Investigating the relationship between gut microbiota and animal behaviour

Thesis submitted in accordance with the requirements of the University of Liverpool for the degree of Doctor in Philosophy by Chloe Elizabeth Heys.

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Abstract: Investigating the relationship between gut microbiota and animal behaviour

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An individual's gut microbiota is increasingly recognised as having a key role in many host behaviours. In insects, the environment largely determines the host gut microbiota, with the majority ingested through the diet. In this thesis, I examine a series of host-gut microbiota relationships within three species of *Drosophila* with varied ecologies. Initially, I analyse the current methods used to eliminate the gut microbiota in *Drosophila melanogaster,* key to studying host-microbiota relationships and providing a foundation for this thesis. I then use this information to assess the role of the gut microbiota as an honest signal in age-based mate choice in *Drosophila pseduoobscura*. Finally, I examine the role the gut microbiota plays in specialisation in *Drosophila sechellia*, through adaptation to its toxic host plant, *Morinda citrifolia*.

To attribute a specific behaviour to the gut microbiota, it must first be removed. However, removal can have serious physiological side effects on the host organism, and the most effective and least detrimental method of doing this is widely debated. I analyse commonly used methods of removing the gut microbiota in *Drosophila melanogaster* and find the addition of low-dose streptomycin to the dietary media is more effective and has fewer physiological effects than other methods such as egg dechorionation or rearing on a sterile diet.

Female *Drosophila pseudoobscura* are known to discriminate between males based on age. This may occur through the alteration of the cuticular hydrocarbon (CHC) profile of males, which can alter due to a varied gut microbiota caused by a varied diet. I determine that the gut microbiota influences female preference for older males and is a key component of attractiveness to females.

I examine the role of the gut microbiota in *Drosophila sechellia*, in adaptation to its toxic host plant, *Morinda citrifolia,* and characterise the gut microbiota of this *Drosophilid* species for the first time. Rearing flies on *M. citrifolia*, a standard laboratory diet, and an additional salak (*Salacca zalacca*) fruit that lacks the toxic compounds present in *M. citrifolia,* I find that flies reared on a laboratory diet have a significantly reduced weight. However, there is no impact on development time or subsequent mating behaviours when compared to individuals reared on the wild, *M. citrifolia* diet.

Finally, by creating experimental evolution lines of *D. melanogaster* supplemented with *D. sechellia* gut microbiota, I disentangle the role of pH in shaping the gut microbiota from the co-evolution of the gut microbiota within *D. sechellia. D. melanogaster* are highly averse to the scent of octanoic acid, the main toxic constituent within *M. citrifolia*, whereas *D. sechellia* are highly attracted. After ten generations *D. melanogaster* show significantly less aversity to octanoic acid. I determine that *Lactobacillus plantarum* acts as a detoxifying agent by metabolising octanoic acid, therefore suggesting this

bacterium has been fundamental to the ecological transition and specialisation of *D. sechellia*.

Taken together, the chapters of this thesis further uncover the role of host-microbiota interactions in important ecological and evolutionary processes within *Drosophila*, from elucidating a principle method in gut microbiota research, to underlying mate choice mechanisms and finally to dietary specialisation.

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I'll conclude this section with the insightful words of my dear friend, Ellie Watts: "when faced with a difficult decision, one must always trust your gut."

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1. Chapter One: General introduction

1.1 The ecology and evolution of host-microbe interactions

There has been an explosion of research into the effect of the microbiota on the host, with the variety of host-microbe interactions incredibly diverse. These interactions can be temporary or permanent, accidental or obligatory, but the outcome often involves either health or disease. As such, the majority of host-microbe interactions occur via two different forms. They can be either pathogenic, as is the case for bacteria such as *Campylobacter sp*. that opportunistically colonise humans, or symbiotic, such as *Vibrio fischeri*, that enables squid to successfully camouflage against their surroundings (Lee & Ruby, 1992; Ruby & Lee, 1998). Examples of microbes enabling host animals to thrive in particular environments, demonstrate both the ecological and evolutionary importance of host-microbiota relationships.

The relationships between microbiota and their host can have profound impacts on the subsequent ecology and evolution of not only the host organism, but surrounding organisms. This can occur in a number of ways. For example, *Arsenophonus nasoniae*, is a predominantly extracellular bacteria that is transmitted from a mother to her offspring via oviposition (Skinner, 1985). This bacterium can manipulate host reproduction by killing males in *Nasonia* wasps, thereby infected mothers only produce females, which can distort sex-ratios of populations (Engelstädter & Hurst, 2009). Other microbes can have a number of intermediate hosts before they reach their final target and therefore microbes that involve the use of more than one host can therefore play a role in the co-evolution of one species with another. For example, potentially the most well documented case of host-manipulation occurs in *Toxoplasma gondii* (e.g. Webster, 2007). Members of the cat family, *Felidae*, are the definitive hosts of *T. gondii* and only here can it undergo full gametogenesis within the intestinal epithelium, before the cat host sheds the oocytes in its faeces for transmission. To get to the definitive

host however, *T. gondii* infects a number of intermediate hosts, one of which is rodents. Infected rodents are shown to display bolder behaviours and are less afraid of cat urine, due to *T. gondii* changing components in the brain. This makes the rodent substantially more likely to encounter a cat, *Felidae*, and be preyed upon, thereby allowing *T. gondii* to complete its transmission to its definitive host.

The origins of host-microbiota studies led to the coining of the hologenome theory of evolution (Rosenberg & Rosenberg, 2008). This theory suggests that the holobiont (the host plus its symbiotic microbiota) acts in consortium with the hologenome, to be considered a single unit of selection in evolution (Rosenberg & Rosenberg, 2008). Rapid changes in the diversity of microbial communities can occur within individuals, which can then act as a driving force in the evolution of the host organism. As such, studies have begun to focus on the role that the microbiota plays on a number of aspects related to the host organism, from host physiology to evolution of whole populations (reviewed in Rosenberg & Rosenberg, 2008). Studies have been conducted in a number of different organisms and examined a variety of host traits, from digestion (e.g. Brune & Ohkuma, 2010) to behaviour (e.g. Sharon et al., 2010).

The microbiota is a complex and diverse ecosystem in which species are argued to be in continually flux (Foster et al., 2017). An individual's microbiota is determined partly by genotype (Early et al., 2017), and parents can transmit microbes to their offspring, that aid them in the early stages of their life (e.g. Freitak et al., 2013). The remaining microbiota is determined by the diet.

In animals, the largest and most diverse population of microbes resides in the gut. The composition of an individual's gut microbiota is determined predominantly by diet (Sharon et al., 2010). It can be argued that the microbial diversity and abundance of microbes that inhabit the gut are due to a coevolution between the microbiota and the host. One way in which this can occur is through the gut-brain axis. This is the signalling that occurs between the gastrointestinal tract and the brain which ensures the proper

maintenance of gut homeostasis as well as a wealth of other mechanisms. This bidirectional communication system occurs via the central nervous system (CNS) and encompasses the brain, spinal cord, the autonomic and enteric nervous systems and the hypothalamic pituitary adrenal axis (Carabotti et al., 2015). Understanding the evolution and maintenance of the gut-brain axis is essential to understanding how and why these complex microbial relationships have formed.

The recent drive in examining host-microbe interactions, has uncovered links between microbiota dysbiosis and physical and mental health implications in mammals. Studies have determined a link between gut microbial composition and obesity (Gérard, 2016), diabetes (Larsen et al., 2010) and anxiety (Desbonnet et al., 2015). In mice, a reduction in gut microbial communities during early post-natal life altered anxiety and cognitive abilities in later life (Desbonnet et al., 2015). Mice have also been used as a model to examine the role of the gut microbiota in autism spectrum disorder (ASD) (de Theije et al., 2014). Intestinal disturbances are frequently reported in ASD patients and changes in the gut microbiota composition are described. In the murine model for ASD, intestinal phenotype and autism-like behaviour is associated with altered microbial colonisation. In humans, studies have shown that dysbiosis in early-life can potentially lead to mental health problems in later life, via production of glucocorticoids (Clark et al., 2014). Therefore, studies that further elucidate the links between a changing gut microbiota and subsequent behaviour, can lead to a greater understanding of these relationships. *Drosophila* have been frequently proposed as an important model organism for studying these interactions between host and the gut microbiota (Buchon, Broderick & Lemaitre, 2013a; Early et al., 2017).

This literature review will focus on the existing knowledge surrounding the role of the gut microbiota on host behaviour. First, I will discuss the role of the microbiota in general on behaviour in a number of different insect species, before focussing more specifically on the components of the *Drosophila* microbiota and finally the *Drosophila* gut microbiota.

1.1.1 Microbiota and behaviour in insects

Insects use a combination of auditory, visual and olfactory cues in order to recognise their conspecifics (reviewed in: Brennan & Kendrick 2006; Hurst 2009), with the latter thought to be the most common mechanism. Recognition of an individual based on olfaction involves the comparison of a label (the odour or cues presented by the individual to be recognised) to a template (a mental representation of cues), which can represent familiar odours of individuals encountered regularly, whether related or not, as well as parental or kin odours. The widespread use of odour cues in insects may be due to the fact they can arise through a variety of different forms. They can be synthesised by the animal itself, be derived from the environment, or arise from the individual's diet.

Recent studies have found a host's behaviour can be significantly altered through changes in diet, due to diet causing alterations in the individual's symbiotic bacteria, which in turn changes the odour profile used in communication (reviewed in Archie & Theis 2011). One way in which this can occur is via the cuticular hydrocarbon (CHC) profile.

The literature on gut microbiota and subsequent behaviour in insects originated with Dodd (1989). Here, *Drosophila pseuoobscura* were reared on either a starch-based or maltose-based diet before being placed into mate choice trials as adults. Dodd (1989) noted that flies preferred to mate with other individuals that were reared on the same diet type, though the gut microbiota was not directly implicated at the time. Further evidence that the gut microbiota plays a pivotal role in mate choice mechanisms was obtained in *Drosophila melanogaster*. Flies were similarly reared on two different diets for a number of generations and were observed to significantly prefer mating with individuals reared on the same diet type, after only one generation (Sharon et al., 2010). The addition of antibiotics to the dietary media abolished this preference, directly implicating the gut microbiota in the

process. In particular, *L. plantarum* was isolated as driving mating preference in this population.

The main way in which insects use olfactory recognition mechanisms is via CHCs. These are heritable, fatty acids used for olfactory based species, sex and kin recognition purposes by many insect taxa (reviewed in Singer, 1998). CHCs cover the surface of terrestrial arthropods, are widely known as sex pheromones and in *Drosophila* are produced by specialised cells known as oenocytes (Ferveur, 2005). They have long been known to be important in sexual signalling in *D. melanogaster* (Jallon 1984) and are also thought to be involved in olfactory kin recognition processes (Lizé et al., 2014; Heys et al., 2018a).

Lizé et al. (2014) were the first to examine kin recognition mechanisms in three different species of *Drosophila* with varied ecologies. In *D. melanogaster*, males of this fruit generalist species invested more in terms of copulation duration, and thus sperm transfer, by mating for significantly longer with partners reared on the same diet type regardless of relatedness. Yet, with the removal of gut microbiota via antibiotic treatment this diet effect was removed, and individuals invested less in matings with related partners. This suggests that two cues have evolved in *D. melanogaster* in order to determine their investment in a mating – the food eating during development, and thus similarities in gut microbiota and relatedness. This was expanded by Heys et al. (2018a; this thesis), who determined that males reproductively invest more in a mating with an unrelated female, in terms of sperm transfer, when the gut microbiota is suppressed via antibiotics. They determined that the cues detected by males regarding relatedness were determined by the CHC profile, with gut microbiota only altering the CHCs that can be expressed in both sexes and not female-specific compounds. It could therefore be suggested that the gut microbiota is masking kin recognition within this species, as when the gut microbiota is intact, no difference in reproductive investment in either related or unrelated partners is observed.

As the gut microbiota, via the CHC profile, has been implicated in determining mate choice behaviours, studies have also begun to uncover the

role the gut microbiota plays in driving reproductive isolation. For example, in leaf beetles, two species, *Chrysochus cobaltinus* and *Chrysochrus auratus*, are known to hybridise. Positive assortative mating between the two species has been observed in the both the laboratory and the field (Peterson et al., 2007). Preference in *C. cobaltinus* males for conspecific females is mediated by sex and species specific CHCs, with CHCs providing signals to guide male mate choice. In two closely related species of *Drosophila*, *Drosophila simulans* and *Drosophila sechellia*, male mate discrimination via variation in CHC profiles has been suggested to be the main driving force in reproductive isolation and thus, speciation, between these two species (Shahandeh, Pischedda & Turner, 2018).

1.2 The *Drosophila* microbiota

Due to its fast life-cycle and ease of culturing, *Drosophila melanogaster* has become a model organism for the study of host-microbiota interactions. The composition of the *Drosophila* microbiota is thought to differ according to genotype (Early et al., 2017), diet (Sharon et al., 2010), laboratory (Chandler et al., 2011) and age (Ren et al., 2007; Wong, Ng & Douglas, 2011). For example, the microbiota of flies has been shown to alter throughout its lifespan, as certain bacteria appear to differ in abundance between young and older adults (Wong, Ng & Douglas, 2011), with the bacterial load found to increase over the whole lifetime of the fly (Ren et al., 2007).

The microbiota of *Drosophila* becomes established via the chorion. The chorion is the protective, outer layer of the egg which is coated with a layer of highly diverse bacteria likely from faecal deposits transmitted from the mother during oviposition (Wong, Ng and Doulgas, 2011). Upon emergence, larvae then ingest this outer layer of the egg and the bacteria coating it, which leads to bacterial colonisation within the fly (Bakula, 1967).

In order to further determine the timescale of microbiota establishment in *Drosophila*, Blum et al. (2013) analysed the microbiota of newly emerged flies that were either tipped onto fresh food daily, or only after seven days. They noted that flies that were transferred after seven days harboured a larger bacterial population than those that were not, with the dynamics similar between males and females. This result suggests that establishment of the *Drosophila* microbiota requires both access to and consumption of exogenous bacteria, which takes time to develop on the medium. They further tested this hypothesis by transferring 16-day-old flies to sterilised media or media inoculated with *L. plantarum* and *Acetobacter pasteurianus*, twice daily. Bacterial populations isolated from flies that were transferred to the sterile media were significantly reduced, although not absent altogether. Therefore, without repeated consumption of exogenous bacteria, the microbial load of *Drosophila* cannot sustain itself, although it does not get fully eliminated.

The general *Drosophila* microbiota tends to consist of acetic acid bacteria, lactic acid bacteria and yeasts (Chandler et al, 2011; Broderick & Lemaitre, 2012; Staubach et al., 2013). A number of studies have reported the main bacterial components as *Enterobacter*, *Lactobacillus* and *Acetobacter* species (Corby-Harris et al., 2007; Cox & Gilmore, 2007; Chandler et al., 2011; Wong, Ng & Douglas, 2011), with the abundances and presence of additional species found to differ between populations. For example, a combination of Tag pyrosequencing of 16S rRNA genes and Sanger sequencing of the laboratory population of *D. melanogaster* of Blum et al. (2013), noted that 94% of the microbiota was comprised of *Acetobacter sp*. with the remaining being *Lactobacillus sp*. In contrast, Staubach et al. (2013) noted similarly high abundance of *Acetobacter* and *Lactobacillus sp.* (86%), with the rest of the microbiota comprising *Enterobacter* (3%) and *Enterococcus* species (2%), including certain pathogenic *Enterococcus* strains.

Like the bacterial components of the *Drosophila* microbiota, yeasts are also predominantly acquired via diet. Yeasts produce esters which attract *Drosophila* (Christiaens et al., 2014; Sciabor et al., 2014) and even induce

oviposition (Becher et al., 2012). They not only provide food, but also provide essential vitamins, sterols and amino acids, and are therefore key components of the *Drosophila* diet (Broderick & Lemaitre, 2012). Here, *Drosophila* tend to avoid underripe fruits and instead prefer overripe fruits, with the majority of species feeding on decaying fruits (Turner & Ray, 2009). As fruit ripens, the cell-wall degrading enzymes and amylases convert the firm, starchy tissue into soft, sugar-rich fruit. The high sugar content supports the bacterial and fungal growth and diversity within *Drosophila* (Barbe et al., 2001; Barata et al., 2012). The dominant yeast species within fruit-feeding *Drosophila* is identified as *Hanseniaspora,* with *Saccharomyces* and *Candida sp*. also characterised. Slight variations occur within flies that are mushroom or flower-feeders (Chandler, Eisen & Kopp, 2012).

The resident microbiota can have a beneficial relationship with *Drosophila* in terms of health and physiology. *D. melanogaster* challenged with *Serratia marcescens*, a *Drosophila* pathogen, exhibited lower mortality when the *Drosophila* diet was supplemented with *L. plantarum* (Blum et al., 2013). Gut bacterial response to trypanosomatid parasites has been well documented in bumble bees, *Bombus terrestris,* (Koch & Schmid-Hempel, 2011) and Tsetse flies, *Glossina morsitans*, (Weiss et al., 2011), and recently the first naturally-occurring trypanosomatid parasite has been determined within *Drosophila*. A parasite of *D. melanogaster* and *Drosophila falleni*, the gut bacteria is thought to play a role in infection response to this parasite (Hamilton et al., 2015).

Endosymbiotic microbes have also been described within *Drosophila*. *Wolbachia*, an obligate endosymbiotic bacterium, is thought to be present in 40% of all insect species (reviewed in Werren, Baldo & Clark, 2008). *Wolbachia* primarily infects the host via vertical transmission and can produce a range of harmful phenotypes. One such phenotype is cytoplasmic incompatibility (CI) (Laven, 1959; Yen & Barr, 1973). When a *Wolbachia* infected male mates with an uninfected female, a form of inherited reproductive failure occurs. It is presumed that *Wolbachia* modify sperm prior to the completion of spermatogenesis, an idea which is further highlighted as

at least two accessory gland proteins (Acp26Aa and Acp36De) transferred to females are unaffected by *Wolbachia* presence (Snook et al., 2000).

Wolbachia can also affect host reproduction in other ways, including male-killing (Hurst et al., 2000), feminization (Asgharian et al., 2014) and parthenogenesis (Huigens et al., 2004). Male-killing *Wolbachia* has been described within *Drosophila* (Hurst et al., 2000). For example, in *Drosophila bifasciata*, the male-killing trait is born to 5-7% of females and is characterised by low egg hatch rates in nearly all female broods, with the trait being inherited by 99% of daughters (Magni, 1953). Similarly, in *Drosophila*, *Wolbachia* have been shown to effect sperm production (Snook et al., 2000), egg production (Hofmann et al., 1990) and longevity (Fry & Rand, 2002). Feminization, the development of genetic males into females, has also been described within *Drosophila* (reviewed in Werren et al., 2008). Despite *Wolbachia* producing a number of phenotypes, the function of each remains the same: to promote the spread and transmission of *Wolbachia* via infected individuals, by distorting the sex ratio (Stouthamer et al., 1999).

