tested against *ebony* males with *ebony* females that were either antibiotic treated (cured - left panel) or not (infected – right panel). There is no association between genotype fitness-ranks across antibiotic treatments (regardless of female status) as indicated by the major crossing over in ranks. Thus antibiotic exposure appears to have altered the male sexual-fitness ranks of fly genotypes.

To assess whether curing flies of their *Wolbachia* infection caused changes in gut biota (Methods: Assay 2, Figure 1) we had to be able to manipulate both in a fully factorial manner. We attempted this using dietary manipulation and also antibiotic treatments with reinfection and then tested to see if our experimental treatments altered gut bacteria as expected (putative treatments were, *Wolbachia* (W) present (+) or absent

(-) and gut bacteria (G) changed (-Δ) or unchanged (+) to generate 4 treatments: –W-ΔG, –W+G, +WΔG and +W+G). To assess the efficacy of treatments, we first converted *P*-values from contingency tests comparing across treatment infection prevalence of the five major bacterial groups found in *D. simulans* to *z*-scores. These were subsequently combined using Stouffer's method (Rosenthal, 1991) and treatment effects on the gut community changes were evaluated by the magnitude of the combined-*z* score. This showed there were significant gut biota differences across the 4 treatments (z = -2.69; P = 0.007). Focused post-hoc tests revealed that the gut biota of antibiotic-treated re-infected flies did not differ from untreated flies reared on the ancestral diet (-W+G = +W+G; z = -0.88; P = 0.38), but the gut bacteria of antibiotic-treated re-infected flies differed from antibiotic-treated flies that were not re-infected (–W+G ≠ –WΔG; z = -2.65; P = 0.008). Furthermore, the gut microbiota of treated but non-re-infected flies did not differ from flies that were placed on the novel diet (–WΔG = +WΔG; z = -0.87; P = 0.40). Importantly, there were significant differences between treatments that putatively altered gut bacteria and those that did not ([–WΔG = +WΔG] ≠ [–W+G = +W+G]; z = -3.27; P < 0.001). Thus the experimental use of antibiotics, diet and reinfection altered gut bacteria and *Wolbachia* infection (see Methods) in a fully factorial manner. As a result we could subsequently compare the fitness of genotypes (isolines) when they were *Wolbachia* free or not and either had altered or unaltered gut bacteria.

GLMM analysis of male fitness (arcsine square-root transformed proportion of offspring sired in competitive mating trials) as a function of changes to gut biota (changed versus unchanged), *Wolbachia* status (infected versus uninfected) (both fixed effects), and genotype (isoline: fitted as a random main effect only), showed that only *Wolbachia* status had a significant effect on male fitness ($F_{1,87}$ = 6.47; P = 0.013). All other effects including the interaction between gut status and *Wolbachia* status were not statistically significant (all F < 1.7; all P > 0.19) (Figure 3). These data were therefore unable to determine the causes of changes in male fitness ranks across the genotypes. However, we did see significant reduction in absolute male fitness with *Wolbachia* infection status indicative of cytoplasmic incompatibility (CI), and this (CI) impedes our ability to ascribe causal factors generating the fitness rank changes (Figure 3).

Supporting this conjecture, averaging genotype fitness/treatment and then using these means to assess the fitness ranks of genotypes across gut-bacterial treatments (ΔG versus +G) revealed that there were no correlations across gut environments (Spearman's rho = 0.29; *P* = 0.33), or when ranked by *Wolbachia* treatments (+/- W) (Spearman's rho = -0.15; *P* = 0.63). This is consistent with previous analyses (Figures 2 & 3) but again changes in the fitness ranks of genotypes could not be definitively assigned to changes in *Wolbachia* or gut microbiota.

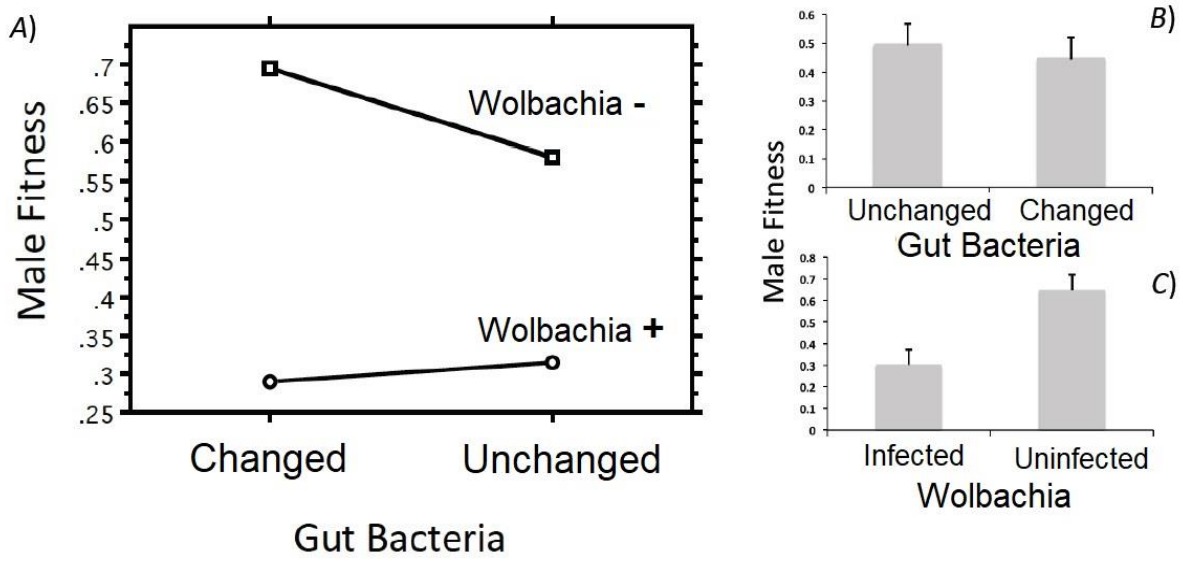

Figure 3. The effects of the gut bacteria and *Wolbachia* infection status on the reproductive fitness of male flies. *A*) shows the non-significant interaction plot (*Wolbachia* x gut bacteria), the right hand-panels the main effects: *B*) – gut bacteria; *C*) – *Wolbachia*. Only the presence or absence of *Wolbachia* had a significant effect on male fitness.

To additionally test whether changes in fitness were primarily due to changes in gut biota or *Wolbachia*, we decided to manipulate both again in a fully factorial manner, but this time using an approach that did not require the use of diet manipulation (Methods: Assay 3). Instead we used antibiotic treatments and reinfection (via collected bacteria). Putative treatments were, *Wolbachia* (W) present (+) or absent (-) and gut bacteria (G) removed (-) or re-infected (+) to generate 4 treatments: –W+G, –W-G, +W+G and +W-G. To mitigate any reproductive effects caused by *Wolbachia* (eg. CI) we used male attractiveness (mating latency) as a measure of fitness (eg.

Taylor et al. 2008; Narraway et al. 2010; Ingleby et al. 2013c). Using a cox proportional hazard model (Kaplan & Meier, 1958; Cox, 1972)with male genotype, gut bacteria treatment, and *Wolbachia* infection status as risk factors and mating as the hazard, allowed us to test which factors impacted attractiveness and also include individuals that did not mate during the observation period (Figure 4). We found genotype (isoline) had a significant effect on male attractiveness (n= 433 X^2=97.82, df=5, p < 0.001), but there was no effect of gut bacteria (X^2=0.19, df=1, p = 0.66) or *Wolbachia* infection status (X^2=0.58, df=1, p = 0.44). However, there was a significant interaction between *Wolbachia* infection status and genotype (X^2=24.86, df=5, p < 0.001), but not between gut bacteria and genotype (X^2= 3.23, df=5, p < 0.66). This finding is consistent with the fitness rank changes documented here and above being caused by changes with *Wolbachia* infection and not altered gut bacteria.

Table 1. ANOVA output from the Cox proportional hazard model with significant p-values in bold.

Predictor	X^2	**DF**	**Pr(>Chisq)**
Isoline	98.195	5	**<0.0001**
Wolbachia	0.584	1	0.44
Gut bacteria	0.194	1	0.66
Isoline : Wolbachia	24.863	5	**<0.0002**
Isoline : Gut bacteria	3.232	5	0.66

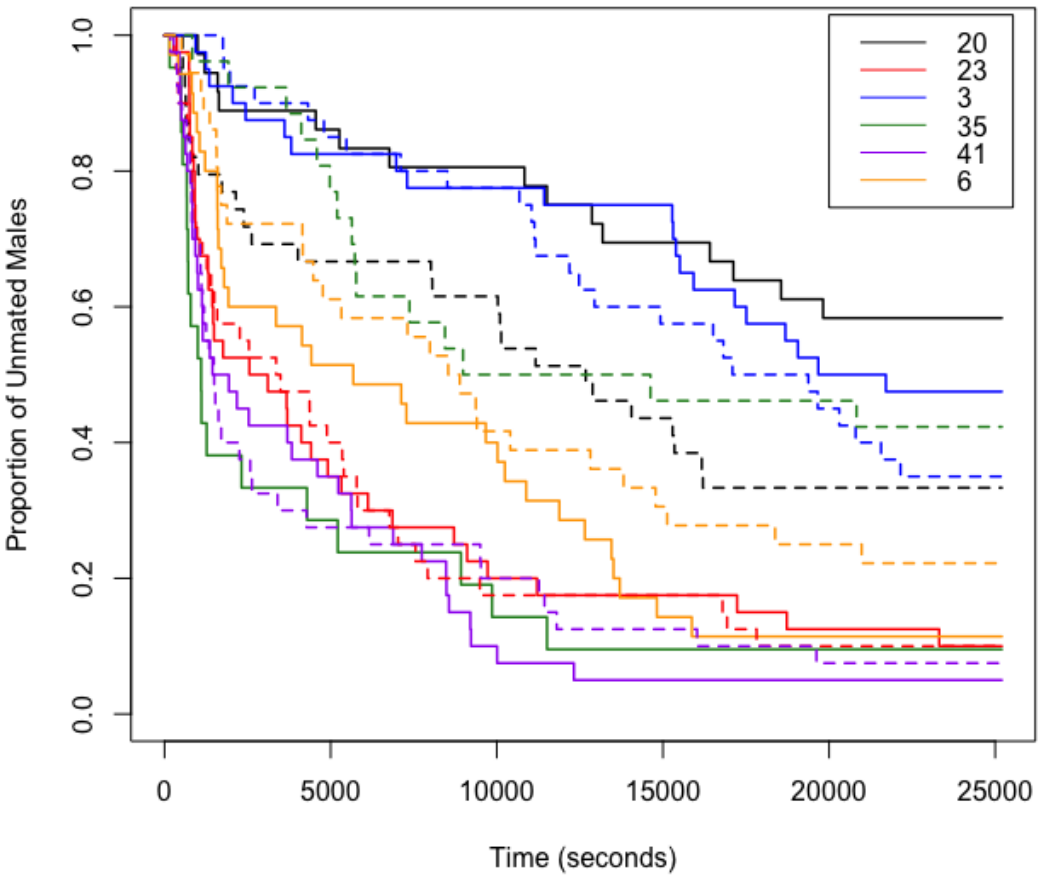

Figure 4. Kaplan-Meier curve showing proportion of unmated males over time separated by genotypes (n=6) and *Wolbachia* infection status with cured (-) a solid line and infected (+) dashed. The steeper the gradient of a curve the more attractive the males (the faster the males of that treatment mated). Male attractiveness effects of *Wolbachia* curing vary across genotypes (isolines) with some likes becoming more attractive after curing and others less so.

Discussion

A broad range of host phenotypes have been attributed to gut bacteria (Coyte et al. 2015) and in flies this varies from effects on development and longevity to mate preferences (Erkosar et al. 2013). In many studies however, antibiotics are employed to manipulate gut biota (reviewed in Erkosar et al. 2013). Antibiotics also kill other infections and in insects, which have served as models to explore the impacts of gut microbiota on hosts (Engel & Moran, 2013), this includes the endosymbiont, *Wolbachia*. Therefore to unequivocally ascribe effects of antibiotic treatment on insect phenotypes to one infection or another is problematic. This is exemplified by our results where *Wolbachia* infection-status, but not altered gut microbiota, drove fitness-rank changes in host genotypes. Since we used isolines (= different genotypes) to explore these relationships, we can ascribe host fitness effects to intergenomic epistasis for fitness between nuclear and cytoplasmic genes. This epistasis occurs despite the fact that all of our stocks were infected with the same *Wolbachia* strain. This in turn suggests the interactive effects we document are potentially widespread, but infrequently detected because they are somewhat cryptic, and they could therefore be important in maintaining genetic variation in host fitness. This may be especially true because *Wolbachia* infections are not always fixed in populations (e.g. Turelli & Hoffmann, 1995), so the conditions necessary for infection-by-host genotype epistasis exist in nature. It should be noted that we cannot definitively rule out other infections/cytoplasmic elements that covaried with *Wolbachia* as the causative agents of the fitness findings. To some extent this does not matter however, as the epistasis for fitness remains, as does the finding that altered gut microbiota did not generate major host fitness effects. Despite these caveates, it appears likely that *Wolbachia* underlie the fitness impacts documented given their general importance for fitness across insects (e.g. Werren, 1997; Werren et al. 2008; Serbus et al. 2008).

This is not the first case of cyto-nuclear epistasis for fitness in *Drosophila*. For example interactions between mito-types and nuclear background affect fitness in *D. melanogaster* (Dowling et al. 2007; Montooth et al. 2010), and in other taxa similar interactions influence a range of traits. For example, mito-nuclear interactions affect metabolic rate and sperm characters in beetles (Dowling et al. 2007; Arnqvist et al. 2010). While mitochondrial-nuclear interactions influencing fitness and metabolism are

perhaps to be expected (Balloux, 2010), the flips in male fitness-ranks due to *Wolbachia* infection-status that we documented are more surprising. Similar effects of *Wolbachia* on mate preference in *Drosophila* have been previously reported (e.g. Koukou et al. 2006; Miller et al. 2010), but this is often when infection induces CI or male killing, and as noted above, these studies frequently do not control for antibiotic impacts on gut bacteria. Nonetheless, the breadth and consequences of genotype-environment and genotype-genotype interactions in sexual selection is only now becoming fully appreciated (Hunt & Hosken, 2014) and the interaction we found here adds to this growing body of work.

It should be noted that we did not investigate changes to the total gut microbiome of *D. simulans*, but instead recorded changes in the prevalence of five key members of the gut community (Wong et al. 2013). While we altered the occurrence of these key species in predictable ways, we did not document significant host-fitness effects of this change. These changes should lead to gut-community restructuring (Coyte et al. 2015), especially because of documented impacts of antibiotics on gut microbiota communities (e.g. Young & Schmidt, 2004). Additionally, there is ample evidence that altering key community members generally, leads to fundamental community restructuring (Paine, 1966; Sanders et al. 2015). So again we expected to see effects, but found none. This has implications for views that hosts and *all* their microbiota are integrated genetic units (Bordenstein & Theis, 2015). We were unable to obtain enough bacteria from the focal fly guts that were dissected from the final assay to reliably identify presence or absence of the 5 most prevalent bacteria in *Drosophila* species (Wong et al. 2013). However, we used the reinfection protocol shown to work by Sharon et al (2010).

One way to reconcile previous findings (e.g. Sharon et al. 2010) with ours is redundancy in the gut community, leading to community and effect stability (Coyte et al. 2015), even though we altered the prevalence of the five most common bacteria found in the host gut. We are currently expanding our investigation of the gut microbiota changes caused by antibiotic treatment beyond these five taxa. And of course it is possible that effects vary across host species. Intriguingly, the mating effects in Sharon et al.'s (2010) study occurred after a single generation of diet change, which appeared to rule out mating impacts due to *Wolbachia*. Is it possible that there

is environment-dependent intergenomic-epistasis between *Wolbachia* and the host? In any case, our data broadly support the notion that variation in host microbiota can alter gene flow between differentially infected host populations (Sharon et al. 2010). However, as we show here, changes in the relative fitness of host genotypes was driven by cytoplasmic rather than gut bacteria.

Conclusions

Our results show that using antibiotics in investigations of the microbiome can be problematic, as they are a blunt tool with multiple impacts. Results additionally suggest that altered gut microbiota may not always have major consequences for hosts, and reinforce the notion that *Wolbachia* are important determinants of host fitness, although in ways that are not always obvious. Furthermore, the inter-genomic epistasis we document may help explain the maintenance of genetic variation for fitness and support the notion that the microbiome generally could influence gene flow.

References

Archie, E.A. & Theis, K.R., 2011. Animal behaviour meets microbial ecology. *Animal Behaviour*, 82(3), pp.425–436.

Arnqvist, G. et al., 2010. Genetic architecture of metabolic rate: Environment specific epistasis between mitochondrial and nuclear genes in an insect. *Evolution*, 64(12), pp.3354–3363.

Balloux, F., 2010. The worm in the fruit of the mitochondrial DNA tree. *Heredity*, 104(5), pp.419–420.

Bordenstein, S.R. & Theis, K.R., 2015. Host biology in light of the microbiome: Ten principles of holobionts and hologenomes. *PLoS Biology*, 13(8).

Brummel, T. et al., 2004. *Drosophila* lifespan enhancement by exogenous bacteria. *Proceedings of the National Academy of Sciences of the United States of America*, 101(35), pp.12974–12979.

Burnet, D.O. & White, M., 1972. *Natural History of Infectious Disease*. Cambridge University Press.

Champion de Crespigny, F.E. & Wedell, N., 2006. *Wolbachia* infection reduces sperm competitive ability in an insect. *Proceedings of the Royal Society of London B: Biological Sciences*, 273(1593), pp.1455–1458.

Champion de Crespigny, F.E. & Wedell, N., 2008. The impact of anaesthetic technique on survival and fertility in *Drosophila*. *Physiological Entomology*, 33(4), pp.310–315.

Cox, D.R., 1972. Regression models and life-tables. *Journal of the Royal Statistical Society: Series B (Methodological)*, 34(2), pp.187–202.

Coyte, K.Z., Schluter, J. & Foster, K.R., 2015. The ecology of the microbiome: Networks, competition, and stability. *Science*, 350(6261), pp.663–666.

David, J.R. et al., 2005. Isofemale lines in *Drosophila*: An empirical approach to quantitative trait analysis in natural populations. *Heredity*, 94(1), pp.3–12.

Dodd, D.M.B., 1989. Reproductive isolation as a consequence of adaptive divergence

in *Drosophila pseudoobscura*. *Evolution*, 43(6), p.1308.

Dowling, D.K. et al., 2007. Intergenomic epistasis for fitness: Within-population interactions between cytoplasmic and nuclear genes in *Drosophila melanogaster*. *Genetics*, 175(1), pp.235–244.

Engel, P. & Moran, N.A., 2013. The gut microbiota of insects - diversity in structure and function. *FEMS Microbiology Reviews*, 37(5), pp.699–735.

Erkosar, B. et al., 2013. Host-intestinal microbiota mutualism: “learning on the fly.” *Cell Host and Microbe*, 13(1), pp.8–14.

Fox, J. & Weisberg, S., 2018. Package “car”, An R companion to applied regression. *R Documentation, Sage*.

Hoffmann, A. & Parsons, P., 1988. The analysis of quantitative variation in natural populations with isofemale strains. *Genetics Selection Evolution*, 20(1), p.87.

Hunt, J. & Hosken, D.J., 2014. *Genotype-by-Environment Interactions and Sexual Selection*,

Ingleby, F.C., Hosken, D.J., et al., 2013b. Genotype-by-environment interactions for cuticular hydrocarbon expression in Drosophila simulans. *Journal of Evolutionary Biology*, 26(1), pp.94–107.

Ingleby, F.C., Hosken, D.J. & Hunt, J., 2014. Sexual selection and genotype-by-environment interactions in Drosophila cuticular hydrocarbons. In: Hunt, J. & Hosken, D.J., (eds). *Genotype-by-Environment Interactions and Sexual Selection*. pp. 265–281.

Ingleby, F.C., Hunt, J. & Hosken, D.J., 2013a. Genotype-by-environment interactions for female mate choice of male cuticular hydrocarbons in *Drosophila simulans*. *PLoS ONE*, 8(6).

Ingleby, F.C., Hunt, J. & Hosken, D.J., 2013c. Heritability of male attractiveness persists despite evidence for unreliable sexual signals in *Drosophila simulans*. *Journal of Evolutionary Biology*, 26(2), pp.311–324.

Kaplan, E.L. & Meier, P., 1958. Nonparametric estimation from incomplete observations. *Journal of the American Statistical Association*, 53(282), pp.457–

481.

Koukou, K. et al., 2006. Influence of antibiotic treatment and *Wolbachia* curing on sexual isolation among *Drosophila melanogaster* cage populations. *Evolution*, 60(1), p.87.

Lane, D.J., 1991. 16S/23S rRNA Sequencing. *Nucleic acid techniques in bacterial systematics*, pp.115–175.

Leclaire, S., Nielsen, J.F. & Drea, C.M., 2014. Bacterial communities in meerkat anal scent secretions vary with host sex, age, and group membership. *Behavioral Ecology*, 25(4), pp.996–1004.

Martinez, J. et al., 2014. Symbionts commonly provide broad spectrum resistance to viruses in insects: a comparative analysis of *Wolbachia* strains. *PLoS Pathogens*, 10(9).

Miller, W.J., Ehrman, L. & Schneider, D., 2010. Infectious speciation revisited: Impact of symbiont-depletion on female fitness and mating behavior of *drosophila paulistorum*. *PLoS Pathogens*, 6(12).

Montooth, K.L. et al., 2010. Mitochondrial-nuclear epistasis affects fitness within species but does not contribute to fixed incompatibilities between species of *Drosophila*. *Evolution*, 64(12), pp.3364–3379.

Narraway, C. et al., 2010. Genotype-by-environment interactions for female preference. *Journal of Evolutionary Biology*, 23(12), pp.2550–2557.

Nguyen, T.T.X. & Moehring, A.J., 2015. Accurate alternative measurements for female lifetime reproductive success in *Drosophila melanogaster*. *PLoS ONE*, 10(6).

O'Neill, S.L. & Karr, T.L., 1990. Bidirectional incompatibility between conspecific populations of *Drosophila simulans*. *Nature*, 348(6297), pp.178–180.

Paine, R.T., 1966. Food web complexity and species diversity. *The American Naturalist*, 100(910), pp.65–75.

Powell, J.R., 1997. *Progress and Prospects in Evolutionary Biology: The Drosophila Model*. Oxford University Press, New York.

R Core Team, 2019. R: A language and environment for statistical computing. R

Foundation for Statistical Computing, Vienna, Austria. https://www.R-project.org/.

Rosenthal, R., 1991. *Meta-analytic Procedures for Social Research*. Sage Press.

RStudio Team., 2016. RStudio: Integrated development for R. RStudio, Inc., Boston, MA URL http://www.rstudio.com/.

Sanders, D., Kehoe, R. & van Veen, F., 2015. Experimental evidence for the population-dynamic mechanisms underlying extinction cascades of carnivores. *Current Biology*, 25.

Serbus, L.R. et al., 2008. The genetics and cell biology of *Wolbachia* -host interactions . *Annual Review of Genetics*, 42(1), pp.683–707.

Sharon, G. et al., 2010. Commensal bacteria play a role in mating preference of *Drosophila melanogaster*. *Proceedings of the National Academy of Sciences*, 107(46), pp.20051–20056.

Starr, D.J. & Cline, T.W., 2002. A host-parasite interaction rescues *Drosophila* oogenesis defects. *Nature*, 418(6893), pp.76–79.

Taylor, M.L., Wedell, N. & Hosken, D.J., 2008. Sexual selection and female fitness in *Drosophila simulans*. *Behavioral Ecology and Sociobiology*, 62(5), pp.721–728.

Theis, K.R. et al., 2013. Symbiotic bacteria appear to mediate hyena social odors. *Proceedings of the National Academy of Sciences of the United States of America*, 110(49), pp.19832–19837.

Turelli, M. & Hoffmann, A.A., 1995. Cytoplasmic incompatibility in *Drosophila simulans*: Dynamics and parameter estimates from natural populations. *Genetics*, 140(4), pp.1319–1338.

Turnbaugh, P.J. et al., 2009. A core gut microbiome in obese and lean twins. *Nature*, 457(7228), pp.480–484.

Werren, J.H., 1997. Biology of *Wolbachia*. *Annual review of entomology. Vol. 42*, pp.587–609.

Werren, J.H., Baldo, L. & Clark, M.E., 2008. *Wolbachia*: Master manipulators of

invertebrate biology. *Nature Reviews Microbiology*, 6(10), pp.741–751.

Wong, A.C.-N., Chaston, J.M. & Douglas, A.E., 2013. The inconstant gut microbiota of *Drosophila* species revealed by 16S rRNA gene analysis. *The ISME Journal*, 7(10), pp.1922–1932.

Young, V.B. & Schmidt, T.M., 2004. Antibiotic-associated diarrhea accompanied by large-scale alterations in the composition of the fecal microbiota. *Journal of Clinical Microbiology*, 42(3), pp.1203–1206.

Zhou, W., Rousset, F. & O'Neill, S., 1998. Phylogeny and PCR-based classification of *Wolbachia* strains using wsp gene sequences. *Proceedings of the Royal Society B: Biological Sciences*, 265(1395), pp.509–515.

Chapter 3

The effect of host genotype on cytoplasmic incompatibility

Abstract

Wolbachia pipientis is a maternally transmitted endosymbiont that infects roughly 20% of arthropods. *Wolbachia* can affect their host reproduction in a number of ways that increase their transmission**.** One of these effects is Cytoplasmic incompatibility (CI), which causes the embryonic death of offspring when infected males mate with uninfected females. Bidirectional CI can occur when males and females are infected with different strains of *Wolbachia*. This means that *Wolbachia* infected females have higher relative fitness compared to uninfected females. It also means that *Wolbachia* infected males have lower fitness than their uninfected counterparts. This should mean there is selection for males to overcome these costs. It is not clear what effect host genotype has on *Wolbachia's* ability to impact their host. Here we tested whether different *Drosophila simulans* genotypes infected with the same strain of *Wolbachia* experienced different levels of CI when mating with testers infected with a different strain. We found evidence of bidirectional CI in our strains of flies as infected focal males did worse than uninfected males when mating with tester females infected with another infection, while infected and uninfected focal females did equally poorly when mated with males infected with another *Wolbachia* strain. In females we found genotype and *Wolbachia* infection status interact to influence the magnitude of CI. In some lines *Wolbachia* infection lowers the level of CI females experience and in other lines it increases the level. This is potential evidence of *Wolbachia* and its host coevolving in some lines to limit the impact of bidirectional CI on their fitness. In males we found that different genotypes suffer different levels of CI. This may be evidence that some lines are evolving in response to the selection that CI imposes on their reproductive success. Our work may help to explain why mixed infection populations persist despite models predicting *Wolbachia* infection should spread to fixation.

Introduction

Wolbachia are a genus of intercellular bacteria that have high prevalence across arthropods and some nematodes, and one species, *Wolbachia pipientis,* is estimated to infect 20% of arthropod species (Werren 1997). However, infection estimates wildly vary and are based on limited observations (Hilgenboecker et al., 2008). In most cases, *Wolbachia* are maternally transmitted through the cytoplasm of the egg and so males represent evolutionary dead-ends in transmission terms. This transmission is similar to other cytoplasmic elements like mitochondria, chloroplasts and other cytoplasmically inherited microorganisms. In mitochondria, maternal transmission generates the mother's curse (Gemmell et al. 2004), where mitochondrial mutations deleterious to males are expected to accumulate. This is in sharp contrast to mutations affecting female fitness. Similar processes should also occur in *Wolbachia* where mutations that increase transmission will accumulate for even if these reduce male fitness.

Wolbachia have evolved to affect host reproduction in a number of ways in order to increase infection transmission. These effects can include male killing, male feminisation, induction of parthenogenesis and cytoplasmic incompatibility. Parthenogenesis, male killing and feminisation, all generate a female-biased sex ratio in the offspring of infected females, which in turn increases the transmission rate of the *Wolbachia* parasite. Generating a female-biased sex ratio in the offspring of infected females is beneficial to *Wolbachia* as only females are able to pass on the infection. This means that male production not only wastes resources and increases competition for them, but also produces potential mates for uninfected females. The *Wolbachia* parasite causes male killing in a variety of ways either through defects in male embryos that lead to either death (Riparbelli et al. 2012) or targeting specific masculinising genes (Fukui et al. 2015). Male killing is common in species that exhibit high levels of sibling egg cannibalism as killing male embryos provides food for female offspring and reduces the risk of these daughters being eaten by their brothers (Jiggins et al. 2000). Feminisation of genetic males will double the number of possible offspring that can transmit the *Wolbachia* infection. The mechanisms behind feminisation of genetic males are different for isopods and insects (Vandekerckhove et al. 2003; Narita et al. 2007). Both male killing and feminisation use similar mechanisms and

male killing often results from incomplete feminisation of males (Werren et al. 2008). The fitness benefits of *Wolbachia* induced parthenogenesis are not only in producing solely female broods but also in removing the requirement of mating. This means that *Wolbachia* can spread to fixation within a population without causing host extinction (Werren et al. 2008).

Cytoplasmic incompatibility (here after CI) is different from these population-feminising mechanisms. It acts by causing the embryonic death of offspring when infected males mate with uninfected females. So CI is unidirectional in its action: infected females are not affected but uninfected females are (Figure 1). The mechanism behind CI involves a "modification-rescue" system (Werren, 1997) where *Wolbachia* modifies the sperm of infected males and then rescues them in infected, but not uninfected, females. The result is a reduction in offspring production for uninfected females within a population. Thus, the relative prevalence of infected individuals increases. Additionally, males infected with CI-inducing *Wolbachia* will have lower fitness than uninfected males (Figure 1). CI can also occur between different strains of *Wolbachia,* and in these instances is called Bidirectional CI, which is caused by strain specific modification and rescue genes.

Much research has studied the effects of *Wolbachia* infection on hosts, however there has been less effort devoted to potential influences of host genotype on infection outcomes. This is surprising as host genotype can change whether *Wolbachia* infection causes unidirectional or bidirectional CI (Raychoudhury & Werren, 2012). This, combined with the increased host fitness that comes with reducing the level of CI suffered, should lead to strong selection for reducing the severity of CI. In addition, we know that host genotype has pronounced effects on other infections. There is genetic variation in resistance both within and between populations *to* a bacterial parasite in *Daphnia magna* for example (Ebert et al. 1998), and genetic background influences the magnitude of infection by intracellular protozoan parasites in chickens (Bumstead & Millard, 1992). Here, variation in parasite load across hosts starts to occur between 4 and 5 days after infection, suggesting variation in the rate of immune response across hosts (Blake et al. 2006).

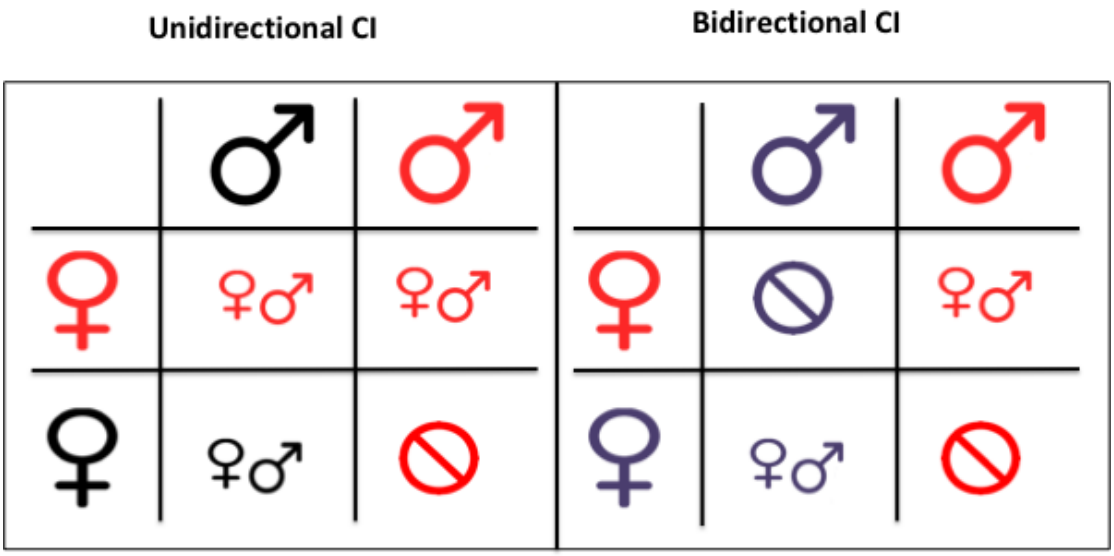

Figure 1. Illustration of unidirectional (left) and bidirectional cytoplasmic incompatibility. Red and purple symbols represent infection with different strains of *Wolbachia.* Black symbols represent uninfected individuals. The ⃠ symbol represents cytoplasmic incompatibility. As seen with unidirectional CI, infected males mating with uninfected females cause CI, which means no/fewer offspring, but all other matings produce viable young. In bidirectional CI males infected with different strains of *Wolbachia* cause CI when mating with the females of the alternate strain. Both types of CI result from attempts by the parasite to increase relative transmission rates regardless of impacts on the host.

The ability of a host to overcome infection requires recognition of the infection and an effective response. *Drosophila*, like other invertebrates, rely on a cellular and humoral immune responses to clear infections. The cellular reaction consists of the release of haemocytes (immunosurveillance cells) which phagocytise or encapsulates invasive cells (Fauvarque & Williams, 2011). The humoral reaction involves the release of antimicrobial peptides (AMPs) from the fat body into the blood (Yi et al. 2014). *Drosophila melanogaster* genotype impacts the bacterial load after infection (Lazzaro et al. 2006) and there is no correlation between genotypes across different bacteria types. This suggests genetic variation in immunity is dependent on pathogen type. Understanding the evolutionary consequences of host-parasite interactions require understanding how infection affects fitness across hosts. This is especially true for CI

inducing *Wolbachia* infecting mosquitos (*Aedes aegypti*) that have recently been employed to control wild populations and slow the spread of disease (Hoffmann et al. 2011; Iturbe-Ormaetxe et al. 2011; Zabalou et al. 2004). Such controls may not work as well as they could if there was variation across host genotypes in suppressing CI for example.

We previously found evidence consistent with bidirectional CI in *Drosophila simulans* where absolute offspring numbers were lower when males were infected with *Wolbachia* (chapter 2). However, we could not determine whether changes in male fitness ranks were affected by variation in CI level that were dependent on male genotype (Chapter 2). The *Wolbachia* infecting our focal fly genotypes (isolines: (David et al. 2005)) were found to all belong to the same strain, whereas our tester population was infected with a separate strain (using multilocus sequence typing (MLST))(Unpublished Data). As a result, any differences in the level of CI we notice across isolines would indicate that host genotype influences *Wolbachia's* ability to manipulate its host. We tested this possibility here.

Materials and methods

Drosophila simulans isolines (David et al. 2005) used in this experiment were originally collected from Greece (Ingleby et al. 2013) and were maintained for > 45 generations with full-sib matings (n= 25 brothers and 25 sisters/isoline). Thus, each isoline could be considered as being distinct genotypes (David et al., 2005). We used the *Wolbachia* curing methodology (Chapter 2) to generate cured and infected treatments of each isoline. All stocks were reared on a standard cornmeal-based Jazzmix diet (hereafter Jazzmix) (supplied by Applied Scientific, UK) at 25°C on a 12:12 hour light:dark cycle (unless stated otherwise). All matings were with stock *ebony* flies naturally infected with *Wolbachia* (from Tucson stock centre). The *ebony* flies were infected with a different strain of *Wolbachia* to our experimental flies, which allows us to test for both CI and bidirectional CI. These flies had been housed in 30cmX30cmX30cm cages with free-mating and fed *ad libitum* on Jazzmix food.

To generate experimental flies, 25 males and females from each isoline (both infected and uninfected), were placed in a large vial and allowed to egg lay for three days. *Ebony* tester flies were set up in the same way with replicates of 25 males and females from the stock population placed in large vials to egg lay for 3 days. The offspring from these vials were then collected as virgins every 6 hours and stored in vials of 5 individuals of the same sex and treatment. These virgin flies were then allowed to age for 3-5 days and then each focal isoline fly was paired with one tester ebony mate and they were watched to ensure they mated - any unmated flies were discarded – (N = 6 males and 6 females from each isoline-infection combination). Once the mating ceased the female was placed in a laying vial allowed to lay for 24 hours, with a new laying substrate provided after 12 hours. The total number of eggs laid was counted immediately (at 12 and 24 hours) and again after ~18 hours to determine the number of unhatched eggs. This gave the total eggs laid and eggs laid that did not hatch.

Data were analysed in Rstudio version 1.1383 (RStudio Team, 2016) using R version 3.6.2 (R Core Team, 2019). Proportions of hatched eggs were compared between *Wolbachia* infection status and genotype (isoline) with GLMs fit with *Wolbachia*, isoline, and their interaction as fixed effects for both focal males and females separately. We ran general linear models with a quasi-binomial error structure to control for over dispersion. We also tested total number of eggs laid across *Wolbachia* infection status and genotype by running GLMs with *Wolbachia* infection status, genotype and the interaction between them as fixed effects for focal males and females separately again. This time the GLMs used a quasi-Poisson error structure again to control for over dispersion. Fixed effects were tested for significance using the Anova function in the car package (Fox & Weisberg, 2018). Rank changes in hatchability with *Wolbachia* infection across Isoline were analysed using a Spearman's rank correlations. This is because variance partitioning approaches can miss significant crossing over (Lewontin, 2006) being primarily designed to detect main effects. These rank correlations will not be able to determine significance of rank changes but will reveal if the hatchability ranks are consistent across treatments.

Results

To test for CI/bidirectional CI we compared the proportion of unhatched eggs from focal males and females either *Wolbachia* infected or cured when mating with tester flies infected with a different *Wolbachia* strain than our focal flies. We found a significant increase in the proportion of unhatched eggs when *Wolbachia* infected focal males mated with *Wolbachia* infected *ebony* females compared to uninfected focal males (X^2=207.9*, df=1, p < 0.001*) (Figure 2). This is consistent with bidirectional CI where infected males have lower fitness mating with females infected with another parasite strain, but uninfected males never need sperm rescue. We find no *Wolbachia* effects on the proportion of hatched eggs in focal females (X^2= *0.31, df=1, p = 0.55*). However overall the proportion of unhatched eggs was high across female treatments (*Wolbachia* infected 78% and uninfected 79% of eggs didn't hatch). Again this is consistent with CI/bidirectional CI because regardless of the female's infection status they did not do well (Figure 2). Overall these results are consistent with bidirectional CI broadly as males infected with either *Wolbachia* strain appear to cause a reduction in hatchability when mating with females infected with the other strain.

We found that genotype (isoline) had a significant effect on proportion of eggs that did not hatch for both focal males (X^2=48.94*, df=17, p < 0.001*) and focal females (X^2= *48.12, df=18, p < 0.001*). To test if genotype affected the level of CI we assessed whether there was an interaction between the *Wolbachia* infection status and the isoline of the focal individual influencing the proportion of unhatched eggs. There was no significant interaction between focal male's *Wolbachia* infection and genotype (isoline) (X^2= 20.33*, df=17, p = 0.26*). This is consistent with genotype not impacting CI levels. All lines show a drop in hatchability when focal males are *Wolbachia* infected. However there is variation in the magnitude of the reduction (Figure 3). This is further evident when comparing the rank changes in average hatchability across isolines (genotype) (Figure 4). There was no correlation between the ranks of each genotype in their egg hatching success across *Wolbachia* infection treatments (Spearman's rho = 0.16; *P* = 0.54). This suggests despite no significant interaction between genotype and infection status, the fitness impact of CI may vary across genotypes.

For focal females we find a significant interaction between genotype and *Wolbachia* infection on proportion of unhatched eggs laid by focal females (X^2= *33.27, df=18, p = 0.016*) (Figure 3). This suggests that female's genotype impacts the level of CI despite seeing no effect of *Wolbachia* on hatchability across all isolines. We again find no correlation between hatchability ranks for focal females across the isolines when infected or cured of *Wolbachia* (Spearman's rho = 0.18; *P* = 0.47) (Figure 4).

We also tested whether any of the effects we saw could be caused by changes in egg laying rates caused by focal males and females. There was also no effect of the focal males *Wolbachia* infection (X^2= *0.004, df=1, p = 0.95*), genotype (X^2= *10.41, df=17, p = 0.88*) or interaction of genotype by *Wolbachia* infection (X^2= *12.79, df=17, p = 0.75*) on total number of eggs laid by the tester female. Similarly we see no effect of the focal females *Wolbachia* infection status (X^2= *0.99, df=1, p = 0.32*), genotype (X^2= *25.78, df=18, p = 0.10*) or an interaction between the two (X^2= *26.35, df=18, p = 0.09*) on their total number of eggs laid. This suggests that all the significant effects we find above are caused by changes in CI and not sperm limitation or other effects that may accompany an increase in egg laying rates by females (i.e. inefficiency in fertilization with faster egg-laying for example).

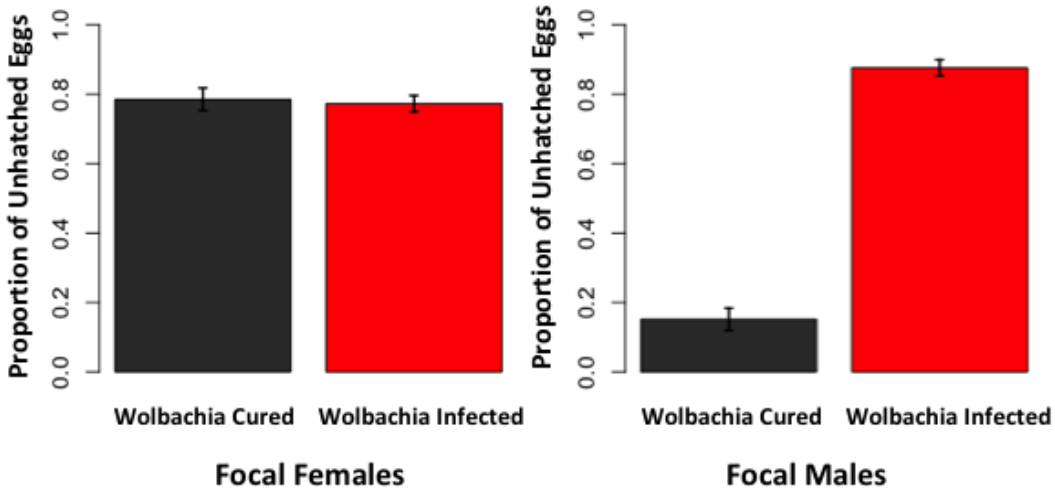

Figure 2. Proportion of unhatched eggs averaged and standard error bars across *Wolbachia* cured (grey) and *Wolbachia* infected (red) treatments for focal females (left panel) and focal males (right panel). The proportion of developmental failures was highest for infected males, which is consistent with bi-directional CI (tester females

were infected with another *Wolbachia* strain). There was no treatment effect for females, despite high failure rates, which again is consistent with CI and bi-directional CI (infected females (bidirectional CI effect) do as poorly as uninfected females (CI effect)).

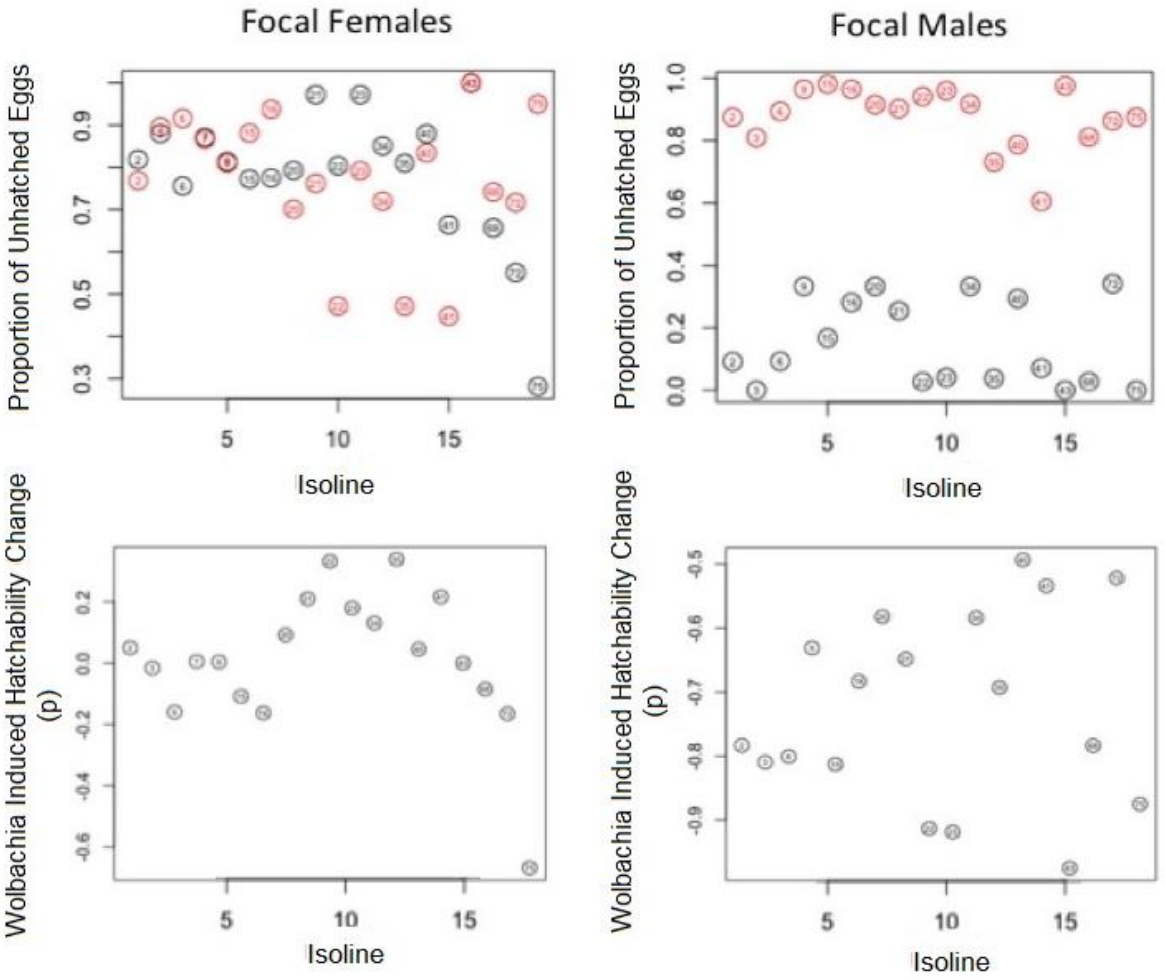

Figure 3. Proportion of unhatched eggs by genotype (isoline) for females (top left panel) and males (top right panel). Red circles are infected (*Wolbachia* +) black circles are cured (*Wolbachia* -). The change in hatchability with infection status by genotype for females (bottom left panel) and males (bottom right panel). An increase in the proportion of hatched eggs when the focal fly is *Wolbachia* infected will result in a positive value. The interaction between genotype and *Wolbachia* infection status can be seen in females where the proportion of unhatched eggs is greater with *Wolbachia* infection in some lines and lower in others. In males the bidirectional CI is obvious with

more unhatched eggs (proportion of total eggs) in infected than cured males regardless of genotype. In the lower plot for males the variation in CI level across genotypes is more obvious.

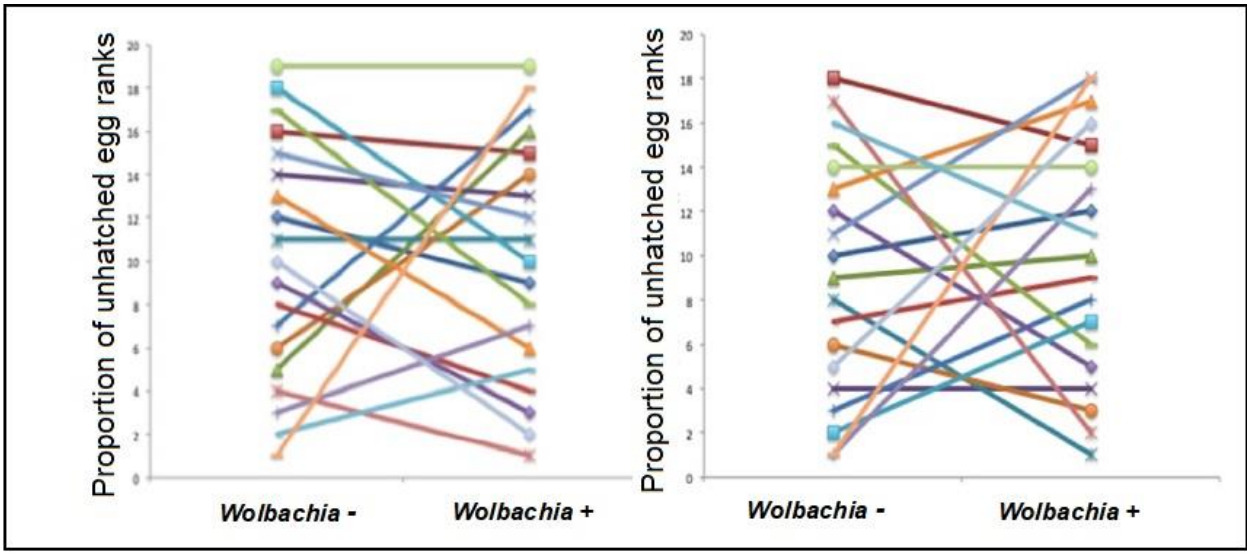

Figure 4. Isoline ranks in the relative proportion of un-hatched egg (1 = lowest) of female (left panel) and male (right panel) genotypes (isolines) either cured (*Wolbachia* -) or infected (*Wolbachia* +) when paired with *ebony* mates that were infected (+) with another *Wolbachia* strain. There is no association between genotype ranks across antibiotic treatments as indicated by the crossing over in ranks.

Discussion

We previously (Chapter 2) found evidence of possible bidirectional cytoplasmic incompatibility and we were able to confirm this phenotype here. Results are consistent with bi-directional CI because infected focal males did worse than uninfected males when mating with tester females infected with another infection, while infected and uninfected focal females did equally poorly when mated with males infected with another *Wolbachia* strain. These findings can only be fully explained with single strain CI and bi-directional CI when two strains are present.

We did not find a significant interaction between genotype and *Wolbachia* infection on the proportion of unhatched eggs in males although there was some evidence of crossing over of genotype hatchability ranks across *Wolbachia* infection statuses. We did find an interaction between genotype and *Wolbachia* infection on the proportion of

unhatched eggs in females that was also accompanied by a change in genotype hatchability ranks with *Wolbachia* infection status. Given that variance partitioning approaches like GLMs can miss significant interactions as they are primarily designed to detect main effects (Lewontin, 2006), the lack of rank correlations may be indicative of host genotype affecting CI broadly although this needs further investigation as this is only the lack of correlation and not a statistically significant change.

Despite that, these findings do not provide a completely compelling case for male host-genotype impacting the level of CI caused by *Wolbachia* infection. Nonetheless, the crossing over of genotype hatchability (fitness) ranks is present (as per Chapter 2) which means that we can't rule out male-host genotype effects on CI. Changes in genotype fitness effects previously found (Chapter 2) may in part be influenced by changes in CI across host genotype similar to possible effects noted here. This is consistent with host genotype influencing the effects of infectious disease (Hall & Ebert 2012; Idaghdour et al. 2012). However, infectious diseases are generally horizontally transmitted and evolutionarily benefit from increasing the production of infectious elements, which in turn increases host mortality or reduces host reproduction (or both). With vertically/maternal transmitted infections the dynamic between host and parasite changes. Vertically transmitted infections usually maximise their fitness by maximising the fitness of their host (Ewald, 1987). In maternal transmitted infections this is only true for female hosts. Nevertheless, CI will still significantly reduce the fitness of infected males in any population of mixed infection so we should expect selection to favour reducing this cost. The rank-crossover suggests there is genetic variation to overcome CI fitness reductions and there is also evidence that *Wolbachia* infection is not universally costly (Teixeira et al. 2008). The molecular basis of the "modification-rescue" mechanisms of CI is not well understood (Zabalou et al. 2008). This makes understanding the mechanisms that males evolve to overcome CI difficult. We know that *Wolbachia* infection increased mating rate in *D. simulans* (De Crespigny et al. 2006) and this reduces the level of CI for males (Karr et al. 1998). In our design males only mated once, however, increased sperm production rate may have a similar effect on CI levels.

In females there were no changes in hatching success based on *Wolbachia* infection. However, the low level of hatching success across both infected and uninfected

individuals is consistent with bidirectional CI. It is unsurprising that we found a genotype effect on hatching success as this has been documented before in *Drosophila melanogaster* (Chow et al. 2010; Delbare et al. 2017). It was more interesting to see an interaction between *Wolbachia* infection status and host genotype on hatchability in focal females despite not seeing any main effect of host infection alone, although this latter affect is probably due to the CI/bidirectional CI we jointly incorporated in the study design. In any case, it seems that the genotype of the female host determines the impact of CI (broadly) experienced depending on the *Wolbachia* strain. We were able to rule out changes rates of egg-laying as a potential confounder of this effect, but if should be noted that *Wolbachia* infection can influence females in so many different ways that disentangling the cause behind this interaction is difficult. Nonetheless, this is potentially another example of epistasis between *Wolbachia* and host that warrants further investigation. Wolbachia-host genetic interactions have been detected before. In *D. simulans* isolines with a *si*III mitochondrial haplogroup received a dramatic fitness benefit when infected *Wolbachia* whereas isolines with different mitochondrial haplogroups saw no effect of *Wolbachia* infection (Dean, 2006). In this example *Wolbachia* infection provided a competitive fitness advantage in some *D. simulans* isolines. Increased hatching rate with *Wolbachia* infection would provide a fitness advantage. Our isolines could potentially be differencing in their mitochondrial haplogroups and this may be interacting with *Wolbachia* infection to impact our female's fitness.

Our results appear to show that the genetic background of female *D. simulans* will determine the level of CI they suffer and there may be potential effects in males. In females this suggests that *Wolbachia* and its host are co-evolving where some lines *Wolbachia* infection helps to prevent the negative fitness effects cause by other CI inducing strains. In males it appears some lines are better able to overcome the costs of infection. Host and parasite will be co-evolving over time where there will be selection for both to maximise their fitness. In vertically transmitted parasites this often leads to selection for reduced antagonism (Lipsitch et al. 1996; Stewart et al. 2005). In maternally transmitted parasites, like *Wolbachia,* this should be the same in females. In males there may be potential selection for reduced antagonism despite males being evolutionary dead ends, especially where there are high levels of inbreeding and infection impacts male fertility (Wade & Brandvain, 2009). Alternatively,

this may be evidence that males of different backgrounds evolve different levels of immunity to the *Wolbachia* induced CI. The population dynamics of *Wolbachia* when modelled generally predict that *Wolbachia* should spread to or close to fixation in CI inducing strains due to the fitness of infected females relative to uninfected (Hoffmann & Turelli 1997). In nature we find that over time *Wolbachia* frequency reaches a stable equilibrium at intermediate frequencies (Turelli & Hoffmann, 1995). Our findings may explain why *Wolbachia* infection does not always reach fixation if in some genetic backgrounds males are evolving to reduce the level of CI they suffer. Future models should include changes in CI rates across males when estimating population dynamics of *Wolbachia* infection. Our results are important for future work using CI inducing *Wolbachia* to control wild mosquito (*Aedes aegypti*) populations (Hoffmann et al. 2011; Iturbe-Ormaetxe et al. 2011; Zabalou et al. 2004). These controls may not work as well as they could due to variation across host genotypes in suppressing CI.

Conclusions

We found bidirectional cytoplasmic incompatibility in the *Wolbachia* strains that infect both our focal genotypes and tester stocks. We also found some evidence suggestive of host genotype affecting levels of CI. The evidence of an effect of male host genotype on the level of CI was not completely conclusive. Further work could evolve males in mixed infection populations too determine whether this can generate selection to overcome CI. Female's genotype was related to variation in the effects of *Wolbachia* infection. We are unable to unequivocally ascribe the causes of these effects, but they're consistent with CI. There is however, no overall effect of *Wolbachia* infection on hatchability across genotypes, which suggests that research that has previously not found effects of infection, may have missed potential epistatic effects. These results should inform future research into the use of *Wolbachia* in biological control. Further work could explore what causes the effects we find here, and also where this interaction with host genotype is present in other *Wolbachia* influence reproductive traits.

References

Blake, D.P. et al., 2006. *Eimeria maxima*: The influence of host genotype on parasite reproduction as revealed by quantitative real-time PCR. *International Journal for Parasitology*, 36(1), pp.97–105.

Bumstead, N. & Millard, B.J., 1992. Variation in susceptibility of inbred lines of chickens to seven species of *Eimeria*. *Parasitology*, 104(3), pp.407–413.

Chow, C.Y., Wolfner, M.F. & Clark, A.G., 2010. The genetic basis for male x female interactions underlying variation in reproductive phenotypes of *Drosophila*. *Genetics*, 186(4), pp.1355–1365.

De Crespigny, F.E.C., Pitt, T.D. & Wedell, N., 2006. Increased male mating rate in *Drosophila* is associated with *Wolbachia* infection. *Journal of Evolutionary Biology*, 19(6), pp.1964–1972.

David, J.R. et al., 2005. Isofemale lines in *Drosophila*: An empirical approach to quantitative trait analysis in natural populations. *Heredity*, 94(1), pp.3–12.

Dean, M.D., 2006. A *Wolbachia*-associated fitness benefit depends on genetic background in *Drosophila simulans*. *Proceedings of the Royal Society B: Biological Sciences*, 273(1592), pp.1415–1420.

Delbare, S.Y.N. et al., 2017. Roles of female and male genotype in post-mating responses in *Drosophila melanogaster*. *Journal of Heredity*. pp. 740–753.

Ebert, D., Zschokke-Rohringer, C.D. & Carius, H.J., 1998. Within- and between-population variation for resistance of *Daphnia magna* to the bacterial endoparasite *Pasteuria ramosa*. *Proceedings of the Royal Society B: Biological Sciences*, 265(1410), pp.2127–2134.

Ewald, P.W., 1987. Transmission modes and evolution of the parasitism-mutualism continuuma. *Annals of the New York Academy of Sciences*, 503, pp.295-306.

Fauvarque, M.O. & Williams, M.J., 2011. *Drosophila* cellular immunity: A story of migration and adhesion. *Journal of Cell Science*, 124(9), pp.1373–1382.

Fox, J. & Weisberg, S., 2018. Package “car”, An R companion to applied regression. *R Documentation, Sage*.

Fukui, T. et al., 2015. The endosymbiotic bacterium *Wolbachia* selectively kills male hosts by targeting the masculinizing gene. *PLoS Pathogens*, 11(7).

Gemmell, N.J., Metcalf, V.J. & Allendorf, F.W., 2004. Mother's curse: The effect of mtDNA on individual fitness and population viability. *Trends in Ecology and Evolution*, 19(5), pp.238–244.

Hall, M.D. & Ebert, D., 2012. Disentangling the influence of parasite genotype, host genotype and maternal environment on different stages of bacterial infection in *Daphnia magna*. *Proceedings of the Royal Society B: Biological Sciences*, 279(1741), pp.3176–3183.

Hoffmann, A.A. et al., 2011. Successful establishment of *Wolbachia* in *Aedes* populations to suppress dengue transmission. *Nature*, 476(7361), pp.454–459.

Hoffmann, A.A. & Turelli, M., 1997. Cytoplasmic incompatibility in insects. In: O'Neill S.L., Hoffman, A.A. & Werren, J.H., (eds). *Influential Passengers: Inherited Microorganisms and Arthropod Reproduction*, Oxford University Press, Oxford, UK, pp.42–80.

Idaghdour, Y. et al., 2012. Evidence for additive and interaction effects of host genotype and infection in malaria. *Proceedings of the National Academy of Sciences*, 109(42), p.16786 LP-16793.

Ingleby, F.C., Hunt, J. & Hosken, D.J., 2013. Genotype-by-environment interactions for female mate choice of male cuticular hydrocarbons in *Drosophila simulans*. *PLoS ONE*, 8(6).

Iturbe-Ormaetxe, I., Walker, T. & O'Neill, S.L., 2011. *Wolbachia* and the biological control of mosquito-borne disease. *EMBO Reports*, 12(6), pp.508–518.

Jiggins, F.M., Hurst, G.D. & Majerus, M.E., 2000. Sex-ratio-distorting *Wolbachia* causes sex-role reversal in its butterfly host. *Proceedings of the Royal Society B: Biological Sciences.*, 267(August 1999), pp.69–73.

Karr, T.L., Yang, W. & Feder, M.E., 1998. Overcoming cytoplasmic incompatibility in *Drosophila*. *Proceedings Of The Royal Society B: Biological Sciences*, 265(1394), pp.391–395.

Lazzaro, B.P., Sackton, T.B. & Clark, A.G., 2006. Genetic variation in *Drosophila*

melanogaster resistance to infection: A comparison across bacteria. *Genetics*, 174(3), pp.1539–1554.

Lewontin, R.C., 2006. The analysis of variance and the analysis of causes. *International Journal of Epidemiology*, 35(3), pp.520–525.

Lipsitch, M., Siller, S. & Nowak, M.A., 1996. The evolution of virulence in pathogens with vertical and horizontal transmission. *Evolution*, 50(5), p.1729.

Narita, S. et al., 2007. Unexpected mechanism of symbiont-induced reversal of insect sex: Feminizing *Wolbachia* continuously acts on the butterfly *Eurema hecabe* during larval development. *Applied and Environmental Microbiology*, 73(13), pp.4332–4341.

R Core Team, 2019. R: A language and environment for statistical computing. R Foundation for Statistical Computing, Vienna, Austria. https://www.R-project.org/.

Raychoudhury, R. & Werren, J.H., 2012. Host genotype changes bidirectional to unidirectional cytoplasmic incompatibility in *Nasonia longicornis*. *Heredity*, 108(2), pp.105–114.

Riparbelli, M.G. et al., 2012. *Wolbachia*-mediated male killing is associated with defective chromatin remodeling. *PLoS ONE*, 7(1).

RStudio Team., 2016. RStudio: Integrated development for R. RStudio, Inc., Boston, MA URL http://www.rstudio.com/.

Stewart, A.D., Logsdon, J.M. & Kelley, S.E., 2005. An empirical study of the evolution of virulence under both horizontal and vertical transmission. *Evolution*, 59(4), pp.730–739.

Teixeira, L., Ferreira, Á. & Ashburner, M., 2008. The bacterial symbiont *Wolbachia* induces resistance to RNA viral infections in *Drosophila melanogaster*. *PLoS Biology*, 6(12), pp.2753–2763.

Turelli, M. & Hoffmann, A.A., 1995. Cytoplasmic incompatibility in *Drosophila simulans*: Dynamics and parameter estimates from natural populations. *Genetics*, 140(4), pp.1319–1338.

Vandekerckhove, T.T.M. et al., 2003. Evolutionary trends in feminization and

intersexuality in woodlice (*Crustacea, Isopoda*) infected with. *Behaviour*, 133, pp.61–69.

Wade, M.J. & Brandvain, Y., 2009. Reversing mother's curse: Selection on male mitochondrial fitness effects. *Evolution*, 63(4), pp.1084–1089.

Werren, J.H., 1997. Biology of *Wolbachia*. *Annual Reviews in Entomology. Vol. 42*, pp.587–609.

Werren, J.H., Baldo, L. & Clark, M.E., 2008. *Wolbachia*: Master manipulators of invertebrate biology. *Nature Reviews Microbiology*, 6(10), pp.741–751.