Maternally-transmitted endosymbionts have been implicated in host defence strategies, for example by providing protection against parasitoid wasps in the case of aphids associated with *Buchnera* (Oliver et al., 2003) and fungal pathogens when aphids are associated with *Acyrthosiphon pisum* (Scarborough et al., 2005). Aside from affecting *Drosophila* reproduction, *Wolbachia pipientis* has also been shown to protect *D. melanogaster* flies from RNA viruses by reducing mortality (Hedges et al., 2008). Similar to *Wolbachia*, *Spiroplasma* is a maternally-transmitted *Drosophila* endosymbiont which is present in both cells and the hemocoel (Williamson, 1965). *Spirosplasma* infects *Drosophila neotestacea* and has been implicated in the defensive strategy of *D. neotestacea* when parasitized by the nematode *Howardula aoronymphium* (Jaenike & Perlman, 2002; Jaenike et al., 2010). *H. aoronymphium* is a nematode that commonly attacks mushroom-feeding *Drosophila* species. Mated female *H. aoronymphium* infect *D. neotestacea* larvae and when in the adult stage, they release offspring and are transmitted to the *D. neotestacea* offspring via oviposition. Severe infections can reduce adult survival, male mating ability and can even

cause sterility in females (Jaenike & Perlman, 2002). When *D. neotestacea* are infected with *Spiroplasma*, the growth of *H. aoronymphium* is impaired, preventing severe infections and limiting transmission from mother to offspring (Jaenike et al., 2010).

1.3 The *Drosophila* gut

1.3.1 *Drosophila* gut structure

When examining the composition of the gut microbiota, it is important to note which component of the gut is being studied. As the *Drosophila* gut is separated into distinct sections that have different functions, determining the correct section to study is essential for understanding host-gut microbiota interactions. The structure of the *Drosophila* gut is similar to other insects, in that it consists of a simple epithelium surrounded by visceral nerves, muscles and tracheae (Lemaitre & Miguel-Aliaga, 2013). This digestive epithelium is then divided into three sections: the foregut, midgut and hindgut (e.g. Demerec, 1950) (Figure 1). Each of these sections are from different developmental origins, with both the foregut and hindgut of ectodermal origin and the midgut from endodermal. The foregut is comprised of the pharynx, oesophagus and the crop – a structure only found in the adult form (Stoffolano & Haselton, 2013). The cardia, located at the junction of the foregut and midgut, is a specialised structure that functions as a sphincter to regulate food passage into the midgut (Buchon et al., 2013b). The central part of the cardia is called the proventriculus, which is responsible for synthesising a peritrophic membrane that wraps around food passing through the digestive tract (Terra, 2001).

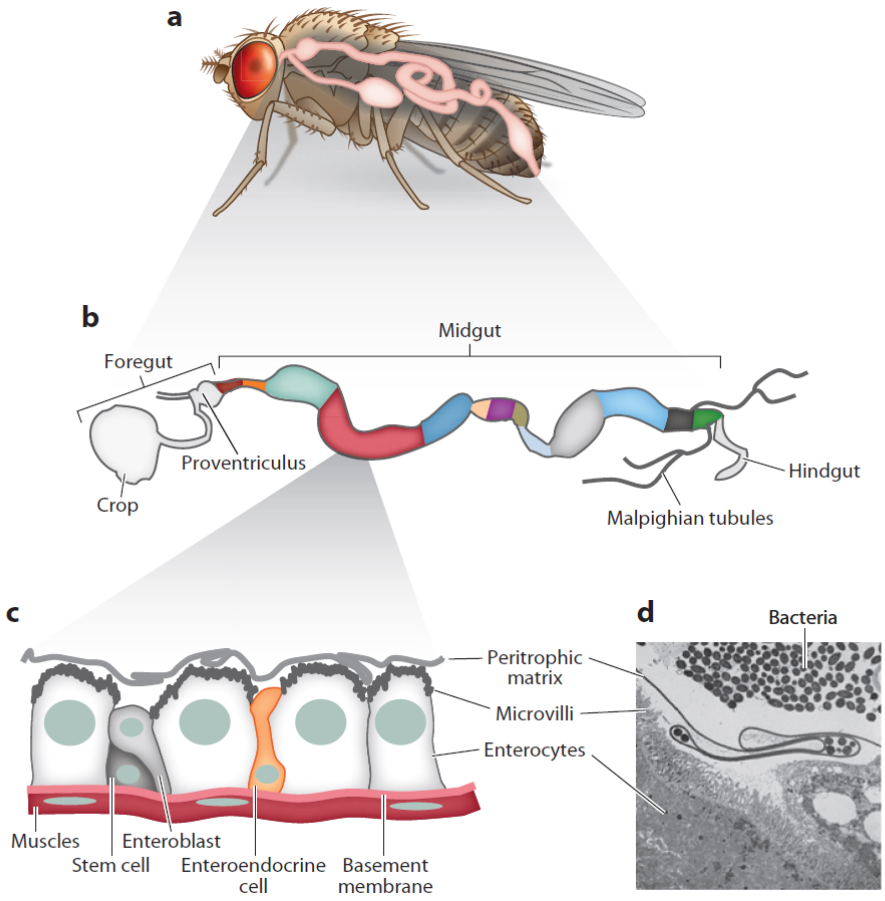

Figure 1. Diagrammatic representation of the organisation of the *Drosophila* gut (Lemaitre & Miguel-Aliaga, 2013). (a) Representation of the gut within the body cavity (Buchon et al., 2013). (b) The digestive tract is separated into three distinct sections – the foregut, midgut and hindgut. (c) The midgut is comprised of epithelium surrounded by two layers of visceral muscles. (d) Electron microscopy demonstrating the response of the gut to infection with pathogenic bacteria, *Erwinia carotovora* (Acosta et al., 2007). Here the peritrophic matrix is forming a physical barrier against the infection.

The midgut is one of the largest insect organs and is the predominant site for digestion and nutrient absorption (Buchon et al., 2013b). It is further divided into three segments: the anterior, middle and posterior segments. Notably, the middle midgut contains a distinct pool of cells known as copper cells (Dubreuil, 2004). These cells are strikingly similar to the parietal cells present in the mammalian gut which are responsible for the secretion of stomach acid, and likely serve a similar function in *Drosophila* as they do in mammals.

The midgut is also the site at which the Malpighian tubules branch. These act as the renal system in insects by secreting fluid and controlling neurohormones (Maddrell, 1981; Maddrell & O'Donnell, 1992). Following the midgut is the hindgut, which functions mainly to concentrate excrement prior to expulsion through water reabsorption (Cognigni et al., 2011).

The midgut consists of a simple epithelium which is renewed every 1-2 weeks by intestinal stem cells (ISCs) which cover the basal surface of the gut epithelium (Micchelli & Perrimon, 2006; Ohlstein & Spradling, 2006). These ISCs undergo either asymmetric or symmetric divisions and their activity is influenced by both environmental factors and the metabolic state of the host (reviewed in Jiang and Edgar, 2011; Singh et al., 2011). Concentrated populations of different ISCs exist within different regions of the gut. For example, the copper-cell region is maintained by its own specific population of ISCs (Strand & Micchelli, 2011). Similarly, the proventriculus has another distinct subpopulation that is solely responsible for renewal of the foregut-midgut junction (Singh et al., 2011).

Interestingly, the *Drosophila* gut varies in structure depending on the stage in the life cycle. These three distinct structures are known as the larval gut, the transient pupal gut and the adult gut (e.g. Singh et al., 2011). After both larval and pupal metamorphosis, the midgut degenerates to form meconium, which is shortly expelled following eclosion. Each stage of the life cycle has its own distinct characteristic. For example, the crop, which occurs in the foregut and is responsible for food storage, is only present in the adult form and its evolutionary origins are thought to be explained by the difference in feeding behaviours of the adult and larval forms (Stoffolano & Haselton, 2013). *Drosophila* larvae feed continuously via the ingestion of solid food courtesy of their mouth hooks, compared to the adult form that infrequently ingests liquids via the proboscis. This dramatic change in feeding behaviours is thought to be responsible for the formation of the crop – enabling food to be stored for longer periods of time so the adult does not have to continuously feed. Features specific to the larval form include the presence of four gastric ceca. These are found in the anterior midgut which is the predominant site for digestion and absorption within the midgut region

(Nation, 2002). It is likely that these differences in anatomical and genetic structure of the digestive tract have arisen through changes in the different dietary habits of the life cycle stages (Lemaitre & Miguel-Aliaga, 2013).

1.3.2 *Drosophila* gut microbiota

In invertebrates, the diversity of the gut microbiota that has been characterised is thought to be between one and two orders of magnitude lower than that of mammals (Dillon & Dillon, 2004; Dunn & Stabb, 2005; Behar et al., 2008; Lehman et al., 2009; Robinson et al., 2010; Wong, Ng & Douglas, 2011). The diversity of the *Drosophila* gut microbiota is similarly low and consists of only a few bacterial genera (e.g. Wong, Ng & Douglas, 2011). However, it is found to greatly vary according to diet (Sharon et al., 2010), strain and laboratory (e.g. Chandler et al., 2011). Gut associated microbes are acquired upon hatching from the egg (Bakula, 1967). During oviposition, females defecate onto the surface of the eggshells, which the larvae consume upon hatching. The gut microbiota can be separated into two distinct categories: autochthonous and allochthonous microbiota. The autochthonous microbiota is classed as resident microbiota, whereas allochthonous microbes are non-resident forms that can vary greatly according to diet or social environment (Wong, Ng & Douglas, 2011).

Feeding patterns within *Drosophilids* greatly vary. The majority are generalists, feeding on the decaying fruit of an array of plant species (Atkinson & Shorrocks, 1977). Other dietary substrates include flowers (Brncic, 1983), mushrooms (Jaenike et al., 1983), leaves (Carson, 1971) tree sap fluxes (Throckmorton, 1975) and soil (Heed, 1977). *Drosophila suzukii* on the other hand, feeds solely on ripening fruit, due to the females possessing a unique, serrated ovipositor that enables them to lay their eggs into the majority of soft-skinned fruits, such as strawberries or blueberries (Hauser, 2011). Unique specialisms have also evolved within *Drosophila*. *D. sechellia*, for example, is a specialist of the toxic host plant, *M. citrifolia* – a

fruit that is toxic to other *Drosophilids* (Jones, 2005). Similarly, *Drosophila pachea* feeds on the rotting stems of the cactus, *Lophocerus schottii* (Heed & Kircher, 1965).

A varied diet is often accompanied by a varied gut microbiota. However, the microbiota of wild flies has, until recently, been somewhat unstudied and laboratory reared flies typically are seen to have a less-diverse bacterial community (e.g. Broderick et al., 2004). For example, studies using *D. melanogaster* have characterised the gut microbiota of laboratory-reared flies and noted a lack of diversity (e.g. Brummel et al., 2004; Ryu et al., 2008). Although the same taxa are found in most studies, there is thought to be a "lab-effect" – that different microbial communities are found across different laboratories due to the varied methods in the rearing and culturing of flies (Ren et al., 2007; Heys et al., 2018b). Studies analysing the gut bacterial communities of wild populations of flies that feed on different diets, noted that flies collected from fruit-feeding hosts exhibited lower bacterial diversity, compared to mushroom or flower-feeding flies (Chandler et al., 2011). These studies confirm that although wild flies have a more diverse microbiota, the most commonly associated microbiota remain consistent with those in the laboratory, indicating that natural populations of *Drosophila* have a low bacterial diversity and richness (Broderick & Lemaitre, 2012).

The most common bacterial communities inhabiting the *Drosophila* gut are *Lactobacillus sp., Acteobacter sp.* and *Enterobacter sp.* Commonly found species within these groups include *Acetobacter pomorum*, *Lactobacillus brevis* and *Lactobacillus plantarum* (reviewed in Broderick & Lemaitre, 2012). Typically, Acetobacter and Lactobacillus species are involved in regulating growth and development with the fly, whereas *Enterobacter* is implicated in immune responses. For example, *A. pomorum* is known to 'rescue' the development of larvae when under thiamine-restricted conditions, with thiamine (vitamin B_1) essential to healthy growth and development (Sannino et al., 2018). Similarly, *L. plantarum* has been shown to protect against colonisation of pathogens in the gut (Ryu et al., 2008), by digesting sugars to produce lactic acid, which inhibits the growth of non-commensal organisms and promotes the growth of *Lactobacilli* that thrive in low pH conditions (e.g.

Kleerebezem et al., 2003). It is also responsible for promoting larval growth when nutrients are scarce (Storelli et al., 2011), and plays a role in mating preferences (e.g. Sharon et al., 2010). *L. brevis* has also been implicated in enhancing larval growth, and so comes of little surprise that it is commonly found within the *Drosophila* microbiota (e.g. Shin et al., 2011).

The physical changes that occur within the *Drosophila* gut during different life stages unsurprisingly alters the gut microbiota. Wong, Ng and Douglas (2011) characterised the gut microbiota of *D. melanogaster* flies at each different life stage and found that although the diversity generally remains the same, the abundance of bacterial species greatly differs. Early-instar larvae were dominated by *Lactobacillus fructivorans*, comprising 80% of sequence reads, with the rest of the bacterial species representing between 1-9%. Species identified include *Acetobacter tropicalis, A. pomorum, L. brevis and L. plantarum*. The bacterial species composition changes when individuals reach third-instar larvae, with *L. plantarum* becoming the most dominant species. At the pupal stage, the predominant bacterial species is *A. tropicalis*, which is thought to be responsible for maintaining mechanical functions within the gut, potentially through the production of polysaccharides (Kounatidis et al., 2009). *Staphylococcus sp.* was also identified at the pupal stage, which was not present during any other time. As sexually mature adults between 3-7 days old, both males and females are dominated by *L. fructivorans* (60% and 92% respectively), although males contain higher abundances of *L. brevis* and *A. tropicalis*. Bacterial composition changes again with the age of adults, with *A. pomorum* becoming the dominant species in both males and females (82% and 74% respectively).

Recently, studies have been exploring the role of microbe-microbe interactions in determining the composition of the gut microbiota (Hughes et al., 2014; Newell & Douglas, 2014). For example, in insects, *Anopheles* mosquitoes are naturally uninfected by *Wolbachia*. One reason for this absence is that the native mosquito microbiota is a barrier to vertical transmission of *Wolbachia* (Hughes et al., 2014). The presence of a specific bacterium, *Asaia sp*. prevents the stable transmission and maintenance of *Wolbachia* within the host. This suggests that incompatibility between the

microbiota and *Wolbachia* may explain why some insect hosts do not carry *Wolbachia* in the wild. Further, in *D. melanogaster*, microbial interactions between *Lactobacillus* and *Acetobacter* are proven to reduce triglyceride number, although the exact mechanisms are yet to be determined (Newell & Douglas, 2014).

1.4 Outline of this thesis

In this thesis, I will discuss the role of the gut microbiota in mate choice mechanisms and specialisation within *Drosophila*. I will evaluate the function and role of the gut microbiota in a number of different species of *Drosophila*, with varying ecologies and diet types, but with similar mating systems. For example, *D. melanogaster* and *D. pseudoobscura* are food generalists, whereas *D. sechellia* is a fruit specialist. I determine the efficacy of current methods used to suppress the gut microbiota in *D. melanogaster*, a widely used model for studying host-microbe relationships. I examine the effect of the gut microbiota on mediating age-based cues used when selecting a mate in *D. pseudoobscura*. I also examine the effect of a changing gut microbiota via diet in *D. sechellia*, and further examine the potential role it plays in adaptation to its toxic fruit host, *M. citrifolia*. Finally, I provide evidence that the gut microbiota is responsible for the evolved resistance to octanoic acid that occurs within *D. sechellia* and propose that the gut microbiota has enabled specialisation in this species.

Chapter Two: The effect of gut microbiota elimination in *Drosophila melanogaster*: A how-to guide for host-microbiota studies

Investigating the role of the microbiota on aspects on host behaviour and physiology relies on the ability to remove or suppress the host-microbiota.

There has been an explosion of research into host-microbiota studies, but no attention has focussed on the methods used to suppress host microbiota. In this study, commonly used methods are evaluated using the model organism, *Drosophila melanogaster*. Current methods in use include egg dechorionation, an axenic (sterile) diet or addition of low-dose streptomycin to host diet. I test the efficacy of these methods at removal of the host microbiota, in a fully factorial design, and assess the implications these have on host life history traits, including the risk of death before adulthood, weight and survival. We conclude that the addition of low-dose streptomycin to the dietary media is the most effective at removing the gut microbiota and has less detrimental effects on the host.

Chapter Three: *Drosophila* sexual attractiveness in older males is mediated by their microbiota

Females of the fruit fly species, *Drosophila pseudoobscura*, are known to prefer to mate with older rather than younger males, but little is known as to how females detect this age cue. Previous studies have shown that gut microbiota alters in complexity with age. Cuticular hydrocarbons (CHCs) are used in sexual signalling in *Drosophila* and CHC odour profiles are known to alter according to the individuals gut microbiota. Here, I examine the role that the gut microbiota plays in influencing female preference in this species and find that an intact microbiota is a key component of attractiveness in older males. I propose that this is due to the gut microbiota providing an honest signal used by females to assess male age.

Chapter Four: A potential role for the gut microbiota in the specialisation of *Drosophila sechellia* to its toxic host, *Morinda citrifolia*

The sister species of *Drosophila melanogaster, Drosophila sechellia* is a specialist living exclusively on the *Morinda citrifolia* fruit. *M. citrifolia* is both highly acidic and toxic to most *Drosophila* species, yet *D. sechellia* has evolved resistance to this fruit. *M. citrifolia's* toxicity is due to the presence of both octanoic and hexanoic acids. In the laboratory *D. sech*ellia is reared on a standard, formulated *Drosophila* diet, with no attention paid to the effect this may have on the gut microbiota. Therefore, we characterise the gut microbiota of *D. sechellia* for the first time on its ancestral diet and compare it to a formulated laboratory diet. We then begin to elucidate the role that the gut microbiota plays in adaptation to a novel host plant, by comparing the gut bacterial diversity and abundance with a population of *D. sechellia* reared on a salak fruit diet, similar in nutritional properties to *M. citrifolia*, but without the toxins. This enables us to determine whether it is pH shaping the microbiota, or whether the components of the microbiota act as a detoxifying agent in order to adapt to this host.

Chapter Five: Gut microbiota and octanoic acid detoxification in *Drosophila sechellia* and *Drosophila melanogaster*

Morinda citrifolia contains high levels of the toxic compounds, octanoic and hexanoic acid, that *Drosophila sechellia* has evolved resistance to. How *D. sechellia* has become adapted to this toxic fruit is unknown. Here we assess the role of the gut microbiota in resistance to octanoic acid – the primary toxic compound in *M. citrifolia* and highly repellent to other *Drosophilids*. We further examine the role of gut microbiota in the ecological transition of *D. sechellia* to its toxic host by directly comparing the gut microbiota with its sister species, *Drosophila melanogaster*. We find that *D. melanogaster* that have been reared on a diet supplemented with *D. sechellia* gut bacteria become less averse to octanoic acid after only ten generations. We present this as the first step in ecological transition to a novel, toxic host, and suggest the gut microbiota acts as a detoxifying agent within this species.

Chapter Six: General conclusions

A general conclusion that encompasses the ideas presented in all other chapters and provides suggestions for future research.