Yi, H.Y. et al., 2014. Insect antimicrobial peptides and their applications. *Applied Microbiology and Biotechnology*, 98(13), pp.5807–5822.

Zabalou, S. et al., 2004. *Wolbachia* induced cytoplasmic incompatibility as a means for insect pest population control. *Proceedings of the National Academy of Sciences of the United States of America*, 101(42), pp.15042-15045.

Zabalou, S. et al., 2008. Multiple rescue factors within a *Wolbachia* strain. *Genetics*, 178(4), pp.2145–2160.

Chapter 4

The effects of natural and sexual selection on the gut microbiome of *Drosophila simulans.*

Abstract

It is increasingly clear that the microbiota have important fitness consequences for hosts. The diversity of these microbial communities and the vast array of genes present in the microbiome have led to the suggestion we should study the combined genetic material of the host's genome and microbiome (the 'holobiome'). For the holobiome to be a useful evolutionary measure, selection must act on the microbial communities whose genes make up the microbiome. However, it is not clear how natural and sexual selection, two main mechanisms of organic evolution, affect the microbiome and whether they act on it antagonistically or not. Here we evolve population of *Drosophila simulans* under either elevated or relaxed, natural and sexual selection in a fully factorial design for 38 generations. We sequenced the gut microbiome of pools of males and females from each population and compared alpha and beta diversity changes across selection regimes. We found that males evolving under increased sexual selection had a more diverse gut microbiome. The gut bacterial communities of both males and females changed across sexual selection treatments. The changes in males were more likely to be functionally significant than in females. We found no effects of changing the strength natural selection on the gut microbiomes of either males or females. There was also no interaction between natural and sexual selection on the microbiomes of males or females. This is the first example of sexual selection altering gut microbial communities over evolutionary time and has important consequences of our understanding of host microbiota interactions.

Introduction

The collection of bacteria, archaea, fungi, protists, and viruses that colonise the surfaces and cells of multicellular organisms are termed the microbiota. It is increasingly clear that the microbiota has important fitness consequences for their host (Archie & Theis, 2011). The bacteria of the microbiota are an important component of an animal's physiology. Symbiotic bacteria can be commensal, mutualistic or pathogenic in their relationship with their host and often these relationships can by dynamic (Sachs et al. 2011). There can be more bacterial cells living on and in an organism than host cells. For example, the number of bacterial cells in the human body is estimated to at least equal that of their host (Sender et al. 2016). These bacterial communities can be incredibly diverse. The number of genes present in the gut microbiome (the genomes of the symbiotic microbiota) of humans outnumber that of their host one hundred to one (Gill et al. 2006). With the vast array of genes that make up animals microbiome some have suggested that we study the combined genetic material of host's genomes and their symbionts known as the 'Holobiome' (Guerrero et al. 2013). This idea requires that the microbiome is made up of the genes of microbial communities that persist over time and are subject to selection.

Sexual selection and natural selection are the two main mechanisms of organic evolution. Sexual selection can be thought of as variation in reproductive success, while natural selection is essentially all other fitness components (Andersson, 1994). Natural selection acts on traits that alter the survivorship, fecundity and fertility of individuals (Endler, 1986). Sexual selection acts through two mechanisms; mate competition (usually male-male competition) and mate choice (usually female choice) (Andersson, 1994). Mate competition can occur both before and after copulation, with individuals competing for access to a mate or their gametes compete for access to the gametes of the opposite sex. Mate choice involves one sex choosing (actively or passively) to mate with certain individuals of the opposite sex. Sexual selection frequently leads to exaggerated traits either used in competition for mates or as displays/signals used in mate choice (Andersson, 1994). The physical and biological environment are the common causes of natural selection (Endler, 1986). Sexual selection and natural selection can both act on the same traits and the evolutionary outcomes will depend on the nature or their interaction (Blows, 2002). This interaction

can either be antagonistic where traits favoured by sexual selection are detrimental to survival or reinforcing where sexually selected trait benefit survival.

Often exaggerated sexual signals are constrained by natural selection, as they are costly to produce and maintain and will reduce the survival of an individual. For example, predators and parasites have been shown to often exploit sexual signals to locate or catch their prey/hosts. This has been shown across a range of classes taxa including birds (Møller & Nielsen, 1997), fish (Endler, 1980), amphibians (Tuttle et al. 1982; Ryan et al. 1981) and insects (Zuk et al. 2006; Hosken et al. 1994). In these instances predators or parasites exploit the morphology, colour/pattern and auditory nature of sexual signals. The exaggerated tail feathers of male barn swallows (*Hirundo rustica*) that are preferred by females also caused an increase in predation rate(Møller & Nielsen, 1997). The spot pattern and colouration that makes male guppies (*Poecilia reticulata*) more attractive also makes them easier targets of predation (Endler, 1980). Where loud calls of male neotropical frogs (*Physalaemus pustulosus*) helps to attract mates it also makes individuals more locatable by predators (Tuttle et al. 1982; Ryan et al. 1981). Male field crickets (*Teleogryllus oceanicus*) call to attract their mates however a parasitic wasp also uses this call to locate hosts (Zuk et al. 2006).

There is also evidence that sexual selection is adaptive. Hamilton and Zuk (1982) suggested males with elaborate signals and displays will prove relative resistance to parasites compared with less showy males. The importance of parasites in sexual selection has been discussed since initially they could be important in the evolution of female choice. Evidence of a positive correlation between sexually selected traits and parasite immunity has been found in three-spined sticklebacks (*Gasterosteus aculeatus*) where females preferentially mated with males that had redder throats and those males also had lower parasite load and greater resistance (Milinski & Bakker, 1990; Folstad et al. 1994). The offspring of redder males were more resistant to parasites showing that the females are receiving indirect benefits from their mate choice (Barber et al. 2001). Experimental evolution experiments in *Drosophila melanogaster* also found that selection for resistance to a parasitoid wasp (*Asobara tabida*) generated a correlated response in reproductive fitness: resistant males also enjoyed greater mating success (Rolff & Kraaijeveld, 2003). This shows the

importance of understanding how natural and sexual selection interact on important phenotypic traits.

There are a number of ways the microbiota can influence their host's fitness that are potentially under natural and sexual selection. Symbiotic bacterial communities have been shown to benefit their host's immune function through a number of mechanisms. Symbiotic gut bacteria form stable communities that can resist colonisation by pathogens (Freter, 1955). These communities can also directly kill or inhibit the growth of these pathogens through the production of organic compounds or metabolites (Pultz et al. 2005; Hammami et al. 2013; Cherrington et al. 1991). As demonstrated above, parasite immunity can be under both natural and sexual selection. The gut microbiota can also play a role in their host's metabolism. Different bacterial communities change their host's ability to harvest energy from their diet (Turnbaugh et al. 2006). The symbiotic bacteria in the gut also help to break down in accessible nutrient sources into more readily absorbable metabolites (Tremaroli & Bäckhed, 2012). Improved energy uptake will provide benefits for survival, fecundity, mate competition and courtship. Fighting weapons and courtship displays are energetically costly (Clark, 2012; Somjee et al. 2018). There is also predicted to be a trade-off in the energy investment in somatic maintenance and reproduction (Reznick, 1985). Increased energy uptake should limit the extent of this trade-off although the nutritional composition of the diet will also dictate the level of this trade-off (Rapkin et al. 2018).

The microbiota can also impact their host's behaviour in a number of ways. Gut bacteria have been found to synthesise neurotransmitters, that through various pathways, can affect the nervous system and brain (Forsythe et al. 2010). The removal or alteration of the gut microbiota can cause an increased stress response in mice (Sudo et al. 2004). More diverse microbiota were associated with an increase in learning and memory behaviour in mice (Li et al. 2009). Changes in the microbiota can also alter brain development problems in mice and can cause a reduction in adult motor function (Heijtz et al. 2011). These behavioural changes are likely to have impacts on all aspects of animal's fitness. Both sexual selection and natural selection act on cognitive ability. Male *Drosophila melanogaster* from populations that evolved under reduced sexual selection intensity had a reduced cognitive ability compared to control males (Hollis & Kawecki, 2014).

Symbiotic bacteria play an important role in many sexual signals. In pied fly catchers (*Ficedula hypoleuca*) feather degrading bacteria have been shown to break down the white feathers used in sexual signals faster than the dark melanised flight feathers (Ruiz-De-Castañeda et al. 2012). While symbiotic bacteria living in the uropygial gland of hoopoes (*Upupa epops*) have been shown to defend against feather degrading bacteria (Martín-Vivaldi et al. 2009; Ruiz-Rodriguez et al. 2009). This should mean that sexual selection should favour an increase in the presence of these symbiotic bacteria. The microbiota of organisms has also been shown to influence their olfactory cues via the secondary metabolites they produce (Bienenstock et al. 2018). These olfactory cues are used in mate choice (Sharon et al. 2010) and kin recognition (Lizé et al. 2013). In *D. melanogaster* mating preferences for individuals raised on the same diet were removed after antibiotic treatment (Sharon et al. 2010). Individuals had different cuticular hydrocarbon (CHC) profiles across diets and the removal of the microbiota reduced these differences and the total CHC quantity. CHCs are important in sexual selection and function as sexual pheromones (Blows, 2002; Cobb & Ferveur, 1995; Ingleby, 2015). CHCs also play an important role in other naturally selected traits such as desiccation resistance (Hadley, 1981). In *Drosophila simulans* there were antagonistic evolutionary responses to natural and sexual selection in male CHC profiles (Sharma et al. 2012). Environmental temperature changes were uses to manipulate the opportunity for natural selection and enforced monogamy was used to reduce the level of sexual selection.

It is clear that the microbiota can impact their host's phenotype and behaviour in a variety of ways that have fitness consequences. It is not clear how natural and sexual selection will affect the microbiota and whether they act on it antagonistically or not. For the 'holobiome' to be a useful evolutionary measure one of the requirements is that selection acts upon the microbial communities (Guerrero et al. 2013). The relationship that natural and sexual selection have is also important as it will determine the strength of selection on the microbiota (Blows, 2002). Previous work has found that altering the level of natural selection by raising or lowering the environmental temperature has strong effects on the microbiota in *D. melanogaster* (Moghadam et al. 2018). Higher development temperature led to an increase in the prevalence of *Acetobacter* and lower temperatures an increase in *Wolbachia*. This is particularly interesting as a lack of *A. pomorum* was previously associated with smaller body sizes

and slower growth rate (Shin et al. 2011), while *Wolbachia* can cause a range of host fitness effects (Werren et al. 2008). Moghadam et al. (2018) used extreme temperatures (13^{o}C and 31^{o}C) for their low and high temperature treatments, respectively. These temperatures have been shown to cause male sterility and substantially reduced growth rates. As these temperatures are beyond where populations could be sustainable, any microbiome changes are not evolutionary significant.

Changing the environmental temperature will increase the opportunity for natural selection, as organisms are generally adapted to their thermal environment. Temperature has a wide range of effects on organisms including changing metabolic (Gillooly et al. 2001), desiccation (Parsons, 1980) and development rates (Zuo et al. 2012). Ectotherms are particularly sensitive as their body temperature changes with the environmental temperature. Body temperature will impact the rate of biological processes and biochemical reactions. For example, this means that metabolic rate increases exponentially with body temperature (Gillooly et al. 2001) Performance of ectotherms usually increases with temperature until it reaches a peak then steeply declines close to lethal temperatures (Huey & Kingsolver, 1993). Once environmental temperatures exceed the optimal temperatures for an organism their fitness starts to decline. This should mean that there is strong natural selection for any traits that minimise these fitness costs. An extreme example of this is in *Drosophila simulans* where males raised in temperatures of 28^{o}C or above are sterile (Chakir et al. 2002). This means that there should be selection for increased thermal tolerance or behavioural changes to mitigate the environmental temperature changes. Evidence of this is that *Drosophila melanogaster (*a closely related species to *D. simulans)* is more prevalent at lower latitudes with higher temperatures and male sterilisation happens at 30^{o}C (Parsons, 1973).

Despite the variety of ways the microbiota can influence sexually selected traits we do not know to what extent sexual selection acts on the microbiota. We know that changing the opportunity for natural selection through environmental temperature manipulation can cause microbiota changes (Moghadam et al. 2018). Here we test how altering the levels natural and sexual selection *Drosophila simulans* evolve under will impact their gut microbiota. We used *D. simulans* as the previous work comparing

the effects of natural and sexual selection intensity found an antagonistic effect on fly phenotypes (Sharma et al. 2012), but it is not clear if this extends to the microbiome. *D. simulans* have historically evolved at a more temperate climate (Chakir et al. 2002); this means that increasing temperatures by a few degrees should provide a stressful environment and impose stronger natural selection. We know that female mate preference is influenced by the microbiota in *D. melanogaster* (Sharon et al. 2010). We also know female mate preference is heritable and can evolve in *D. simulans* (Sharma et al. 2010). This means that natural (temperature) and sexual selection may act antagonistically on the gut microbiota of *D. simulans* or that female preference will evolve to prefer males with gut profiles more adapted to their temperature environment.

Materials and methods

In order to test the impacts of both temperature and sexual selection intensity on the gut microbiota of *D. simulans* we established selection lines from a founding stock population. This stock population was established from 20 iso-female lines collected from a wild population at Tuncurry, Eastern Australia in 2004. The stock was kept for approximately 5 years in population cages of 800–1000 flies, with overlapping generations and free mate choice. This population was reared on '*Drosophila* quick mix medium' (BLADES BIOLOGICAL, Kent, UK) and maintained at 25 °C under a 12:12 h light:dark cycle.

The selection lines were established by creating replicate experimental population of flies with either relaxed (-) or elevated (+) sexual selection (SS) and either the standard rearing (-) or an elevated (+) temperature environment (T), in a fully factorial design. This generated four treatments: –SS/–T, –SS/+T, +SS/–T and +SS/+T. We established three replicates of each experimental treatment for a total of 12 populations. The elevated temperature treatments were reared at 27°C while the standard rearing temperature treatments were kept at 25°C. Our temperature treatments were used to alter the level of natural selection. As the elevated temperature (27°C) will be more stressful the opportunity natural selection in those populations will be higher. In the elevated sexual selection treatment each female was

housed with 4 males whereas in the relaxed sexual selection treatment individual males and females were housed together. At total of 60 females were used to propagate the elevated sexual selection treatment and 64 females were used for the relaxed sexual selection treatment. These different female numbers were used in an attempt to equalise the effective population size (N_e) as there are a higher number of males present in the elevated sexual selection treatment (discussed in: Sharma et al. 2012). To avoid any incubator effects, one replicate population per selection regime was reared in each of three incubators per temperature treatment. Selection lines were maintained using the protocol outlined in Fig. 1. (Sharma et al. 2012; Archer et al. 2015) and mirrored the protocol of Archer et al. (2015), Sharma et al. (2012) and House et al. (2013).

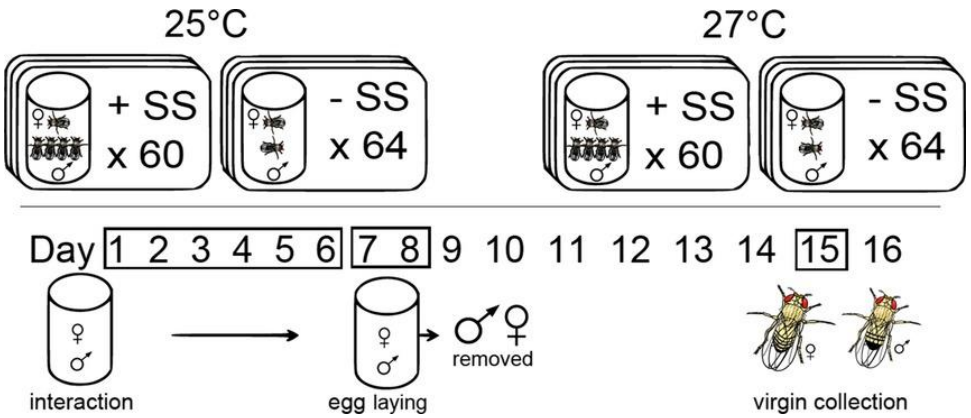

Figure 1. Flies were housed for 6 days in 'interaction vials' before being transferred to 'laying vials' for 2 days. Adults were then discarded and virgin offspring collected from 7 days after and pooled by sex for each replicate population of each selection line. Individuals were selected haphazardly from these pools to propagate the next generation. Any excess virgins were placed in 2ml Eppendorf tubes and stored at -80°C. Figure originally from Sharma et al. (2012) and Archer et al. (2015).

Gut dissections and 16s sequencing

After 38 generations of evolving in their respective selection lines, flies were haphazardly selected from the virgins not used for the next generation to be stored at -80^{o}C. These flies were stored in single sex Eppendorfs separated by replicate and

treatment. These flies were thawed at room temperature before being dissected. The guts were aseptically dissected out of 10 males and 10 female flies per replicate, per treatment. The flies were dissected under sterile conditions next to a flame to minimise contamination. Dissected guts were stored in 100ul nuclease free water before being extracted. DNA was extracted from the guts by 3 freeze thaw cycles and hand homogenising after which the Qiagen blood and tissue DNA extraction kit was used. DNA was eluted into 100ul of elution buffer and was then quantified using a Nanodrop Microvolume Spectrophotometers before being sent for 16s amplicon sequencing using the illumina miseq (Illumina Inc., San Diego, CA, USA). The v3-v4 region was amplified by PCR using universal primers (Table 1). Pools were checked on the Bioanalyser High sensitivity DNA chip to ensure primer dimers had been removed and sequenced using paired ends reads. The raw sequence data were processed using the Quantitative Insights Into Microbial Ecology (*QIIME2*) software pipeline. Dada2 was run on the data for QC filtering and we used a de novo OTU picking process.

Data Analysis

The biom files were exported into RStudio version 1.2.5033 (RStudio Team, 2016) using R version 3.6.2 (R Core Team, 2019). These data were then analysed using the *phyloseq* (McMurdie & Holmes 2013) and *vegan* (Oksanen et al. 2013) packages. All plots were created using *ggplot2* (Wickham, 2016). Male and female data was analysed separately to control for repeated sampling within the same population. The sequences were rarefied to even depths. The alpha diversities were plotted across the four selection regimes. The alpha diversity measures were used to compare the number and evenness of OTUs present in each treatment. We measured the alpha diversity of our samples in a number of ways. When mentioning species below we are referring to operational taxonomic units or OTUs as v3-v4 16s sequencing only provides us with Genus level identification. We calculated observed number of species (species richness), which was a simple count of OTUs after our samples have been controlled for sampling depth. The Chao1 index is an estimate of the expected species richness of our samples and other qualitative measures of species richness however takes into account the rarity of species gives more weight to less abundant species (Chao, 1984). Both the Shannon-Weaver and Simpson diversity indexes take into account the species richness, but also the relative abundance of each species. The

Shannon-Weaver index places a greater weight on species richness and the Simpson index is more weighted towards species evenness (Kim et al. 2017). Multiple measures of microbial diversity are used to analyse treatment effects and these multiple comparisons may increase the likelihood of a type one error. To analyse the Shannon-Weaver, Simpsons, Observed and Chao1 measures of species diversity we ran general linear models (GLMs) for the male and female data separately with the alpha diversity measure as our dependent variable, temperature, the level of sexual selection and the interaction terms between them as our fixed effects in our models. The Chao1, Shannon and Simpson diversity index used a gamma error structure while for observed number of species a Quasipoisson distribution was used to control for over-dispersion. All the GLMs were then analysed using the ANOVA function in the car package (Fox & Weisberg, 2018) in RStudio.

Multivariate statistics were conducted via the adonis function from the R package *Vegan v2.5-6* to analyse microbial beta-diversity to compare the diversity in microbial communities between temperature and sexual selection treatments. We ran permutational multivariate analysis of variance (PerMANOVA) using distance matrices with the adonis function to test the homogeneity of dispersion using different distance matrices. We tested the difference between bacterial communities across temperature treatments and sexual selection. We analysed males and females separately as we sequenced the gut microbiome of males and females from each population the measures are not independent. We used the Bray-Curtis distance measure to test dissimilarity between treatments and a weighted UniFrac distance measure that accounts for phylogenetic distances where branches are weighted by relative abundance (Lozupone et al. 2006). Both these measure will compare how the microbial composition of each sample vary however we are aware multiple comparisons of bacterial community composition may increase our chances of a type one statistical error however we feel the different nature of the measures justifies for the associated risk. The Bray-Curtis distance measure accounts for the large number of zero values that are common in this type of 16s sequencing. The weighted UniFrac distance measure accounts for the phylogenetic similarity of different OTUs and their relative abundances, which may determine the functional significance of these microbial differences. Bray-Curtis dissimilarity and UniFrac weighted distances were also used for distance-based ordination plots using the ggplot2 package (Wickham,

2016). Both ordination plots function in the same way where the distance between the data points corresponds to the relative distances between samples bacterial composition.

Table 1. V3-V4 primer sequences used for DNA amplification and sequencing of the 16s gene.

Direction	Primer	Primer sequence
Forward	**16S V3-V4 (341F)**	**5'-TCGTCGGCAGCGTCAGATGTGTATAAGAGACAG CCTACGGGNGGCWGCaG**
Reverse	**16s V3-V4 (785R)**	**5'-GTCTCGTGGGCTCGGAGATGTGTATAAGAGACA GGACTACHVGGGTATCTAATcC**

Results

To test for differences in alpha diversity across temperatures and sexual selection intensities, we ran GLMs for both males and females separately with the temperature and sexual selection intensity as fixed effects, as well as all the interaction between them. Sexual selection intensity has a significant effect on the Shannon-Weaver's diversity index (*f*= 5.11*, df=1, p*=0.045*)* in males*,* where populations with higher/elevated sexual selection intensity had higher gut microbial diversity. However, sexual selection had no significant effect on the Shannon-Weaver's diversity index in females (*f*= 1.99*, df=1, p*=0.186*).* There were no significant effects of sexual selection on any of our other alpha diversity measures for males or females (Table 2). There was also no effect of the temperature populations evolved at on any of our measures of alpha diversity across both males and females (Table 2). The interaction between temperature and sexual selection treatments also had no effect on any of our alpha diversity measures (Table 2). Overall, the trend across all alpha diversity indexes is in males evolving under stronger sexual selection and weaker natural selection (25^{o}C) to have a more diverse microbiome however these interaction are not significant (see Figure 2).

To test the sexual selection and temperature effects on population gut microbial beta diversity we used two distance measures. We tested the Bray-Curtis dissimilarity between males and females separately of each population evolving at 25^{o}C and 27^{o}C at elevated or relaxed sexual selection as well as testing if there were any interactions between sexual selection and temperature. We found a significant difference between the bacterial communities across sexual selection treatments in females but not males. There was no difference between the gut microbial communities across temperature treatments for either males or females. The interaction between temperature and sexual selection was not significant either (Table 3). The ordination plots for the bray distance measure also show a separation between the two sexual selection treatments for the females (Figure 3).

The weighted UniFrac distance matrix takes into account the similarity of the phylogenies and the relative abundance of them. We found that with this beta diversity measure, the gut microbiota was significantly different across sexual selection treatments for males but not females and did not vary across temperature treatments and there was no interaction between them in either males or females (Table 4). The ordination plot for the weighted UniFrac distance measure shows a greater separation between the 2 sexual selection treatments in males than females and no real separation between temperature treatments (Figure 4).

Table 2. General Linear models of alpha diversity measures for males and females. The only significant result we found was sexual selection strength impacts males' microbial diversity when using the Shannon-Weaver index. We found that evolving under higher sexual selection intensity results in males having higher microbial diversity. We did not find any significant effect of our treatments across any of the other measures of alpha diversity in males or females.

Observed OTUs Males	Sum Sq	Df	F value	Pr(>F)
Sexual Selection	111.72	1	1.4005	0.262
Temperature	52.61	1	0.6595	0.434
Sexual Selection : Temperature	249.98	1	3.1337	0.104
Residuals	877.46	11		

Observed OTUs Females	Sum Sq	Df	F value	Pr(>F)
Sexual Selection	108.46	1	0.9693	0.346
Temperature	24.13	1	0.2157	0.651
Sexual Selection : Temperature	0.39	1	0.0035	0.954
Residuals	1230.86	11		

Chao1 Males	Sum Sq	Df	F value	Pr(>F)
Sexual Selection	0.27187	1	1.3828	0.264
Temperature	0.12338	1	0.6276	0.445
Sexual Selection : Temperature	0.57366	1	2.9178	0.116
Residuals	2.16265	11		

Chao1 Females	Sum Sq	Df	F value	Pr(>F)
Sexual Selection	0.26479	1	1.0068	0.337
Temperature	0.0526	1	0.2	0.663
Sexual Selection : Temperature	0.00019	1	0.0007	0.979
Residuals	2.89292	11		

Shannon-Weaver Males	Sum Sq	Df	F value	Pr(>F)
Sexual Selection	0.61891	1	5.1066	0.045 *
Temperature	0.2517	1	2.0768	0.177
Sexual Selection : Temperature	0.30493	1	2.516	0.141
Residuals	1.33317	11		

Shannon-Weaver Females	Sum Sq	Df	F value	Pr(>F)
Sexual Selection	0.20326	1	1.9933	0.186
Temperature	0.35014	1	3.4337	0.091 .
Sexual Selection : Temperature	0.0859	1	0.8424	0.378
Residuals	1.1217	11		

Simpson males	Sum Sq	Df	F value	Pr(>F)
Sexual Selection	0.22532	1	2.5444	0.139
Temperature	0.14669	1	1.6564	0.225
Sexual Selection : Temperature	0.20687	1	2.336	0.155
Residuals	0.97411	11		

Simpson Females	Sum Sq	Df	F value	Pr(>F)
Sexual Selection	0.00546	1	0.1261	0.729
Temperature	0.13691	1	3.1591	0.103
Sexual Selection : Temperature	0.04192	1	0.9672	0.347
Residuals	0.47672	11		

Table 3. Result from the ADONIS analysis of the Bray-Curtis distance matrices across sexual selection and temperature treatments for males (top panel) and females (bottom panel). The results show that sexual selection treatment had a significant impact on the gut microbiome in females however not males. The temperature treatments did not have significantly different gut microbial communities for either males or females. There is also no significant interaction between temperature and sexual selection treatments for either males or females.

Bray-Curtis Males	Df	SumsOfSqs	MeanSqs	F.Model	R2	Pr(>F)
Temperature	1	0.25427	0.25427	1.2169	0.08691	0.226
SexualSelection	1	0.28035	0.28035	1.3417	0.09582	0.163
Temperature:SexualSelection	1	0.30158	0.30158	1.4433	0.10308	0.133
Residuals	10	2.08955	0.20896		0.71419	
Total	13	2.92575			1	

Bray-Curtis Females	Df	SumsOfSqs	MeanSqs	F.Model	R2	Pr(>F)
Temperature	1	0.3286	0.3286	1.4983	0.09028	0.182
SexualSelection	1	0.5806	0.58058	2.6473	0.1595	0.012 *
Temperature:SexualSelection	1	0.3184	0.31838	1.4517	0.08747	0.172
Residuals	11	2.4124	0.21931		0.66276	
Total	14	3.64			1	

Table 4. Result from the ADONIS analysis of the weighted UniFrac distance matrices across sexual selection and temperature treatments for males (top panel) and females (bottom panel). The results show that sexual selection treatment had a significant impact on the gut microbiome in males but not females. The temperature treatments did not have significantly different gut microbial communities for either males or females. There is also no significant interaction between temperature and sexual selection treatments for either males or females.

Weighted UniFrac Males	Df	SumsOfSqs	MeanSqs	F.Model	R2	Pr(>F)
Temperature	1	0.03064	0.030637	0.68239	0.04948	0.754
SexualSelection	1	0.09241	0.09241	2.05831	0.14926	0.040 *
Temperature:SexualSelection	1	0.04713	0.04713	1.04977	0.07612	0.364
Residuals	10	0.44896	0.044896		0.72514	
Total	13	0.61913			1	

Weighted UniFrac Females	Df	SumsOfSqs	MeanSqs	F.Model	R2	Pr(>F)
Temperature	1	0.08716	0.087155	1.4566	0.09442	0.21
SexualSelection	1	0.11688	0.116876	1.9534	0.12662	0.144
Temperature:SexualSelection	1	0.06084	0.060842	1.0169	0.06591	0.361
Residuals	11	0.65817	0.059833		0.71304	
Total	14	0.92304			1	

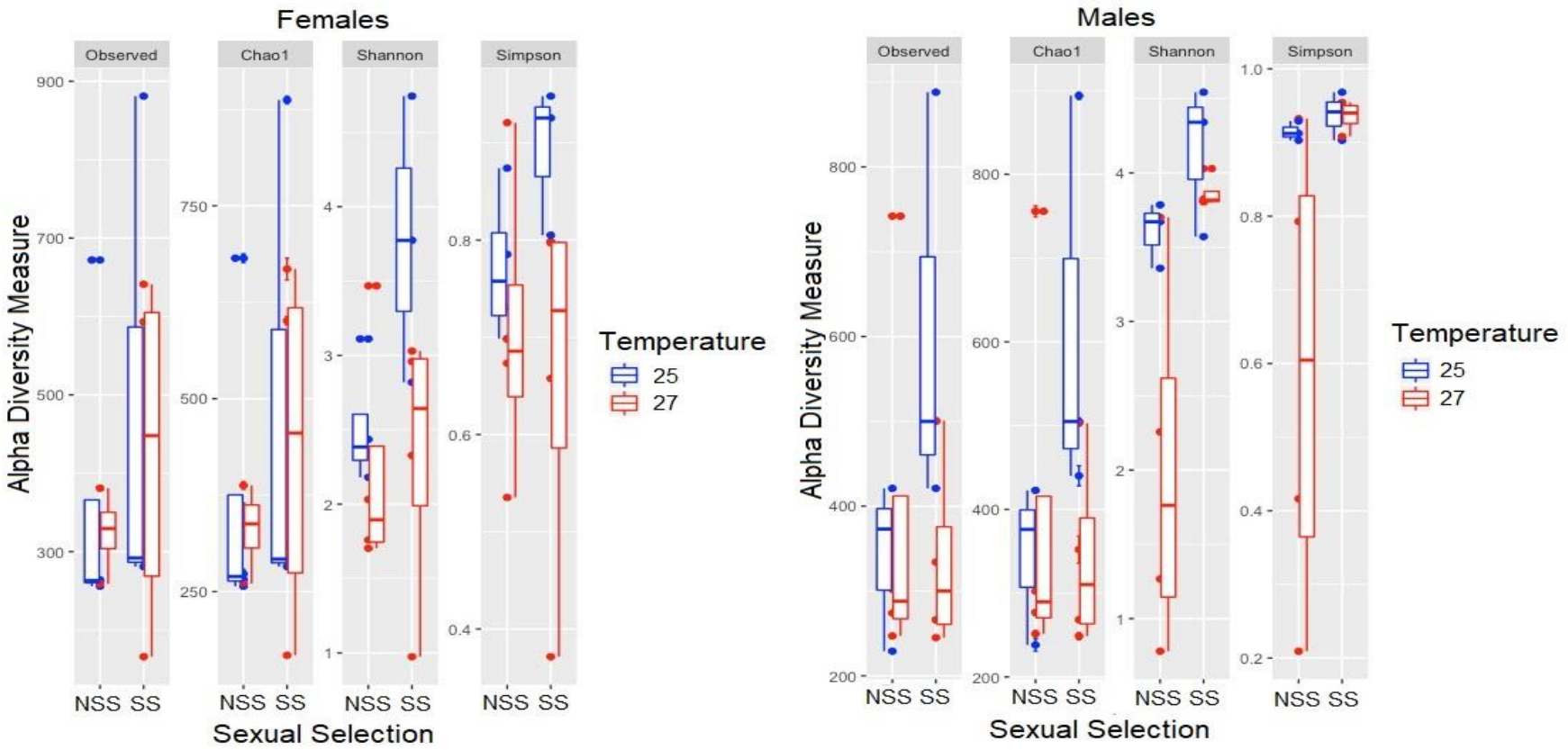

Figure 2. Boxplots of alpha diversity measures separated by sexual selection and temperature treatment for males (right) and females (left). Each panel from left to right shows a separate diversity measure listed at the top. In each panel the 2 left boxplots identified as 'nss' have evolved under relaxed sexual selection and the 2 on the right identified as 'ss' have evolved under elevated sexual selection. The red boxplots represent the values for populations evolving at 27ºC and the blue boxplots represent the populations evolving at 25ºC. Higher sexual selection intensity appears to be associated with an increase in male gut microbial diversity across most measure however this is only significant for the Shannon-Weaver diversity index. Higher temperatures seem to be associated with lower gut microbial diversity however, these effects were not significant.

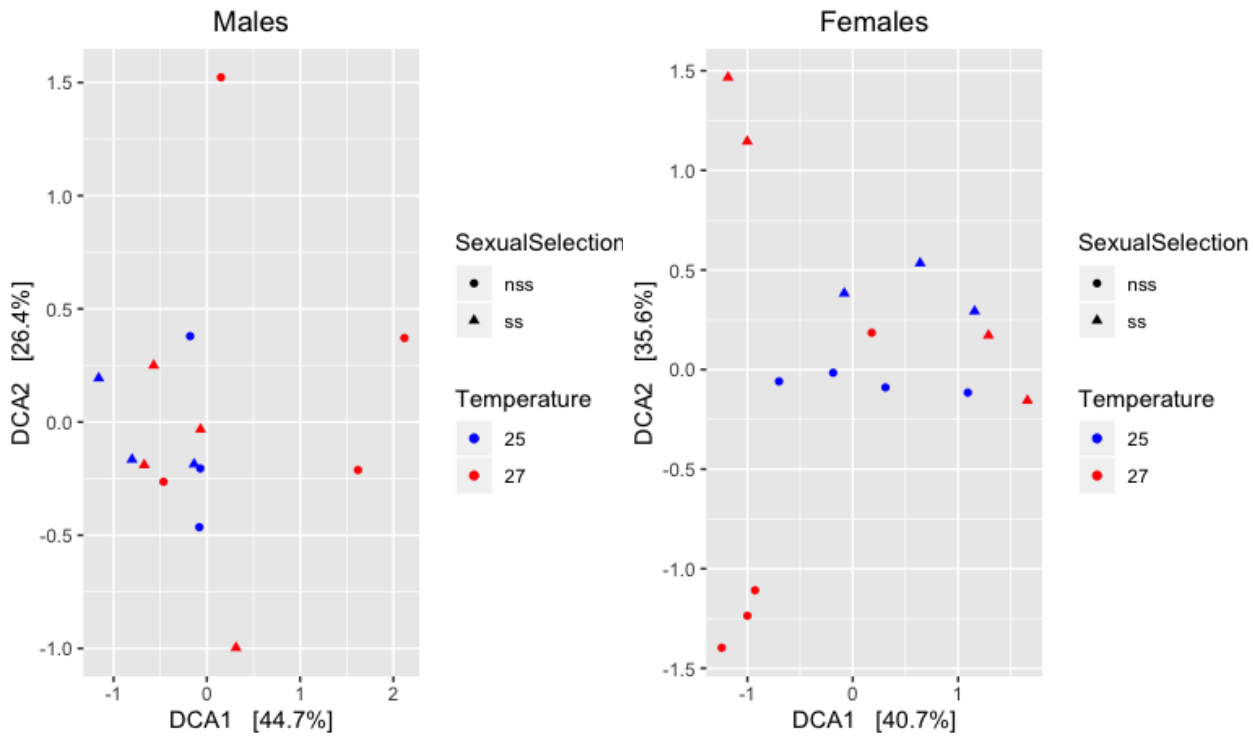

Figure 3. The Bray-Curtis distance based ordination analysis of gut communities from flies evolving at 25ºC (blue symbols) and 27ºC (red symbols). Circular data points have evolved under relaxed sexual selection (nss) and triangular data points have evolved under elevated sexual selection (ss). Results for males are plotted on the left and females on the right. The male plot does not appear to show any clustering by treatment. This suggests with this beta diversity measure neither of our treatments are having a consistent effect on male gut microbial communities. The female plot shows separation between the sexual selection treatments and some grouping across the temperature treatments. This appears to show sexual selection consistently alters female microbial communities.

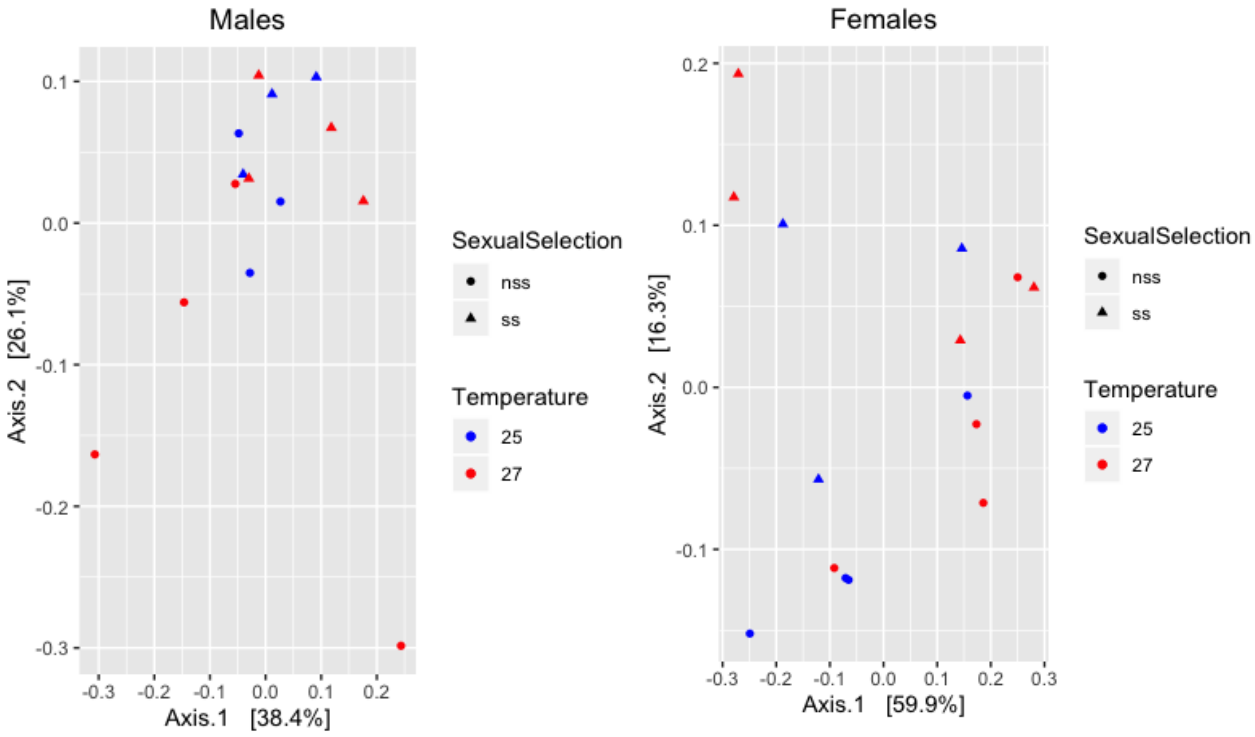

Figure 4. The weighted UniFrac distance based ordination analysis of gut communities from flies evolving at 25ºC (blue symbols) and 27ºC (red symbols). Circular data points have evolved under relaxed sexual selection (nss) and triangular data points have evolved under elevated sexual selection (ss). Results for males are plotted on the left and females on the right. Both graphs show some separation of two sexual selection treatments however they do not solely cluster together and exhibit crossing over that suggests other factors are impacting the gut microbial communities. The male plot shows greater separation across the sexual selection treatments suggesting that the sexual selection has a greater impact on male microbial communities with this measure of diversity.

Discussion

Our results have shown that the sexual selection strength *Drosophila simulans* evolve at changes their gut microbiomes. This is the first time sexual selection has been shown to cause evolutionary changes to the gut microbiome. We found that the temperature environment/natural selection strength *D. simulans* evolve at does not alter their gut microbiome. There was also no significant interaction between natural

selection (temperature) and sexual selection on the microbiome in *D. simulans*. We found that males that evolved under increased sexual selection strength had significantly increased microbiome diversity when using the Shannon-Weaver diversity index. Sexual selection had no effect on any of our other alpha diversity measures in males or females. Across our two beta diversity measures we find that evolving under different strengths of sexual selection changes both male and female bacterial communities. We are aware of the risks of using multiple measures of diversity however finding effects of sexual selection treatment using both alpha and beta diversity measures suggests that sexual selection is having an impact of the gut microbiomes in our treatments. We found that females gut microbiomes differ across sexual selection treatments when we use the Bray-Curtis distance measure and males using a weighted UniFrac distance measure. The weighted UniFrac measure, where we found the male effect, accounts for the phylogenetic similarity of the OTUs in each community and their relative abundances. The Bray-Curtis measure, where we found a sexual selection effect in females, will only compare the abundance and presence of OTUs. This means that in males sexual selection treatment may be more likely to have a phylogenetically significant effect on gut microbiomes, these may be more phenotypically significant however further investigation would be needed to confirm this. In females their gut microbial communities change in their composition, but these changes may potentially be less likely to be biologically significant as the changed OTUs are potentially closer related. We found no effect of temperature treatment on the gut microbiome across any of our alpha or beta diversity measures. It is important to note that as our measures are based on pooled gut samples from a population we cannot distinguish if diversity changes are at the population or individual level.

The impacts of sexual selection intensity on the gut microbial communities appear to be significant. Alpha diversity estimates show that the observed species (OTU) richness and the estimated Chao1 richness did not significantly differ across the sexual selection intensities in either males or females. This supports the idea that increasing the number of females in each generation by four, controlled for potential differences in effective population size (N_e)(Sharma et al. 2012). Our finding that when using the Shannon-Weaver diversity index alpha diversity increases with an increase

in sexual selection strength in males suggests that sexual selection is acting on the gut microbiome. As we find effects with the Shannon-Weaver index and not with the Simpson's index means that communities have a richer and more even microbiome, however, this difference is more weighted to richness (Kim et al. 2017). This may mean that increased sexual selection strength selects for males with increased gut microbial diversity or that there is higher variation amongst males. The former would suggest that increased gut bacterial diversity increases male's reproductive success and the later would indicate there is variation in preferred gut types in males. Further work should investigate both these potential options. This increase in bacteria diversity may be due to the link between gut microbiota and CHC profiles, where removing gut bacteria reduces the differences in five mating related CHCs (Sharon et al. 2010). The fact we only found a response in males suggests that these alpha diversity changes are having a functional effect. This would make sense as our elevated sexual selection regime impose stronger selection on males than femalesWhen measuring beta diversity differences we were looking at the differences in the makeup of each bacteria community. Beta diversity looks at whether the species and their individual abundances are different across populations. We analysed the beta diversity across treatments by using two distance measures. The Bray-Curtis distance measure looks at the dissimilarity between communities and is able to handle the large number of zero values common in this type of sequencing. The weighted UniFrac distance measures account for the phylogenetic similarity and is weight by the abundance of species. The Bray-Curtis measure will allow us to look at if communities differ between treatments and the weighted UniFrac measure may tell us more about if there are functionally significant differences. Our results show that populations evolving under either elevated or relaxed sexual selection intensity have different gut microbial communities across both beta diversity measures. Males and females each had changes in their microbiome when using different distance measures.

When using Bray-Curtis dissimilarity we found a significant difference in female's bacterial communities across the two sexual selection treatments. The weighted UniFrac distance analysis however found microbiome differences across our sexual selection treatments in males. These finding suggest that sexual selection is altering the microbiome of both males and females in different ways. The changes in the

makeup of female's microbiomes are likely to be between more closely related OTUs. This means that while the microbial community changes, these changes are potentially less likely to be functionally significant. In males we only find changes when using the weighted UniFrac measure, this suggests that the changes in the microbiome are not as great in numerical terms as they are in females. However, the changes are between more distantly related OTUs. This could means these changes are more likely to be functionally significant and would be an important avenue for future research. Changes in certain phylum of bacteria can be associated with strong fitness effects. For example, age related changes in a specific phylum in the gut microbiota correlates with aging and mortality in humans and *D. melanogaster* (Clark et al. 2015; Claesson et al. 2012). In *D. melanogaster* changes in sexual signals correlated with changes in the prevalence of *Lactobacillus* spp. in the gut microbiota (Sharon et al. 2010).

Our increased sexual selection treatment would impose stronger selection on males than females, as males would be subject to both mate competition and mate choice. It is also worth noting that the increased sexual selection treatment will potentially expose females to greater levels of male harm and harassment, which could reduce female longevity. However we would still expect functional changes in the gut microbiota driven by sexual selection to be more likely in males. This was found previously in *D. simulans* where evolving under elevated sexual selection resulted in significant changes in male CHC profiles but not in females (Sharma et al. 2012). As gut microbiota changes have been found to influence CHC profiles they may be one mechanism that sexual selection is acting on the gut microbiota (Sharon et al. 2010). The gut microbiota can have a wide range of fitness effects on their host so sexual selection may be acting on a number of these fitness effects. For example, increases in the energy harvesting capacity caused by the gut microbiota(Turnbaugh et al. 2006) could provide benefits in both mate competition and sperm competition as both are potentially energetically costly (Bretman et al. 2013). The microbiota can also have immune function effects and selection on increased parasite immunity in *D. melanogaster* was associated with an increase in male mating success (Rolff & Kraaijeveld, 2003). *D. melanogaster* evolving under weaker sexual selection have been found to have a reduced cognitive ability (Hollis & Kawecki, 2014). This suggests that sexual selection acts on cognitive ability and the gut microbiota have been shown

to affect their host's cognition and behaviour (Forsythe et al. 2010; Li et al. 2009; Heijtz et al. 2011).

We did not find a significant effect of temperature/natural selection on the gut microbial diversity of males or females across any of our diversity measures. This is surprising as higher and more stressful temperature environments have previously been show to decrease microbiota diversity in several other ectotherms including the eastern red-backed salamander (*Plethodon cinereus),* common lizard (*Zootoca vivipara*) and northern leopard frog (Lithobates pipiens) (Fontaine et al., 2018; Kohl & Yahn, 2016; Bestion et al., 2017). Previous work on the closely related *Drosophila melanogaster* found development temperature altered gut microbial community composition (Moghadam et al. 2018). There are a few potential explanations we did not find differences in the microbiota of populations evolving at different temperatures. The previous work that found significant temperature based microbiota changes was carried out in *D. melanogaster* (Moghadam et al. 2018) which despite being closely related has previously been shown to differ in a number of ways to *D. simulans*. *D. simulans* are more sensitive to high temperatures with temperature induced male sterility happening above 28^{o}C compared to 30^{o}C in *D. melanogaster* (Chakir et al. 2002). The different response we find in the gut microbiota evolving at different temperatures compared to Moghadam et al. (2018) may be due to differences between *simulans* and *melanogaster* in their response to temperature changes. We also used less extreme temperatures in our study that are stressful but below the level of thermal sterilisation. This may be another reason why gut bacterial communities did not differ across thermal environments as was found in Moghadam et al. (2018). The final explanation could be that as we are testing pooled individuals within a population the diversity of the population does not decrease but individuals may have experienced reduced gut bacterial diversity.

A further explanation may be that our work allowed populations to develop at their temperatures for 38 generations and so should show the long-term consequences of changing temperatures on an ectotherms microbiota. Our results are less clear than previous work and may demonstrate that populations are evolving to minimise the effects of changing environment on their gut microbial communities. Previous work

only changed the temperature of development and so did not allow any evolution of the *D. melanogaster* in response to their climate changing their gut microbiota (Moghadam et al. 2018). As the effects of the gut microbiome are so wide reaching and important for fitness, large environment driven changes could select for a response in the host to negate these changes. This would mean that the populations raised at higher temperatures have evolved to offset short term costs of temperature induced microbiota changes. We know that a hosts gut environment imposes selection for certain bacterial community compositions, where reciprocal transplant of gut bacteria between zebrafish and mice found bacterial communities mimicked the composition of their original guts (Rawls et al. 2006). The idea of hosts evolving to offset the temperature induced gut changes would require further work looking at gut biota differences across generations.

Our alpha diversity plots appear to show in males a trend for increased natural selection limiting the diversity increases associated with stronger sexual selection. This is in line with classical ideas of sexual selection being balanced by natural selection (Andersson, 1982; Kirkpatrick, 1982). However, we found no significant interaction between temperature (natural) and sexual selection on gut microbial communities. This may be because we found no overall temperature effects on the gut microbiome. It may be possible that with this experimental design the strength of sexual selection is so high that any antagonistic effects of natural selection are unable to offset the effects of sexual selection. Our study did not find an interaction between sexual selection and natural selection on gut microbial diversity, however, the possibility for antagonistic selection between sexual and natural selection warrants further study.

If the ‘holobiome’ is going to be a useful evolutionary measure one of the main requirements is that selection is acting on a host’s microbial communities. Our work is the first instance of showing sexual selection altering the gut microbiota of organisms in evolutionary terms. Our work has shown the evolutionary response of gut profiles to varied sexual and natural selection intensity. We show that increased sexual selection is associated with both a changed and more diverse gut microbiome. As gut microbial profiles are heritable both through vertical transmission (Bright & Bulgheresi,

2010) and through the gut environment shaping bacterial communities (Rawls et al. 2006), we can look at the importance of 'holobiome' in terms of sexual selection theory.

Research into the importance of organisms' microbiome has so far focused on changing the microbiota of individuals and measuring responses. This is the case whether researching gut microbial effects on development rate (Newell & Douglas, 2014), where removing gut bacteria reduced development rate, or on metabolism (Wong et al. 2014), where microbial removal highlighted changes in nutrient uptake. The same methodology has been used in testing microbial effects on mate choice (Sharon et al. 2010) where removal and inoculation with different bacterial communities changed mating preference. Our research show that hosts and their microbiota are evolving together and the evolutionary history of the experimental animals will potentially determine the effects of microbiota removal on these fitness measures. Future work should account for the strength of selection their organisms have been evolving under when designing this type of experiment.

Conclusions

Overall, it appears that evolving under different sexual selection intensities causes changes in the gut microbiota within populations. Natural selection (temperature changes) does not have a significant effect on gut microbial communities. Increasing the strength of sexual selection appears to increase gut microbial diversity of males but not females when using an alpha diversity measure. We found the gut microbial communities of males and females both change with increased sexual selection intensity. The microbiome of males changes in a potentially more functionally significant way than that of females. This is likely due to the strength of sexual selection being higher in males. The functional differences of these changes need further investigation and future work should asses if more phylogenetically distant changes in gut microbiota have greater phenotypic significance. We did not find an interaction between natural and sexual selection on the gut microbiome. This is the first experimental evolutionary response of gut microbiota to sexual selection. Gut microbial communities may play an important role in mediating sexually selected traits or may be directly selected on for the indirect benefits that they can provide to

offspring. Finally, future work should test if these responses are caused by evolved changes in the gut environment of individuals promoting certain bacterial communities, or if these changes are cause by vertical and horizontal transmission within the selection populations.

References

Andersson, M., 1994. Sexual selection. Princeton University Press; Princeton, NJ.

Andersson, M., 1982. Sexual selection, natural selection and quality advertisement. *Biological Journal of the Linnean Society*, 17(4), pp.375–393.

Archer, C.R. et al., 2015. Sex-specific effects of natural and sexual selection on the evolution of life span and ageing in *Drosophila simulans*. *Functional Ecology*, 29(4), pp.562–569.

Archie, E.A. & Theis, K.R., 2011. Animal behaviour meets microbial ecology. *Animal Behaviour*, 82(3), pp.425–436.

Barber, I. et al., 2001. Indirect fitness consequences of mate choice in sticklebacks: Offspring of brighter males grow slowly but resist parasitic infections. *Proceedings of the Royal Society B: Biological Sciences*, 268(1462), pp.71–76.

Bestion, E. et al., 2017. Climate warming reduces gut microbiota diversity in a vertebrate ectotherm. *Nature Ecology and Evolution*, 1(6).

Bienenstock, J., Kunze, W.A. & Forsythe, P., 2018. Disruptive physiology: olfaction and the microbiome–gut–brain axis. *Biological Reviews*, 93(1), pp.390–403.

Blows, M.W., 2002. Interaction between natural and sexual selection during the evolution of mate recognition. *Proceedings of the Royal Society B: Biological Sciences*, 269(1496), pp.1113–1118.

Bretman, A. et al., 2013. Costs and benefits of lifetime exposure to mating rivals in male *Drosophila melanogaster*. *Evolution*, 67(8), pp.2413–2422.

Bright, M. & Bulgheresi, S., 2010. A complex journey: Transmission of microbial symbionts. *Nature Reviews Microbiology*, 8(3), pp.218–230.

Chakir, M. et al., 2002. Male sterility thermal thresholds in *Drosophila: D. simulans* appears more cold-adapted than its sibling *D. melanogaster*. *Genetica*, 114(2), pp.195–205.

Chao, A., 1984. Non-parametric estimation of the classes in a population. *Scandinavian Journal of Statistics*, 11(4), pp.265–270.

Cherrington, C.A. et al., 1991. Short-chain organic acids at pH 5.0 kill *Escherichia coli* and *Salmonella spp*. without causing membrane perturbation. *Journal of Applied Bacteriology*, 70(2), pp.161–165.

Claesson, M.J. et al., 2012. Gut microbiota composition correlates with diet and health in the elderly. *Nature*, 488(7410), pp.178–184.

Clark, C.J., 2012. The role of power versus energy in courtship: What is the “energetic cost” of a courtship display? *Animal Behaviour*, 84(1), pp.269–277.

Clark, R.I. et al., 2015. Distinct shifts in microbiota composition during *Drosophila* aging impair intestinal function and drive mortality. *Cell Reports*, 12(10), pp.1656–1667.

Cobb, M. & Ferveur, J.F., 1995. Evolution and genetic control of mate recognition and stimulation in *Drosophila*. *Behavioural Processes*, 35(1–3), pp.35–54.

Endler, J.A., 1986. *Natural Selection in the Wild.* Princeton University Press: Princeton, N.J., Usa.

Endler, J.A., 1980. Natural selection on color patterns in *Poecilia reticulata*. *Evolution*, 34(1), p.76.

Folstad, I. et al., 1994. Sexually selected color in male sticklebacks: A signal of both parasite exposure and parasite resistance? *Oikos*, 69(3), p.511.

Fontaine, S.S., Novarro, A.J. & Kohl, K.D., 2018. Environmental temperature alters the digestive performance and gut microbiota of a terrestrial amphibian. *Journal of Experimental Biology*, 221(20).

Forsythe, P. et al., 2010. Mood and gut feelings. *Brain, Behavior, and Immunity*, 24(1), pp.9–16.

Fox, J. & Weisberg, S., 2018. Package “car”, An R Companion to Applied Regression. *R Documentation, Sage*.

Freter, R., 1955. The fatal enteric cholera infection in the guinea pig, achieved by inhibition of normal enteric flora. *Journal of Infectious Diseases*, 97(1), pp.57–65.

Gill, S.R. et al., 2006. Metagenomic analysis of the human distal gut microbiome.

Science, 312(5778), p.1355 LP-1359.

Gillooly, J.F. et al., 2001. Effects of size and temperature on metabolic rate. *Science*, 293(5538), p.2248 LP-2251.

Guerrero, R., Margulis, L. & Berlanga, M., 2013. Symbiogenesis: The holobiont as a unit of evolution. *International Microbiology*, 16(3), pp.133–143.

Hadley, N.F., 1981. Cuticular lipids of terrestrial plants and arthropods: a comparison of their structure, composition, and waterproofing function. *Biological Reviews*, 56(1), pp.23–47.

Hamilton, W.D. & Zuk, M., 1982. Heritable true fitness and bright birds: A role for parasites? *Science*, 218(4570), pp.384–387.

Hammami, R. et al., 2013. Anti-infective properties of bacteriocins: An update. *Cellular and Molecular Life Sciences*, 70(16), pp.2947–2967.

Heijtz, R.D. et al., 2011. Normal gut microbiota modulates brain development and behavior. *Proceedings of the National Academy of Sciences of the United States of America*, 108(7), pp.3047–3052.

Hollis, B. & Kawecki, T.J., 2014. Male cognitive performance declines in the absence of sexual selection. *Proceedings of the Royal Society B: Biological Sciences*, 281(1781).

Hosken, D.J. et al., 1994. Localisation of insect calls by the bat *Nyctophilus geoffroyi* (*Chiroptera: Vespertilionidae*): A laboratory study. *Australian Journal of Zoology*, 42(2), pp.163–176.

House, C.M. et al., 2013. Sexual and natural selection both influence male genital evolution. *PLoS ONE*, 8(5).

Huey, R.B. & Kingsolver, J.G., 1993. Evolution of resistance to high temperature in ectotherms. *The American Naturalist*, 142, pp.S21–S46.

Ingleby, F.C., 2015. Insect cuticular hydrocarbons as dynamic traits in sexual communication. *Insects*, 6(3), pp.732–742.

Kim, B.R. et al., 2017. Deciphering diversity indices for a better understanding of

microbial communities. *Journal of Microbiology and Biotechnology*, 27(12), pp.2089–2093.

Kirkpatrick, M., 1982. Sexual selection and the evolution of female choice. *Evolution*, 36(1), p.1.

Kohl, K.D. & Yahn, J., 2016. Effects of environmental temperature on the gut microbial communities of tadpoles. *Environmental Microbiology*, 18(5), pp.1561–1565.

Li, W. et al., 2009. Memory and learning behavior in mice is temporally associated with diet-induced alterations in gut bacteria. *Physiology and Behavior*, 96(4–5), pp.557–567.

Lizé, A., McKay, R. & Lewis, Z., 2013. Gut microbiota and kin recognition. *Trends in Ecology and Evolution*, 28(6), pp.325–326.

Lozupone, C., Hamady, M. & Knight, R., 2006. UniFrac - An online tool for comparing microbial community diversity in a phylogenetic context. *BMC Bioinformatics*, 7.

Martín-Vivaldi, M. et al., 2009. Antimicrobial chemicals in hoopoe preen secretions are produced by symbiotic bacteria. *Proceedings of the Royal Society of London B: Biological Sciences*, 277(1678), pp.123–130.

McMurdie, P.J. & Holmes, S., 2013. Phyloseq: An R package for reproducible interactive analysis and graphics of microbiome census data. *PLoS ONE*, 8(4).

Milinski, M. & Bakker, T.C.M., 1990. Female sticklebacks use male coloration in mate choice and hence avoid parasitized males. *Nature*, 344(6264), pp.330–333.

Moghadam, N.N. et al., 2018. Strong responses of *Drosophila melanogaste*r microbiota to developmental temperature. *Fly*, 12(1), pp.1–12.

Møller, A.P. & Nielsen, J.T., 1997. Differential predation cost of a secondary sexual character: Sparrowhawk predation on barn swallows. *Animal Behaviour*, 54(6), pp.1545–1551.

Newell, P.D. & Douglas, A.E., 2014. Interspecies interactions determine the impact of the gut microbiota on nutrient allocation in *Drosophila melanogaster*. *Applied and Environmental Microbiology*, 80(2), pp.788–796.

Oksanen, J. et al., 2013. *Package vegan: Community ecology package*, Version 2. Available at: http://CRAN.R-project.org/package=vegan.

Parsons, P.A., 1973. Genetics of resistance to environmental stresses in *Drosophila* populations. *Annual Review of Genetics*, 7(1), pp.239–265.

Parsons, P.A., 1980. Parallel climatic races for tolerances to high temperature-desiccation stress in two *Drosophila* Species. *Journal of Biogeography*, 7(1), p.97.

Pultz, N.J. et al., 2005. Mechanisms by which anaerobic microbiota inhibit the establishment in mice of intestinal colonization by Vancomycin-resistant *Enterococcus* . *The Journal of Infectious Diseases*, 191(6), pp.949–956.

R Core Team, 2019. R: A language and environment for statistical computing. R Foundation for Statistical Computing, Vienna, Austria. https://www.R-project.org/.

Rapkin, J. et al., 2018. The geometry of nutrient space–based life-history trade-offs: Sex-specific effects of macronutrient intake on the trade-off between encapsulation ability and reproductive effort in decorated crickets. *American Naturalist*, 191(4), pp.452–474.

Rawls, J.F. et al., 2006. Reciprocal gut microbiota transplants from zebrafish and mice to germ-free recipients reveal host habitat selection. *Cell*, 127(2), pp.423–433.

Reznick, D., 1985. Costs of reproduction: an evaluation of the empirical evidence. *Oikos*, 44(2), p.257.

Rolff, J. & Kraaijeveld, A.R., 2003. Selection for parasitoid resistance alters mating success in *Drosophila*. *Proceedings of the Royal Society B: Biological Sciences*, 270, pp.S154-S155.

RStudio Team, 2016. RStudio server: Integrated Development for R. *RStudio, Inc., Boston, MA*.

Ruiz-De-Castañeda, R. et al., 2012. Bacterial degradability of an intrafeather unmelanized ornament: A role for feather-degrading bacteria in sexual selection? *Biological Journal of the Linnean Society*, 105(2), pp.409–419.

Ruiz-Rodriguez, M. et al., 2009. Symbiotic bacteria living in the hoopoe's uropygial gland prevent feather degradation. *Journal of Experimental Biology*, 212(22), pp.3621–3626.

Ryan, M.J., Tuttle, M.D. & Taft, L.K., 1981. The costs and benefits of frog chorusing behavior. *Behavioral Ecology and Sociobiology*, 8(4), pp.273–278.

Sachs, J.L., Essenberg, C.J. & Turcotte, M.M., 2011. New paradigms for the evolution of beneficial infections. *Trends in Ecology and Evolution*, 26(4), pp.202–209.

Sender, R., Fuchs, S. & Milo, R., 2016. Revised estimates for the number of human and bacteria cells in the body. *PLoS Biology*, 14(8).

Sharma, M.D., Hunt, J. & Hosken, D.J., 2012. Antagonistic responses to natural and sexual selection and the sex-specific evolution of cuticular hydrocarbons in *Drosophila simulans*. *Evolution*, 66(3), pp.665–677.

Sharma, M.D., Tregenza, T. & Hosken, D.J., 2010. Female mate preferences in *Drosophila simulans*: Evolution and costs. *Journal of Evolutionary Biology*, 23(8), pp.1672–1679.

Sharon, G. et al., 2010. Commensal bacteria play a role in mating preference of *Drosophila melanogaster*. *Proceedings of the National Academy of Sciences*, 107(46), pp.20051–20056.

Shin, S.C. et al., 2011. *Drosophila* microbiome modulates host developmental and metabolic homeostasis via insulin signaling. *Science*, 334(6056), pp.670–674.

Somjee, U. et al., 2018. The hidden cost of sexually selected traits: The metabolic expense of maintaining a sexually selected weapon. *Proceedings of the Royal Society B: Biological Sciences*, 285(1891).

Sudo, N. et al., 2004. Postnatal microbial colonization programs the hypothalamic-pituitary-adrenal system for stress response in mice. *Journal of Physiology*, 558(1), pp.263–275.

Tremaroli, V. & Bäckhed, F., 2012. Functional interactions between the gut microbiota and host metabolism. *Nature*, 489(7415), pp.242–249.

Turnbaugh, P.J. et al., 2006. An obesity-associated gut microbiome with increased

capacity for energy harvest. *Nature*, 444(7122), pp.1027–1031.

Tuttle, M.D., Taft, L.K. & Ryan, M.J., 1982. Evasive behaviour of a frog in response to bat predation. *Animal Behaviour*, 30(2), pp.393–397.

Werren, J.H., Baldo, L. & Clark, M.E., 2008. *Wolbachia*: Master manipulators of invertebrate biology. *Nature Reviews Microbiology*, 6(10), pp.741–751.