2. Chapter Two: The effect of gut microbiota elimination in *Drosophila melanogaster*: A how-to guide for host-microbiota studies

2.1 Abstract

In recent years, there has been a surge in interest in the effects of the microbiota on the host. Increasingly, we are coming to understand the importance of the gut microbiota in modulating host physiology, ecology, behaviour, and evolution. One method utilized to evaluate the effect of the microbiota is to suppress or eliminate it, and compare the effect on the host with that of untreated individuals. In this study, we evaluate some of these commonly used methods in the model organism, *Drosophila melanogaster*. We test the efficacy of a low-dose streptomycin diet, egg dechorionation, and an axenic or sterile diet, in the removal of gut bacteria within this species in a fully factorial design. We further determine potential side effects of these methods on host physiology by performing a series of standard physiological assays. Our results showed that individuals from all treatments took significantly longer to develop, and weighed less, compared to normal flies. Males and females that had undergone egg dechorionation weighed significantly less than streptomycin reared individuals. Similarly, axenic female flies, but not males, were much less active when analysed in a locomotion assay. All methods decreased the egg to adult survival, with egg dechorionation inducing significantly higher mortality. We conclude that low-dose streptomycin added to the dietary media is more effective at removing the gut bacteria than egg dechorionation and has somewhat less detrimental effects to host physiology. More importantly, this method is the most practical and reliable for use in behavioural research. Our study raises the important issue that the efficacy of and impacts on the host of these methods, requires investigation in a case by case manner, rather than assuming homogeneity across species and laboratories.

2.2 Introduction

In the past few years, there has been an explosion of interest in the gut microbiota, and the myriad ways in which it affects host processes from modulating immune responses (Round & Mazmanian, 2009) to mate selection (Lizé, McKay & Lewis, 2014). To date, using a Web of Science search, there have been 4,617 articles published on the gut microbiota, across diverse species (search terms: gut microbio* under TITLE). Of that number, 3,281 (71%) of these were published in the last four years. However, there is little consensus regarding the most effective method for eliminating the gut microbiota, despite its importance for our understanding of the effects the gut microbiota may have on the host.

Drosophilid species, particularly *Drosophila melanogaster,* have become an important model for examining how changes to, or differences in, the gut microbiota affect the host, for example, by regulating intestinal regeneration (Buchon, Broderick & Lemaitre, 2013a), or through driving mating preferences (Sharon et al., 2010). For such studies to be considered reliable, effective methods of altering the gut microbiota must be utilised in concordance with a given study system.

In *Drosophila*, there are two particularly common methods of altering gut bacterial communities: supplementing dietary media with antibiotics, or creating sterile or axenic flies using egg dechorionation. The protective outer layer of the egg, the chorion, is coated with highly diverse bacteria transmitted largely from faecal deposits from the mother during oviposition (Wong, Ng & Douglas, 2011). Emerging larvae then ingest the chorion and the bacteria coating it, forming the basis of their microbial community (Bakula, 1967). Removal of this embryonic chorion using bleach creates axenic, or microbe-free, adults. Supplementing the dietary media with antibiotics is a considerably simpler method. Here, a broad-spectrum antibiotic such as streptomycin or tetracycline is added to the diet; some studies use a combination of antibiotics in order to remove the microbiota (Sharon et al., 2010; Sharon et al., 2011).

Both the use of antibiotics and dechorionation of the egg are widely applied, and widely criticised. Therefore, evaluating the efficacy of current methods and how they impact the study organism is vital for the investigation of host-microbiota relationships. Some recent publications have favoured the use of antibiotics (Sharon et al., 2010; Sharon et al., 2011). Yet while broad spectrum antibiotics are active against a wide range of bacterial species, they also act on host enzymes and mitochondrial proteins by inhibiting synthesis, and/or nucleic acid metabolism and repair (Broderson et al., 2000). In pseudoscorpions, this has been shown to reduce sperm viability, and the effect can be passed down generations (Zeh et al., 2012). The repeated use of broad-spectrum antibiotics also has severe consequences in other organisms. For example, in humans long-term antibiotic use is thought to correlate, directly or indirectly, with diseases such as type-2 diabetes and early-life obesity (Blaser & Falkow, 2009). Egg dechorionation in egg-laying animals is thought to be a less hazardous method of eliminating gut bacteria. However, studies comparing this with antibiotic treatment have only ever used harsher antibiotics such as chlortetracycline or rifampicin, and in high concentrations (Ridley et al., 2012). The impacts on the host of tetracycline use have been fairly well documented (e.g. O'Shea & Singh 2015; Zeh et al. 2012), yet to date, little attention has focussed on low-dose streptomycin.

In this study we analysed the efficacy and the physiological effects on the flies, of the most common methods used to eliminate the resident host gut microbiota in *D. melanogaster*. We compared flies reared via a range of methods, in a factorial design: those reared on streptomycin, egg dechorionated individuals, and flies reared on an axenic diet (Figure 1). Parallel to bacterial analyses determining the effectiveness of the techniques in eliminating the gut microbiota, we conducted a series of standard physiological assays in order to test the effect of each treatment on the overall health and fitness of the fly host. We measured the zrisk of death before adulthood (Tantway & El-Helw, 1970), adult weight (Partridge & Fowler, 1993), egg to adult survival, and how adults responded to stress. In the natural environment, the ability of *D. melanogaster* to develop more quickly on the limited food source of rotting fruit is beneficial to survival, as it

ensures an individual can achieve pupation before the food source is exhausted (Nunney, 1996). This pressure is also increased if multiple females lay eggs on the same fruit. Thus, measuring the risk of death before adulthood is a fundamental assay of an individual's physiological fitness. Similarly, size directly correlates with mating success in *Drosophila*, with larger males being more successful (Partridge & Farquhar, 1983). Starvation assays measure how long a fly can survive when deprived of nutrition (Service et al., 1985), whilst locomotion assays such as the Rapid Iterative Negative Geotaxis (RING) assay (Gargano et al., 2005) measure the innate escape response, where individuals ascend the walls of a container after being knocked to the bottom. From these results we suggest addition of antibiotics to the diet is the most effective method for eliminating the gut microbiota in our *Drosophila* system, with the least deleterious effects for the host. We note that this method is both more practical and reliable when conducting behavioural experiments, as, when using axenic individuals, there is a high likelihood of introducing external bacteria through the very nature of manipulating the study organisms. Our results demonstrate the importance of considering the potential impacts of each method with respect to the host organism studied, and the target research area.

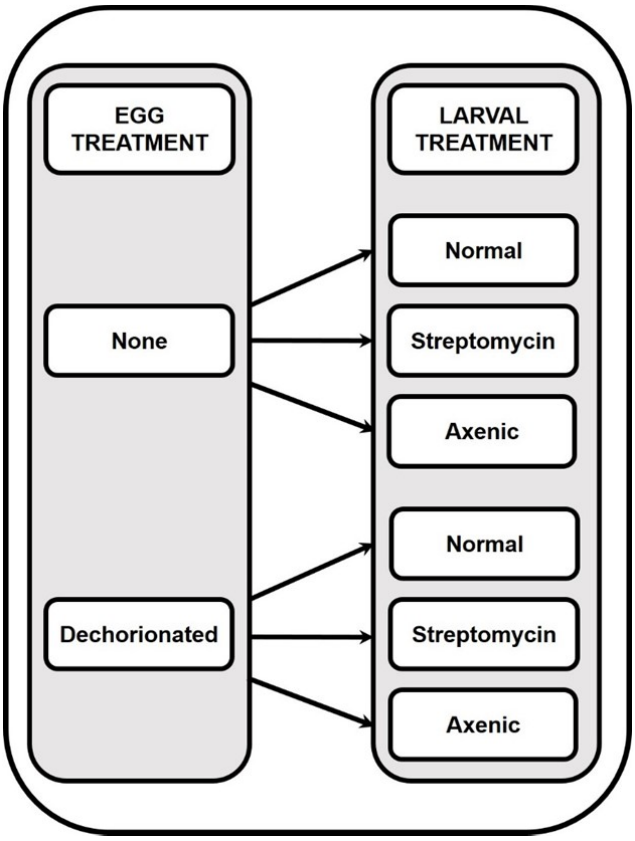

Figure 1. Schematic representation of our 2x3 factorial design of egg and larval treatments. Physiological assays were conducted on flies from each treatment type.

2.3 Materials and Methods

2.3.1 Fly stocks

Wild-type, *Wolbachia*-free *D. melanogaster* stocks were isolated from an outbred population collected in Lyon. Flies were reared at 25°C under a 12:12hour light:dark cycle. Recently mated females were placed into vials containing 25ml standard yeast-cornmeal diet (for 1l of water: 85g of sugar, 60g of corn, 20g of yeast, 10g of agar and 25ml of nipagin), and left to lay eggs for twenty-four hours. The following day, the females were removed and eggs were collected using a fine paint-brush. The eggs and their successive developing larvae were then assigned to one of the six treatments (Figure 1). Henceforth we abbreviate our treatments as outlined in table 1.

Once eggs had been harvested and a treatment assigned (for example, dechorionated or not), they were placed into vials at a standard density of fifty per vial. Eggs that were not subjected to the dechorionation, were still physically manipulated in the same way, but without the chemical treatment. Thus, we controlled for any potential effects of physically manipulating the eggs, across all treatments. Eggs were then left to hatch, and the emergent larvae left to develop. At eclosion, newly emerged adults were isolated using an aspirator and separated according to sex. Males and females were stored separately in groups of ten in vials containing 25ml of the diet on which they were reared as larvae.

Table 1. Treatment abbreviations used throughout, and sample sizes for each.

Treatments	Abbreviations	Egg to adult survival / Development time	Weight		Starvation		Response to stress (RING assay)	
			males	females	males	females	males	females
Eggs reared normally Larvae reared normally	N-Norm	1100/438	68	87	97	104	35	25
Eggs reared normally Larvae reared on streptomycin food	N-Strep	1100/353	62	62	90	95	25	25
Eggs reared normally Larvae reared on axenic food	N-Ax	1400/188	53	59	60	51	25	25
Eggs dechorionated Larvae reared normally	D-Norm	1050/152	80	67	54	52	30	25
Eggs dechorionated Larvae reared on streptomycin food	D-Strep	1100/127	68	56	53	54	25	25
Eggs dechorionated Larvae reared on axenic food	D-Ax	1450/82	50	50	51	53	25	25

2.3.2 Experimental treatments

Normal diet

Eggs assigned to a normal diet treatment were transferred into vials containing 25ml standard yeast-cornmeal diet at 25°C and left to develop.

Diet containing streptomycin

Once harvested from the stock vials, eggs were then transferred into vials containing 25ml standard yeast-cornmeal diet that had been supplemented with streptomycin at a concentration of 400µg/ml, as is common in the literature (Lizé, McKay & Lewis, 2014; Sharon et al., 2010). Upon cooling, 4ml of a solution composed of 10g of streptomycin in 100ml of ethanol was added per litre of food. Food was then dispensed into vials with 25ml in each.

Axenic diet

An axenic diet was produced by autoclaving vials of standard yeast-cornmeal diet, without the addition of nipagin, for 20 minutes at 120°C. Nipagin was added once the media had cooled to 65°C. Any manipulation of the axenic diet was conducted under a laminar flow cabinet to ensure sterility. 25ml of the media was then dispensed into sterile vials.

Egg dechorionation

Eggs were gently harvested using a sterile paintbrush and placed onto a piece of fine cloth mesh. They were then placed into a strainer and washed with sterile, deionized water once. They were then immersed in a 10% sodium hypochlorite solution for five minutes (Ridley et al., 2012). The eggs were washed three more times with sterile, deionized water and then carefully removed using a sterile paintbrush and placed onto the desired food treatment. All work was conducted under a laminar flow cabinet to ensure sterility. Eggs from all treatments were subjected to the physical manipulation utilised during the egg dechorionation treatment, but without the addition of bleach, in order to control for any deleterious effects of the action.

2.3.3 Physiological assays

Risk of death before adulthood

Once treated, eggs were placed into the assay and the number of days for these eggs to emerge as newly eclosed adults was counted. Vials were

checked at three time points within each day – 9am, 12pm and 5pm – and the cumulative number of adults emerged from each time point was scored. Emergent adult flies from each time point were removed from the vial and placed into a fresh vial of their corresponding diet treatment.

Egg to adult survival

Each vial was set up to contain fifty eggs so that the number of flies that reach adulthood could be counted. Vials were checked at three time points within each day – 9am, 12pm and 5pm – and the cumulative number of alive, newly eclosed adult flies was counted. Emergent flies were then removed from the vial and placed into a fresh vial of their corresponding diet treatment. This was repeated daily until it was there were no live larvae or pupae left in the vial. The mortality rate was then calculated from the number of flies that had reached adulthood compared to the number of eggs set up.

Adult weight

Vials were checked daily at three time points – 9am, 12pm and 5pm – and any newly emerged, virgin adults were isolated and separated according to sex. They were placed into vials at a standard density of ten per vial and left for two hours to allow their wings to dry out and inflate. Flies reared in the egg dechorionated egg treatments and the axenic larval treatments were always manipulated within the laminar flow cabinet in order to prevent contamination. Two hours later, vials were placed into the freezer at -18°C and left overnight. The following morning, individuals were collected from the freezer using a Kahn balance and their weight was recorded (in mg) to four decimal places. Male and female measurements for each treatment were recorded and analysed separately.

Starvation resistance

Newly emerged, virgin adults were isolated and separated according to sex. Flies reared in the egg dechorionated egg treatments and the axenic larval treatments were always manipulated within the laminar flow cabinet in order to prevent contamination. Flies were placed into vials at a standard density of ten per vial and left to mature for two days. After this time, they were transferred to a fresh vial containing 10ml of non-nutritional agar in order to prevent desiccation. Fresh agar was used to prevent microorganismal growth – no bacterial and fungal growth was observed during the course of the experiment. Flies were left in these vials to acclimatise for 24 hours and then the starvation assay was started. The time to starvation death was measured by monitoring the flies every 8 hours – at 8am, 4pm and 12am. Here the number of dead flies were counted and the starvation assay continued until there were no more living flies. This assay was conducted at 25°C. Male and female measurements for each treatment were recorded and analysed separately.

Locomotion – RING (Rapid Iterative Negative Geotaxis)

Newly emerged, virgin adults were isolated and separated according to sex. Flies reared in the dechorionated egg treatments and the axenic larval treatments were always manipulated within a laminar flow cabinet in order to prevent contamination. Flies were placed into vials at a standard density of ten per vial and left to mature for two days. After this time, flies were placed into fresh vials containing 10ml of the diet type on which they were reared. Five vials were then placed into an apparatus similar to that described by Gargano et al. (2005) and Nichols et al. (2012), and flies were left to acclimatise for thirty minutes. After this time, the apparatus was rapped sharply on the work surface three times in rapid succession in order to initiate the negative geotaxis response. After a three second rest, a photograph was taken of the vials, recording the flies' position within the vial, and thus their negative geotaxis response to the stimulus. After a one-minute rest, the procedure was repeated. This procedure was repeated five times in total for each set of flies, resulting in five digital images for each vial. This

assay was performed at 25°C. Male and female measurements for each treatment were recorded and analysed separately.

Digital images were later analysed manually by measuring the distance each fly had travelled following the tapping stimulus. All ten flies in each vial were measured across the five digital images generated. An average distance travelled value was then created for each vial and statistical analysis performed.

2.3.4 Bacterial analysis

In order to quantify the bacterial load within flies reared on each treatment, and therefore the efficacy of each treatment, we cultured the bacteria present in both the whole gut and the whole fly. Newly emerged, virgin adults were isolated and separated according to sex. Flies reared in the dechorionated egg treatments and the axenic larval treatments were always manipulated within a laminar flow cabinet in order to prevent contamination. Flies were placed into vials at a standard density of ten per vial and left to mature for two days.

Gut bacterial analysis

Following maturation, adults were isolated using gas anaesthesia and surface sterilised in 70% ethanol, rinsed in distilled water and air-dried. The head was then removed. Three guts were dissected into each Eppendorf containing 500µl of sterile PBS (Phosphate Buffered Saline solution). An equal number of males and females were used in order to ensure there were no sex-specific differences in the bacterial content. Gut tissue was homogenised with a sterile plastic pestle. 100µl of gut homogenate was pipetted onto MRS (de Man, Rogosa and Sharpe) agar and spread-plated using a sterile glass loop. Plates were left to air dry aseptically, before being closed and sealed with parafilm. Plates were incubated at 25°C for 72 hours, and bacterial load was quantified by performing CFU (Colony Forming Unit) counts.

Whole fly bacterial analysis

Following maturation, flies were isolated using gas anaesthesia and placed into a sterile Eppendorf containing 500µl sterile PBS. Three flies were added into each Eppendorf. An equal number of males and females were used in order to ensure there were no sex-specific differences in the bacterial content. The whole-fly solute was then homogenised using a sterile, plastic pestle. 100µl of the whole-fly solute was pipetted into the centre of a petri dish containing MRS media and spread across the plate using a sterile glass loop. The plate was left to dry close to the flame before being closed and sealed using parafilm. Plates were incubated at 25°C for 72 hours and then checked for bacterial growth. Bacterial load was quantified by performing CFU counts.

Single colonies were isolated using a sterile 1µl loop and placed into an Eppendorf with 10µl sterile water. PCR amplification was performed in a 25µl reaction volume consisting of 10µl nuclease-free water, 13µl Taq green master mix, 0.5µl of forward primer 27F (5'- AGAGTTTGATCMTGGCTCAG-3') and reverse primer 1492R (5′-GGTTACCTTGTTACGACTT-3') and 1µl of template DNA. Thermal cycling was performed for 90 seconds at 95°C as initial denaturation, followed by 35 cycles of 30 sec at 95°C for denaturation, 30 sec at 55 °C as annealing, 90 sec at 72 °C for extension, and final extension at 72 °C for 5 min. 1500 bp 16S PCR products were purified with Ampure beads and subjected to Sanger sequencing. The resulting sequences were identified using NCBI BLAST against the nt database (Altschul et al., 1990).

2.3.5 Statistical analysis

Sample sizes are given in table 1. All analyses were performed in R (3.1.3) (Ihaka & Gentleman, 1996), and the effects of egg (dechorionation or not) and larval treatments (Normal, Axenic, and Streptomycin) were studied in addition to their interactions. Egg to adult survival, weight and response to

stress (RING assay) were analyzed by fitting a General Linear Model with binomial, Gaussian, and Gaussian distributions respectively. Weight data were Box-Cox transformed to improve normality of the GLM residuals. All GLMs were followed by an ANOVA to test for global effects, and *post hoc* multiple comparisons between treatments were conducted using Tukey's HSD tests. Following these general GLMs, sexes were studied separately for weight and response to stress (starvation and RING assay).

Cox Proportional-Hazard Regressions for survival were used to assess variation in development time, measured as the risk of death before adulthood, and survival under starvation. Survival analysis involves the modelling of time to event data, with death being considered the 'event'. Death and development failure of flies was used as the 'event' for survival data, and risk of death before adulthood data, respectively. The *Survdiff* function was used to assess differences between two or more survival curves according to egg and larval treatments. The *coxph* function was used to assess differences between treatments. This allowed treatments to be compared in a pairwise fashion, to ascertain whether all treatments differed, or whether any significant differences observed were derived from a single treatment.

2.4 Results

2.4.1 Risk of death before adulthood

Globally, egg dechorionation (*Surdiff,* χ^1_1=473, P<0.001) and larval treatments (*Surdiff,* χ^1_2=726, P<0.001) altered the risk of death before adulthood (Figure 2). When compared to N-Norm flies, egg dechorionation (*Coxph*, β±S.E.=0.068±0.101, Z=-26.305, P_z<0.001), and larval treatments (*Coxph*, Ax, β±S.E.=0.091±0.093, Z=-25.603, P_z<0.001, Strep, β±S.E.=0.089±0.077, Z=-31.110, P<0.001) increased the risk of death before adulthood. Moreover, egg dechorionation and larval treatment effects interacted with each other (*Coxph*, D-Ax, β±S.E.=1.461±0.173, Z=2.187, P_z=0.028, D-Strep, β±S.E.=8.406±0.143, Z=14.875, P_z<0.001). Thus,

removing or altering the microbiota increased the risk of death before adulthood.

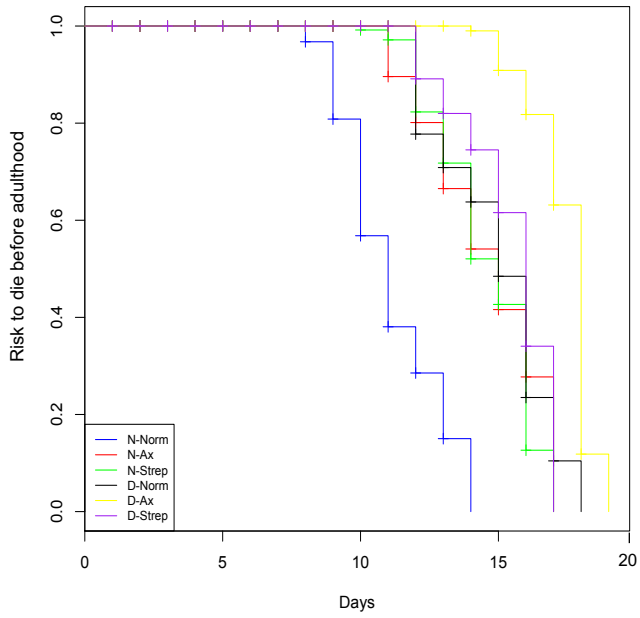

Figure 2. Risk of death before adulthood measured over time in days when eggs were dechorionated (D), or not (N), and when larvae were reared in a conventional diet (Norm), an axenic diet (Ax), or an antibiotic-supplemented diet (Strep).

2.4.2 Egg to adult survival

Globally, across all treatments, dechorionation ($P<0.001$) and larval treatments ($P<0.001$) affected egg to adult survival both as factors and via interaction ($P=0.024$) (Figure 3). More specifically, larval treatments (Ax, and Strep) significantly increased mortality during development compared to Norm when eggs were intact (N-Norm - N-Ax: $P<0.001$, N-Norm – N-Strep: $P<0.001$, N-Ax – N-Strep: $P<0.001$). In dechorionated eggs, only the Ax treatment increased mortality during development compared to Norm and Strep (D-Norm – D-Ax: $P<0.001$, D-Norm – D-Strep: $P=0.434$, D-Ax – D-Strep: $P=0.011$). Furthermore, egg dechorionation also increased mortality

within larval treatments (N-Norm - D-Norm: P<0.001, N-Strep – D-Strep: P<0.001, and N-Ax – D-Ax: P<0.001).

In this assay it should be noted that egg to adult survival for non-dechorionated eggs and conventionally reared larvae is quite low (mortality rate of 60%) compared to previous studies where egg to adult viability is approximately 100% (Kristensen et al., 2015). However, as non-dechorionated eggs were manipulated the same way as dechorionated eggs, but without the chemical agents to remove the chorion, we are confident that the results are comparable.

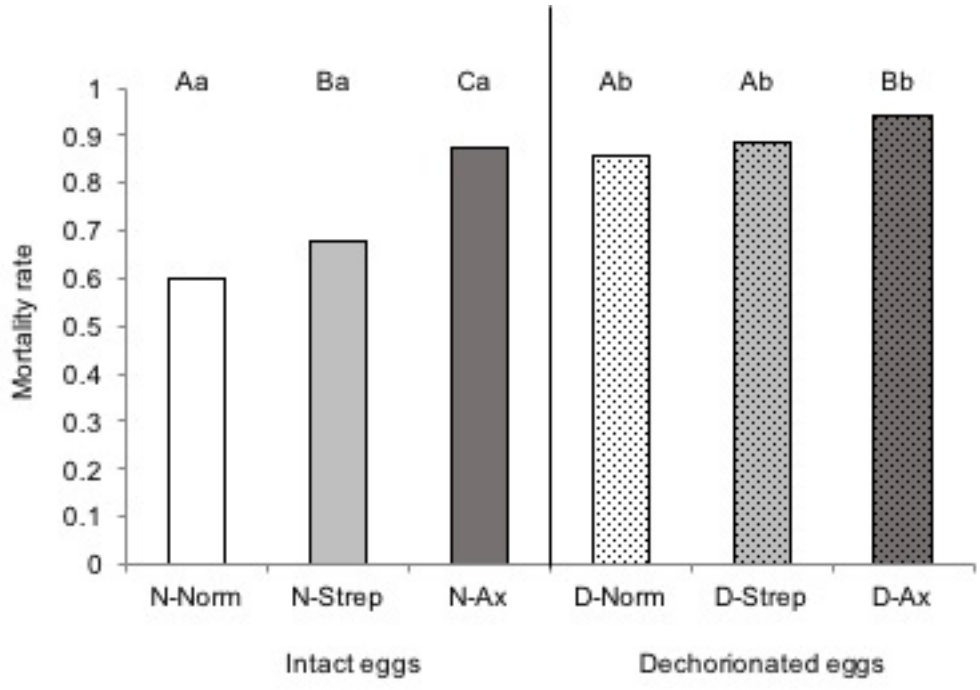

Figure 3. Egg to adult survival measured as mortality rate when eggs were dechorionated (D) or not (N), and when larvae were reared conventionally (Norm), or with the antibiotic streptomycin (Strep), or with axenic media (Ax). Different uppercase letters represent significant differences between larval treatments within egg treatment, while different lowercase letters represent significant differences within larval treatment between egg treatments.

2.4.3 Weight

Unsurprisingly, adult males were always found to weigh less than adult females across all treatments (P<0.001). When males and females are treated separately, dechorionation (P<0.001) and larval treatments (P<0.001) affected male adult weight both as factors and via interaction (P=0.024) (Figure 4). In intact eggs, Ax and Strep larval treatments significantly decreased male adult weight compared to Norm (N-Norm – N-Ax: P<0.001, N-Norm – N-Strep: *P*<0.001, N-Ax – N-Strep: *P*=0.011). By contrast, in dechorionated eggs, Ax treatment increased male adult weight when compared to Norm (D-Ax – D-Norm: P=0.011), and Strep (D-Ax – D-Strep: P<0.001), while Strep decreased male adult weight when compared to Norm (D-Strep – D-Norm: P<0.001). Furthermore, egg dechorionation also decreased male adult weight within larval treatments (N-Norm – D-Norm: P<0.001, N-Strep – D-Strep: P=0.020), except for Ax (N-Ax – D-Ax: P=928).

In females, dechorionation (P<0.001) and larval treatments (P<0.001) affected female adult weight both as factors and via interaction (P<0.001) (Figure 4). In intact eggs, Ax and Strep larval treatments significantly decreased female adult weight compared to Norm (N-Norm – N-Ax: P<0.001, N-Norm – N-Strep: P<0.001), while Ax had no effect on female adult weight compared to Strep (N-Ax – N-Strep: P=0.372). In dechorionated eggs, only the Strep larval treatment significantly decreased female adult weight compared to Norm (D-Norm – D-Strep: P=0.019), or Ax (D-Ax – D-Strep: P=0.009), while Ax larval treatment had no significant impact on female adult weight (D-Norm – D-Ax: P=0.997). Furthermore, egg dechorionation decreased female adult weight within the Norm treatment (N-Norm – D-Norm: P<0.001), while increasing it within the Ax treatment (N-Ax – D-Ax: P=0.006), but egg dechorionation had no effect within the Strep treatment (N-Strep – D-Strep: P=448).

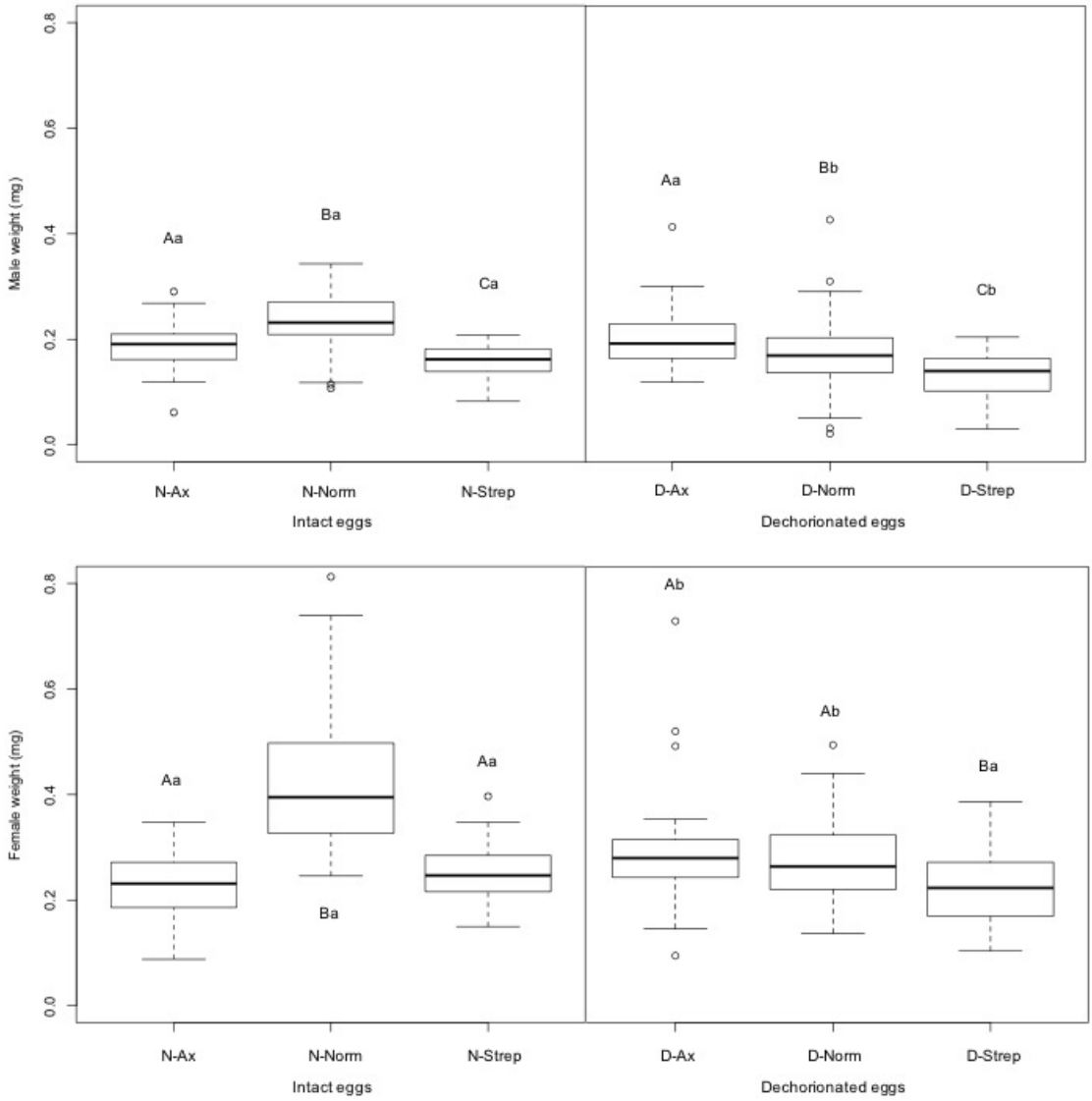

Figure 4. Boxplot of adult male and female weight according to egg treatments (dechorionated (D) or not (N), and larval treatments (conventionally reared (Norm), axenic medium (Ax), or antibiotic-supplemented medium (Strep)). Different uppercase letters represent significant differences between larval treatments within egg treatment, while different lowercase letters represent significant differences within larval treatment between egg treatments.

2.4.4 Starvation

As expected, males and females did not react the same way to starvation stress, with males dying more quickly than females (*Coxph*, β±S.E.=0.424±0.193, Z=-4.431, P_z<0.001). Thus, males and females were analysed separately.

In females, egg dechorionation (*Surdiff,* χ^1_2=117, P<0.001) as well as larval treatments (*Surdiff,* χ^2_3=90.6, P<0.001) affected female survival (Figure 5a). Egg dechorionation increased female resistance to starvation (*Coxph*, β±S.E.=0.508±0.172, Z=-3.918, P_z<0.001). Axenic rearing of the larvae had no significant impact on female resistance to starvation when compared to conventionally reared larvae (*Coxph*, β±S.E.=1.379±0.172, Z=1.864, P_z=0.062). In contrast, antibiotic rearing of the larvae decreased female resistance to starvation when compared with conventionally reared larvae (*Coxph*, β±S.E.=2.092±0.144, Z=5.122, P_z<0.001).

In males, egg dechorionation had no significant impact on male resistance to starvation (*Surdiff,* χ^1_2=1.1, P=0.291) (Figure 5b). In contrast, larval treatments affected male resistance to starvation (*Surdiff,* χ^2_3=450, P<0.001), with axenic rearing of the larvae (*Coxph*, β±S.E.=2.191±0.257, Z=3.050, P_z=0.002) in addition to antibiotic rearing of the larvae (*Coxph*, β±S.E.=2.162±0.245, Z=3.146, P_z=0.001), increasing male resistance to starvation when compared to conventionally reared larvae.

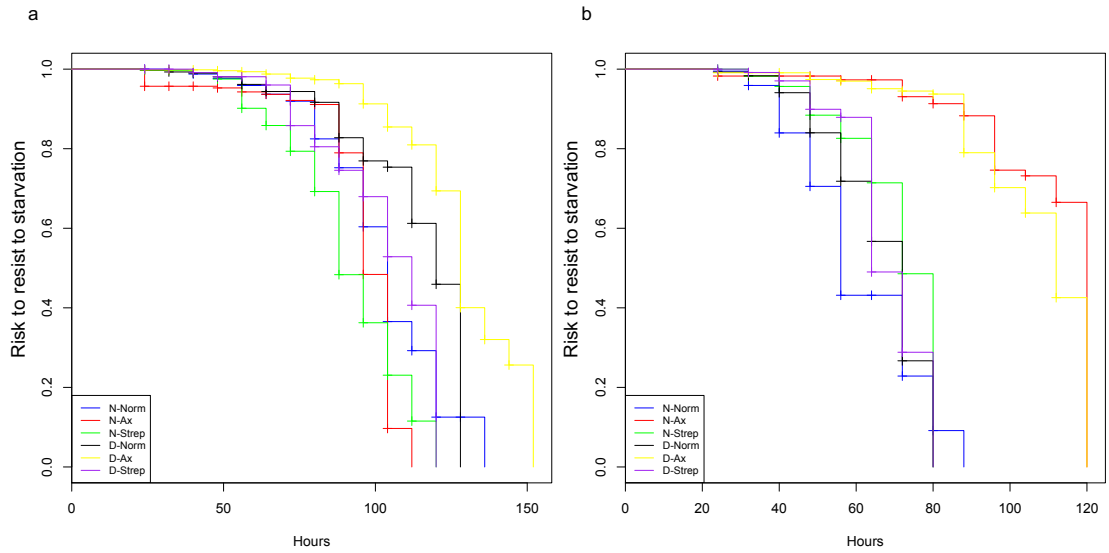

Figure 5. Female (a) and male (b) survival curves representing the risk to resist starvation over time in hours when eggs were dechorionated (D), or not (N), and reared as larvae in a conventional diet (Norm), an axenic diet (Ax), or an antibiotic-supplemented diet (Strep).

2.4.5 Response to stress (RING assay)

Global effects show that sex (P=0.311) had no significant effect on fly locomotion. However, sex interacted significantly with larval treatments (P<0.001) in determining fly locomotion. Therefore, we treated males and females separately.

In males, larval treatments (P=0.001) affected their locomotion as a factor and via an interaction with egg treatments (P=0.001), while egg treatment as a factor had no significant effect on male locomotion (P=0.988) (Figure 6). In intact eggs, Ax and Strep larval treatments had no significant effect on male locomotion (N-Norm – N-Ax: P=0.913, N-Norm – N-Strep: P=0.051, N-Strep – N-Ax: P=0.518). By contrast, in dechorionated eggs, Ax significantly reduced male locomotion compared to Norm (D-Ax – D-Norm: P=0.001), or Strep (D-Ax – D-Strep: P=0.006), while no significant effects on male

locomotion was found for Strep when compared to Norm (D-Norm – D-Strep: P=0.999). Furthermore, egg dechorionation had no effect on male locomotion within larval treatments (N-Norm – D-Norm: P=0.090, N-Ax – D-Ax: P=0.153, N-Strep – D-Strep: P=0.990).

In females, larval treatments (P=0.001) affected their locomotion as a factor and via an interaction with egg treatments (P=0.004), while egg treatment as a factor had no significant effect on female locomotion (P=0.139) (Figure 6). In intact eggs, Ax larval treatment significantly reduced female locomotion when compared to Norm (N-Norm – N-Ax: P<0.001), and Strep (N-Strep – N-Ax: P=0.001), while Strep had no effect on female locomotion when compared to Norm (N-Norm – N-Strep: P=0.999). In dechorionated eggs, both Ax and Strep larval treatments significantly reduced female locomotion compared to Norm (D-Ax – D-Norm: P<0.001, D-Strep – D-Norm: P<0.001, D-Ax – D-Strep: P<0.001). Furthermore, egg dechorionation had no effect within Norm and Ax larval treatments (N-Norm – D-Norm: P=0.545, N-Ax – D-Ax: P=0.829), while it significantly decreased female locomotion within the Strep larval treatment (N-Strep – D-Strep: P=0.032).