Wong, A.C.N., Dobson, A.J. & Douglas, A.E., 2014. Gut microbiota dictates the metabolic response of *Drosophila* to diet. *Journal of Experimental Biology*, 217(11), pp.1894–1901.

Zuk, M., Rotenberry, J.T. & Tinghitella, R.M., 2006. Silent night: Adaptive disappearance of a sexual signal in a parasitized population of field crickets. *Biology Letters*, 2(4), pp.521–524.

Zuo, W. et al., 2012. A general model for effects of temperature on ectotherm ontogenetic growth and development. *Proceedings. Biological sciences*, 279(1734), pp.1840–1846.

Chapter 5

The effects of microbiota across different genotypes on male competitive fitness, female choosiness and reproductive output.

Abstract

The exosymbiotic bacteria that inhabit the internal and external surfaces of animals play an important role in their host's fitness. The number of bacterial cells can often outnumber that of their hosts. These bacteria play a role in their host's immunity, development, physiology and behaviour. Symbiotic bacteria have also been found to influence a range of sexual signals. Most previous studies have compared germ-free individuals to standard individuals to find evidence of microbial influence on their hosts. Here we use a novel treatment where we alter the microbial profile of different *Drosophila simulans* genotypes and test the fitness effects across a range of measures compared to the removal of or original microbiota. We found a significant interaction between microbiota and genotype on male body size. Our microbial treatments did not influence male competitive reproductive success, female choosiness or body size. When comparing the effects on female reproductive output across genotypes simply removing the microbiota had no effects compared to the original microbiota fitness ranks, however, when we altered the microbiota fitness ranks were significantly changed. This shows that host genotype and the microbiota are interacting. When looking for microbiota fitness effects it is important that we test across a range of hosts. Future work should also attempt to use more biologically relevant microbial treatments rather than just comparing individuals with an intact microbiota to germ-free individuals.

Introduction

Exosymbiotic bacteria are understood to be an important physiological feature of animals. These bacteria live on the surface (skin, feathers, scales, feathers and exoskeletons) or in reproductive tracts, specialised preening or olfaction glands and digestive tracts of their host. The number of symbiotic bacteria an organism hosts often out-number host somatic cells - for example in humans there are ten times more bacteria than somatic cells (Savage, 1977). This specific example means that the number of symbiotic bacterial genes outnumber human genes 100 to 1 (Yang et al. 2009). This demonstrates the potential there is for symbiotic bacteria to have physiological or behavioural effects on their host. Symbiotic bacteria can interact with their hosts in a number of ways; they can benefit their host (i.e. a mutualism), have no fitness effect on their host (i.e. have a commensal relationship), or, negatively affect their host (i.e. act as parasites) (Dimijian, 2000). To date the majority of research has focused on parasitic relationships as often these have the most dramatic or obvious effects on host fitness. In many cases it is now understood that many of these interactions are dynamic between bacteria and host. However, symbiotic bacteria have been shown to have wide ranging fitness effects on their host.

Symbiotic bacteria have been shown to be important in improving host immune function. One mechanism of this is that commensal bacteria in the gut form fairly stable communities that resist the colonisation of the gut by pathogens (Freter, 1955). Gut symbionts have also been shown to directly inhibit the growth of pathogens and even kill them (Pultz et al. 2005). Some of the mechanisms behind these direct effects are either be the production of bacteriocins (Hammami et al. 2013) or the type VI secretion system (Russell et al. 2014). Both of these mechanisms involve by-products of bacterial competition from the commensal bacteria, which helps to stop the colonisation of the gut by pathogens. Immune benefits from symbiotic bacteria are not limited to the gut microbiota. In amphibians the symbiotic skin-bacteria help to defend again the pathogenic chytrid fungus (*Batrachochytrium* dendrobatidis) (Woodhams et al. 2007). Metabolites produced by symbiotic bacteria can also help to protect the host against infection by bacterial parasites. Some symbiotic anaerobic bacteria produce

short-chained fatty acids (SCFAs) which have been shown to inhibit the growth of or kill pathogens (Cherrington et al. 1991; Bohnhoff et al. 1964).

Symbiotic gut bacteria can also impact their host's metabolism. The SCFAs produced by some symbiotic gut microbiota can regulate host metabolism (Bjursell et al. 2011; Bellahcene et al. 2013). For example, obese and lean identical twins vary in their core microbiome (Turnbaugh et al. 2009). The gut microbiota from obese individuals have also been shown to increase energy harvesting capacity in mice (Turnbaugh et al. 2006). Gut bacterial induced gut inflammation has also been shown to have detrimental metabolic effects in mice (Lam et al. 2012). The mechanisms behind microbiota effects on host metabolism are not fully understood. However, gut microbiota can break down inaccessible nutrient sources into more easily absorbable metabolites (Tremaroli & Bäckhed, 2012). These metabolites can also effect host neuroendocrine systems, which can have behavioural and development effects in mice (Bravo et al. 2011; Heijtz et al. 2011). These changes induce anxiety like behaviours and change motor activity.

Metabolites and symbiotic bacterial by-products also influence the olfactory communication and signalling in a range of mammals. Changes in the anal glad bacterial communities co-vary with social odour in meerkats (*Suricata suricatta)* (Leclaire et al. 2017) and hyenas (Theis et al. 2013). Bacterial communities in European badger's (*Meles meles)* (Sin et al. 2012) and meerkat's (*Suricata suricatta)* (Leclaire et al. 2014) scent glands correlate with individual-specific traits such as age, sex, condition and reproductive status. These are examples of symbiotic bacteria potentially mediating important signalling pathways. However, once again all these examples show correlation and not causation. In greater sac-winged bats (*Saccopteryx bilineata*), males have a pouch like scent organ that is used in courtship and these are morphologically different to females' pouches. The bacteria present in wing sacs varied across the sexes with males having much less diverse microbiota inhabiting their wing sacs compared to females (Christian et al. 2005). Males will clean and refill their wing sacs daily and this may be in order to maintain a highly specialised microbial community (Voigt, 2002). Bacterial communities in the uropygial (preen) glands of hoopoes (*Upupa epops*) protect against feather degrading bacteria (Ruiz-

Rodriguez et al. 2009) and the white feathers used in sexual signalling are broken down faster than the darker, more melanised feathers (Ruiz-De-Castañeda et al. 2012). Males with larger preen glands are able to better maintain these sexual signals (Ruiz-Rodríguez et al. 2015). This means that symbiotic bacteria are responsible for the honesty and the maintenance of the sexual signal. Gut microbiota have also been shown to influence signalling in invertebrates. In *Drosophila melanogaster* symbiotic bacteria have been shown to alter cuticular hydrocarbon (CHC) profiles (Sharon et al. 2010). CHCs are important sexual signals (Howard & Blomquist, 2005) and have been shown to evolve in divergent ways under different levels of sexual selection (Sharma et al. 2012). This means that in *Drosophila* symbiotic bacteria are responsible for certain mating signals and once the symbiotic bacteria are removed mating signals change (Sharon et al. 2010).

Symbiotic bacteria impact sexual selection in a number of ways. As shown above symbiotic bacteria cause changes in important olfactory and visual sexual signals. Gut bacterial changes have also been shown to influence development rates in *D. melanogaster* (Shin et al. 2011) and some of these changes appear to be cause by *Lactobacillus plantarum* modulating hormone signalling (Storelli et al. 2011). Interestingly, Sharon et al. (2010) found that flies raised on different diets greatly varied in the prevalence of *L. plantarum* in their guts. Males inoculated with different bacterial species varied in their mating duration (Morimoto et al. 2017). This can have important effects on male competitive ability as increased mating duration can result in a greater competitive mating success (Bretman et al. 2009). However, this change in mating duration was likely caused by the inoculation with *Acetobacter pomorum* causing a reduction in male condition (Morimoto et al. 2017). This study manipulated individual symbiotic bacteria species which is likely to not be biologically relevant as bacteria within a community interact and likely multiple species are responsible for many of the fitness effects described above.

Much of the research into the fitness effects of symbiotic bacteria can be classified in to two groups: they either look for an association between the gut profiles of individuals in different groups, or they remove bacteria and compare germfree individuals to standard individuals. The problem with the first approach is that correlation does not

necessarily mean causation. The problem with removing bacteria or creating germfree individuals is that this is does not tell us much other than that symbiotic bacteria are important for a range of functions that are all linked.

In the study conducted here, we take another approach. We used *Drosophila simulans* as our model to test for effects of symbiotic bacteria on host sexual-fitness. Rather than the usual remove/retain bacteria then compare host fitness, we introduced another treatment, which tests if altering the microbial profiles of individuals has different effects compared to simply removing their microbiota. Thus, we had three treatments: gut bacteria removed, removed and a re-infected with original gut bacteria and removed and subsequently inoculated with a novel bacterial community. We previously found that evolving under higher or lower intensities of sexual selection cause the evolution of divergent gut microbiomes in *D. simulans* (Chapter 4). This suggests that the symbiotic gut bacteria play a role in sexual selection in this species. As the gut bacterial communities appear to evolve with a population, we wanted to test if different genotypes responded differently to a novel symbiotic gut microbiota. Host genotype has been shown to influence their gut microbiome (Spor et al. 2011). Reciprocal microbial transplants between zebrafish and mice have shown that microbial communities return to a structure similar to the original host, but the bacterial species composition of these communities remain altered (Rawls et al. 2006). We tested whether manipulating the microbiota of *D. simulans* impacts sexually selected fitness-linked traits and if these effects are genotype dependent.

Materials and methods

Drosophila simulans isolines used in this experiment were originally collected from Greece (Ingleby et al. 2013) and were maintained for > 45 generations with full-sib matings (n= 25 brothers and 25 sisters/isoline). Thus, each isoline could be considered as being distinct genotypes (David et al. 2005). All stocks were reared on a standard cornmeal-based Jazzmix diet (hereafter Jazzmix) (supplied by Applied Scientific, UK) at 25°C on a 12:12 hour light:dark cycle. Each isoline was then split into one of three gut microbial treatments. For all treatments, founding isolines were allowed to egg lay for 24h on apple juice agar (50% apple juice, 50% DiH_2O by liquid volume and 1.2%

agar powder) supplemented with yeast paste to encourage egg laying. These eggs were then collected had their chorion removed through washing in a 50% bleach solution in order to remove any gut bacteria from the eggs (Chapter 2). These clean eggs were then split into each of the three gut microbial treatments (Figure 1). The bacteria used to generate these treatments were collected using the methods outlined in Chapter 2 - from food vials that 25 males and females interacted on for 3 days. One treatment was inoculated with gut bacteria collected from self-isoline to provide a treatment re-infected with their original gut bacteria (+ gut bacteria). The second treatment was inoculated with sterile PBS to provide a treatment with washed gut bacteria (- gut bacteria). The final treatment was inoculated with gut bacteria from *Drosophila pseudoobscura* raised on a separate diet type, this would provide a treatment with a new gut bacterial profiles (Δ gut bacteria). The *D. pseudoobscura* were originally collected from Lewiston, Montana, in 2008 (Price et al. 2014) and maintained as an isoline for >80 generations on a diet medium of rolled oats, brown sugar, dried yeast, agar, nipagin, proprionic acid and water (Shorrocks, 1972).

After treatment appropriate inoculation, the eggs from each bacterial treatment were allowed to develop and adult flies collected as virgins <6 hours after eclosion. These virgin flies were then housed by isoline and treatment in single sex vials of up to 10 individuals until they were between three and six days old. For all fitness assays tester flies came from a population of *ebony* (a recessive, phenotypic body-colour mutant) flies. The *ebony* stock populations (stocks from Tucson stock centre) were maintained at the standard conditions described above (*ca*. 800 flies/cage). To collect tester flies 25 male and 25 female ebony flies were allowed to egg lay for 2 days in each large vial containing 30ml Jazzmix. The eggs were left to develop until virgin males and females were collected every 6 hours and stored in single sex groups of 10. These flies were left to age for 3-5 days and used as tester mates for both males and females.

Male fitness assay

We used competitive male reproductive output (the number of offspring sired by focal males competing against two *ebony* males for access to two *ebony* females) as a measure of male fitness. We set up 5 replicates/isoline per treatment (for a total of 270

flies). Each focal male was housed with two *ebony* males and two *ebony* females for 48 hours, then males were removed and females were moved into fresh egg-laying vials for 48 hours and then again for 72 hours. All fly transfers were performed without anaesthesia to avoid any negative effects on female fecundity (Champion De Crespigny & Wedell, 2008). Offspring from each vial were counted on the 8th day after the first eclosions. This measure has been shown to be a good proxy for lifetime productivity from a single copulation (Taylor et al. 2008; Nguyen & Moehring, 2015). We counted the number of *wild-type* and *ebony* offspring to determine parentage. We scored the proportion of offspring that were sired by the focal males (*wild-type*). The focal males had wing measurements taken after being removed from their mating vials to determine if the gut bacterial treatments had an effect on male size as this may reflect changes in condition across treatments.

Male fitness analysis

All data analyses were carried out in RStudio version 1.1383 (RStudio Team, 2016) using R version 3.6.2 (R Core Team, 2019). To analyse the effects of gut bacteria on the proportion of offspring sired by the focal male we used a GLM fit with quasi-binomial error structure. Full models were fit with gut bacterial treatment, genotype (isoline) and the interaction between them as fixed effects. The interaction was included to see if the gut bacterial effects were dependent on the genetic background of the fly. Fixed effects were then tested for significance using the Anova function in the car package (Fox & Weisberg, 2011). Rank changes in the proportion of offspring sired by the focal male with gut bacteria treatment across Isolines were analysed using a Spearman's Rho correlation coefficient. To test if gut bacterial treatment had an effect on male size we averaged the wing measurements of each fly to obtain an average wing size for each male. We then used a GLM fit with a Gaussian error structure. The full models were fit again with gut bacterial treatment, genotype (isoline) and the interaction as fixed effects. The fixed effects were again were then tested for significance using the Anova function in the car package.

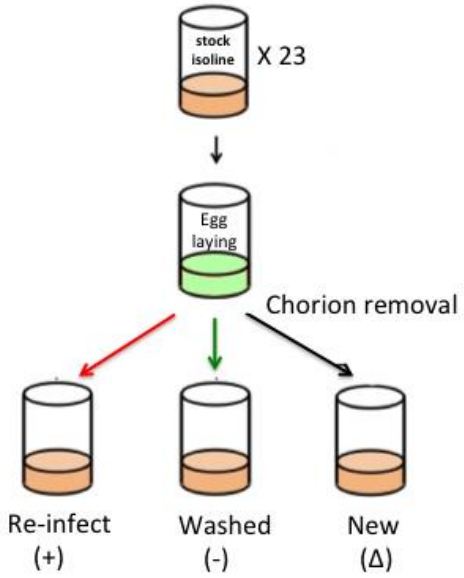

Figure 1: Illustration of the bacterial treatment protocol. Males and females from each isoline were transferred on to separate apple juice agar for egg laying. Those eggs were then collected and de-chorionated using a 50% bleach solution. These eggs were then split into one of 3 treatments for each isoline. One third were re-infected with the original gut microbiota from their matching isoline. The next group were inoculated with sterile PBS to provide a washed treatment. The final treatment was inoculated with a new gut microbiota collected from *D. pseudoobscura* raised on a different diet.

Female latency to mate assay

We used female's latency to mate with an *ebony* male as a measure of female choosiness. In *Drosophila* females have complete control over whether or not they choose to mate (Spieth, 1974; Lasbleiz et al. 2006). As there is not any forced copulation in *D. simulans* the time a female takes to mate can be used as an estimate of choosiness (Taylor et al. 2008; Narraway et al. 2010; Sharma et al. 2010; Ingleby et al. 2013). We provided each female with a single male and timed how long from introduction it took for copulation to occur. We set up 5 females per treatment for each isoline (for a total of 345 flies) in individual vials 24 hours before the introduction of a male. An *ebony* male was placed into each vial ~1 hour after lights on in the incubators. The time that each male was introduced into each vial was recorded and then investigators blind to the treatments of each female watched the vials until mating

started. We recorded the time that mating started and then allowed mating to conclude before the vials were moved.

Female choosiness analysis

To assess whether changes in gut biota causes changes in female choosiness, we used Kaplan-Meier curves to visualize the data and analysed differences in female choosiness using a cox proportional hazard model (Kaplan & Meier, 1958; Cox, 1972) with isoline and gut bacteria treatment as co-variates and mating being the hazard. This allows us to tests which factors impact the time to the event and also include individuals that did not mate during the observation period. We then tested the significance and interactions of all the risk factors using the ANOVA function in the car package (Fox & Weisberg 2011) of RStudio version 1.1383 (RStudio Team, 2016) using R version 3.6.2 (R Core Team, 2019).

Female fitness assay

To test if altering females gut biota also impacted their fitness we tested their reproductive output from the matings in the choosiness assay above. After the copulations ended the male was removed from the vial and the female allowed to egg lay for 48 hours until being moved to a fresh vial and egg-laying for another 48 hours and then moving to a final egg-laying vial for 72 hours. After the final transfer the females were removed and their wings measured to provide a body size estimate. The eggs were allowed to develop and the total offspring counted from each vial 8 days after the first eclosion. All transfers were carried out without anaesthesia to limit fitness effects of the transfers.

Female fitness analysis

To test the gut bacterial effects on female fitness we used a GLM fit with a quasi-Poisson error distribution to compare the total number of offspring across treatments. Full models were fit with gut bacterial treatment, genotype (isoline) and the interaction as fixed effects. The model was the tested for significance as above using the Anova

function in the car package (Fox & Weisberg 2011) of RStudio version 1.1383 (RStudio Team, 2016) using R version 3.6.2 (R Core Team, 2019). We analysed female size in the same way as for male size to test for any gut bacterial effects on female condition.

Results

To test the effects of the three gut bacterial treatments on male fitness we used male competitive reproductive output as our measure of fitness. We found that Gut bacterial treatment had no effect on male competitive reproductive output (F= 1.10*, df=2, p = 0.33)* with proportion of offspring sired by our focal male being ranging from 0.24 to 0.33 (Figure 2). We found that genotype (isoline) had a significant effect on male competitive reproductive output (F= 1.93*, df=18, p = 0.015)*. There was no significant interaction between the effects of genotype and gut bacterial treatment on male competitive reproductive output (F= 0.98*, df=35, p = 0.81*). However, we detected crossing-over in the average fitness ranks of each isoline across the three gut bacterial treatments (Figure 3). This suggests that the effects of gut treatment may depend on the genetic background of the individual although there is no significant interaction. To test this we averaged genotype fitness for each gut bacterial treatment and then using these means to assess the fitness ranks of genotypes across gut bacterial treatments. This revealed that there were no correlations between the new (Δ) and re-infected (+) gut bacteria treatments (Spearman's rho = 0.28; P = 0.27), the re-infected (+) and the washed (-) gut bacterial treatments (Spearman's rho = 0.42; P = 0.08) or between the new (Δ) and washed (-) bacterial treatments (Spearman's rho = 0.03; P = 0.91).

We used wing measurements as a proxy for body size and tested the effects of gut bacterial treatment, genotype (isoline) and their interaction on male body size. We found that genotype had a significant effect on male body size (X^2=214.1*, df=15, p < 0.001*). Gut bacterial treatment did not significantly affect male body size in a consistent way across genotypes (isolines) (X^2=0.28*, df=2, p = 0.87*). We do find a significant interaction between gut bacterial treatment and genotype (isoline) on male body size (X^2=61.6*, df=30, p < 0.001)* meaning the gut bacterial treatment effects depend on the genetic background or original gut bacterial profile of an individual (Figure 4). We also measure female body size and analysed the effects of gut bacterial

treatment, genotype and their interaction in the same way as for males. We found that genotype (isoline) had a significant effect on the body size of females (X^2=90.42*, df=16, p < 0.001*). We found that gut bacterial treatment did not significantly alter female body size (X^2=2.491*, df=2, p = 0.288*). There was also no significant interaction between gut bacterial treatment and genotype (isoline) (X^2=42.49*, df=32, p = 0.102*) despite finding similar patterns to the male body size when plotted by genotype and bacterial treatments (Figure 4).

To test if altering the gut bacterial profile of females changes their choosiness we used latency to mate with an *ebony* male as a measure of choosiness. We found that gut bacterial treatment had no effect on female choosiness (X^2=3.11*, df=2, p = 0.21).* This is also shown in the Kaplan-Meier curve (Figure 5) where the time individuals that had their bacteria removed and inoculated with sterile PBS seem to be the least choosey however the differences are minimal. We also found that a female's genotype (isoline) did not affect choosiness (X^2=24.94*, df=22, p = 0.30).* We also found no interaction between gut bacterial treatment and genotype on this measure of female choosiness (X^2=43.48*, df=44, p = 0.49).*

The successfully mating females were allowed to egg lay for 7 days and their total reproductive output for this time was measured. The total number of offspring that eclosed from the egg lay vials was recorded for each female that successfully mated. We found that genotype (isoline) had a significant effect on female reproductive output (*F*=3.80*, df=22, p < 0.001)*. Gut bacterial treatment had no effect on female reproductive output (*F*=1.89*, df=2, p = 0.15)* (Figure 2). We also found the interaction between gut bacteria treatment and genotype (isoline) had no effect on female reproductive output (*F=1.16, df=44, p = 0.24).* As above for male competitive reproductive success we averaged genotype fitness across our gut bacterial treatments and compared these averages. Again we see crossing over in the average fitness ranks of each isoline across the three gut bacterial treatments (Figure 3). When we compare the genotype fitness ranks across treatments we find no correlation between re-infection (+) and new (Δ) gut bacterial treatments (Spearman's rho = 0.40; *P* = 0.06) or between new (Δ) and washed (-) gut treatments (Spearman's rho = 0.21; *P* = 0.34). There is however significant correlation between re-infection (+) and

washed (-) gut bacterial treatments (Spearman's rho = 0.600; *P* = 0.002). This suggests that the effects of gut treatment may depend on the genetic background of the individual but only when changing the gut bacteria not simply removing it.

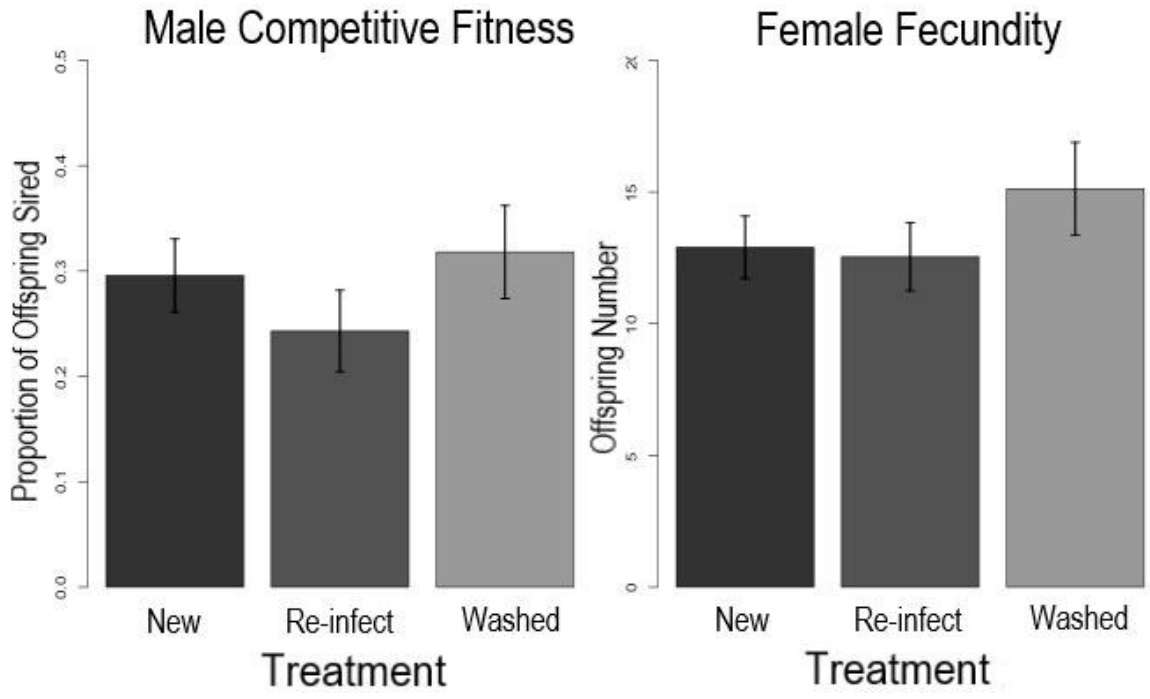

Figure 2. The effects of bacterial treatment on measure of male (left panel) and female fitness (right panel). All treatments had their gut bacteria removed. The Washed treatment were inoculated with sterile PBS (-), Re-infect were inoculated with their original gut bacteria (+) and New were inoculated with a novel gut bacteria from a different species of *Drosophila* (Δ). The mean proportion of offspring sired by the focal male with standard error bars across the three gut bacterial treatments averaged across isolines for the male fitness assay (left panel). The plot shows that gut bacterial treatment doesn't significantly impact male competitive reproductive success however surprisingly the treatment where flies are re-infected with their original gut bacteria their average fitness is lowest and when inoculated with sterile PBS they have the highest average fitness. The mean female offspring production over 7 days averaged across isolines for each bacterial treatment with standard error bars (plotted on the right). Again the plot shows no significant effect of gut bacterial treatment on female fitness. Surprisingly we again see females inoculated with sterile PBS in the washed (-) bacterial treatment have the highest average fitness.

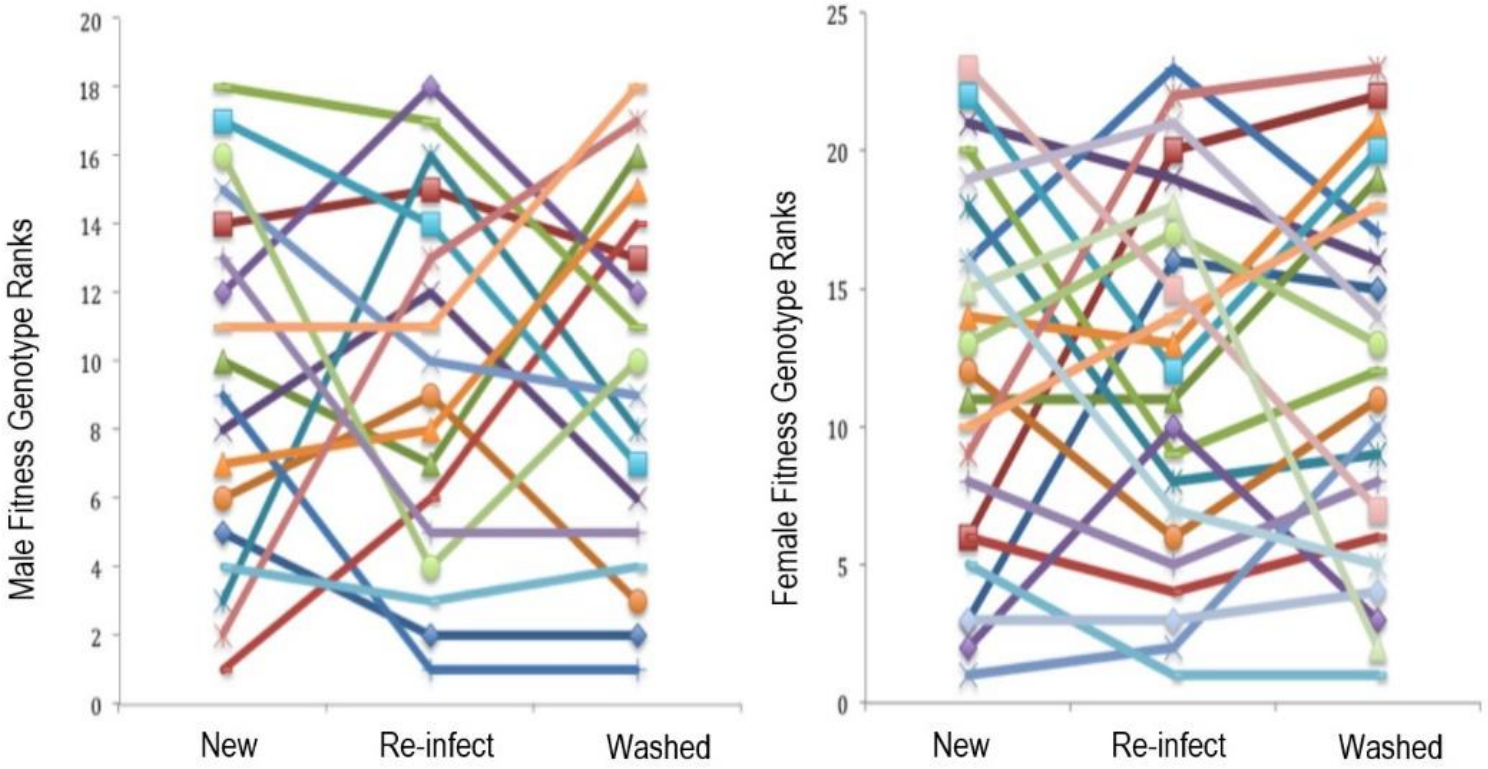

Figure 3. The relative competitive male-fitness ranks of genotypes (isolines) on the left and female offspring production on the right with either new (Δ), re-infect (+) or washed (-) gut bacterial treatments. There is no association between genotype fitness-ranks across gut bacterial treatments as indicated by the major crossing over in ranks for males. This suggests changing gut bacterial profile fundamentally alters the male sexual-fitness ranks of fly genotypes. Female genotype fitness ranks cross over however there is significant correlation between the re-infect and washed treatments. This suggests the gut bacterial treatment genotype differences only apply when being inoculated with new bacteria.

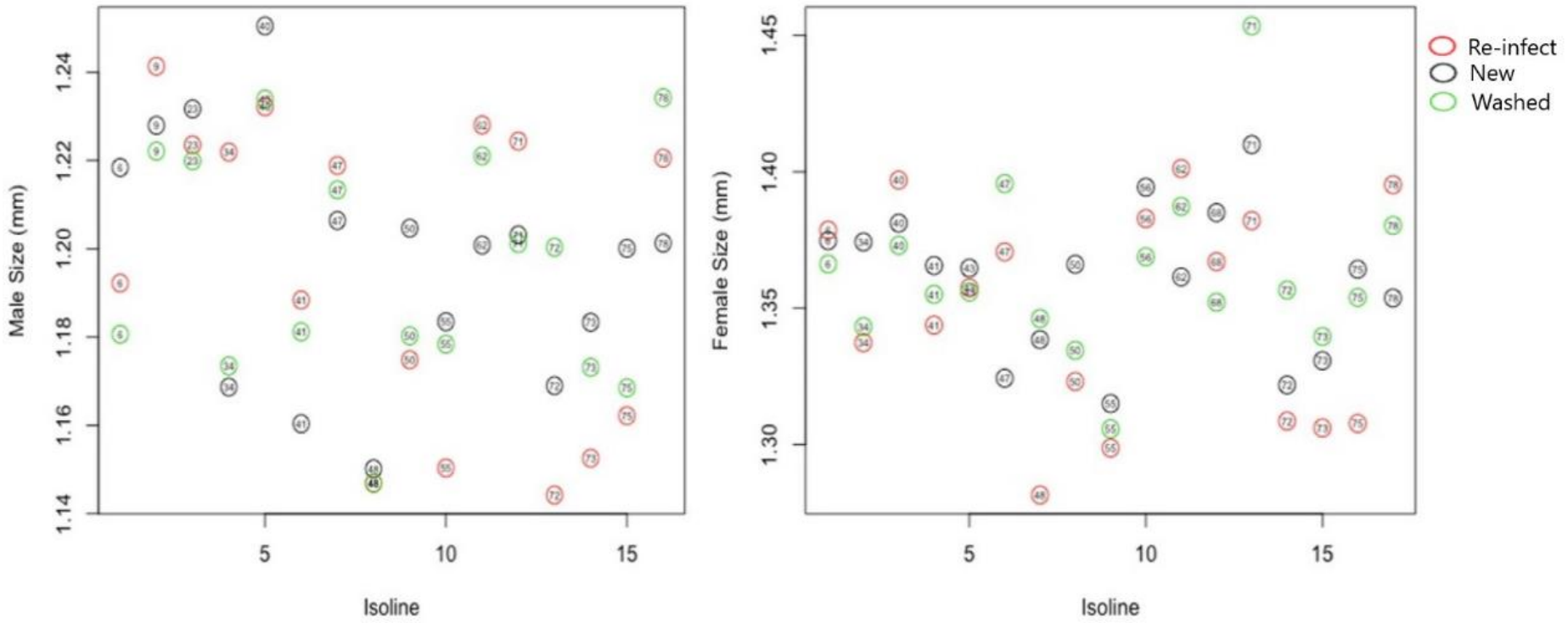

Figure 4. Body size plotted across gut bacteria treatment and genotype (isoline). Gut bacterial treatments are shown with the different coloured data points where black circles represent the new (Δ) gut treatment of inoculation with a novel gut microbiota, red circles represent the re-infect with their original gut microbiota (+) gut treatment and green circles represent the washed (-) gut bacterial treatment where the gut microbiota a removed. Male body size is plotted on the left and female body size on the right. The plots show the gut bacterial treatments have different effects on body size across the genotypes (isolines) however this effect is only significant in males. In some instances removing or changing the gut bacterial profile causes flies to be larger and in some instances smaller.