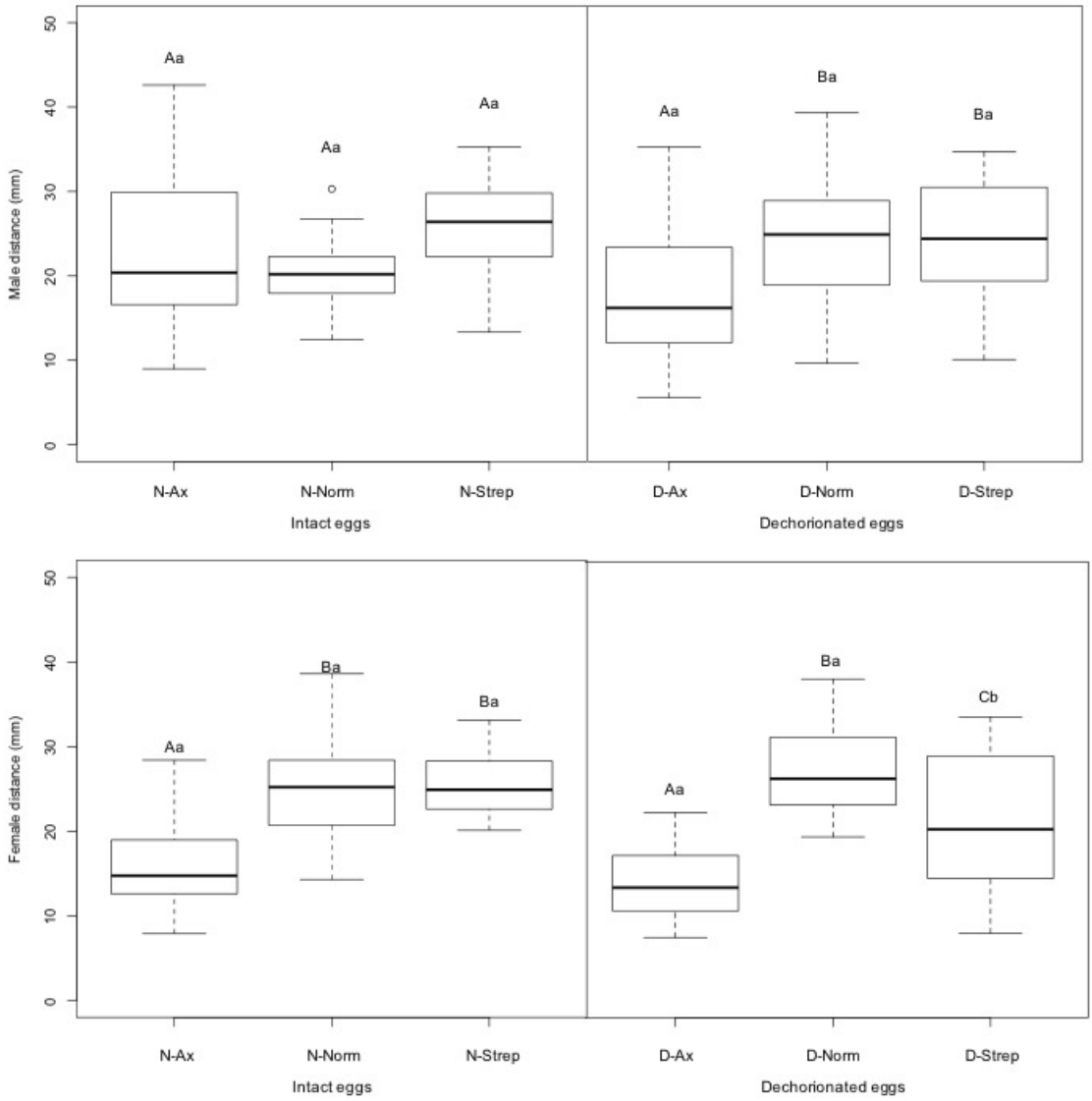

Figure 6. Boxplot of male and female locomotion, measured as distance travelled (RING) according to egg treatments (dechorionated (D) or not (N), and larval treatments (conventionally reared (Norm), axenic medium (Ax), or antibiotic-supplemented media (Strep)). Different uppercase letters represent significant differences between larval treatments within egg treatment, while different lowercase letters represent significant differences within larval treatment between egg treatments.

2.4.6 Bacterial analysis

In order to assess the efficacy of each treatment in eliminating the gut microbiota, we dissected the midgut of adult *D. melanogaster* and used spread plates on to MRS media to determine the contents. We analysed the bacterial content of the midgut as this is one of the only larval structures that stays intact during pupation. It is known that a sharp decrease in bacterial density occurs 24 hours after pupation, only increasing again after 48 hours (Storelli et al., 2011), but the midgut is contained and develops within a transient pupal epithelium (Takashima et al., 2011). As the midgut remains unchanged during pupation whilst almost all other structures are histolyzed, the midgut is an accurate representative of the gut bacterial content and diversity within an adult *Drosophila*. We also analysed the bacterial content of the whole fly in a similar manner in order to determine whether our treatments affected the whole host microbiota. We used Colony Forming Unit (CFU) counts to measure the bacterial load of flies from each treatment in triplicate by taking the average, which is a standard measure of estimating bacterial load (Nadkani et al., 2002). Only one species of bacteria was detected across all treatments and Sanger sequencing identified this as *Lactobacillus brevis*.

We discovered bacterial colony growth on all plates from all treatments, except those from flies reared on the streptomycin diet alone. In the case of the latter, there were zero colonies present on all spread plates containing the dissected midgut. For D-Strep flies, only one out of three replicate midgut plates contained any colony growth (Table 2), with the other two replicates containing zero colonies. This is likely an anomaly due to potential contamination of the media during spread plating, or transfer of bacteria from other parts of the fly during midgut dissection.

The results for the midgut contrast with the results of the whole fly spread plates, in which colony growth occurs on all replicates for both the N-Strep and the D-Strep flies (Table 2), though it can be noted that these results are considerably lower compared to all other treatments. Considerably more colonies were found for the whole fly spread plates for each treatment in

comparison to the midgut contents. The highest number of colonies was found on the normal treatment, which is to be expected (Table 2). Yet similar numbers of bacterial colonies were found for the whole fly plates from the axenic and the egg dechorionated, axenic treatment.

Table 2: Number of bacterial colonies

Treatments	Origin of bacteria	Average number of bacterial cells per gut in each replicate
N-Norm	Gut	3.1x10^1
	Gut	5.9x10^1
	Gut	6.2x10^1
	Whole fly	4.5x10^2
	Whole fly	3.8x10^2
	Whole fly	6.3x10^2
N-Strep	Gut	0
	Gut	0
	Gut	0
	Whole fly	2.1x10^1
	Whole fly	2.8x10^1
	Whole fly	1.0x10^2
N-Ax	Gut	1.8x10^2
	Gut	3.3x10^2
	Gut	2.0x10^2
	Whole fly	5.5x10^2
	Whole fly	5.7x10^2
	Whole fly	4.4x10^2
D-Norm	Gut	5.5x10^1
	Gut	2.8x10^1
	Gut	8.3x10^1
	Whole fly	1.4x10^2

	Whole fly	7.3x10^1
	Whole fly	6.4x10^2
D-Strep	Gut	0
	Gut	0
	Gut	0.4x10^1
	Whole fly	0
	Whole fly	4.5x10^1
	Whole fly	2.1x10^1
D-Ax	Gut	0.04x10^1
	Gut	1.0x10^1
	Gut	7.6x10^1
	Whole fly	6.2x10^2
	Whole fly	5.3x10^2
	Whole fly	4.5x10^2

2.5 Discussion

Effectively eliminating the resident gut microbiota is essential to the study of host-microbiota interactions, through which we can gain a greater understanding of a species' fundamental ecology. From the array of physiological assays conducted, it is clear that manipulating the microbiota has a profound effect on the overall health of the host. This is particularly true for the risk of death before adulthood and adult weight; individuals from all treatments took significantly longer to develop, and weighed less, compared to normal flies. This is hardly surprising considering the gut microbiota is known to affect a wealth of host developmental and physiological processes (Sommer & Backhed, 2013). In *D. melanogaster*, a

symbiotic relationship exists between the fly and its gut microbe, *Acetobacter pomorum* (Shin et al., 2011). Acetic acid produced by the alcohol dehydrogenase of *A. pomorum* initiates insulin signalling and thereby tunes the homeostatic signalling of the fly, controlling a variety of factors including developmental rate and body size.

In terms of mortality rate of individuals, considerably fewer flies survived to adulthood when reared on axenic and streptomycin diets compared with normal flies. Sterilisation of the diet by rendering it axenic had the most profound effect on egg to adult survival. Removal of the egg chorion also increased mortality rate in all larval treatments (Norm, Strep, and Ax). Dechorionation involved the use of bleach and alcohol to remove the chorion, which acts as a barrier to the environment, and protects against dehydration in insects such as coleopterans (Biémont, Chauvin & Hamon, 1981) and dipterans (Klowden, 2013). Thus, dechorionation in itself (i.e. the absence of the barrier) might explain the higher mortality rate observed. Sterilisation or antibiotic supplementation of the diet kills all or part of the bacteria present in the diet that are ingested by the flies. These bacteria could be used as a food source by the flies and/or help the flies in digesting complex carbohydrates present in the diet, as shown by previous studies (Storelli et al., 2011; Wong et al., 2015). Some of the treated flies could thus have died due to poor nutrition and/or inability to develop through their life cycle. Our findings contrast to previous studies that found that dechorionation had no effect on survivorship from egg to adulthood (Ridley, Wong & Douglas, 2013). The stark differences in these results highlight the importance for individual laboratories to evaluate the impacts of the methods employed to remove or alter the microbiota in their experiments. Such differences in results are likely due to the ability of different strains of *D. melanogaster*, for example, wild type compared to laboratory strains, to cope with environmental stressors.

Fly responses to starvation were sexually dimorphic. Males exhibited higher resistance to starvation and thus survival when reared in a diet free of or with reduced bacterial load (the axenic, antibiotic treatments). Egg dechorionation had no effect on male resistance to starvation. In contrast, females exhibited

increased resistance to starvation when their eggs were dechorionated; being reared in an axenic diet had no effect, and an antibiotic-supplemented diet decreased female resistance to stress. From these results, it is clear that antibiotic has some deleterious effects on females when they are faced with starvation, and some beneficial effects on male resistance to starvation. Thus, there is a contradictory effect of antibiotic according to sex. Egg dechorionation and axenic rearing of the larvae increased resistance to starvation in females and males respectively. However, depending on sex, removal of bacteria could be beneficial when starving. Different scenarios possibly explain this. Bacteria residing in the guts need to feed in order to develop and may compete with the host for nutritional resources. An alternative explanation is that some bacteria may have deleterious effects on the host, and in their absence the flies are healthier.

The presence/absence of bacteria in the diet during development of the fly also altered locomotion in relation to sex, while egg dechorionation had no impact. Females showed a decrease in their level of activity when reared in an axenic and/or antibiotic-supplemented medium. This result demonstrates that bacterial feeding by females during development is essential for activity levels. Males are less affected by the absence of bacteria during development. Potentially females' needs are higher than males due to egg production; bacteria may participate in this process either through the digestion of nutrients, or through the hormonal pathway. Indeed, *Lactobacillus plantarum* is known to control hormonal growth signalling (Storelli et al. 2011). It could be that the symbiosis between the fly and their gut microbiota is tighter in females than males, rendering females more susceptible to the absence of bacteria during development.

In addition to determining deleterious effects of treatments on the overall health and physiology of the fly, a key part of this study was confirmation of the effectiveness of each treatment. Our results showed that flies reared on a streptomycin diet had their gut bacteria completely eliminated; no bacteria were present on the plates. This result remained fairly consistent for the egg dechorionation, streptomycin treatment, in which two of the replicates were devoid of bacteria. One of these replicates, however, did contain some

bacteria, though at low titre, and is likely to have resulted from contamination from another part of the *Drosophila* during dissection. The treatments containing streptomycin did however, still possess substantial amounts of bacteria when the whole fly was analysed, although less than the normal flies. This is to be expected, as adding streptomycin to the dietary media was designed to specifically eliminate the gut microbiota, rather than the entire *Drosophila* microbiota. Both treatments reared on axenic media contained similar numbers of colonies to the normal flies. Across all treatments, we identified the bacteria present as *Lactobacillus brevis*, a bacterium that has been previously found to dominate in flies with reduced bacterial diversity, as a result of being reared on a sterile diet (Broderick, Buchon & Lemaitre, 2014).

An essential aspect of behavioural experiments relies on the ability to easily manipulate individuals when conducting an experimental design. In *Drosophila*, and other insect research, aspirators are commonly used to move individuals between treatments, as it allows for individuals to be manipulated without the use of carbon dioxide anaesthesia, which has been shown to negatively impact on mating behaviour in some species (e.g. Verspoor et al. 2015). Producing axenic or egg dechorionated individuals inhibits this ability to aspirate flies directly, in order to prevent external bacteria being transmitted onto the fly or their immediate environment, which could potentially confound experimental results. Therefore, we propose that the purpose of the experiment be an integral factor when considering which gut microbiota elimination method to choose; based on our results we would suggest that the addition of streptomycin to the dietary media is the most favourable for behavioural research.

D. melanogaster is one of the most useful and powerful models to study host-microbiota interactions. The fly harbours differing levels of bacterial diversity depending on rearing condition (for example natural *versus* laboratory), but overall this diversity is disproportionately lower than in mammals. Thus, the fly is a highly convenient model for evaluating interactions between bacteria, and between bacteria and the host, and how these interactions effect the host. To date, most studies of the interactions of

D. melanogaster with its microbiota have focussed on the molecular dialog between them (Storelli et al., 2011; Lhocine et al., 2008); Buchon et al., 2009). Our study highlights the need to take into account not only the molecular dialog, but also the final phenotypic effects of the interaction between the host and its microbiota, in terms of host fitness traits, as these could have strong evolutionary implications for host populations. It also demonstrates that the addition of streptomycin to the larval growth media effectively eliminates the resident bacteria within the *Drosophila melanogaster* gut whilst resulting in the fewest non-specific, deleterious effects in our host organism. However, it is important to consider that microbiota even within the same species/strains can differ between laboratories, so evaluating individual methods is necessary for a robust experimental design. Of equal importance is the consideration of the type of experiment performed. Adding low-dose streptomycin to the dietary media is the most reliable and practical method of eliminating the gut bacteria, whilst still allowing easily manipulation of the host for behavioural experiments, and without introducing external bacteria. This method has the potential for widespread use for elucidating the understanding of host-microbiota systems, not only in *Drosophila*, but across all other insect systems.

3. Chapter Three: *Drosophila* sexual attractiveness in older males is mediated by their microbiota

3.1 Abstract

Females of many species discriminate between males on the basis of age. However, the reasons and mechanisms behind these choices are not well understood, with several competing theories and little consensus. One factor increasingly shown to be a vital component of mate choice is the microbiota carried by individuals. In this study we examine whether the microbiota influences female preference for older males in the fruit fly *Drosophila pseudoobscura*. We find that an intact microbiota is a key component of attractiveness in older males. However, we found no evidence that this decrease in older male attractiveness was simply due to impaired microbiota generally reducing male quality. Instead, we propose that the microbiota underlies an honest signal used by females to assess male age, and that impaired microbiota disrupt this signal. This suggests that age-based preferences may break down in environments where the microbiota is impaired, for example when individuals are exposed to extreme temperatures, naturally occurring antibiotics, or in animals reared in laboratories on antibiotic supplemented diet.

3.2 Introduction

Choosing the right mate can have a major impact on a female's fitness (Trivers, 1972). Where males only provide sperm to females, females often choose mates in order to gain genetic benefits for their offspring (Byers and Waits, 2006; Suzaki et al., 2013). One key factor that can influence the value of a male as a mate, and hence his mating success, is his age, and females in many species show preferences for males of particular ages (e.g. Jones, Balmford and Quinell, 2000; López, Aragón and Martin, 2003; Kleindorfer 2007). However, there are several competing theories that suggest different reasons for how and why male quality will vary with age, and hence the age

preference females should show. For example, older males may be genetically superior to young males as they are proven survivors, potentially indicating that they possess fewer maladaptive alleles (Manning, 1985; Brooks and Kemp, 2001). Another suggestion is that signals of quality are more reliable in older males (Proulx, Day and Rowe, 2002). Alternatively, older males might experience negative impacts of pleiotropic genes that enhance their success when younger, but reduce their fertility and reproductive rate when older (Bonduriansky and Brassil, 2002; Price and Hansen, 1998). A build-up of harmful germ-line mutations in older males could also reduce their offspring's fitness (Hansen and Price, 1995).

Currently there is no consensus on whether females should prefer older or younger males, nor how they can judge male age. The experimental data also has not reached a consensus. For example, female preference for old males has been documented in a number of species of *Drosophila* (Moulin et al., 2001; Avent, Price and Wedell, 2008; Somashekar and Krishna, 2011), with preference for both young males (Wedell and Ritchie, 2004) and males of an intermediate age (Jones, Balmford and Quinell, 2004) shown in a variety of other insects. Within the Dipterans, experimental work has found female preference for young males (e.g. Shelly, Edu and Pahio, 2011; Papanastasiou et al., 2001), and old males (e.g. Avent, Price and Wedell, 2008), while some Coleopteran females prefer males of intermediate age (e.g. Liu et al., 2011).

Similar variation in whether older or younger males are preferred mates is also seen in the *Gryllus* genera of field crickets, for example (Verburgt, Ferreira and Ferguson, 2011). At present, conflicting theories and a lack of empirical evidence means we have limited ability to predict when preference for older or younger males will evolve in a species. Moreover, one can question why females evolve preferences for particular male ages. For example, a preference for older males could be a true preference, with females benefiting from mating with older males, and using some honest signal of male age to make their choice. Alternatively, older males may simply be better at harassing or manipulating females into mating, despite this not benefitting the female.

Recently it has become increasingly clear that an individual's microbiota can have a major impact on attractiveness. The microbiota consists of the symbiotic and commensal bacteria associated with a host that live on and within them. Although microbiota can refer to all type of micro-organisms associated with a host or a particular environment (yeast, fungus, etc.), most authors restrict its definition to bacteria (e.g. Brucker and Bordenstein, 2012), and we will use this definition throughout this article. Within the microbial community, recent studies have stressed the importance of gut microbiota in particular, on the evolution of mate preferences (Markov et al., 2009; Sharon et al., 2010; Sharon et al., 2013; Lizé, McKay and Lewis, 2014; Najarro et al., 2015). The microbiota community associated with *Drosophila* is subject to spatio-temporal variations (Chandler et al., 2011; Wong, Chaston and Douglas, 2013), and has been shown to change through development as well as ageing (Wong, Ng and Douglas, 2011). In *Drosophila melanogaster*, the presence of bacteria during early or late adulthood has contradictory effects, either enhancing or decreasing adult longevity respectively (Brummel et al., 2004). In addition, an age-related deterioration of gut homeostasis occurs during natural aging, which is affected by the presence of (Buchon et al., 2009) and variation within (Buchon et al., 2009); Ryu et al., 2008) the *Drosophila* gut microbiota community. Thus, the microbiota may be particularly likely to impact on age-based preferences. However, it is currently unknown whether the microbiota does play a role in age preferences.

In the fruit fly *Drosophila pseudoobscura* females prefer to mate with older males (Avent, Price and Wedell, 2008). In this species, females are quicker to accept matings when courted by an older male, and in trials where old and young males compete for a mating, the older males typically win (Avent, Price and Wedell, 2008; but see Dhole and Pfennig, 2014). Older males also copulate for longer and probably invest more sperm in matings with females (Avent, Price and Wedell, 2008), and females produce more offspring from mating with older males, although not extremely old males (Dhole and Pfennig, 2014). There are two potential reasons for this mating bias. Older males may be more experienced, faster or more dominant and so have a

competitive advantage over younger males, and be better at courting a female to mate with them. However, it could be that this apparent preference exhibited for older males is a true female choice, reflecting female use of an honest signal to assess male age. In this species, the evolution of diet induced mate preferences has been demonstrated, with both males and females performing assortative mating with individuals that has evolved on the same diet for several generations in the laboratory (Dodd, 1989). Thus, mating preferences appear to be complex in this species, entailing different factors that could all be influenced by the microbiota associated with the species.

In this study we examined whether the microbiota associated with a fly underlies the preference for older males in *D. pseudoobscura*. Preference was measured in both no choice (single male) and choice (two males) competitive mating trials, where the microbiota was either intact or impaired. We hypothesised that females would prefer to mate with older males, but that this preference would disappear when the microbiota is impaired. However, males with impaired microbiota might be poor at acquiring mates simply because an impaired microbiota is costly, resulting in male physical impairment. To test this possibility, we also examined whether suppression of the microbiota impacted on standard measures of male *Drosophila* activity and competence.

3.3 Materials and Methods

D. pseudoobscura were collected in Show Low, Arizona in 2008, with offspring from approximately 70 wild caught females combined to produce a mixed outbred population. Flies were maintained in the laboratory at a population size of 400 adults per generation for approximately 50 generations. All flies were kept and reared at 22°C on a 12:12 hour light:dark cycle. Flies were kept in standard 75x25mm *Drosophila* vials containing 25ml of standard *Drosophila* food composed of yeast/agar/maize/sugar. Flies were moved to new vials every 4 days.

3.3.1 Manipulation of the microbiota

The microbiota was impaired via the addition of the antibiotic streptomycin (4ml of 10g streptomycin/100ml ethanol solution per litre of growth medium) to the growth medium. Adding antibiotic to dietary medium is a common method to suppress insect microbiota (e.g. Sharon et al., 2010; Lin et al., 2015), and has few side effects in *Drosophila* (Graf and Benz, 1970; Heys et al., 2018b) when used at low concentrations. This strain of *D. pseudoobscura* does not carry any bacterial endosymbionts.

In order to determine that the microbiota is in fact impaired, we analysed the *D. pseudoobscura* gut bacterial content. Here, the whole gut of males from both the old and young treatments from both the normal and streptomycin supplemented diet, were dissected into 250µl BHI (Brain Heart Infusion) liquid media. The gut-solute was then transferred into a 1.5ml Eppendorf tube and disrupted by hand using a sterile plastic pestle. From this solution, 100µl was placed in the centre of a petri dish containing BHI agar. A sterile glass loop was then used to spread the solute across the whole plate. This was repeated three times for both ages from both the normal and streptomycin diets. The plates were then incubated at 25°C for 72 hours, after which, the plates were checked for bacterial growth and CFU (colony forming units) counts were then performed to quantify the bacterial load.

3.3.2 Preference in no choice mating trials

Recently mated females were placed on 25ml of either standard diet (here, named Strep-) or diet containing streptomycin (Strep+) and allowed to oviposit to form two populations. At eclosion, virgin males were isolated twice daily from each diet type to form the 'old' male experimental treatment (ten days old). Eight days later, further virgin males were collected to form the 'young' male treatment (two days old). Virgin females were collected from a separate set of vials that did not contain streptomycin. Mating trials were staggered over several days in order to ensure a high replication rate and reliability. Isolated virgin males were left to mature on the same food medium on which they were reared (either Strep+ or Strep-). Males were kept singly

to avoid any potential effects of male-male interactions (Lizé et al., 2012; Lizé et al., 2014). Females were stored in groups of ten on food without streptomycin until they were four days old. Following maturation, the mating trials were conducted. Virgin females were gently aspirated onto 15ml of standard food media and allowed to rest overnight. Mating trials were conducted during the morning, as this is when *D. pseudoobscura* is the most active in the wild (Dobzhansky and Epling,1944). Either an 'old' or 'young' male was then gently aspirated into the vial and the observations begun. We recorded whether or not copulation occurred, in addition to mating latency (time elapsed between male introduction and copulation), and the duration of copulation. In this experiment, female preference is reflected by the mating latency (the time it takes a male from being placed in the vial to start mating with the female), which is a commonly used indicator of female preference in *Drosophila* (e.g. Somashekar and Krishna, 2011; Speith, 1974; Economos et al., 1979; Noor and Coyne, 1996; Lefranc and Bundgaard, 2000; Verspoor, Cuss and Price, 2015; Prathibha, Krishna and Jayaramu, 2011).

3.3.3 Preference in choice competitive mating trials

In order to measure female preference when males can compete for matings, we set up trials as above, but placed two males in the vial with each female, one old male and one young one. We recorded whether or not copulation occurred, in addition to the mating latency, and the duration of copulation. Wing clipping was used in order to distinguish between the two males. This is a standard technique used in *Drosophila* research that allows the simple and accurate detection of an individual (Ehrmann, 1966; Ehrmann and Petit, 1968; Byrant, Kence and Kimball, 1980; Knoppien, 1984). Two days before the mating trials, virgin males were isolated under ice anaesthesia and a small section of the distal end of one wing was cut off. All males were wing clipped, with half clipped on the left wing, half clipped on the right, and side was randomised across treatment in order to remove any potential bias, although wing clipping has been shown to have no effect on mating propensity in *D. pseudoobscura* (Dodd, 1989).

3.3.4 Measurement of male activity

Differences in male performance could be due to the result of the different treatments and males with impaired microbiota are physically impaired and thus less able to court the female. To test this possibility we ran an independent test of male speed and responsiveness, the Rapid Iterative Negative Geotaxis (RING) test (Nichols, Becnel and Pandey, 2012). The RING test examines the climbing speed of flies after being knocked to the bottom of a vial, and provides a simple, repeatable and accurate measure of activity speed, which correlates well with other measures of activity and physical ability (Nichols, Becnel and Pandey, 2012). Newly emerged virgin adult males were isolated and gently aspirated into vials containing 15ml of either Strep+ or Strep- food, according to the diet on which they were reared, at a standard density of ten per vial. Following a ten-day maturation period, flies were transferred to a vial containing 15ml standard food media as before, placed in the RING apparatus and left to acclimate for 15-20 minutes. The apparatus was then sharply tapped three times on the counter, knocking all flies to the bottom of the vial, and a picture taken following a three second period. The flies were then left to rest for one minute, and the steps repeated, five times in total. Subsequently, each photo was examined, and the height climbed by each fly in each photo was calculated from the height of the vial and the proportion climbed by the fly above the level of the food, was calculated. Care was taken to ensure that each vial contained an identical height of food. Our measure of activity was the mean height climbed by the flies in each vial over the five trials. This generated an overall mean distance climbed for both the Strep+ and Strep- flies allowing comparisons in the overall physical condition of ten-day old virgin males, both with intact or impaired microbiota.

3.3.5 Data analysis

Data for the single male trials were analysed in R2.15.0 (R Foundation for Statistical Computing, Vienna, Austria) using generalised linear models. As

the latency data was not normally distributed, data were square-root transformed and analysed using a quasibinomial error structure with a logit link. In each case a maximal model was constructed, and then non-significant factors removed in a stepwise process to give the minimum adequate model. Mating success in the two male trials was analysed using binomial tests.

3.4 Results

3.4.1 Preference in no choice mating trials

Males with impaired microbiota had a significantly longer mating latency (F test: $F_{1,153}$=6.592, P=0.011) than males with intact microbiota (Figure 1). Age had no significant effect on copulation latency (F test: $F_{1,152}$=0.022, P=0.883), regardless of whether the microbiota was intact or impaired (F test: $F_{1,151}$=0.009, P=0.924).

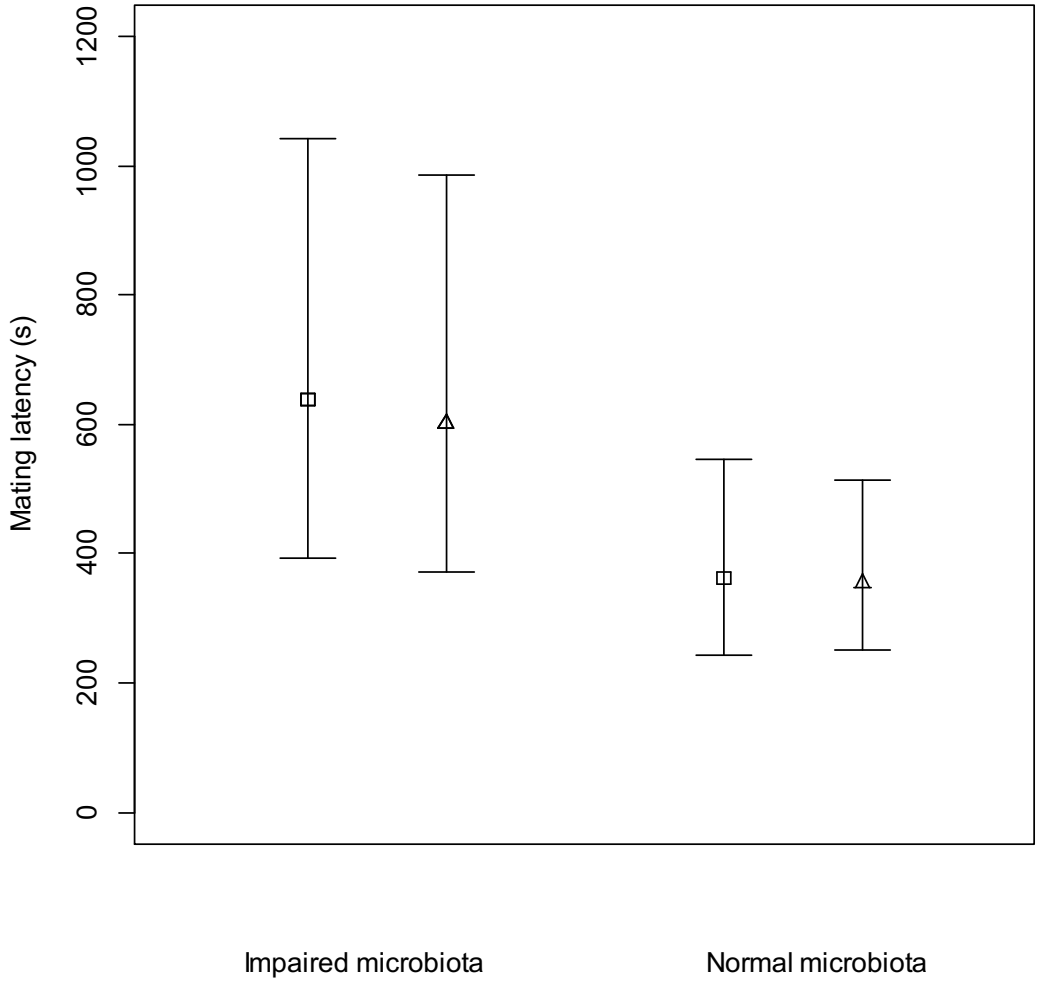

Figure 1. Mean copulation latency and 95% confidence intervals of old (square markers) and young (triangular markers) males, with either their microbiota impaired or intact (Normal) when placed with a single female.

Copulation duration was directly affected by male age (Figure 2), with older males copulating for significantly longer than young males (F test: $F_{1,154}$=44.71, P<0.001). Whether the microbiota was intact or impaired had no significant effect on copulation duration (F test: $F_{1,153}$=0.181, P=0.671), nor did the interaction between age and microbiota (F test: $F_{1,152}$=0.142, P=0.707).

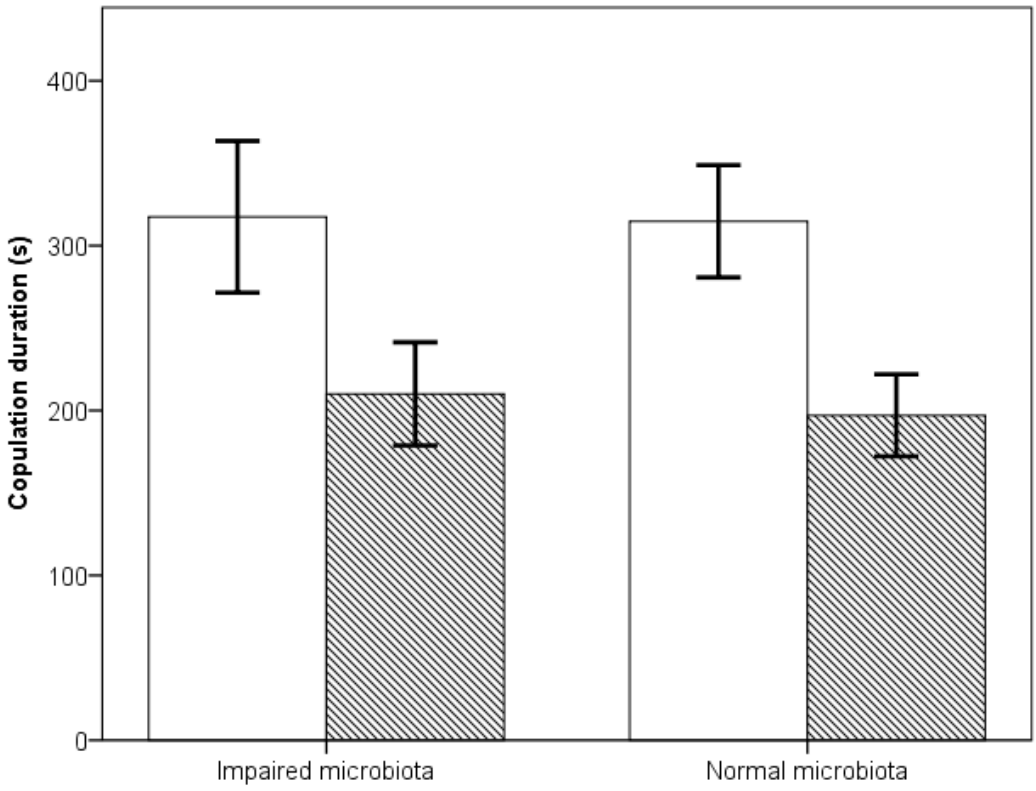

Figure 2. Mean copulation duration and 95% confidence intervals of old (open bars) and young (hatched bars) males, with either their microbiota impaired or intact when placed with a single female.

3.4.2 Preference in choice competitive mating trials

In this experiment where a female had to choose between an old and a young male with an intact microbiota, older males gained significantly more matings than their younger counterparts (number of trials: 52, number won by old male: 38, number won by young male: 14; binomial test; $P<0.001$). However, when the microbiota was impaired there was no difference in the success of old and young males (number of trials: 28, number won by old male: 15, number won by young male: 13; binomial test; $P=0.425$).

3.4.3 RING test of male activity

Males with impaired microbiota exhibited significantly higher upwards movement in the RING test than males with intact microbiota (impaired microbiota: mean±SD=18.3±7.1mm; intact microbiota: mean±SD=8.9±4.2mm; t-test: t=6.005, df=41.281, P<0.001); (Figure 3).

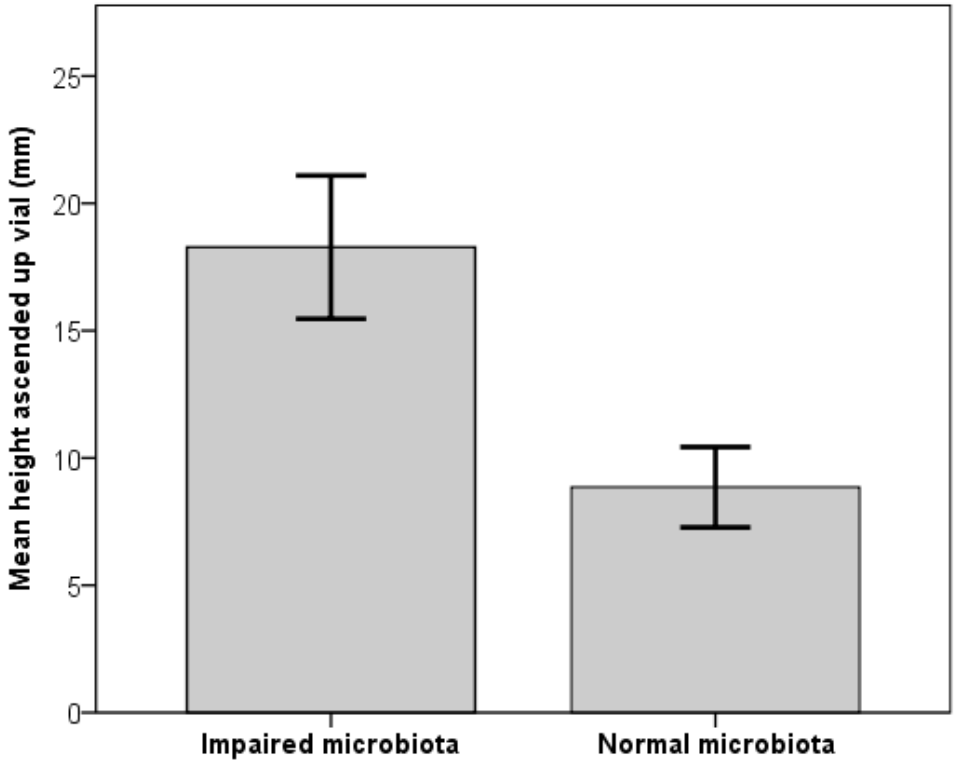

Figure 3. Mean height males climbed up a vial after being knocked to the base, using the RING test, with either their microbiota impaired (Strep+) or intact (Strep-). Error bars show 95% confidence intervals.

3.4.4 Gut microbiota

Plates containing the gut of flies from the normal diet had substantial colony growth (Table 1). There was a stark difference in the number of bacterial colonies present between the old and young males from the normal diet treatment. There were no bacterial colonies present on plates that contained flies reared on dietary media that was supplemented with streptomycin

(Table 1). This was the case for both the old and young treatments. This suggests that the gut microbiota has been impaired.

Table 1. Bacterial colony counts of the whole gut from male flies of both old and young ages, from both normal and antibiotic-supplemented diets.

Diet	**Age**	**Replicate**	**Number of colonies**
Normal	Young	1	1
Normal	Young	2	3
Normal	Young	3	2
Antibiotic	Young	1	0
Antibiotic	Young	2	0
Antibiotic	Young	3	0
Normal	Old	1	6396
Normal	Old	2	8528
Normal	Old	3	12428
Antibiotic	Old	1	0
Antibiotic	Old	2	0
Antibiotic	Old	3	0

3.5 Discussion

Our results confirm that female *D. pseudoobscura* prefer older males in two-male choice trials. But surprisingly, this female preference for older males disappears when the males' microbiota is impaired. In no choice mating trials, we found no significant difference in how quickly old and young males were able to begin mating with a female, contrary to previous studies.

However, we found that microbiota impaired males, whether old or young, took more time to initiate mating in these no choice mating trials. These results suggest that an impaired microbiota makes males less attractive, and that this prevents females from expressing their preference for older males. Perhaps the simplest explanation for this would be that the impaired microbiota causes males to develop poorly, making them inadequate mates with limited ability to locate and court females. However, we found that suppression of the microbiota of old males had no negative impact on a simple test of physical fitness. Indeed, males with an impaired microbiota actually scored higher in the test used. Hence it is unlikely that impaired microbiota simply reduces male ability to locate and court females. Instead, we suggest that females can detect male age in older males by an honest signal, and that this honest signal is lost when males' microbiota is impaired.

One potential issue with our methodology is that we did not directly measure the impact of streptomycin on the male microbiota community in intact and antibiotic exposed flies. However, our objectives were not to show a particular correlation between a given microbiota community and sexual attractiveness through ageing in males. Instead we wished to demonstrate that a simple impairment of this community, through antibiotic treatment during development, can have wider effects later in life, particularly in the context of sexual attractiveness. Previous work has clearly demonstrated that antibiotic supplemented diets alter *Drosophila* microbiota (Sharon et al., 2010; Koukou et al., 2006; Miller, Erhman and Schneider, 2010; Storelli et al., 2011). Hence it is highly likely that our antibiotic exposed males did indeed have impaired microbiota.

A second potential concern is whether streptomycin exposure has any direct relevance to *Drosophila* in nature. The efficiency of antibiotics as antibacterials/antimicrobials has been widely recognised for centuries, and in human populations tetracycline supplemented diets have been used since 350–550 AD (Bassett et al., 1980; Nelson et al., 2010; Aminov, 2010). Hence it is possible that some populations of human associated *Drosophila* species may too have been exposed to antibiotics for centuries. In recent decades the release of antibiotics into the environment has become a major concern

(Kümmerer, 2003), and it is increasingly likely that *Drosophila* will encounter antibiotics in nature. In our experiment, we deliberately choose to use streptomycin as antibacterial, as it is naturally produced by a soil bacterium: *Streptomyces griseus* (Emerson de Lima Procópio et al., 2012). Moreover, the antibiotic properties of *Streptomyces sp* have been shown to be used by some insects as for example in the solitary digger wasp *Philanthus triangulum* to avoid fungal contaminations of their broods (Kaltenpoth et al., 2005). *Drosophila pseudoobscura* larvae are thought to feed on leaf litter among other food sources (Kaltenpoth et al., 2005), and thus might potentially be exposed to *Streptomyces* in the wild.