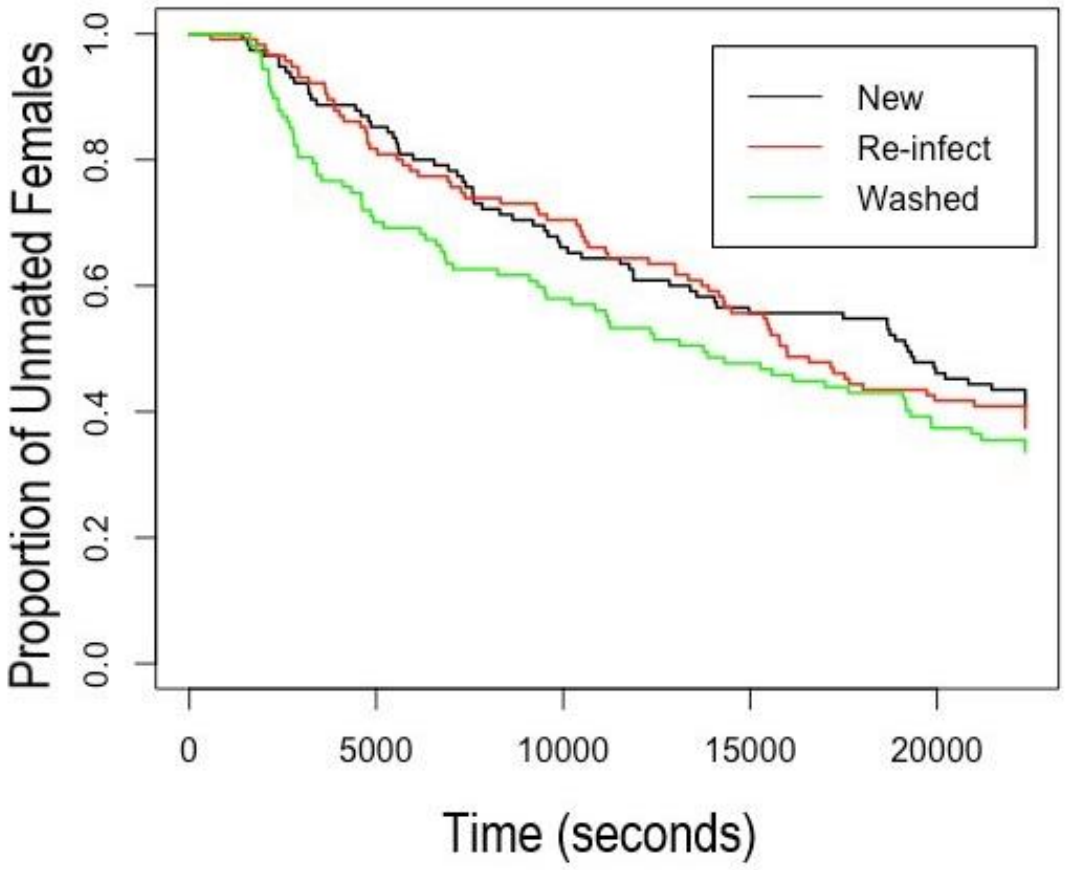

Figure 5. Kaplan-Meier curve plotted for time until females mate with a stock *ebony* male across the three gut bacterial treatments. The y-axis shows the proportion of females that have mated plotted against time on the x-axis. Females with the new gut bacterial treatment (Δ) are plotted in black, females with the re-infect treatment (+) are plotted in red and the washed bacterial treatment (-) are plotted in green. The plot shows how close the three bacterial treatments track in terms of female choosiness with the washed (-) treatment only being slightly less choosey.

Discussion

Changing the composition of individual's microbiota did not consistently affect male competitive reproductive success across all genotypes. The competitive reproductive success of males appeared to vary across genotypes (isoline), however, there was no significant interaction between genotype and bacterial treatment on male competitive reproductive fitness. When we plotted the averaged male fitness ranks of each isoline

across the three bacterial treatments we found crossing over of these ranks. We also found that there was no correlation between the male fitness ranks across any of the bacterial treatments. It has been suggested GLMs and analyses of variance are relatively poor at detecting genotype by environmental interactions (Lewontin, 2006), although, finding no significant correlation between the genotype fitness ranks across treatments does not mean the crossing-over of fitness ranks is statistically significant. This may be evidence that the impact of the gut microbiota on male reproductive success is dependent of the male's genotype. The effects of bacterial treatment on male body size depend of the genotype on the male. This means that manipulating or removing the symbiotic bacteria causes larger males in some isolines and smaller males in others. Overall, we found no effect of bacterial treatment independently on male body size.

Across all our measures of female fitness we found no effect of bacterial treatment. Female choosiness and offspring production did not vary across our symbiotic bacterial treatments. There was no significant interaction between genotype and bacterial treatment on either of these fitness measures either. When comparing female offspring production fitness ranks of isolines across treatments we found the there is a significant correlation between these ranks of washed (-) and re-infected (+) treatment females. There was no correlation between either re-infected (+) and new (Δ) or washed (-) and new (Δ) treatment females. This suggests that and symbiotic bacterial effects on female offspring production depend on the genetic background of the female and require manipulation with a novel bacterial community not just the removal the symbiotic bacteria although this would need further work to identify a significant interaction between genotype and microbiota on female fitness using this novel bacterial treatment. We did not find any effects of bacterial treatment on female body size or any interaction between bacterial treatment and genotype although this interaction was only marginally not significant. Only genotype had a significant effect on body size in females suggesting that males and females respond differently to gut microbiota changes.

We found no consistent effect of microbiota treatment on our measure of male reproductive success. We would expect to see flies with different symbiotic bacterial

communities perform differently in male competitive fitness and have varied female choosiness as diet induced assortative mating is caused by diet caused gut bacterial changes in *D. melanogaster* (Sharon et al. 2010). This means that *D. melanogaster* are making mating choices based on gut bacteria profiles, which may be inducing CHC profile changes. Therefore, removing or changing the symbiotic bacterial communities should cause significant changes in males' competitive ability. We may not see these consistent effects of the gut microbiota on male competitive ability as the scale and nature of the effects depend on the host's genetic background.

We were unable to find a significant interaction between gut bacterial treatment and genotype on male reproductive success. This may be due to cytoplasmic incompatibility (CI) between the *Wolbachia* strains that infect the focal flies and the *ebony* testers. CI causes offspring to fail to develop when *Wolbachia* infected males mate with females not infected with that *Wolbachia* strain. This may be the reason focal males only sire around 30% of offspring across treatments despite females normally preferring *wild-type* males (Sharma et al. 2010) and *wild-type* males outcompeting *ebony* males (Archer et al. 2017). CI causing offspring to fail to develop would reduce the proportion of offspring sired by focal males and this would make any interaction difficult to detect. We find that male competitive fitness isoline (genotype) ranks vary across bacterial treatments. With the potential CI effects on offspring production and the difficulties that GLMs have in detecting genotype by environment interactions this suggests that there may still be an interaction between bacterial treatment and genotype on male competitive fitness. This is important, as most research looking into symbiotic bacterial fitness effects uses mixed populations, individual genotypes or do not address the genotype of their focal animals. This could potentially lead to studies missing important fitness consequences of symbiotic bacteria as they interact with the host's genotype. As we previously found that gut bacterial profiles evolve with varying sexual selection intensity (Chapter 4) it is likely that each isoline (genotype) has co-evolved with its symbiotic bacteria despite not evolving under sterile conditions.

The direction and magnitude of the fitness consequences of symbiotic bacteria removal or modification could depend on a number of factors. Firstly, if more of the

symbiotic bacteria were parasitic and costly in certain isolines then the removal or alteration of these communities would be more beneficial than in lines with lower proportions of parasitic symbionts. Again, the opposite is true where removing more mutualistic communities from certain isolines would be more harmful than removing the less beneficial bacterial communities in other lines. The extent to which each isoline's host selective process alters the composition of their microbiota may also explain why the fitness effects of bacterial treatments vary across genotypes. If some lines gut environment more strongly select for beneficial gut bacterial communities then removing or altering their gut microbiota may not have as detrimental effects on their fitness. This however, would not explain why some lines have improved fitness when their bacterial communities are removed.

We found no consistent effect of bacterial treatment across genotypes on either male or female size measures. This is surprising as the presence of *A. pomorum*, a common gut bacterial species across lab reared *Drosophila* species (Broderick & Lemaitre, 2012), is able to influence both the growth rate and body size or *D. melanogaster* via the insulin signalling pathway (Shin et al. 2011). We would therefore expect to see removal of the symbiotic bacterial communities reduce the body size of our flies. However, we did find a significant interaction between microbiota treatment and genotype on male body size. This suggests that the body size effects of microbiota treatment seem to depend on the male's genetic background. The reasons for this interaction are likely to be similar to the reasons listed above where the nature of male's gut microbial communities changes across genetic backgrounds. We did not find a significant interaction between bacterial treatment and genotype on female body size although this was only marginally not significant. This may mean that males and females react differently to changes in their microbiota. Trans-generational microbiota effects on body size have been found to impact daughters and not sons in *D. melanogaster* (Morimoto et al. 2017). This provides evidence that microbiota changes impact males and females differently.

We found no effect of gut microbiota treatment on female mating latency which is a component choosiness. Choosiness in females can vary depending on a wide range of factors including female condition (Judge et al. 2014) and male encounter rate

(Kokko & Rankin, 2006). Our body size measurements suggest female condition is not drastically changing across gut microbiota treatments, which may explain why we do not find any choosiness effects. The gut microbiota has been found to alter their host behaviour in mice (Neufeld et al. 2011). In *D. melanogaster* the removal of the gut microbiota has been shown to increase females walking speed and daily activity (Schretter et al. 2018). This increased activity may alter females mate encounter rate in wild flies and so alter levels of choosiness. In our treatments populations have not evolved with their gut microbiota treatments and this might explain why we did not find females changing their mating behaviour.

We would expect changing symbiotic bacterial communities would alter female reproductive output as bacterial supplementation of females diet has been shown to significantly improve ovary size, egg number and slightly improve fecundity (Qiao et al. 2019). We did not find any microbiota effects on female body size in our treatments and this may explain why we don't find any effect on female reproductive success. A potential explanation for our results not reflecting what other studies found is that the symbiotic bacterial communities are returning to close to what their original communities were even after their bacterial treatments. We know that host's gut environments favour specific bacterial communities and the specific composition of these bacterial communities (Rawls et al. 2006). In this example, when the gut bacterial communities are reciprocally crossed between mice and zebrafish the community structures return resemble their original bacterial community. The new transplanted species however remain which shows that both the available bacteria and the host selective process are important. It may be that in our treatments the symbiotic bacterial communities can return to a similar structure as before treatment.

One of the interesting observations from our results is that the fitness ranks of female 7-day offspring production significantly correlated across the washed (-) and re-infection (+) treatments. This may be evidence that when we simply remove the symbiotic bacterial communities hosts are able to return to closer to their original microbiota than if we inoculate them with a novel bacterial community. This also shows that comparing the fitness consequences of maintaining or removing the microbiota may not actually reveal the microbiota's importance. Potential work needs to use novel

bacterial communities to understand the importance of animal's microbiota. The same applies when interpreting the effects on individual bacteria on fitness. We don't fully understand how changing the abundance or presence of one species of bacteria will change the entire community structure. This makes ascribing fitness effects to individual bacteria species difficult and ill advised.

We were unable to test the changes in bacterial communities by our bacterial treatments, as we could not isolate sufficient bacterial DNA quantities for confirmatory PCR analysis. We did find that across our bacterial treatments development rates varied with the washed (-) bacterial treatment developing much slower than our other two treatments and the new (Δ) bacterial treatment developing slightly faster than our re-infected (+) treatment. We also found that there was a significant interaction between genotype and bacterial treatment on male body size and the interaction was only marginally not significant in females. This gives evidence that the bacterial treatments did create flies with different symbiotic bacterial communities. The method of bacterial removal and reinfection has also previously been used in Sharon et al. (2010), but we cannot be 100% certain these were effective.

The importance of an individual's microbiota on their fitness is becoming increasingly apparent. The microbiota have effects on the development rate/body size (Shin et al. 2011), metabolism (Wong et al. 2014), kin recognition (Lizé et al. 2013) and immune function (Pickard et al. 2017). The importance of symbiotic bacteria on sexual selection are difficult to ascribe as sexual selection acts on such a range of traits that the responses to these changes are likely to be complex. We used male competitive reproductive success as that measure encompasses many aspects of sexual selection. The males have to compete for access to females and their sperm potentially compete for access to eggs as well as having to court females. This is a useful measure, however, as it is also complex and encompasses many male traits it may make inferring the mechanisms behind fitness changes difficult. So, where changing the symbiotic bacteria of an individual may make them better at competing against rival males, it may also make them less attractive and so these traits may balance each other out. We could only measure the total fitness effects of the microbiota changes. Our measure of female choosiness also does not account for

potential changes in male investment based on male mate choice. Previously, gut bacteria has been found to be involved in male mating investment (Lize et al. 2014). If males are responding differently to females based on female bacterial treatment any changes in choosiness may be obscured.

Previous work hasn't looked at how microbial changes affect different genotypes differently. We found that the interaction between symbiotic bacteria and genetic background may be important. The causes of these interactions are not yet known, however, if it is caused by variation between the level of mutualism or parasitism in the bacterial communities of isolines then certain lines may have evolved "better" microbiota. The other option is that different genotypes vary in their response to microbiota changes. Understanding how an individual's genotype and microbiota interact is important to better understanding the importance of microbiota in general. Future research needs to account for how different genetic backgrounds will respond to microbiota changes and acknowledge any fitness effects may be specific to the genetic background they used. This could also have health and medical implications as microbiota treatments are being tested for multiple diseases (Vivarelli et al. 2019; Aggeletopoulou et al. 2019) and neurodevelopmental disorders (Kang et al. 2019). If the effects of altering an individual's microbiota depend on their genotype then these treatments may need to be tailored to each patient or trials need to include genetically diverse patients.

Conclusions

Individual's microbiota plays an important role on their phenotype and behaviour. We tested how microbiota changes interact with host genotype in their effects on sexual selection. We found a mixed response in the fitness traits we measured across symbiotic bacterial treatments. We found no overall fitness effects of the bacterial treatments on any of our fitness measures. We found a significant interaction between genotype and bacterial treatment on male body size. We also found that genotype fitness ranks changed across bacterial treatment for male competitive reproductive success, potentially indicating that again host genotype influences the importance of microbiota changes. We found bacterial treatment did not differentially alter female

choosiness across genotypes. We found no significant interaction between bacterial treatment and genotype on female offspring production. For female offspring production we found simply removing the symbiotic bacteria did not change genotype fitness ranks but inoculation with a novel bacterial profile did. This highlights the importance of research that changes individual's microbiota rather than testing the fitness consequences of removing symbiotic bacteria. Our results also provide the first example of a microbiota/genotype interaction on both body size. These interactions were present despite not finding effects of microbiota changes when averaged across genotypes. Future research should account for these genotype/microbiota interactions. We are only starting to understand the magnitude of the effects microbiota have on their hosts however understanding the interactions between hosts and their microbiota will be essential.

References

Aggeletopoulou, I. et al., 2019. The role of the gut microbiota in the treatment of inflammatory bowel diseases. *Microbial Pathogenesis*, 137.

Archer, C.R. et al., 2017. The *Drosophila simulans* Y chromosome interacts with the autosomes to influence male fitness. *Journal of Evolutionary Biology*, 30(10), pp.1821–1825.

Bellahcene, M. et al., 2013. Male mice that lack the G-protein-coupled receptor GPR41 have low energy expenditure and increased body fat content. *British Journal of Nutrition*, 109(10), pp.1755–1764.

Bjursell, M. et al., 2011. Improved glucose control and reduced body fat mass in free fatty acid receptor 2-deficient mice fed a high-fat diet. *American Journal of Physiology - Endocrinology and Metabolism*, 300(1).

Bohnhoff, M., Miller, C.P. & Martin, W.R., 1964. Resistance of the mouse's intestinal tract to experimental salmonella infection. *The Journal of Experimental Medicine*, 120(5), pp.817–828.

Bravo, J.A. et al., 2011. Ingestion of Lactobacillus strain regulates emotional behavior and central GABA receptor expression in a mouse via the vagus nerve. *Proceedings of the National Academy of Sciences of the United States of America*, 108(38), pp.16050–16055.

Bretman, A., Fricke, C. & Chapman, T., 2009. Plastic responses of male *Drosophila melanogaster* to the level of sperm competition increase male reproductive fitness. *Proceedings of the Royal Society B: Biological Sciences*, 276(1662), pp.1705–1711.

Broderick, N.A. & Lemaitre, B., 2012. Gut-associated microbes of *Drosophila melanogaster*. *Gut Microbes*, 3(4).

Champion De Crespigny, F.E. & Wedell, N., 2008. The impact of anaesthetic technique on survival and fertility in *Drosophila*. *Physiological Entomology*, 33(4), pp.310–315.

Cherrington, C.A. et al., 1991. Short-chain organic acids at pH 5.0 kill *Escherichia coli* and *Salmonella spp*. without causing membrane perturbation. *Journal of Applied Bacteriology*, 70(2), pp.161–165.

Christian, C.V., Barbara, C. & Stephanie, S., 2005. Bats, bacteria, and bat smell: Sex-specific diversity of microbes in a sexually selected scent organ. *Journal of Mammalogy*, 86(4), pp.745–749.

Cox, D.R., 1972. Regression models and life-tables. *Journal of the Royal Statistical Society: Series B (Methodological)*, 34(2), pp.187–202.

David, J.R. et al., 2005. Isofemale lines in *Drosophila*: An empirical approach to

quantitative trait analysis in natural populations. *Heredity*, 94(1), pp.3–12.

Dimijian, G.G., 2000. Evolving together: The biology of symbiosis, Part 1. *Baylor University Medical Center Proceedings*, 13(3), p.217a–226.

Fox, J. & Weisberg, S., 2018. Package “car”, An R companion to applied regression. *R Documentation, Sage*.

Freter, R., 1955. The fatal enteric cholera infection in the guinea pig, achieved by inhibition of normal enteric flora. *Journal of Infectious Diseases*, 97(1), pp.57–65.

Hammami, R. et al., 2013. Anti-infective properties of bacteriocins: An update. *Cellular and Molecular Life Sciences*, 70(16), pp.2947–2967.

Heijtz, R.D. et al., 2011. Normal gut microbiota modulates brain development and behavior. *Proceedings of the National Academy of Sciences of the United States of America*, 108(7), pp.3047–3052.

Howard, R.W. & Blomquist, G.J., 2005. Ecological, behavioral, and biochemical aspects of insect hydrocarbons. *Annual Review of Entomology*, 50(1), pp.371–393.

Ingleby, F.C., Hunt, J. & Hosken, D.J., 2013. Genotype-by-environment interactions for female mate choice of male cuticular hydrocarbons in *Drosophila simulans*. *PLoS ONE*, 8(6).

Judge, K.A., Ting, J.J. & Gwynne, D.T., 2014. Condition dependence of female choosiness in a field cricket. *Journal of Evolutionary Biology*, 27(11), pp.2529–2540.

Kang, D.W. et al., 2019. Long-term benefit of microbiota transfer therapy on autism symptoms and gut microbiota. *Scientific Reports*, 9(1).

Kaplan, E.L. & Meier, P., 1958. Nonparametric estimation from incomplete observations. *Journal of the American Statistical Association*, 53(282), pp.457–481.

Kokko, H. & Rankin, D.J., 2006. Lonely hearts or sex in the city? Density-dependent effects in mating systems. In *Philosophical Transactions of the Royal Society B: Biological Sciences*. pp. 319–334.

Lam, Y.Y. et al., 2012. Increased gut permeability and microbiota change associate with mesenteric fat inflammation and metabolic dysfunction in diet-induced obese mice. *PLoS ONE*, 7(3).

Lasbleiz, C., Ferveur, J.F. & Everaerts, C., 2006. Courtship behaviour of *Drosophila melanogaster* revisited. *Animal Behaviour*, 72(5), pp.1001–1012.

Leclaire, S. et al., 2017. Social odours covary with bacterial community in the anal secretions of wild meerkats. *Scientific Reports*, 7(1).

Leclaire, S., Nielsen, J.F. & Drea, C.M., 2014. Bacterial communities in meerkat anal

scent secretions vary with host sex, age, and group membership. *Behavioral Ecology*, 25(4), pp.996–1004.

Lewontin, R.C., 2006. The analysis of variance and the analysis of causes. *International Journal of Epidemiology*, 35(3), pp.520–525.

Lize, A., McKay, R. & Lewis, Z., 2014. Kin recognition in *Drosophila*: the importance of ecology and gut microbiota. *ISME J*, 8(2), pp.469–477.

Lizé, A., McKay, R. & Lewis, Z., 2013. Gut microbiota and kin recognition. *Trends in Ecology and Evolution*, 28(6), pp.325–326.

Morimoto, J., Simpson, S.J. & Ponton, F., 2017. Direct and trans-generational effects of male and female gut microbiota in *Drosophila melanogaster*. *Biology Letters*, 13(7).

Narraway, C. et al., 2010. Genotype-by-environment interactions for female preference. *Journal of Evolutionary Biology*, 23(12), pp.2550–2557.

Neufeld, K.M. et al., 2011. Reduced anxiety-like behavior and central neurochemical change in germ-free mice. *Neurogastroenterology & Motility*, 23(3), pp.255--e119.

Nguyen, T.T.X. & Moehring, A.J., 2015. Accurate alternative measurements for female lifetime reproductive success in *Drosophila melanogaster*. *PLoS ONE*, 10(6).

Pickard, J.M. et al., 2017. Gut microbiota: Role in pathogen colonization, immune responses, and inflammatory disease. *Immunological Reviews*, 279(1), pp.70–89.

Price, T.A.R. et al., 2014. Does polyandry control population sex ratio via regulation of a selfish gene? *Proceedings of the Royal Society B: Biological Sciences*, 281(1783).

Pultz, N.J. et al., 2005. Mechanisms by which anaerobic microbiota inhibit the establishment in mice of intestinal colonization by Vancomycin-resistant *Enterococcus*. *The Journal of Infectious Diseases*, 191(6), pp.949–956.

Qiao, H. et al., 2019. Gut microbiota affects development and olfactory behavior in *Drosophila melanogaster*. *Journal of Experimental Biology*, 222(5).

Rawls, J.F. et al., 2006. Reciprocal gut gicrobiota transplants from zebrafish and mice to germ-free recipients reveal host habitat selection. *Cell*, 127(2), pp.423–433.

Ruiz-De-Castañeda, R. et al., 2012. Bacterial degradability of an intrafeather unmelanized ornament: A role for feather-degrading bacteria in sexual selection? *Biological Journal of the Linnean Society*, 105(2), pp.409–419.

Ruiz-Rodriguez, M. et al., 2009. Symbiotic bacteria living in the hoopoe's uropygial gland prevent feather degradation. *Journal of Experimental Biology*, 212(22), pp.3621–3626.

Ruiz-Rodríguez, M. et al., 2015. Bacteria and the evolution of honest signals. The case of ornamental throat feathers in spotless starlings. *Functional Ecology*, 29(5), pp.701–709.

Russell, A.B. et al., 2014. A type VI secretion-related pathway in bacteroidetes mediates interbacterial antagonism. *Cell Host and Microbe*, 16(2), pp.227–236.

Savage, D.C., 1977. Microbial ecology of the gastrointestinal tract. *Annual Review of Microbiology*, 31(1), pp.107–133.

Schretter, C.E. et al., 2018. A gut microbial factor modulates locomotor behaviour in *Drosophila*. *Nature*, 563(7731), pp.402–406.

Sharma, M.D., Hunt, J. & Hosken, D.J., 2012. Antagonistic responses to natural and sexual selection and the sex-specific evolution of cuticular hydrocarbons in *Drosophila simulans*. *Evolution*, 66(3), pp.665–677.

Sharma, M.D., Tregenza, T. & Hosken, D.J., 2010. Female mate preferences in *Drosophila simulans*: Evolution and costs. *Journal of Evolutionary Biology*, 23(8), pp.1672–1679.

Sharon, G. et al., 2010. Commensal bacteria play a role in mating preference of *Drosophila melanogaster*. *Proceedings of the National Academy of Sciences*, 107(46), pp.20051–20056.

Shin, S.C. et al., 2011. Drosophila microbiome modulates host developmental and metabolic homeostasis via insulin Signaling. *Science*, 334(6056), p.670 LP-674.

Sin, Y.W. et al., 2012. Molecular characterization of the microbial communities in the subcaudal gland secretion of the European badger (*Meles meles*). *FEMS Microbiology Ecology*, 81(3), pp.648–659.

Spieth, H.T., 1974. Courtship behavior in *Drosophila*. *Annual Review of Entomology*, 19(1), pp.385–405.

Spor, A., Koren, O. & Ley, R., 2011. Unravelling the effects of the environment and host genotype on the gut microbiome. *Nature Reviews Microbiology*, 9(4), pp.279–290.

Storelli, G. et al., 2011. *Lactobacillus plantarum* promotes *Drosophila* systemic growth by modulating hormonal signals through TOR-dependent nutrient sensing. *Cell Metabolism*, 14(3), pp.403–414.

Taylor, M.L., Wedell, N. & Hosken, D.J., 2008. Sexual selection and female fitness in *Drosophila simulans*. *Behavioral Ecology and Sociobiology*, 62(5), pp.721–728.

Theis, K.R. et al., 2013. Symbiotic bacteria appear to mediate hyena social odors. *Proceedings of the National Academy of Sciences of the United States of America*, 110(49), pp.19832–19837.

Tremaroli, V. & Bäckhed, F., 2012. Functional interactions between the gut microbiota and host metabolism. *Nature*, 489(7415), pp.242–249.

Turnbaugh, P.J. et al., 2009. A core gut microbiome in obese and lean twins. *Nature*, 457(7228), pp.480–484.

Turnbaugh, P.J. et al., 2006. An obesity-associated gut microbiome with increased capacity for energy harvest. *Nature*, 444(7122), pp.1027–1031.

Vivarelli, S. et al., 2019. Gut microbiota and cancer: From pathogenesis to therapy. *Cancers*, 11(1).

Voigt, C.C., 2002. Individual variation in perfume blending in male greater sac-winged bats. *Animal Behaviour*, 63(5), pp.907–913.

Wong, A.C.N., Dobson, A.J. & Douglas, A.E., 2014. Gut microbiota dictates the metabolic response of *Drosophila* to diet. *Journal of Experimental Biology*, 217(11), pp.1894–1901.

Woodhams, D.C. et al., 2007. Symbiotic bacteria contribute to innate immune defenses of the threatened mountain yellow-legged frog, *Rana muscosa*. *Biological Conservation*, 138(3–4), pp.390–398.

Yang, X. et al., 2009. More than 9,000,000 unique genes in human gut bacterial community: Estimating gene numbers inside a human body. *PLoS ONE*, 4(6).

Chapter 6

How *Wolbachia* and microbiota impact female latency to mate and body size across genotypes.

Abstract

Female mate choice is an essential part of sexual selection. Female preference can lead to selection on males to evolve exaggerated sexual traits beyond their naturally selected optima. The strength of this selection will depend on the level of female choosiness. One key aspect of female choosiness is female's latency to mate, as choosiness relates to the time individuals spend assessing potential mates. Female latency to mate can vary dependent on their environment, population dynamics or own condition. Microbial symbionts can alter the levels of female's choosiness in a number of ways. Parasites are able to disrupt female's ability to choose between partners or reduce the resources they are able to allocate to mate choice. *Wolbachia* are a wide spread endosymbiont of insects and can have many reproductive effects on their host. Many studies into these reproductive effects compare cured individuals to *Wolbachia* infected individuals but do not account for the effects curing has on other symbiotic bacteria. Here we assess the effects of *Wolbachia* infection and other non-cytoplasmic microbial symbionts on female latency to mate in *Drosophila simulans*. We used female latency to mate as it is an important aspect of female choosiness and will have mate choice implications. We found *Wolbachia* infection status and host genotype interactions on female latency to mate, with no effects of non-cytoplasmic microbiota treatment. We found these effects did not appear to be caused by changes in female condition. *Wolbachia* infection appears to reduce the negative effects of microbiota removal on female condition. This appears to show the importance of the interactions between host genotype and symbionts when assessing the fitness effects of the microbiota.

Introduction

Darwin (1871) first suggested sexual selection as an explanation for the wide array of traits that were apparently not favoured by natural selection. Sexual selection can be thought of as variation in reproductive success as opposed to natural selection affecting all other fitness components (Hosken & House, 2011), but is should be noted that sexually selected traits are almost certainly subjected to natural selection. Mate choice is a major mechanism of sexual selection. Mate choice can occur in both males and females, however females are usually the choosier sex. Female choice occurs when females either actively or passively choose to mate with a certain sub-set of males. Female choice can lead to males evolving elaborate displays or signals that make them more attractive to females (Andersson, 1994). Cryptic female choice occurs after mating, where the females' reproductive tract preferentially favour the gametes of certain males. Cryptic female choice can cause reproductive tracts to evolve that select for the gametes of specific males (Birkhead & Pizzari, 2002).

It has been suggested that males originally evolve these conspicuous sexual signals to exploit pre-existing female sensory bias (Ryan, 1998). However, for female preference for these traits to be maintained, females must gain fitness benefits from their choices. The benefits of mate choice for elaborate sexual signals are not always clear, as these traits in their sons are often costly to produce or maintain. In some instances females gain direct benefits (Møller & Jennions, 2001). Females that mate with preferred males may benefit from increased fertility and fecundity through nuptial gifts, improved breeding territory, parasite avoidance or increased paternal care (Andersson 1994). The magnitude of the direct benefits gained by females during mate choice vary depending on the kind of direct benefit measured (Møller & Jennions 2001). Females may also gain indirect genetic benefits for their offspring via mate choice. These genetic benefits would manifest as either increased offspring longevity or production of more attractive sons (Fisher 1930; Hamilton & Zuk, 1982; Heywood, 1989).

Mate choice can potentially provide indirect and direct benefits in terms of parasite avoidance. Hamilton and Zuk (1982) first suggested the importance of parasites in the

evolution female choice. As parasites reduce the fitness of their host, females would gain indirect fitness benefits for their offspring when choosing to mate with males that carry resistance genes. Hamilton and Zuk (1982) proposed that conspicuous sexual traits would be signalling parasite resistance of males and so female's offspring would inherit these 'good genes'. Females will also gain direct benefits by mating with males that are not infected with parasites by decreasing the risk of direct transmission. Research has focused on the link between parasites and sexual signals. Sexually selected signals in males have been shown to negatively correlate with parasite load and positively correlate with parasite resistance in birds, fish and reptiles (Møller 1990; Clayton 1991) (Milinski & Bakker 1990; Folstad et al. 1994; Molnár et al. 2013). Female *Drosophila melanogaster* prefer to mate with males that have been selected for higher parasite resistance (Rolff & Kraaijeveld, 2003). However, this study did not expose experimental flies to parasites and so the underlying mechanism behind the preference for parasite resistant males is unclear.

For exaggerated sexual signals to evolve, females need to show a preference for the trait, however, the strength of selection on the trait will depend on how choosey the females are (Jennions & Petrie, 1997). Female mate choice speed or latency to mating is an aspect of female choosiness. Females that mate faster are potentially mating with a suboptimal male as their opportunity of encounter multiple males is reduced. Faster mating decisions have been shown to increase the instances of mating with lower quality partners in sand gobies (*Pomatoschistus minutus*) (Pauli & Lindstrom, 2021). Parasites also play a role in female choosiness with parasitized females being less choosey. This has been shown in upland bullies (*Gobiomorphus breviceps*) where heavily parasitized females made less mate inspections before mating (Poulin, 1994). There are a number of ways that parasites can alter female choosiness. If females are parasitized they will not gain direct benefits from the avoidance of mating with infected males. This means they should be less choosey in avoiding parasitized males. The costs of parasitism may limit the energy and time individuals can expend on mate assessment (Poulin & Vickery, 1996). This means that a female's condition could alter their level of choosiness. This is found in crickets (*Gryllus pennsylvanicus*) where low condition females are less choosey than higher condition individuals (Judge et al. 2014). Parasites may also alter their host's ability to distinguish between mates. One

example of this is where parasitism by eye flukes reduces the vision of female sticklebacks (*Gasterosteus aculeatus*) (Owen et al. 1993), which make mate choice decisions based on male colouration difficult (Milinski & Bakker, 1990).

Parasites may also alter the density or sex ratio of a population, which again could influence female choosiness. At higher population densities, females should be more choosey, especially if mating is costly (Kokko & Rankin, 2006). This prediction was supported in dung flies (*Sepsis cynipsea*) where at higher population densities females were more resistant to matings (Martin & Hosken, 2003) although mating resistance does not always equate to choosiness. One example of an endosymbiotic parasite is *Wolbachia pipientis,* an intracellular bacteria of that has been found in >20% of arthropods (Werren & Windsor 2000), which can have wide ranging fitness effects on their host. *Wolbachia* can cause male killing (Hurst & Jiggins, 2000) or feminisation (Rigaud, 1997), which can reduce population densities and skew the sex ratio towards females. As *Wolbachia* are maternally transmitted they can directly increase their transmission rate by creating a more female biased population. *Wolbachia* can also cause cytoplasmic incompatibility between infected males and uninfected females that will again reduce population densities (Hoffmann & Turelli, 1997). Females that are not infected with *Wolbachia* however, do not preferentially mate with uninfected males, despite potential fitness costs if mating with infected males in both *Drosophila simulans* and flour beetles (*Tribolium confusum*) (Champion De Crespigny & Wedell, 2007; Wade & Chang, 1995; Hoffmann et al. 1990). *Wolbachia* can, however, have further fitness effects on their host's reproductive traits and behaviour. Wild female *Drosophila simulans* infected with *Wolbachia* originally had a 10% lower fecundity (Hoffmann et al. 1990) now after 20 years of coevolution infected females are now 15% more fecund (Weeks et al. 2007). In the closely related sister species *Drosophila melanogaster* the fecundity effect of *Wolbachia* infection varied across strains with some infected females having increased fecundity (Fry et al. 2004). We have previously found that *Wolbachia* infection alters the attractiveness of male *D. simulans* differently across genetic backgrounds (Chapter 2). Ingleby et al. (2013) found genetic correlations between female preference and male attractiveness (measured by cuticular hydrocarbon profiles) in *D. simulans*. However, the Ingleby et al. (2013) study did not look for correlation in female choosiness and male attractiveness. In *D.*

melanogaster there is a positive genetic correlation between male attractiveness and female choosiness (Ratterman et al. 2014), although choosiness was measured as the standard deviation in female mating latency across male genotypes. This means that females that vary more in their latency to mate across males were deemed as more choosey. If there is genetic correlation between son's attractiveness and daughter's choosiness then we may expect to find *Wolbachia* changing the levels of choosiness in *D. simulans* dependent on genetic background.

Many of the previous studies of *Wolbachia*-induced reproductive effects compare the responses of cured and infected individuals. This curing process involves antibiotic treatment over multiple generations. We now know eukaryotes are host to a wide range of microbial symbionts (Archie & Theis 2011). These communities are described as that host's microbiota, and when antibiotics are used to cure individuals of their *Wolbachia* infection, they also remove other bacterial components the host's microbiota, and since the microbiota can have wide ranging host fitness effects, we are also impacting hosts in ways we do not fully understand.

The microbiota that inhabit the gastrointestinal tract of organisms have been shown to affect immune (Macpherson & Harris, 2004) and metabolic function (Turnbaugh et al. 2006), as well as affecting hosts behaviour (Cryan & Dinan, 2012). Germ free mice (lacking their microbiota) have immunological defects in their intestines (Macpherson & Harris, 2004) and have impaired immune responses to pathogens (Hentges, 2018). The amount of energy harvest from food by lean mice increased when their gut microbiota was substituted with that from obese individuals (Turnbaugh et al. 2006). The gut-brain axis provides bidirectional communication that uses neural, hormonal and immunological pathways (Mayer, 2011). Germ free mice display lower levels of anxiety like behaviours (Neufeld et al. 2011). When germ free mice from different strains are given cognitive tests they perform in a similar way to when they are colonised by their original microbiota, however, when they are colonised by the microbiota of the alternate mouse strain their behaviour profile is more similar to that of their donors (Bercik et al. 2011). The mechanisms by which the microbiota can alter their host's behaviour are not fully understood, however it likely involves various mechanisms of communication including immune activation, as well as the production

of metabolites and neurometabolites (Cryan & Dinan 2012). Microbiota-induced cognition and behavioural changes may have wide ranging fitness consequences. Cognitive changes may potentially alter females mating decision or ability to optimally choose a mate. Microbiota induced changes could also affect the mating investment, courtship and mating behaviour of males.

The microbiota can also have an impact on their host's sexual signals. Feather degrading bacteria (FDB) maintain the honesty of sexual signals of some birds by breaking down white feathers used as sexual signals faster than darker feathers (Ruiz-Rodríguez et al. 2015). This makes larger more conspicuous white areas more costly to maintain meaning only higher quality males can afford to express them. Symbiotic bacteria in the preen (uropygial) glands of birds help to protect against these FDB (Ruiz-Rodriguez et al. 2009). In *D. melanogaster* the microbiota are responsible for diet induced mating preferences, where individuals preferentially mate within diet treatment unless their microbiota were removed (Sharon et al. 2010). The presence of an intact microbiota has also been found to disrupt males' ability to recognise kin in *D. melanogaster* (Heys et al. 2018). Males invest more sperm when mating with unrelated females compared to sisters, but only if the females have their microbiota removed or the females are raised on a different diet. Other aspects of the microbiota may cause many of the phenotypic and behavioural effects previously attributed to *Wolbachia*.

We asked if there were *Wolbachia* or other non-cytoplasmic microbiota effects on female's latency to mate. We have used female latency to mate as a measure of female choosiness. As female choosiness relates to the time spent assessing a potential mate, measuring latency to mate will give us an estimate of the time females are spending making mating decisions (Jennions & Petrie, 1997). Female latency to mate will not be only a measure of female choosiness and will also reflect the female's propensity to mate and quality of their potential mate. Despite females not having an active choice between males, using stock ebony tester males should minimise the variation in attractiveness across potential mates. This should maximise the portion of female latency to mate which reflects the individual female's choosiness. To test this we used the model species *D. simulans* as we have previously found *Wolbachia* alters

male attractiveness dependent on their host's genetic background (Chapter 2). As male attractiveness has been shown to positively genetically correlate with female choosiness (Ratterman et al. 2014) we may also find that *Wolbachia* alters female choosiness differently across genotypes. We used iso-genetic strains (isofemale lines, hereafter referred to as genotypes or isolines), as they are powerful way to assess naturally occurring genetic variation in a population and allow repeatable measures of a range of fixed genotypes (David et al. 2005). These isofemale lines were naturally infected with the same strain of *Wolbachia* so we could test if any choosiness effects were dependent on the female's genetic background in the absence or presence of *Wolbachia*. To be able to determine if changes in female's latency to mate were caused by *Wolbachia* curing and not by changes in the hosts' non-cytoplasmic microbiota, we used a microbiota removal and reinfection protocol. We also wanted to test if any changes in female's latency to mate were due to changes in condition. We used female body size measurements to test if our experimental treatments had an effect on their condition. In *Drosophila melanogaster* wing size was smaller in flies raised on a nutritionally limited diet or at stressful temperature suggesting wing size will provide a measure of body condition (De Moed et al. 1997).

Materials and methods

Drosophila simulans isolines used in this experiment were originally collected from Greece (Ingleby et al. 2013) and were maintained for > 45 generations with full-sib matings (n= 25 brothers and 25 sisters/isoline). Thus, each isoline could be considered as a distinct genotypes (David et al. 2005). All stocks were reared on a standard cornmeal-based Jazzmix diet (hereafter Jazzmix) (supplied by Applied Scientific, UK) at 25°C on a 12:12 hour light:dark cycle (unless stated otherwise).

Wolbachia and microbiota manipulation

We randomly selected 6 naturally *Wolbachia* infected isofemales (isolines) from the lines established from wild caught females (Chapter 2) and established sub-lines that were exposed to one of 4 experimental treatments. All isolines were naturally infected with the same strain of *Wolbachia.* This means any changes in the effects of infection

on female choosiness across isolines will be due to changes in host genotype and not variation in the strain of *Wolbachia.* The isolines and treatment sub-lines are the same as used in Chapter 2 for the third assay. In all treatments, the experimental flies had their non-cytoplasmic microbiota removed by dechorionating their eggs and then were either re-infected with their original bacteria from the matching isoline (re-infected) or inoculated with sterile PBS as a control (removed) to provide the microbiota +/- treatments. Flies were *1*) treated with antibiotics and had their gut bacteria removed (–W-G), *2*) treated with antibiotics and re-infected (–W+G), *3*) untreated but had their gut bacteria removed (+W-G) or *4*) untreated and re-infected (+W+G) – where W = Wolbachia (+ or -) and G is gut bacteria, removed (-) or removed and then re-infected (+). *Wolbachia* curing involved rearing flies on Drosophila Quick Mix Media Blue (Blades Biological) treated with 0.03% tetracycline hydrochloride for two generations. The flies were then allowed to recover on the blue media without tetracycline for 3 generations before being moved back onto Jazzmix for > 5 generations before being used in experiments to avoid and diet effects. After this process each isolines infection status was confirmed by PCR using the specific *Wolbachia* surface protein (wsp) primers *wsp* 81F and *wsp* 691R (Zhou et al. 1998; Duffy et al. 2019).

For our non-cytoplasmic microbiota manipulation, we used the same protocol as Sharon et al. (2010). The experimental flies from both sub-treatments of each isoline (W+/W-) had their gut bacteria removed by dechorionating their eggs using a 50% bleach solution. Then, 20 eggs were distributed into each small vial containing 7ml Jazzmix food. Half of these vials were then inoculated with 100ul of the gut bacteria collected from the matching isoline as the + microbiota treatment. The other half were inoculated with 100ul sterile PBS as the – microbiota treatment. Gut bacteria for inoculation was collected as in chapter 2*.* Briefly 25 male and 25 female flies from each isoline were allowed to live for 6 days on Jazzmix food. After this, the flies were removed and 10ml sterile PBS added to each vial and then vortexed for 10 seconds, before 5 ml of this solution was pipetted out into an Eppendorf for each isoline. These vials were then spun at a low speed (100 × *g*) for 10 minutes so the sediment settled and the supernatant was moved into a fresh Eppendorf and spun at high speed (16,000 × g) for 2 minutes to pellet the bacteria. The supernatant and was then discarded and the pellet washed in sterile PBS. This washed bacteria was pelleted

again and the pellet re-suspended in a total of 500ul PBS. This was stored for < 6 hours between 2^{o}c and 8^{o}c, before it was added to washed eggs from the matching isolines.

Female latency to mate assay post **Wolbachia** *and microbiota manipulation*

To test the effects of *Wolbachia* and gut microbiota on female latency to mate we compared the 4 treatments, –W-M, –W+M, +W-M and +W+M across the 6 randomly selected isolines. Choosiness relates to the time and effort females spend assessing a potential mate and so measuring latency to mate will give us an estimate of the time females are spending making mating decisions (Jennions & Petrie, 1997). In *D. simulans* there is no forced copulation with sexually mature females (Spieth, 1974). Female latency to mate will depend on both female choosiness and the male's attractiveness. By standardising the tester males presented to females we were able to use latency to mate as a measure of female choosiness as is common in *Drosophila* studies (Spieth, 1974; Narraway et al. 2010; Ingleby et al. 2013). To collect the focal females of each treatment the eggs from the gut treatment process above were allowed to develop and females collected as virgins every 6 hours. These virgin males were stored in vials of 5-10 individuals and allowed to mature for 3-6 days. Tester flies came from a population of *ebony* (a recessive, phenotypic body-colour mutant) flies, the stock populations were established (stocks from Tucson stock centre) and maintained on a standard diet of Jazzmix in a 30x30x30cm population cage of ~800 flies with overlapping generations. All our tester males were naturally *Wolbachia* infected. *Ebony* males are more aggressive (Søndergaard, 1986) and often suffer courtship defects. This means females preferentially mate with *wild-type* males in competitive environments (Sharma et al. 2010). We used *ebony* tester males as a standard control male so that our latency to mate measures reflected female choosiness and not variation in male attractiveness. To collect tester males, 25 male and 25 female *ebony* flies were allowed to egg lay for 2 days in each large vial containing 30ml Jazzmix. The eggs were left to develop until female virgins were collected as virgins every 6 hours and stored in groups of 10. These flies were then aged for 3-5 days. Focal females were stored individually for >12 hours prior to the experiment starting. Individual tester males were introduced to the focal females one

hour after the incubator lights came on. Observations were conducted blind to experimental treatments to record the time from introduction until mating (mating latency) and mating duration. The focal flies were then removed and stored at -20 until a subset of 5 females for each of the 4 treatments were tested for body size with wing measurements. Wings were imaged and then measured using ImageJ (Schneider et al. 2012).

Analyses

All analyses were performed in RStudio version 1.1.383 (RStudio Team 2016) using R version 3.6.2 (R Core Team, 2019). To assess whether changes in female choosiness were likely to be due to symbiotic microbiota or *Wolbachia,* we used Kaplan-Meier curves to visualize the data and analysed differences in choosiness using a Cox's proportional hazard model (Kaplan & Meier 1958, Cox 1972) with isoline, microbiota treatment, and *Wolbachia* infection status as co-variates and time to mating being the hazard. This approach allows us to tests which factors impact the time to the event and also include individuals that did not mate during the observation period. We then tested the significance and interactions of all the risk factors using the ANOVA function in the *car* package (Fox & Weisberg 2018).

To analyse the effects of microbiota and *Wolbachia* infection status on female body size we used general linear models (GLMs) with a Gaussian error structure. Full models were fit with microbiota treatment, *Wolbachia* infection status, isoline and the interactions between them as explanatory variables. To test the effects of microbiota removal and *Wolbachia* infection status on female body size across genotypes, we used a GLM fit with a Gaussian error structure. We used wing size measures as a proxy of female body size (Gilchrist & Partridge, 1999). Full models were fit with isoline (genotype), *Wolbachia* infection status, microbiota treatment and the interactions between them as explanatory variables. We then tested for significance using the ANOVA function in the *car* package (Fox & Weisberg 2018).

Results

We used female's latency to mate with a tester male as a measure of females choosiness in order to test how an individual's microbiota and *Wolbachia* infection status affect female choosiness. We found that genotype (isoline) had a significant effect on female latency to mate (X^2=19.95, df=5, p = 0.001). Neither *Wolbachia* infection status (X^2=0.85, df=1, p = 0.36) nor microbiota treatment (X^2=1.91, df=1, p = 0.17) had a significant effect on female latency to mate consistently across all genotypes (isolines). However, we found a significant interaction between female genotype and *Wolbachia* infection status on female latency to mate (X^2=17.24, df=5, p = 0.004) suggesting *Wolbachia* caused choosiness effects depend on the females' genotype (Figure 1). We did not find a significant interaction between female genotype and microbiota treatment (X^2= 3.334, df=5, p = 0.649) suggesting that removal of females' microbiota did not significantly impact on their choosiness (Figure 2).

We also tested how changes in microbiota and *Wolbachia* infection status impact female size across different genotypes. Changing the female's microbiota had a significant effect on body size (Table 1). We found that removal of a female's microbiota significantly reduced their body size (Figure 3). There was no interaction between microbiota treatment and genotype on female body size (Table 1). We found no effect of *Wolbachia* infection status on female body size (Table 1) and the interaction between *Wolbachia* infection and genotype was marginally not significant (Table 1). Genotype had a significant effect on female body size however did not interact with any other variable significantly (Table 1). We did find that *Wolbachia* infection status and microbiota treatment interact in their effect on female body size (Figure 4). We found that the effect of microbiota on female body size was much greater when females were cured of *Wolbachia* (-).

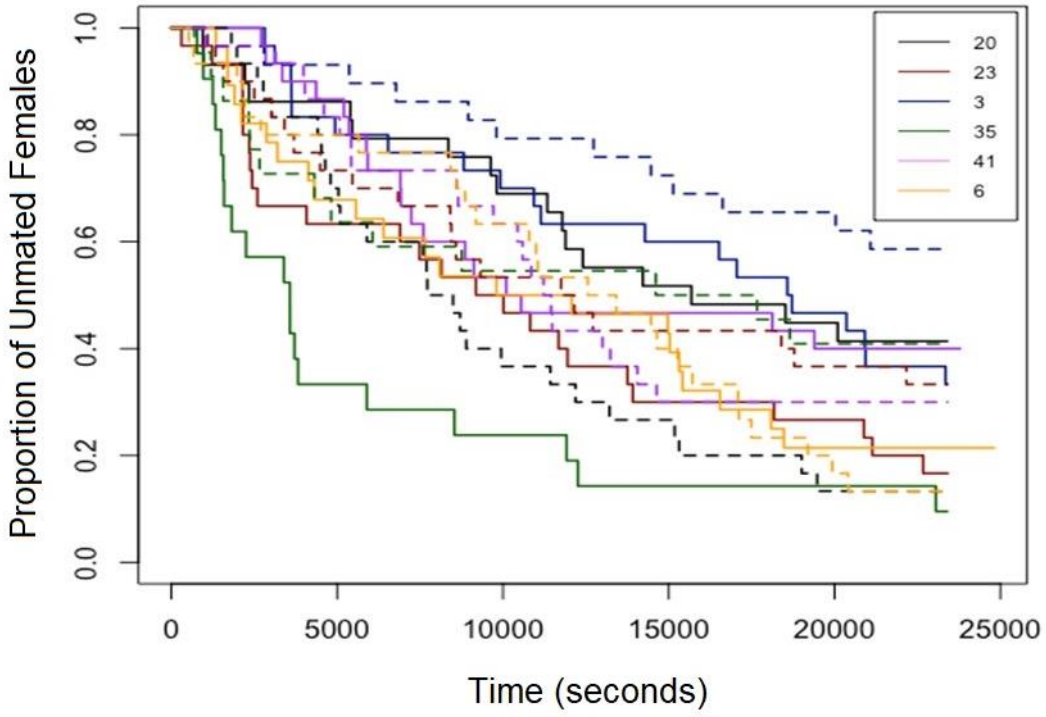

Figure 1. Kaplan-Meier curve showing proportion of unmated females over time separated by genotypes (n=6) with the *Wolbachia* cured treatment (-) plotted with a dashed line and infected (+) with a solid line. The less steep the gradient of a curve the choosier the females (the slower the females of that treatment mated). Female latency to mating effects of *Wolbachia* curing vary across genotypes (isolines) with some lines becoming more choosey after curing and others less so.

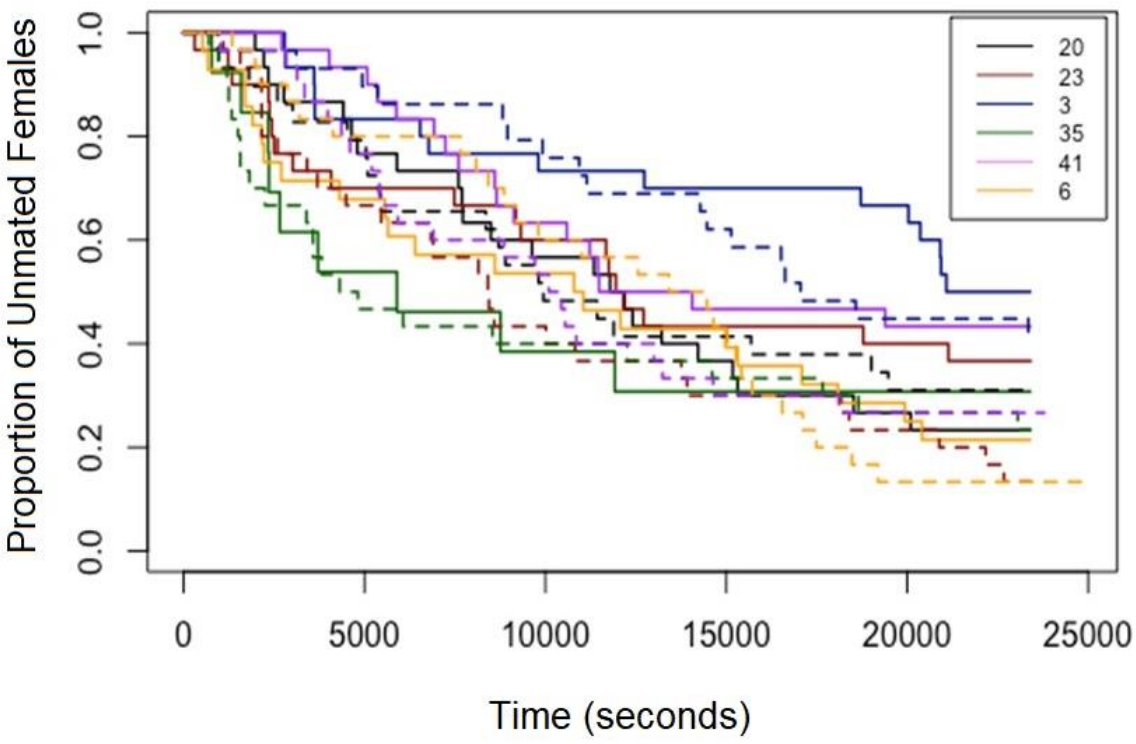

Figure 2. Kaplan-Meier curve showing proportion of unmated females over time separated by genotypes (n=6) with the microbiota removed treatment (-) plotted with a dashed line and reinfected (+) with a solid line. The less steep the gradient of a curve the choosier the females (the faster the females of that treatment mated). Microbiota changes do not affect female choosiness independent of genotype (isoline).

Table 1. Output from the GLM testing the effects of *Wolbachia* infection status, microbiota treatment, Isoline and their interactions on female body size.

	Sum Sq	DF	F value	P
Microbiota	0.007724	1	5.39	0.02*
Wolbachia	0.001053	1	0.74	0.39
Isoline	0.053367	5	7.45	<0.0001***
Microbiota:*Wolbachia*	0.005761	1	4.02	0.048*
Microbiota:Isoline	0.007929	5	1.11	0.36
Wolbachia :Isoline	0.016199	5	2.26	0.055.
Microbiota:*Wolbachia* :Isoline	0.008867	5	1.24	0.3
Residuals	0.12891	90		

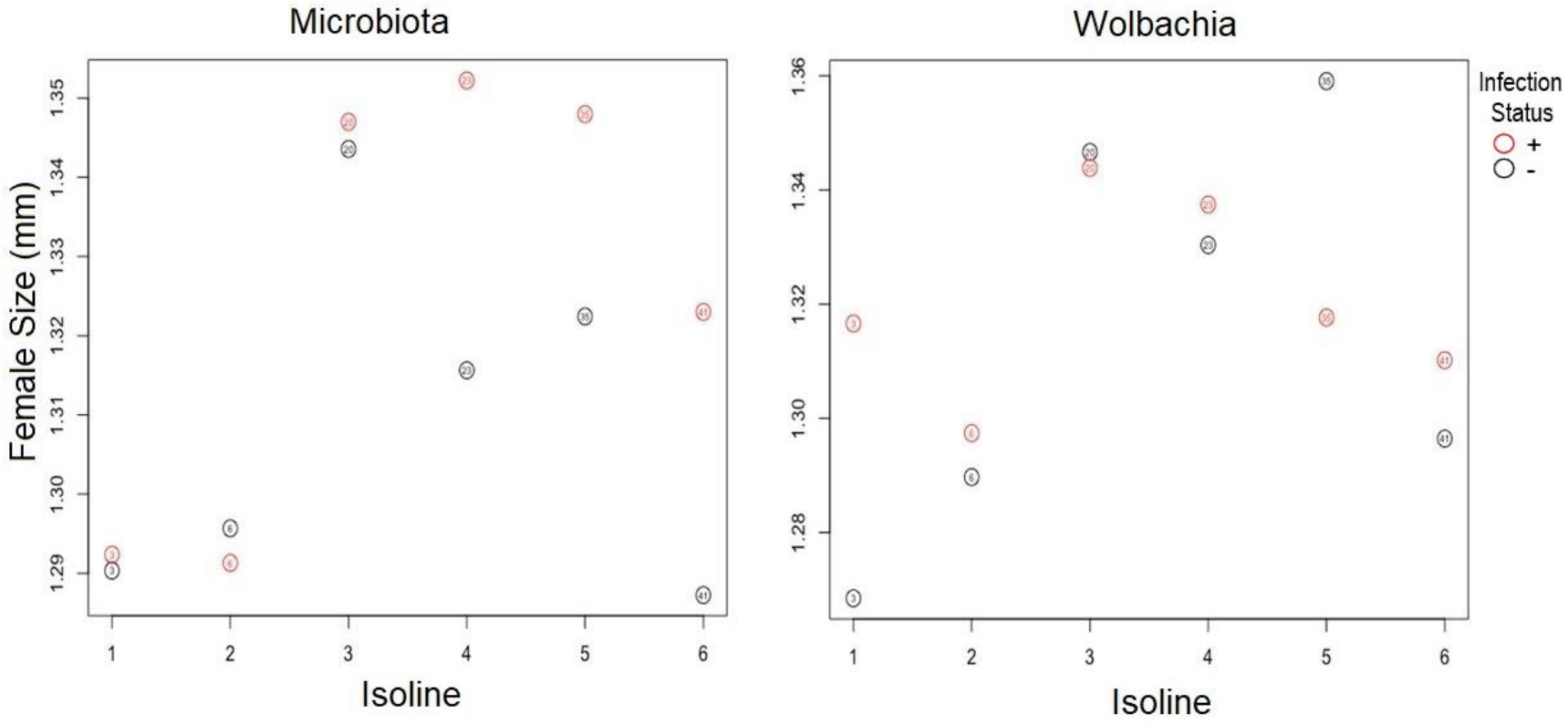

Figure 3. Female size (mm) plotted by genotype (isoline). The left plot shows microbiota + treatment plotted in red and microbiota – plotted in black. The plot shows microbiota – (removal of female microbiota) treatment reducing female body size especially across three of the isolines. The right plot shows *Wolbachia* infected females plotted in red and *Wolbachia* cured females plotted in black. The plot shows that *Wolbachia* infection status does not have a consistent effect on female body size. The effect seems to depend on the genetic background of the female however this interaction is marginally not significant.

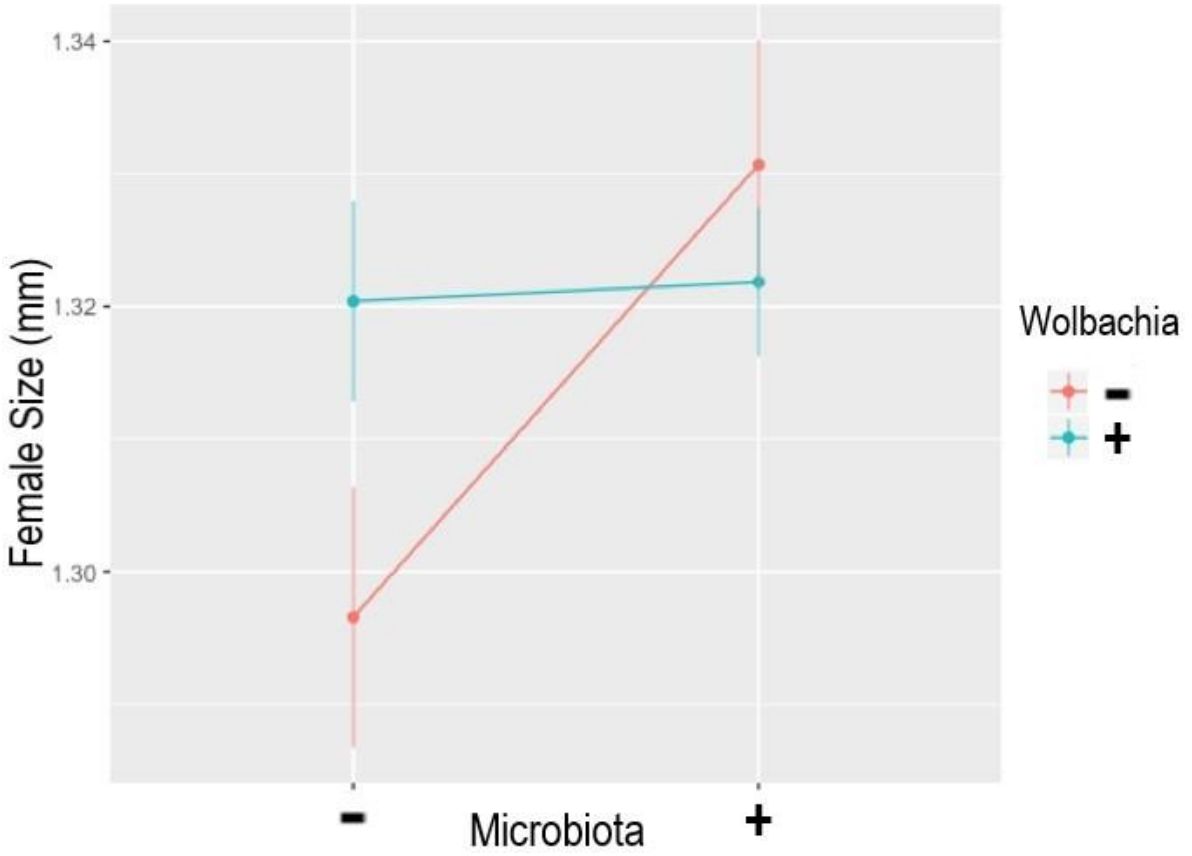

Figure 4. Interaction plot showing the effects of *Wolbachia* infection status and microbiota treatment on female body size with standard error bars plotted. The plot shows that the size difference between microbiota + and microbiota – flies is much greater when females are *Wolbachia* cured (-).

Discussion

We found that females from different genetic backgrounds have different latencies to mate, suggesting they vary in the time they spend mate inspecting. We found no effect of removing the female's microbiota on their latency to mate or an interaction between genetic background and microbiota treatment. This suggests that females' microbiota do not influence female choosiness in *D. simulans* when measured by latency to mate. *Wolbachia* infection did not consistently impact female latency to mate across all genotypes, but the interaction between *Wolbachia* infection status and female genotype affecting mating latency indicates that the effects of infection depend on the host genotype. We found that *Wolbachia* infection made some genotypes more

choosey and others less choosey. This finding is discussed further below, but is indicative of epistasis between the host genome and their bacterial symbiont. We also tested if our different treatments affected body size, one indicator of female condition. We found that female size was affected by genotype and that microbiota removal reduces female body size, whereas *Wolbachia* curing has no effect. There were also no interactions between genotype and microbiota treatment or *Wolbachia* infection status on body size. This suggests that the impacts of the microbiota treatment on condition are not dependent on the genetic background of their host and *Wolbachia* infection is not changing the females' condition. However, we found a significant interaction between *Wolbachia* infection and microbiota removal. *Wolbachia* infection appears to reduce the effects of microbiota treatment on female body size. The microbiota removal-induced reduction on body size is much greater when the females are cured of their *Wolbachia* infection.