Assuming that the fly microbiota was impaired during the experiment, the next key question is whether the change in mating success of old and young males was due to a true change in preference by females, or was simply due to microbiota impaired males being generally damaged. It is often difficult to distinguish true female choice from passive female choice driven by innate differences in males: for example, if a class of males is able to court females more intensely and is more successful in gaining matings, is the male simply overcoming female resistance, or are females choosing this class of male because they gain adaptive benefits? In some models of mate choice, the question is irrelevant, but in others it is important (Brennan and Richard, 2012). In the current study, we used the RING test to give us a general measure of male activity. This measure correlates well with several other standard measures of *Drosophila* vigour and activity (Gargano et al., 2005). As microbiota impaired males performed slightly better in the RING test than normal males, there is little evidence to suggest that the impaired microbiota damaged males in any extensive way. In *Drosophila,* copulation duration is controlled by males, and is generally correlated with male reproductive investment (Price et al., 2008). In this experiment, copulation duration was not altered by the microbiota impairment of males, which adds support to the fact that microbiota impaired males do not suffer physiological alterations that would consequently affect their sexual behaviour. In a previous study, copulation duration was found to vary according to microbiota impairment, and authors acknowledged that the use of antibiotics could have more

general physiological effect on the flies (Lizé, McKay and Lewis, 2014). Our results suggest that streptomycin has little or no effect on male sexual abilities. Taken together, our results suggest that the impaired microbiota may reduce older male success through disrupting some signal females use to assess potential mates.

If females are using some potentially honest signal of male age, which is disrupted by antibiotics, what might this signal be? Perhaps the strongest candidate is the cuticular hydrocarbons (CHCs) that are a key sexual signal in *Drosophila* (Ferveur, 2005) and many other insects (reviewed in Blomquist, 2010). CHCs are widely referred to as sex pheromones, as they communicate essential information to a potential mate. For example, Scott *et al.* (2010) noted that slight changes in the composition of CHC profiles were shown to significantly alter mating success in *Drosophila* species. CHCs are strongly influenced by diet and environment. Ageing has been shown to alter the composition of CHC profiles in both the stingless bee, *Schwarziana quadripunctata* (Nunes et al., 2009), and mosquito species (Hugo et al., 2006). Similarly in *D. melanogaster*, it has been shown that ageing alters a variety of CHC compounds, with consistent variation amongst individuals suggesting that these changes with age are strongly regulated (Kuo et al., 2012). It is possible that a male's CHC profile provides an honest signal of age in *D. pseudoobscura*. However, if the microbiota is impaired, CHC profiles are likely to be altered, and may no longer be used/detected as an honest signal. Testing this hypothesis will require determining the CHC profiles of old and young males, with or without antibiotic exposure.

In choice mating trials the impact of removing the microbiota only negatively affected old males. Copulation duration was similar for old and young males who had their microbiota impaired. However, microbiota impaired old males were no longer preferred by females for mating, compared to old males whose microbiota was kept intact. In *D. melanogaster*, the presence of bacteria in young males increases their longevity, while decreasing it when present in old males (Brummel et al., 2004). Although, longevity effects of the presence/absence of bacteria have not been evaluated in *D. pseudoobscura,* one can envision that the presence/absence of bacteria in male *D.*

pseudoobscura may reflect their age and potential remating probabilities. Therefore, old and young male with impaired microbiota would be perceived as of similar ages by the female, while old males with intact microbiota could be perceived as having a lower probability of remating, and so potentially investing more in each copulation than a young male as they are likely to have fewer remaining opportunities to mate. Indeed, old males copulate for longer than young males regardless of their microbiota status (intact or impaired) in our experiment, and in a previous study (Avent, Price and Wedell, 2008). The impact that this may have on sexual selection in wild *D. pseudoobscura* could be profound. For example, in populations where flies feed on atypical food, or are exposed to extreme temperatures, the normal microbiota may be impaired in males. This would remove the honest signal of old age in this species and potentially allow younger males to gain increased access to females, thereby overcoming the evolution of female choice.

In conclusion, we find that *D. pseudoobscura* males reared on antibiotic supplemented diet have decreased attractiveness to females. This effect is particularly strong in older males, which causes females to lose their preference for them. This change in attractiveness is not simply due to microbiota impaired males having decreased energy or movement ability, because they perform better than normal males in a simple physical test. Instead, we suggest that females are using an honest signal to assess male age, and that impaired microbiota damages this signal in older males. This suggests age-based preferences may break down in environments where the microbiota is impaired by natural antibiotics, unusual diets, temperature extremes, or in animals reared in laboratories on antibiotic supplemented diet.

4. Chapter Four: A potential role for the gut microbiota in the specialisation of *Drosophila sechellia* to its toxic host, *Morinda citrifolia*

4.1 Abstract

Insects adapt to nutritionally poor food sources in a number of different ways. One way in which this can potentially occur is via the gut microbiota which is known to compensate against the harmful implications that a poor diet may have on host physiology. *Drosophila sechellia* is a specialist species and in the wild lives solely on the *Morinda citrifolia* fruit. The toxic compounds within the fruit are toxic to all other species of *Drosophila*, but *D. sechellia* has evolved resistance. The toxic basis of the fruit is caused by octanoic and hexanoic acid, with octanoic acid being the primary constituent. Presence of these compounds within the fruit also cause *M. citrifolia* to have a low, acidic pH at 3.86. A number of studies have aimed to uncover the genetic basis for this evolutionary transition in *D. sechellia*, but none have focussed on the potential role of the gut microbiota. Here, we examine the gut microbiota of wild-type, laboratory reared flies and determine their gut microbiota when reared on the natural host plant, versus a standard *Drosophila* diet. We show a rapid transition in the core bacterial diversity and abundance within this species and discover sole precedence of *Lactobacillus plantarum* when reared on *M. citrifolia*. We also discover that flies reared on a laboratory diet are more likely to carry bacterial pathogens such as *Bacillus cereus*, although their function in *Drosophila* is unknown. We also note the differences that rearing *D. sechellia* on these two diets, and an additional *Salacca zalacca* fruit diet similar in nutritional property to *M. citrifolia* but without the toxins, has on host physiology and behaviour. Flies reared on a laboratory diet have a significantly reduced weight but with no impact on the risk of death before adulthood or subsequent mating behaviours, including mating propensity and mating latency, when compared to the wild, *M. citrifolia* diet. This suggests that rearing flies on these diets has no effect on

mating ability or preference, due to the potential lack of large-scale changes in the scent profile (via cuticular hydrocarbons), which is determined by the gut microbiota. Further work is needed to determine the potential role that the gut microbiota plays in host specialisation within this species, from external factors affecting the gut microbiota, such as pH.

4.2 Introduction

The diversification of ecological niches can lead to an increase in biodiversity and animals employ different tactics in order to adapt to these niches. Some species are generalists, enabling them to thrive in a wide variety of environments, but in doing so they face an increased level of competition (e.g. McArthur, 1972). In contrast, specialists can only thrive in a narrow range of environments but benefit though reduced levels of competition (e.g. McArthur, 1972). The way in which these specialists adapt to life in a novel environment can occur through a number of different ways. One is through the gut microbiota (e.g. Bolnick et al., 2014; Morrow et al., 2015). In insects, for example, members of the order Hemiptera have evolved to feed on plant phloem sap - a nutritionally poor diet due to the grossly unbalanced amino acid composition (e.g. Douglas, 1993; Sandström & Moran, 1999; Sandström, 2000). A number of studies have demonstrated that all phloem feeders within this order possess certain symbiotic bacteria that mitigate the effects of this nutritionally poor diet (Buchner 1965; Gündüz and Douglas, 2009). For example, two specialist species of *Lepidoptera, Hyles euphorbiae* and *Brithys crini*, feed exclusively on latex-rich *Euphorbia sp*. and alkaloid-rich *Pancratium maritimum*, respectively (Vilanova et al., 2016). Metagenomic sequencing has identified that the primary microbiota within the gut is *Entereococcus sp*., which it is predicted to be responsible for mitigating the effects of these nutrient-poor diets to the host.

Similarly, the desert locust, *Schistocerca gregaria*, possesses a gut microbiota that is predominantly acquired from the local environment (Dillon & Charnley, 2002). Here, the gut microbiota forms an additional barrier to protect the host from pathogenic microorganisms – a process known as

colonisation resistance. High levels of antifungal phenols are produced by the gut microbiota under stress, which enables the locusts to be more resistant to attack from opportunistic pathogens (Dillon & Charnley, 2002). In *Drosophila melanogaster*, the importance of a diverse diet in creating and maintaining a diverse gut microbiota has also been documented, as it increases survival and reduces the development time of this species (Rohlfs and Kürschner, 2010).

The fruit, *Morinda citrifolia*, also known as the noni fruit, is the natural host plant of the species *Drosophila sechellia*; a rare specialist that lives exclusively on this host. Endemic to the Seychelles, *D. sechellia* is a member of the *D. melanogaster* subgroup which branches into two complexes (Legal et al., 1994; Legal et al., 1999). The first, the *D. melanogaster* complex, contains the species *Drosophila melanogaster, Drosophila mauritiana, D. sechellia* and *Drosophila simulans*. The second is the *Drosophila yakuba* complex containing the species *Drosophila yakuba, Drosophila tessieri, Drosophila orena* and *Drosophila erecta.* Whilst *D. sechellia* is a fruit specialist and has evolved to exploit the noni fruit, its sister species, *D. mauritiana* and *D. simulans*, are generalist species that are incredibly averse to the scent of noni. This is due to the fact that noni fruit possesses the toxic compounds, octanoic and hexanoic acids (Legal et al., 1994) which are known to repel (Legal et al., 1992) and even kill other *Drosophilid* species (Legal et al., 1994; Legal et al., 1999).

The chemosensory system of *D. sechellia* is adapted to detect the key volatiles released from the noni fruit (Dekker et al., 2006), with individuals able to detect fruit from distances of up to 150m away (R'Kha et al., 1991). Females increase egg production and oviposition upon contact with the fruit (R'Kha et al., 1991; Jones, 2004). Female *D. sechellia* exhibit a much lower reproductive potential than their sister species, *D. melanogaster* (R'Kha et al., 1991, R'Kha et al., 1997). This is in part due to the low number of ovarioles present. It has been suggested that this reduction in ovariole number is due to a negative pleiotropic effect of the genetic changes required for specialisation, also known as the 'cost of resistance,' within this species (Jones, 2004). The presence of L-DOPA, the precursor to dopamine,

in the noni fruit is thought to compensate for the reduction by stimulating egg production and even increasing egg size, and thus overall fitness, within *D. sechellia*. It has therefore been suggested that the need for L-DOPA to ensure successful reproduction in this species has driven *D. sechellia* to become a noni fruit specialist (Lavista-Llanos et al., 2014).

The underlying genetic architecture of how *D. sechellia* is adapted to this toxic host plant is relatively well studied. The gene *desat1* has pleiotropic effects on both the cuticular hydrocarbon (CHC) expression and the odorant binding proteins that are responsible for the perception of pheromones (Labeur et al., 2002). This gene, expressed in both the oenocytes (the source of CHC synthesis) and the head, are linked to the odorant binding proteins, Obp57d and Obp57e (Matsuo et al., 2007). These proteins are not only responsible for taste perception, but they can also change the behavioural response elucidated by the fly in response to the toxins in the fruit (Matsuo et al., 2007). Thus, differential expression of this gene can lead to differences in both detection and taste perception of the toxic compounds found in noni fruit. It is thought that other species of *Drosophila* do not possess this gene and thus are repelled by the noni fruit scent. The *desat1* gene also encodes an enzyme that is involved in the synthesis of CHCs (Billeter et al., 2009).

Previous work has shown that the gut microbiota can alter an individual's CHC profile (Heys et al., 2018a). CHCs are heritable, fatty acids that are widely known as sex pheromones in *Drosophila* (reviewed in Singer, 1998). Due to this, CHCs are thought to alter with mating success within *Drosophila*, with flies expressing a preference for mating with individuals of a similar CHC type (Scott et al., 1988). Alteration of the CHC profile via the gut microbiota has also been implicated in kin recognition in *D. melanogaster* (Lizé et al., 2014; Heys et al., 2018a). Males of this fruit generalist species were shown to invest more when mating with females reared on the same diet type (Lizé et al., 2014). Yet, this effect was removed when the gut microbiota was suppressed via antibiotics. The effect was also linked to changes in the CHC profile (Heys et al., 2018a). As the composition of an individual's CHC profile is altered by changes in the gut microbiota, it can be suggested that the gut

microbiota directly influences CHC composition and resulting behavioural responses of *D. sechellia* to the noni fruit.

Although the genetic adaptations of *D. sechellia* to the toxic compounds present in the noni fruit are fairly well understood, the role the gut microbiota plays in this specialisation has not yet been investigated. Interestingly, Chandler *et al.* (2011) characterised the microbiota of wild *D. sechellia* found feeding on the noni fruit and discovered that the gut is dominated by a single *Lactobacillales* OTU (84%). This demonstrates the very low bacterial community richness and diversity within this species, particularly when it is compared to its sister species, *D. melanogaster*, which exhibit greater diversity and carry the bacterial genera *Lactobacillus*, *Acetobacter* and *Enterococcus* (Ryu et al., 2008; Brummel et al., 2004; Ren et al., 2007; Cox & Gilmore, 2007). Further, the composition of an individual's gut microbiota is known to be influenced by pH (see Overend et al., 2016). Overend et al. (2016) demonstrated that decreasing the pH in certain regions of the *Drosophila* gut can lead to an increased abundance of key members of the gut microbiota – *Lactobacillus* and *Acetobacter*. This raises the question whether the very low bacterial richness found in the *D. sechellia* gut when it is feeding on its acidic natural host diet, *M. citrifolia*, is due to the pH determining the microbiota? Alternatively, potentially the almost exclusive prevalence of this *Lactobacillales* is caused by the specialism of *D. sechellia* to the host plant, and thus the *Lactobacillales* acts as a form of detoxifying agent by metabolising the toxic acids found within the noni fruit.

In this study we investigated the role of the gut microbiota on host specialisation in *D. sechellia*. *D. sechellia* are widely kept in the laboratory, but little attention has been paid to the effect that feeding this specialist species a generalist diet has on the resulting gut microbiota. This study is separated into two sections. In the first, we determined the effect that rearing *D. sechellia* on a standard laboratory diet has on the diversity and richness of the gut microbiota. Flies were first reared on a standard *Drosophila* diet (ASG), then moved onto noni fruit, before being transferred back onto ASG. At each stage, the diversity and abundance of the gut bacteria was measured. In the second section, we disentangled the role of pH on shaping

the gut microbiota, from the toxic compounds present in the noni fruit. We introduced a new dietary treatment, *Salacca zalacca* (hereon known as salak fruit), with similar nutritional and acidic properties to noni fruit but lacking in the toxic compounds - octanoic and hexanoic acid. We determined the effect that these diets - a standard laboratory diet, noni fruit and salak fruit - had on a series of life history traits, larval, pupal and adult weight, the risk of death before adulthood and fecundity. We also examined the effect of these diets on the gut microbiota and any resulting effects on mate choice behaviours, including mating propensity, mating latency and copulation duration. We predicted that the gut microbiota would become more simplified on the noni diet, but not the salak or ASG, which would indicate that the gut microbiota plays a role in specialisation to this diet. We also predicted that any difference observed in mating behaviours would arise through flies being reared on different diets, which in turn alters the gut microbiota and the CHC profile, therefore altering the scent of an individual.

4.3 Materials and methods

4.3.1 The changing gut microbiota of *D. sechellia*

General fly maintenance for all experiments

D. sechellia stocks were obtained from the National Drosophila Species Stock Center located in San Diego. Three lines of outbred flies were utilised (lines 0.21, 0.07 and 0.08), that were collected on Cousin Island, Seychelles in 1980 and maintained in the laboratory ever since. All flies were kept and reared at 25°C on a 12:12hour light-dark cycle. Flies were kept in standard 75x25mm *Drosophila* vials containing 25ml of standard *Drosophila* food composed of yeast/agar/maize/sugar. Flies were moved to new vials every 4 days.

4.3.2 Experimental treatments

Newly emerged, virgin adult flies were obtained from the stock population and transferred to a new vial containing 25ml of a standard *Drosophila* dietary media composed of yeast/sugar/agar/maize (hereon known as ASG). Flies were left to mature on this media for two days before being transferred to a new vial containing the same media (N=30). After one week, two males and two females from different vials from each stock line were isolated using carbon dioxide gas anaesthesia. The protocol below detailing the bacterial analysis was then followed for these individuals, to determine the gut bacterial load and diversity of flies reared on this diet. These flies formed the "ASG 1" treatment.

The remaining flies were then gently aspirated into fresh vials containing 25g of *Morinda citrifolia* fruit and left for one week (N=30). After this time, two males and two females from different vials from each stock line were again isolated and the same bacterial analysis protocol was followed. This enabled us to determine any changes in the gut microbiota in the same population of flies, that were first reared on a different diet. These flies formed the "Noni" treatment.

Similarly, the remaining flies were again, gently aspirated into fresh vials containing 25ml of ASG and left for one week (N=30). After this time, two males and two females from different vials from each stock line were again isolated and the same bacterial analysis protocol was followed. This enabled us to determine any further changes in the gut microbiota, when flies from the same population were transferred between two different diets in a short period of time. These flies formed the "ASG 2" treatment.

4.3.3 Bacterial analysis

Collected flies were first surface sterilised in 70% ethanol, rinsed in distilled water and air dried. The head was then removed. Two guts were dissected into each Eppendorf containing 250µl of sterile LB (Lysogeny Broth) broth (Bertani, 2004). An equal number of males and females were used to ensure

there were no sex-specific differences in the bacterial content. Gut tissue was homogenised with a sterile plastic pestle. 100µl of gut homogenate was pipetted onto BHI (Brain, Heart Infusion) agar (Atlas, 2004) and spread-plated using a sterile glass loop. BHI media was used as it was found to favour greater colony growth. Plates were left to air dry aseptically, before being closed and sealed with parafilm. Plates were incubated at 25°C for 72 hours, and bacterial load was quantified by performing CFU (Colony Forming Unit) counts.

Single colonies were isolated using a sterile 1µl loop and placed into an Eppendorf with 10µl sterile water. PCR amplification was performed in a 25µl reaction volume consisting of 10µl nuclease-free water, 13µl Taq green master mix, 0.5µl of forward primer 27F (5'- AGAGTTTGATCMTGGCTCAG-3') and reverse primer 1492R (5′-GGTTACCTTGTTACGACTT-3') and 1µl of template DNA. Thermal cycling was performed for 90 seconds at 95°C as initial denaturation, followed by 35 cycles of 30 sec at 95°C for denaturation, 30 sec at 55 °C as annealing, 90 sec at 72 °C for extension, and final extension at 72 °C for 5 min. 1500 bp 16S PCR products were purified with Ampure beads and subjected to Sanger sequencing. The resulting sequences were identified using NCBI BLAST against the nt database (Altschul et al., 1990).