The effect of *Wolbachia* infection on female latency to mate appears to depend on host genotype. These changes are not due do variation in *Wolbachia* across isolines, as all of our lines were infected with the same strain. This means that these effects on female choosiness depend on the interaction between the host nuclear and *Wolbachia* cytoplasmic genes. Our results tie in with the finding that *Wolbachia* alters male attractiveness differently across host genotypes (Chapter 2). We can rule out changes in the surface or gut microbiota as our microbiota treatments have controlled for these changes. However, we cannot rule out other intracellular bacterial endosymbionts that co-vary with *Wolbachia* infection.

Parasites are able to manipulate the choosiness of their host in a number of ways. Firstly, the *Wolbachia* infection may be manipulating the female's behaviour and choosiness directly. *Wolbachia* has been found to alter female's reproductive behaviour. Curing *Wolbachia* infection in *D. melanogaster and D. paulistorum* has been shown to reduce mate discrimination with infected individuals showing less assortative mating (Populations et al. 2006; Miller et al. 2010). As antibiotics were used for *Wolbachia* curing in these studies their results may also be explained by changes in other microbiota. However, we also find here that *Wolbachia* altered female mating behaviour. *Wolbachia* infection has also been shown to impact on a variety of other

mating behaviours including female oviposition location (Vala et al. 2004) and increasing male mating rate (Champion de Crespigny et al. 2006). Altering male mating rate within a population may also influence the level of female choosiness. For instance, if the down time between male mating events is lower, females' encounter rate may also increase and their partners may be more sperm depleted (Lefevre & Jonsson, 1962; Markow et al. 1978). Higher male encounter rates could lead to an increase in female choosiness in the same way as increased population density can (Kokko & Rankin, 2006). More sperm depleted partners may select for less choosey females to reduce the risk of being unable to fertilise all of their potential eggs (Puurtinen & Fromhage, 2017). *Wolbachia*-induced behavioural changes in males may be selecting for changes in female choosiness.

A second potential explanation for the changes choosiness may be that *Wolbachia* alters the female's ability to determine male quality. *Wolbachia* accumulate in the nervous tissues of their host, as well as the reproductive tissue. This accumulation may have effects on host cognition, learning and memory. For example, in *Armadillidium vulgare* learning and memory function is significantly reduced with *Wolbachia* infection (Templé & Richard, 2015). In both *D. melanogaster* and *D. simulans, Wolbachia* infection significantly improved long term memory (Bi et al. 2019). The fact that the effects of *Wolbachia* on learning and memory vary across species may indicate that there is epistasis between the *Wolbachia* infection and host nuclear genes. It is possible that the observed changes in female latency to mating are driven by *Wolbachia* induced learning/memory changes in our females. Female *D. melanogaster* with learning and memory mutations alter their response to male courtship songs (Kyriacou & Hall, 1984). Male seminal fluid proteins have also been found to increase female long term memory in *D. melanogaster* (Scheunemann et al. 2019). These seminal proteins evolve to affect females reproductive behaviour to maximise male fitness, so it is likely memory plays a role in female reproduction (Kubli 2003). Further work is needed to test how learning and memory affects female mating behaviour in *D. simulans,* as well as how *Wolbachia* infection alters female memory function across different genetic backgrounds.

Another possible explanation for the observed effect is that the *Wolbachia* infection is costly to some genotypes (isolines) and either beneficial or neutral in others. This would mean the *Wolbachia* infection could produce females with low body condition or fitness in some lines, and higher fitness females in other isolines. Higher fitness females may be more choosey compared with lower fitness counterparts. If *Wolbachia* infection is reducing female condition in some lines and not others, low-condition females may be less able to spend time and energy on assessing mates or making mate choice decisions. To test if *Wolbachia* infection or microbiota removal impacted female condition differently across genetic backgrounds, we compared body size.

We found that *Wolbachia* infection did not significantly impact female body size and there was no interaction with genetic background. This suggests that variable changes in female body condition across genotype were not responsible for the *Wolbachia*-induced changes in choosiness. We did find that microbiota removal significantly reduced female body size and this finding may indicate reduced condition of those females. This shows that removal of the microbiota significantly impacts female *D. simulans* and that it is important to control for other microbiota changes when curing of *Wolbachia* infection. We did not find any effects of microbiota removal on female choosiness in our study, despite the observed body size effects. Interestingly, we found a significant interaction between *Wolbachia* infection status and microbiota treatment on female body size. We found that the removal of the microbiota had a much greater effect on body size in females that were cured of *Wolbachia* compared to infected females. It appears that the *Wolbachia* infection is protecting the females' from the full costs of losing their microbiota. In *D. melanogaster, Wolbachia* infection reduces the biodiversity of the gut microbiota but not the total bacterial load (Ye et al. 2017) indicating that *Wolbachia* infection and the gut microbiota interact. *Wolbachia* seem to be altering the composition of the gut microbiota. The effects of these gut microbiota changes are unknown, however, they do not appear to alter antiviral protection (Ye et al. 2017). *Wolbachia* may be promoting gut bacteria that benefit the development and fecundity of females as *Wolbachia* are maternally transmitted. This means their transmission rate depends on female offspring production. Female body size positively correlates with fecundity in *D. melanogaster* (Lefranc & Bundgaard, 2000). It would therefore be beneficial for *Wolbachia* to influence the gut microbiota in

a way that maximises female size and/or fecundity. This could mean that when we remove the microbiota in our treatments, *Wolbachia*-infected females are better able to acquire a beneficial gut microbiota from their environment than uninfected females. This is currently only a possible explanation and would require further investigation.

Conclusions

We found that removing the non-cytoplasmic microbiota of females reduced their body size, but did not significantly change their latency to mate. The effects of microbiota removal on body size were significantly reduced by the presence *Wolbachia* infection. This suggests that *Wolbachia* and the rest of the microbiota interact in their effects on female phenotype. Interestingly, *Wolbachia* appear to 'save' females from the costs of microbiota removal. These interactions between symbionts on host fitness need further investigation. We found evidence that parasites are potentially altering the choosiness of their host and the scale and direction of these effects depend on the genotype of their host. This has important implications for sexual selection, as changes in female choosiness will alter the strength of sexual selection. Hamilton and Zuk (1982) suggested that females choice may evolve as a mechanism for parasite avoidance. Poulin and Vickery (1996) subsequently suggested that if parasites were reducing female choosiness then the selection for parasite immunity would be much weaker. Although, if parasite resistance is an indicator of 'good genes' it is possible that the assortative mating between individuals with the same infection status may in fact increase the strength of selection (Rolff & Kraaijeveld, 2003). Our results show that the picture is less clear and that there is epistasis between the cytoplasmic *Wolbachia* genes and host nuclear genes. This finding shows that *Wolbachia* infection is increasing the strength of sexual selection in some backgrounds and reducing the strength of selection in others. In order to understand how endosymbiotic parasites are playing a role in sexual selection and specifically in the evolution of female choice, we need to understand how parasites are affecting female choice. We have shown that parasite effects on females vary depending on the female's genetic background. Future work needs to investigate this host-parasite interaction further.

References

Andersson, M., 1994. *Sexual selection*. Princeton University Press; Princeton, NJ.

Archie, E.A. & Theis, K.R., 2011. Animal behaviour meets microbial ecology. *Animal Behaviour*, 82(3), pp.425–436.

Bercik, P. et al., 2011. The intestinal microbiota affect central levels of brain-derived neurotropic factor and behavior in mice. *Gastroenterology*, 141(2).

Bi, J. et al., 2019. *Wolbachia* infection may improve learning and memory capacity of *Drosophila* by altering host gene expression through microRNA. *Insect Biochemistry and Molecular Biology*, 106, pp.47–54.

Birkhead, T.R. & Pizzari, T., 2002. Postcopulatory sexual selection. *Nature Reviews Genetics*, 3(4), pp.262–273.

Champion de Crespigny, F.E., Pitt, T.D. & Wedell, N., 2006. Increased male mating rate in *Drosophila* is associated with *Wolbachia* infection. *Journal of Evolutionary Biology*, 19(6), pp.1964–1972.

Champion De Crespigny, F.E. & Wedell, N., 2007. Mate preferences in *Drosophila* infected with *Wolbachia*? *Behavioral Ecology and Sociobiology*, 61(8), pp.1229–1235.

Clayton, D.H., 1991. The influence of parasites on host sexual selection. *Parasitology Today*, 7(12), pp.329–334.

Cryan, J.F. & Dinan, T.G., 2012. Mind-altering microorganisms: The impact of the gut microbiota on brain and behaviour. *Nature Reviews Neuroscience*, 13(10), pp.701–712.

David, J.R. et al., 2005. Isofemale lines in *Drosophila*: An empirical approach to quantitative trait analysis in natural populations. *Heredity*, 94(1), pp.3–12.

De Moed, G.H., De Jong, G. & Scharloo, W., 1997. Environmental effects on body size variation in *Drosophila melanogaster* and its cellular basis. *Genetical Research*, 70(1), pp.35–43.

Duffy, E. et al., 2019. *Wolbachia* infection can bias estimates of intralocus sexual conflict. *Ecology and Evolution*, 9(1), pp.328–338.

Folstad, I. et al., 1994. Sexually selected color in male sticklebacks: A signal of both parasite exposure and parasite resistance? *Oikos*, 69(3), p.511.

Fry, A.J., Palmer, M.R. & Rand, D.M., 2004. Variable fitness effects of *Wolbachia* infection in *Drosophila melanogaster*. *Heredity*, 93(4), pp.379–389.

Hamilton, W.D. & Zuk, M., 1982. Heritable true fitness and bright birds: A role for parasites? *Science*, 218(4570), pp.384–387.

Hentges, D.J., 2018. Inhibition of shzgella by the normal intestinal flora. In *Bacterial Interference*. pp. 121–132.

Heys, C. et al., 2018. Evidence That the microbiota counteracts male outbreeding strategy by inhibiting sexual signaling in females. *Frontiers in Ecology and Evolution*, 6, pp.1–11.

Heywood, J.S., 1989. Sexual selection by the handicap mechanism. *Evolution*, 43(7), p.1387.

Hoffmann, A.A. & Turelli, M., 1997. Cytoplasmic incompatibility in insects. In: O'Neill S.L., Hoffman, A.A. & Werren, J.H., (eds). *Influential Passengers: Inherited Microorganisms and Arthropod Reproduction*, Oxford University Press, Oxford, UK, pp.42–80.

Hoffmann, A.A., Turelli, M. & Harshman, L.G., 1990. Factors affecting the distribution of cytoplasmic incompatibility in *Drosophila simulans*. *Genetics*, 126(4), pp.933–948.

Hosken, D.J. & House, C.M., 2011. Sexual selection. *Current Biology*, 21(2).

Hurst, G.D.D. & Jiggins, F.M., 2000. Male-killing bacteria in insects: Mechanisms, incidence, and implications. *Emerging Infectious Diseases*, 6(4), pp.329–336.

Ingleby, F.C., Hunt, J. & Hosken, D.J., 2013. Genotype-by-environment interactions for female mate choice of male cuticular hydrocarbons in *Drosophila simulans*. *PLoS ONE*, 8(6).

Jennions, M.D. & Petrie, M., 1997. Variation in mate choice and mating preferences: A review of causes and consequences. *Biological Reviews*, 72(2), pp.283–327.

Judge, K.A., Ting, J.J. & Gwynne, D.T., 2014. Condition dependence of female choosiness in a field cricket. *Journal of Evolutionary Biology*, 27(11), pp.2529–2540.

Kokko, H. & Rankin, D.J., 2006. Lonely hearts or sex in the city? Density-dependent effects in mating systems. *Philosophical Transactions of the Royal Society B: Biological Sciences*, 361(1466), pp.319–334.

Kubli, E., 2003. Sex-peptides: Seminal peptides of the *Drosophila* male. *Cellular and Molecular Life Sciences*, 60(8), pp.1689–1704.

Kyriacou, C.P. & Hall, J.C., 1984. Learning and memory mutations impair acoustic priming of mating behaviour in *Drosophila*. *Nature*, 308(5954), pp.62–65.

Lefevre, G. & Jonsson, U.B., 1962. Sperm transfer, storage, displacement, and utilization in *Drosophila melanogaster*. *Genetics*, 47, pp.1719–1736.

Lefranc, A. & Bundgaard, J., 2000. The influence of male and female body size on copulation duration and fecundity in *Drosophila melanogaster*. *Hereditas*, 132(3), pp.243–247.

Macpherson, A.J. & Harris, N.L., 2004. Interactions between commensal intestinal bacteria and the immune system. *Nature Reviews Immunology*, 4(6), pp.478–485.

Markow, T.A., Quaid, M. & Kerr, S., 1978. Male mating experience and competitive courtship success in *Drosophila melanogaster*. *Nature*, 276(5690), pp.821–822.

Martin, O.Y. & Hosken, D.J., 2003. The evolution of reproductive isolation through sexual conflict. *Nature*, 423(6943), pp.979–982.

Mayer, E.A., 2011. Gut feelings: The emerging biology of gut-"brain communication. *Nature Reviews Neuroscience*, 12(8), pp.453–466.

Milinski, M. & Bakker, T.C.M., 1990. Female sticklebacks use male coloration in mate choice and hence avoid parasitized males. *Nature*, 344(6264), pp.330–333.

Miller, W.J., Ehrman, L. & Schneider, D., 2010. Infectious speciation revisited: Impact of symbiont-depletion on female fitness and mating behavior of *Drosophila paulistorum*. *PLoS Pathogens*, 6(12).

De Moed, G.H., De Jong, G. & Scharloo, W., 1997. Environmental effects on body size variation in *Drosophila melanogaster* and its cellular basis. *Genetical Research*, 70(1), pp.35–43.

Møller, A. & Jennions, M., 2001. How important are direct fitness benefits of sexual selection? *Naturwissenschaften*, 88(10), pp.401–415.

Møller, A.P., 1990. Parasites and sexual selection: Current status of the Hamilton and Zuk hypothesis. *Journal of Evolutionary Biology*, 3(5–6), pp.319–328.

Molnár, O. et al., 2013. Negative correlation between nuptial throat colour and blood parasite load in male European green lizards supports the Hamilton-Zuk hypothesis. *Naturwissenschaften*, 100(6), pp.551–558.

Narraway, C. et al., 2010. Genotype-by-environment interactions for female preference. *Journal of Evolutionary Biology*, 23(12), pp.2550–2557.

Neufeld, K.M. et al., 2011. Reduced anxiety-like behavior and central neurochemical change in germ-free mice. *Neurogastroenterology & Motility*, 23(3), pp.255--e119.

Owen, S.F., Barber, I. & Hart, P.J.B., 1993. Low level infection by eye fluke, *Diplostomum spp*., affects the vision of three-spined sticklebacks, *Gasterosteus aculeatus*. *Journal of Fish Biology*, 42(5), pp.803–806.

Pauli, D.B. & Lindström, K., 2021. Trade-off between mate choice speed and decision accuracy under mating competition in female sand gobies. *J Ethol* **39, pp.**55–64.

Koukou, K. et al., 2006. Influence of antibiotic treatment and *Wolbachia* curing on sexual isolation among *Drosophila melanogaster* cage populations. *Evolution*, 60(1), pp.87–96.

Poulin, R., 1994. Mate choice decisions by parasitized female upland bullies, *Gobiomorphus breviceps*. *Proceedings of the Royal Society B: Biological Sciences*, 256(1346), pp.183–187.

Poulin, R. & Vickery, W.L., 1996. Parasite-mediated sexual selection: Just how choosy are parasitized females? *Behavioral Ecology and Sociobiology*, 38(1), pp.43–49.

Puurtinen, M. & Fromhage, L., 2017. Evolution of male and female choice in polyandrous systems. *Proceedings of the Royal Society B: Biological Sciences*, 284(1851).

R Core Team, 2019. R: A language and environment for statistical computing. R Foundation for Statistical Computing, Vienna, Austria. https://www.R-project.org/.

Ratterman, N.L. et al., 2014. Genetic variation and covariation in male attractiveness and female mating preferences in *Drosophila melanogaster*. *G3: Genes, Genomes, Genetics*, 4(1), pp.79–88.

Rigaud, T., 1997. Inherited microorganisms and sex determination of arthropod hosts. In *Influential Passengers*. pp. 81–101.

Rolff, J. & Kraaijeveld, A.R., 2003. Selection for parasitoid resistance alters mating success in *Drosophila*. *Proceedings of the Royal Society B: Biological Sciences*, 270(SUPPL. 2).

RStudio Team., 2016. RStudio: Integrated development for R. RStudio, Inc., Boston, MA URL http://www.rstudio.com/.

Ruiz-Rodriguez, M. et al., 2009. Symbiotic bacteria living in the hoopoe's uropygial gland prevent feather degradation. *Journal of Experimental Biology*, 212(22), pp.3621–3626.

Ruiz-Rodríguez, M. et al., 2015. Bacteria and the evolution of honest signals. The case of ornamental throat feathers in spotless starlings. *Functional Ecology*, 29(5), pp.701–709.

Ryan, M.J., 1998. Sexual selection, receiver biases, and the evolution of sex differences. *Science*, 281(5385), pp.1999–2003.

Scheunemann, L. et al., 2019. A sperm peptide enhances long-term memory in female *drosophila*. *Science Advances*, 5(11).

Schneider, C.A., Rasband, W.S. & Eliceiri, K.W., 2012. NIH Image to ImageJ: 25 years

of image analysis. *Nature Methods*, 9(7), pp.671–675.

Sharma, M.D., Tregenza, T. & Hosken, D.J., 2010. Female mate preferences in *Drosophila simulans*: Evolution and costs. *Journal of Evolutionary Biology*, 23(8), pp.1672–1679.

Sharon, G. et al., 2010. Commensal bacteria play a role in mating preference of *Drosophila melanogaster*. *Proceedings of the National Academy of Sciences*, 107(46), pp.20051–20056.

Søndergaard, L., 1986. Mating competition in artificial populations of *Drosophila melanogaster* polymorphic for *ebony*: II. A test for minority male mating advantage. *Genetical Research*, 47(3), pp.205–208.

Spieth, H.T., 1974. Courtship behavior in *Drosophila*. *Annual Review of Entomology*, 19(1), pp.385–405.

Stuart Gilchrist, A. & Partridge, L., 1999. A comparison of the genetic basis of wing size divergence in three parallel body size clines of *Drosophila melanogaster*. *Genetics*, 153(4), pp.1775–1787.

Templé, N. & Richard, F.J., 2015. Intra-cellular bacterial infections affect learning and memory capacities of an invertebrate. *Frontiers in Zoology*, 12(1).

Turnbaugh, P.J. et al., 2006. An obesity-associated gut microbiome with increased capacity for energy harvest. *Nature*, 444(7122), pp.1027–1031.

Vala, F. et al., 2004. Wolbachia affects oviposition and mating behaviour of its spider mite host. *Journal of Evolutionary Biology*, 17(3), pp.692–700.

Wade, M.J. & Chang, N.W., 1995. Increased male fertility in *Tribolium confusum* beetles after infection with the intracellular parasite *Wolbachia*. *Nature*, 373(6509), pp.72–74.

Weeks, A.R. et al., 2007. From parasite to mutualist: Rapid evolution of *Wolbachia* in natural populations of *Drosophila*. *PLoS Biology*, 5(5), pp.0997–1005.

Werren, J.H. & Windsor, D.M., 2000. *Wolbachia* infection frequencies in insects: Evidence of a global equilibrium? *Proceedings of the Royal Society B: Biological Sciences*, 267(1450), pp.1277–1285.

Ye, Y.H. et al., 2017. Gut microbiota in *Drosophila melanogaster* interacts with *Wolbachia* but does not contribute to *Wolbachia*-mediated antiviral protection. *Journal of Invertebrate Pathology*, 143, pp.18–25.

Zhou, W., Rousset, F. & O'Neill, S., 1998. Phylogeny and PCR-based classification of *Wolbachia* strains using wsp gene sequences. *Proceedings of the Royal Society B: Biological Sciences*, 265(1395), pp.509–515.

Chapter 7

General Discussion

Symbiotic bacterial communities play an important role in their hosts' fitness. These communities are incredibly abundant and diverse, for example, in humans the number of bacterial cells are estimated to at least match that of their host (Sender et al. 2016). The collection of genes present in these bacterial communities outnumber those of the human genome 100 to 1 (Yang et al. 2009). It is therefore unsurprising that the range of effects these bacteria have on their hosts are also varied. However, the role of these symbiotic bacteria on their host's phenotype have only recently begun to be explored in detail (Archie & Theis, 2011; Ezenwa et al. 2012). Before advances in sequencing technology most research focused on the fitness consequences parasitic infections (Burnet & White, 1972).

Wolbachia pipientis (*Wolbachia*) is an obligate endosymbiotic parasite of arthropods and is incredibly wide spread (Hilgenboecker et al. 2008). *Wolbachia* are able to manipulate their hosts in a number of ways that increase their transmission. *Wolbachia* can cause major changes to reproductive phenotypes in their hosts and also manipulate their behaviour, immunity and other sexually and naturally selected traits (Werren et al. 2008). Often the impacts of *Wolbachia* infection are dependent on both the strain and their host (Iturbe-Ormaetxe & O'Neill, 2007). However, the majority of research into the effects of *Wolbachia* on their hosts' fitness has used antibiotic treatment to remove infection and then compare cured to infected individuals. This antibiotic treatment is likely to not only alter the *Wolbachia* infection status but also the other symbiotic bacterial communities. Therefore, many of the fitness effects previously attributed to *Wolbachia* may in fact be due to other symbiotic bacteria. Despite this, research that attempts to determine the significance of other symbiotic bacteria on fitness traits previously attributed to *Wolbachia* infection is rare. The understanding of how symbiotic bacteria and their host's co-evolve is limited. Most research will test *Wolbachia* or other symbiotic bacteria's effect on a measure of host fitness in one genetic background or one population. In this thesis I have presented

research in which we have investigated the effects of *Wolbachia* and other non endosymbiotic bacteria on a variety of sexually selected traits across different *D. simulans* genotypes. We have also investigated how the gut microbial communities of *D. simulans* respond when evolving under different levels of natural and sexual selection. Despite the phenotypic and behavioral effects bacterial symbionts can have on their hosts little in know about how these communities respond to either natural or sexual selection.

In Chapter 2 we simultaneously explore the impact of gut bacteria and *Wolbachia* on the male sexual-fitness of *Drosophila simulans* genotypes (isolines). We manipulated the symbiotic bacteria of both *Wolbachia* cured and infected flies in multiple ways. We found evidence that our gut microbial treatment was successful. We found no significant interaction between genotype and either *Wolbachia* infection or gut bacterial treatment, so we were still unable to determine the causes of fitness rank changes. We found evidence of bidirectional cytoplasmic incompatibility (CI) in this assay, which may have been obscuring our ability to find significant interactions between genotype and bacterial treatments. This potential fitness effects from using diet to manipulate gut microbiota could also have been influencing our sexual-fitness measures. We used male attractiveness as a measure of male sexual-fitness to avoid any CI effects. We also used a new re-infection and bacterial washing treatment to manipulate the gut bacteria of our flies to control for diet effects. We found a significant interaction between *Wolbachia* infection status and genotype on male attractiveness but no interaction between gut bacterial treatment and genotype. This means that the antibiotic caused changes male fitness ranks were caused by changes in *Wolbachia* infection and not gut bacteria. This also suggests that *Wolbachia* is a potential source of intergenome epistatic fitness-variation.

In Chapter 3 we investigated the potential bidirectional cytoplasmic incompatibility (CI) found in Chapter 2. We tested if there was bidirectional between the *Wolbachia* strain our *D. simulans* isolines were infected with and the strain our tester *ebony* flies were infected with. We also tested whether the level of bidirectional CI changed across either male or female genotypes. We found evidence of bidirectional CI in our strains as infected focal males did worse than uninfected males when mating with tester

females infected with a different strain of *Wolbachia*. While infected and uninfected focal females did equally poorly when mated with males infected with a different *Wolbachia* strain. In females we found a significant interaction between genotype and *Wolbachia* infection status on magnitude of CI. In some genotypes *Wolbachia* infection lowers the level of CI females' experience, and in others it increases the level. This is potential evidence of *Wolbachia* and its host coevolving in some genotypes to limit the impact of bidirectional CI on their fitness. In males we found that different genotypes may suffer different levels of CI, as the genotype fitness ranks do not correlate between *Wolbachia* infected and cured males. This could be evidence that some lines are evolving in response to the selection that CI imposes on the number of offpring they sire. However, the interaction between male genotype and *Wolbachia* infection status was not significant. This work may help to explain why mixed infection populations persist despite models predicting CI inducing *Wolbachia* infection should spread to fixation.

Chapter 4 investigates the effects of evolving under different strengths of natural and sexual selection has on a host's gut microbiome. The combined genetic material of both the host genome and the genes present in the communities that make up the microbiota (microbiome) has been termed the 'holobiome' (Guerrero et al. 2013). Selection must be able to act on a host's symbiotic microbial communities for the holobiome to be a useful evolutionary measure. Understanding how natural and sexual selection effect a host's microbiota and the nature of the relationship between the two mechanisms of selection is important in determining the strength of selection (Blows, 2002). Research into the effects of either natural or sexual selection on the microbiota is limited. Most research has been limited to comparing microbiome changes across within generational environmental manipulation. We evolved populations of *Drosophila simulans* under either elevated or relaxed natural and sexual selection in a fully factorial design for 38 generations. We found that elevated sexual selection resulted in a more diverse gut microbiome in male but not females. And that both male and female gut microbial communities varied across sexual selection intensities. The males gut microbial changes with sexual selection were more phylogenetically distinct than the females suggesting that they are more likely to be functionally significant changes. This is likely due to the strength of sexual selection being higher on males

than females in our treatment. We found significant effects of natural selection on the gut microbiome of males or females across any of our measures of diversity. There was also no interaction between natural and sexual selection on the gut microbiome. This is the first study to find that sexual selection can act on the gut microbial communities and cause changes to these communities over evolutionary time.

In Chapter 5 we tested how the symbiotic bacteria effects host fitness across different genotypes on a range of male and female fitness traits. Most research that manipulates the microbiota to test host fitness effects uses one of two methods. The first method involves comparing individuals with an intact microbiota to individuals that have their microbiota removed. The second method involves manipulating individual bacterial species and comparing fitness effects. Neither of these methods is likely to be biologically relevant as germ-free and monoculture organisms do not exist in nature. In this chapter we introduce a third microbial treatment and test the fitness responses in males and females across *D. simulans* genotypes. Our microbial treatments did not influence female choosiness or body size. When comparing the female genotypes fitness ranks, we found when simply removing the microbiota ranks correlated compared the original microbiota fitness ranks. When we altered the microbiota, fitness ranks were significantly changed. This suggests microbiota fitness effects are not straight forward. This chapter provides further evidence that the host and microbiota are co-evolving and that different genetic backgrounds respond differently to microbiota removal or alteration.

Chapter 6 explores the effects of *Wolbachia* infection and the non-endosymbiotic microbiota on female choosiness and body size across different *D. simulans* genotypes. The level of female choosiness will impact the strength of sexual selection through female choice. Female choosiness can vary dependent on population dynamics, their condition as well as their environment (Kokko & Rankin, 2006). Parasites can also influence the level of female choosiness by either disrupting females ability to choose between partners or reducing the resources they're able to allocate to mate choice. In Chapter 2 we found that *Wolbachia* infection alters male attractiveness dependent on their hosts genetic background. As male attractiveness has been shown to positively genetically correlate with female choosiness (Ratterman

et al. 2014), we may also find that *Wolbachia* alters female choosiness differently across genotypes. To test this we manipulated the *Wolbachia* and gut microbiota using the same methods as in third assay of chapter 2. We used antibiotics to cure *Wolbachia* infection and used the microbiota removal and reinfection protocol to avoid any diet effects. We also compared changes in female body size across treatments to test if any choosiness changes were caused by changes in female condition. We found a significant interaction between *Wolbachia* infection status and female genotype on choosiness levels. This affect did not appear to be caused by changes in female body condition, as there was no interaction between *Wolbachia* infection and genotype on female body size. We found the changes in the non-endosymbiotic microbiota did not effect female choosiness and there was no interaction between this treatment and genotype on choosiness. Microbiota removal caused a reduction in female body size across genotypes. There was a significant interaction between *Wolbachia* infection status and microbiota treatment on female body size where *Wolbachia* infection reduces the negative effects of microbiota removal. This is further evidence that the genetic background of a host plays an important role in the fitness effects of their microbial symbionts.

Overall, this thesis has found evidence that the effects of symbiotic bacteria on sexually selected traits in their host are complex. We have shown that both endosymbiotic and exosymbiotic bacteria are having significant effects on these traits in their hosts. We found that using a blunt tool, such as an antibiotic, to remove specific symbiotic bacteria is likely to alter other bacterial components of the microbiota. This shows the importance of controlling for all changes in the microbiota when investigating the role these symbiotic bacteria play on their hosts' fitness. We have shown for the first time that sexual selection can act on the composition of gut bacterial communities. Symbiotic bacterial communities and their hosts are coevolving and their effects vary across hosts. We find evidence of symbiont-by-genotype epistasis for a range of sexually selected traits in both males and females. Understanding the complex interactions between microbiota and their hosts is essential to better understand the importance of the microbiota in general.

References

Archie, E.A. & Theis, K.R., 2011. Animal behaviour meets microbial ecology. *Animal Behaviour*, 82(3), pp.425–436.

Blows, M.W., 2002. Interaction between natural and sexual selection during the evolution of mate recognition. *Proceedings of the Royal Society B: Biological Sciences*, 269(1496), pp.1113–1118.

Burnet, D.O., & White, M., 1972. *Natural History of Infectious Disease*. Cambridge University Press, Cambridge, UK.

Ezenwa, V.O. et al., 2012. Animal behavior and the microbiome. *Science*, 338(6104), pp.198–199.

Guerrero, R., Margulis, L. & Berlanga, M., 2013. Symbiogenesis: The holobiont as a unit of evolution. *International Microbiology*, 16(3), pp.133–143.

Hilgenboecker, K. et al., 2008. How many species are infected with *Wolbachia*? - A statistical analysis of current data. *FEMS Microbiology Letters*, 281(2), pp.215–220.

Iturbe-Ormaetxe, A. & O'Neill, S.L., 2007. *Wolbachia* – host interactions: connecting phenotype to genotype. *Current Opinion in Microbiology*, 10, pp.221–224.

Kokko, H. & Rankin, D.J., 2006. Lonely hearts or sex in the city? Density-dependent effects in mating systems. *Philosophical Transactions of the Royal Society B: Biological Sciences*. pp. 319–334.

Ratterman, N.L. et al., 2014. Genetic variation and covariation in male attractiveness and female mating preferences in *Drosophila melanogaster*. *G3: Genes, Genomes, Genetics*, 4(1), pp.79–88.

Sender, R., Fuchs, S. & Milo, R., 2016. Revised Estimates for the Number of Human and Bacteria Cells in the Body. *PLoS Biology*, 14(8).

Werren, J.H., Baldo, L. & Clark, M.E., 2008. *Wolbachia*: Master manipulators of invertebrate biology. *Nature Reviews Microbiology*, 6(10), pp.741–751.

Yang, X. et al., 2009. More than 9,000,000 unique genes in human gut bacterial

community: Estimating gene numbers inside a human body. *PLoS ONE*, 4(6).