4.3.4 pH and diet type on behaviour and life history traits

In order to test the effect of acidity on *D. sechellia* life history traits, we constructed three different diets of varying pH. Flies were reared on one of three diets: ASG (for 1l of water: 85g of sugar, 60g of corn, 20g of yeast, 10g of agar and 25ml of nipagin), noni fruit, or salak fruit. *Morinda citrifolia* is the diet of wild *D. sechellia* and has a low, acidic pH of 3.86 due to the high concentrations of both octanoic and hexanoic acids (Legal et al., 1994). The salak fruit diet was used as an alternative diet to the noni fruit, as it also has a low, acidic pH at 3.59, but does not contain the toxic octanoic and hexanoic acids that are present in noni. These two diet types were compared to the

typical laboratory diet of ASG, that has a higher, more alkaline pH than the fruit diets at 5.97.

4.3.5 Risk of death before adulthood

The number of days was measured from day of female oviposition to day of adult emergence. Vials were checked at three time points within each day – 9am, 12pm and 5pm – and the cumulative number of adults emerged from each time point was scored (N_{ASG} = 76; N_{Noni} = 60; N_{Salak} = 125).

4.3.6 Weight at different life stages

In order to accurately determine the effect of acidity on life history, the weights of three different life stages were measured. For larval weight, vials were checked daily during the morning and any third instar larvae present were removed and washed with distilled water in order to remove any excess food. Larvae were grouped according to treatment and placed into the freezer at -18°C for two hours. Later, larvae were removed and weighed using an Ohaus five place balance and their weight was recorded (in mg) to four decimal places (N_{ASG} = 50; N_{Noni} = 50; N_{Salak} = 50).

For the pupal and adult weights, vials were similarly checked daily at three time points – 9am, 12pm and 5pm – to check for any freshly pupated or newly emerged individuals. For the pupae, care was taken to remove pupae from the vials without damaging them (N_{ASG} = 50; N_{Noni} = 50; N_{Salak} = 50). The adult flies were isolated as virgins and separated according to sex. Adults were placed into vials at a standard density of ten per vial and left for two hours to allow their wings to dry out and inflate. Two hours later, vials were placed into the freezer at -18°C and left overnight. Pupae were grouped according to treatment and placed into the freezer at -18°C for two hours. Later, the pupae and adults were removed and weighed using an Ohaus five place balance and their weight was recorded (in mg) to four decimal places. In the adults, male and female measurements for each treatment were

recorded and analysed separately (females: N_{ASG} = 53; N_{Noni} = 50; N_{Salak} = 50; males: N_{ASG} = 45; N_{Noni} = 45; N_{Salak} = 50).

4.3.7 Mate choice assays

In order to ascertain any differences in mate choice behaviours across individuals reared on different diet type and therefore ascertain the effect of gut microbiota on behaviour, we performed a series of mating assays. As our primary focus was to uncover the impact of different diets (ASG, noni and salak) on mating behaviours, we did not perform a fully factorial design and analyse all possible mating combinations. We did, however, ensure that all pairwise comparisons were made. For example, ASG reared flies were placed into separate mating scenarios with a salak partner, an ASG partner and a noni partner.

Recently mated females were placed onto one of three diets and allowed to oviposit to form three populations. At eclosion, virgin males and females were isolated twice daily and separated into fresh vials containing the media they were reared on, according to sex. Females and males were placed into groups of 10 per vial. Flies were left for seven days to ensure full maturation. Following maturation, mating trials were conducted. Virgin females were gently aspirated onto 15ml of neutral food media (comprised of yeast/agar/sugar) and allowed to rest overnight. Mating trials were conducted at 25°C, under a bright light and during the morning, as this is when *Drosophila* are most active (Hardeland, 1972). A male of one of the three diet types was then gently aspirated into the vial and the observations begun. We recorded whether or not copulation occurred, in addition to mating latency (time elapsed between male introduction and copulation), and the duration of copulation. Mating latency is a commonly used indicator of female preference in *Drosophila* (e.g. Lefranc & Bundgaard, 2000; Somashekar & Krishna, 2011; Verspoor, Cuss and Price, 2015; Heys et al., 2018a), with copulation duration used a proxy of male investment (e.g. Friberg, 2006; Bretman, Fricke & Chapman, 2009; Byrne & Rice, 2006). A full list of abbreviations of mating pairs is given below (Table 1). Mating trials were

staggered over several days in order to ensure a high replication rate and reliability (N_{AxA} = 48; N_{NxN} = 50; N_{SxS} = 47, N_{AxS} = 49; N_{NxA} = 49; N_{SxN} = 50).

Table 1. List of abbreviations of mating pairs that are used throughout.

Mating pair	Abbreviation
Salak female x Salak male	SxS
Noni female x Noni male	NxN
ASG female x ASG male	AxA
Salak female x Noni male	SxN
ASG female x Salak male	AxS
Noni female x ASG male	NxA

4.3.8 Fecundity

In order to determine the level of female investment into a particular mating, female fecundity in terms of egg production was measured. This is a commonly used method of assessing female investment in *Drosophila* (e.g. Dhole and Pfennig, 2014). Following copulation, mated females were placed onto 15ml of neutral food medium supplemented with two grains of yeast and left to oviposit at 25°C. Egg production was counted every 24 hours for a total of 72 hours, with the female transferred to a new vial of neutral food medium at each time point. The total egg production for each female over the total 72 hours was then analysed (N_{AxA} = 48; N_{NxN} = 50; N_{SxS} = 47, N_{AxS} = 49; N_{NxA} = 49; N_{SxN} = 50).

4.3.9 Bacterial analysis

The bacterial load of the midgut was extracted as before and similarly quantified using CFU counts. The midgut contents were spread-plated and the different colonies were Sanger sequenced as before.

4.3.10 Statistical analysis

All data were analysed using R (version 3.3.0; R Core Team, 2016). Larval, pupal and adult weight were analysed using separate General Linear Models (GLM). Adult weight was first analysed with both sexes grouped, before further analysis separated according to sex were performed. Mating propensity was analysed using separate ANOVA models. Mating data (copulation duration and mating latency) were analysed using a combined GLM. Fecundity was measured using a combined GLM with adult weight included as a covariate. Variation in the risk of death before adulthood data was analysed via Cox Proportional-Hazard Regressions. Development failure of flies was used as the 'event' for the risk of death before adulthood data. The *Survdiff* function was used to assess differences between two or more survival curves according to treatment. The *coxph* function was used to assess differences between treatments. This allowed treatments to be compared in a pairwise fashion, to ascertain whether all treatments differed, or whether any significant differences observed were derived from a single treatment.

4.4 Results

4.4.1 The changing gut microbiota of *D. sechellia*

Bacterial colony growth was observed in all treatments, with both greater diversity and greater abundance of bacteria found in the ASG 1 and ASG 2 flies (Table 1). Flies analysed from these treatments were found to have *Lactobacillus plantarum*, *Paenibacillus sp*. and *Bacillus cereus* species present. In nearly all of the noni flies only *L. plantarum* was observed, with the exception of minor colony growth in two of the male replicates. Little difference was observed between the three different strains of *D. sechellia,* or between sexes.

Table 2. Number of bacterial colonies isolated from the midgut of adult flies. Flies were first reared on ASG (represented by ASG 1), then moved onto noni fruit (Noni), before being transferred back onto ASG (ASG 2).

Diet	Strain	Replicate	Sex	*L. plantarum*	*Paenibacillus sp.*	*Bacillus cereus*
ASG 1	0.21	1	F	3.12x10^2	0.20x10^1	0
ASG 1	0.21	2	F	1.82x10^2	1.40x10^1	0
ASG 1	0.21	1	M	1.58x10^2	0	0.10x10^1
ASG 1	0.21	2	M	2.50x10^1	0.90x10^1	0
ASG 1	0.07	1	F	5.11x10^3	1.81x10^2	0.50x10^1
ASG 1	0.07	2	F	5.94x10^3	1.23x10^2	0.20x10^1
ASG 1	0.07	1	M	4.88x10^3	5.40x10^1	0.70x10^1
ASG 1	0.07	2	M	3.58x10^3	1.75x10^	2.20x10^1
ASG 1	0.08	1	F	6.25x10^3	2.02x10^2	2.70x10^1
ASG 1	0.08	2	F	4.09x10^3	1.96x10^2	1.50x10^1
ASG 1	0.08	1	M	3.17x10^3	2.70x10^1	0.40x10^1
ASG 1	0.08	2	M	2.89x10^3	8.80x10^1	0
Noni	0.21	1	F	2.72x10^3	0	0
Noni	0.21	2	F	1.78x10^3	0	0
Noni	0.21	1	M	1.62x10^2	0	0
Noni	0.21	2	M	1.59x10^2	0	0
Noni	0.07	1	F	1.43x10^3	0	0
Noni	0.07	2	F	1.34x10^3	0	0
Noni	0.07	1	M	2.55x10^2	1.50x10^1	0
Noni	0.07	2	M	1.92x10^2	0.70x10^1	0
Noni	0.08	1	F	4.5x10^2	0	0
Noni	0.08	2	F	1.81x10^2	0	0
Noni	0.08	1	M	8.10x10^1	0.50x10^1	0
Noni	0.08	2	M	7.20x10^1	0	0
ASG 2	0.21	1	F	1.21x10^3	2.40x10^1	0
ASG 2	0.21	2	F	1.45x10^3	1.20x10^1	0.10x10^1
ASG 2	0.21	1	M	1.22x10^2	0.20x10^1	0.10x10^1
ASG 2	0.21	2	M	2.31x10^2	0.50x10^1	0.20x10^1
ASG 2	0.07	1	F	4.51x10^2	1.50x10^2	0.70x10^1
ASG 2	0.07	2	F	5.22x10^2	8.90x10^2	0.20x10^1
ASG 2	0.07	1	M	2.09x10^3	2.90x10^1	0.60x10^1
ASG 2	0.07	2	M	2.87x10^3	4.60x10^1	1.30x10^1
ASG 2	0.08	1	F	3.22x10^3	1.98x10^2	2.90x10^1
ASG 2	0.08	2	F	2.46x10^3	2.51x10^2	2.20x10^1
ASG 2	0.08	1	M	1.78x10^3	1.12x10^2	0.50x10^1
ASG 2	0.08	2	M	2.34x10^3	1.43x10^2	0.70x10^1

4.4.2 The effect of pH and diet type on behaviour and life history traits

4.4.3 Risk of death before adulthood

No significant difference was observed in the risk of death before adulthood between the ASG or noni treatments (Z_2=0.373, P=0.709) (Figure 1). However, flies reared on salak had a significantly lower risk of death before adulthood than flies reared on noni (Z_2=-2.187, P=0.028). A trend was observed in the risk of death before adulthood between ASG and salak treatments, with salak individuals exhibiting a higher risk of death before adulthood than ASG flies (Z_2=-1.905 P=0.056).

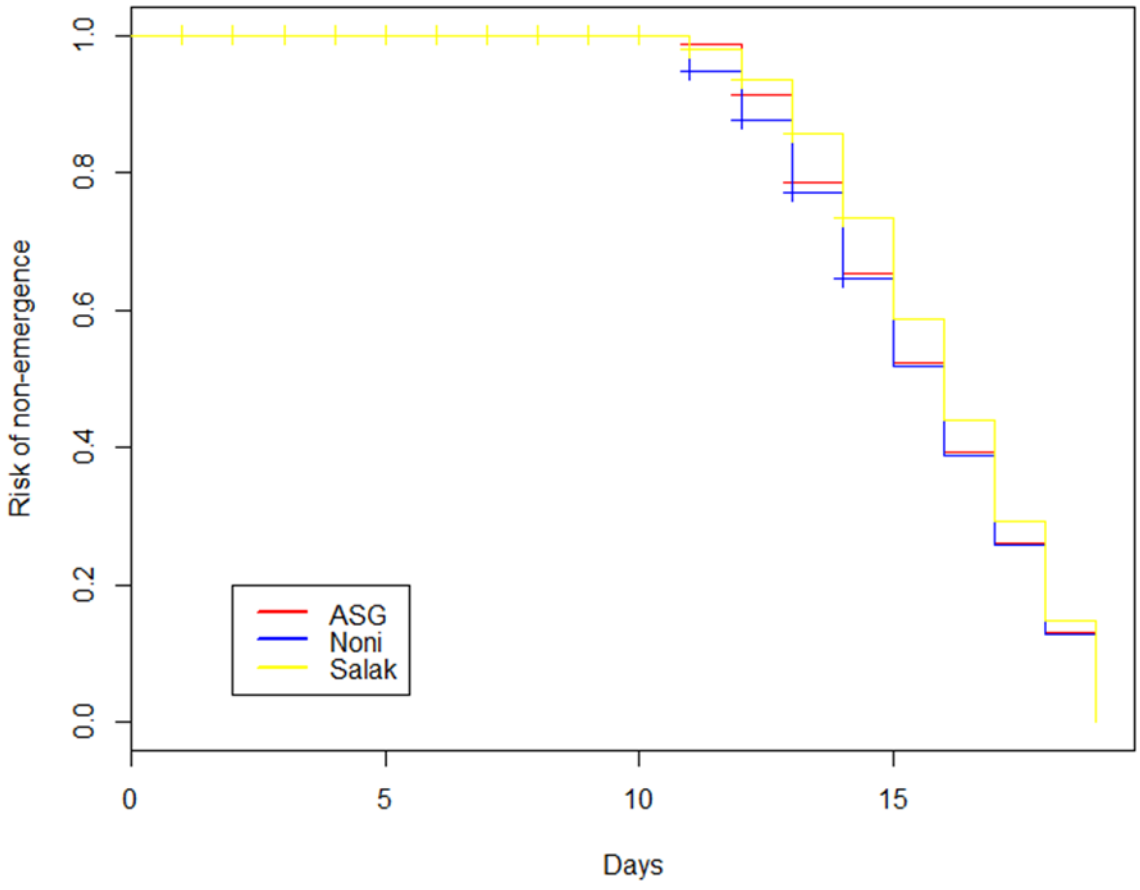

Figure 1. Development time failure, measured in days as the risk to die before adulthood, of *D. sechellia*. Eggs were reared under one of three different dietary treatments - either ASG, noni or salak diets.

4.4.4 Larval weight

No difference was observed in larval weight in any pairwise comparisons across all three treatments: ASG and noni (T_2=0.850, P=0.397), ASG and salak (T_2=0.335, P=0.738), noni and salak (T_2=-0.515, P=0.608) (Figure 2).

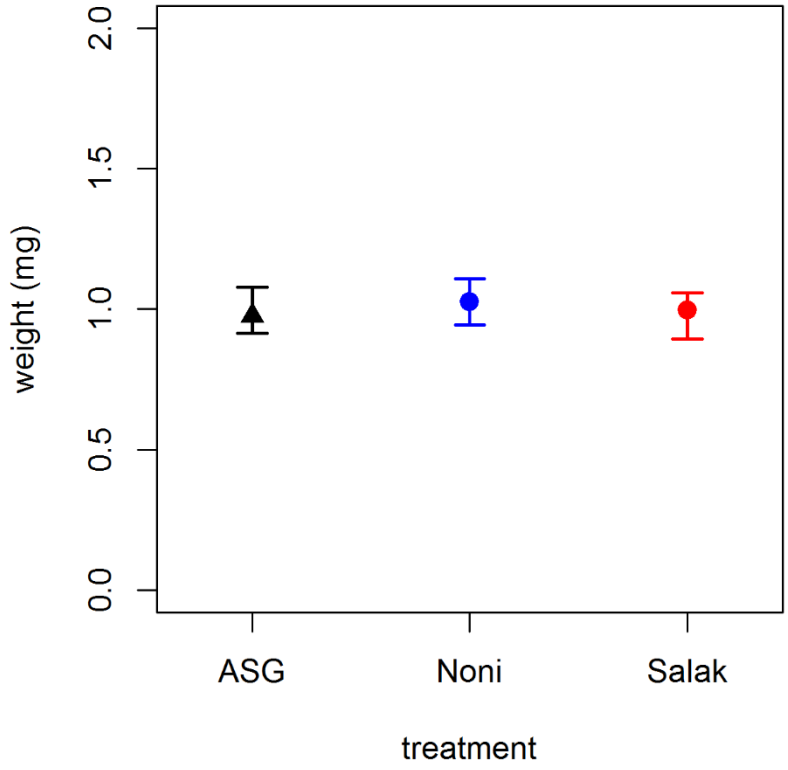

Figure 2. Weight (mg) of third instar *D. sechellia* larvae reared on one of three different diet types - either ASG, noni or salak. No significant differences were found.

4.4.5 Pupal weight

Pupae collected from the noni treatment weighed significantly less than pupae from the ASG treatment (T_2=-8.961, P<0.001) (Figure 3). Similarly, pupae obtained from the salak treatment were found to weigh significantly

less than the pupae from the ASG treatment (T_2=-8.722, P<0.001). No difference in pupal weight was observed between pupae from the noni and salak treatments (T_2=0.239, P=0.812).

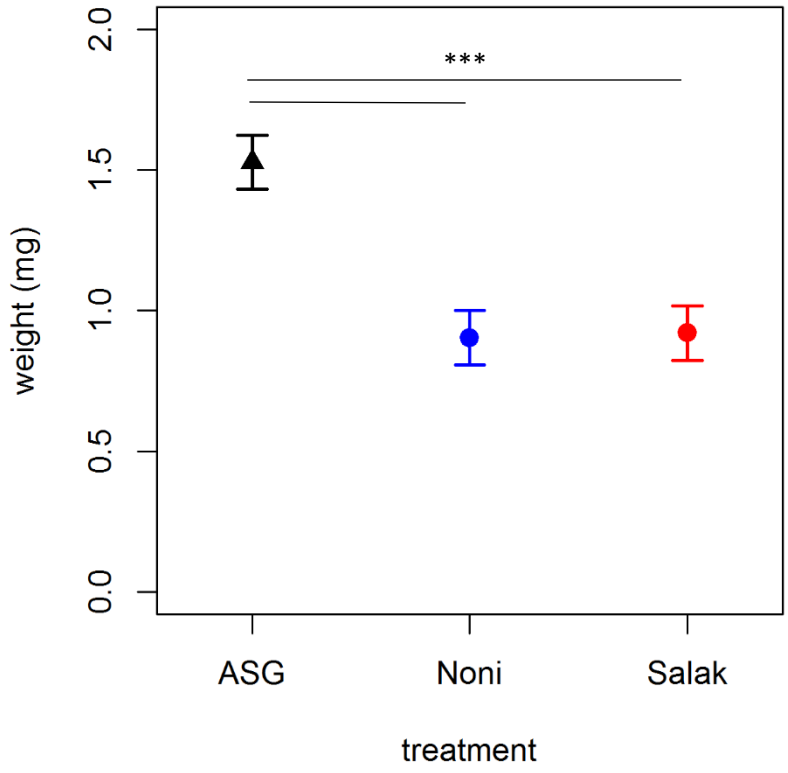

Figure 3. Weights (mg) of *D. sechellia* pupae reared on one of three different dietary treatments - either ASG, noni or salak. Vials were checked at various time points for freshly pupated flies. Significant results are shown with *.

4.4.6 Adult weight

Adult male flies were always found to weigh less than females (P<0.001). When males and females are analysed separately, in male flies, significant differences were found across two comparisons, with both noni males and salak males weighing significantly more than ASG flies (T_2=3.919, P<0.001; T_2=3.115, P=0.002, respectively) (Figure 4). However, no differences were found between the weights of noni and salak males (T_2=0.905, P=0.366). In

females, significant differences were found across all comparisons, with noni females weighing more than ASG females (T_2=19.069, P<0.001) and salak females (T_2=-15.410, P<0.001). Similarly, salak females were also shown to weigh significantly more than ASG females (T_2=3.435, P<0.001).

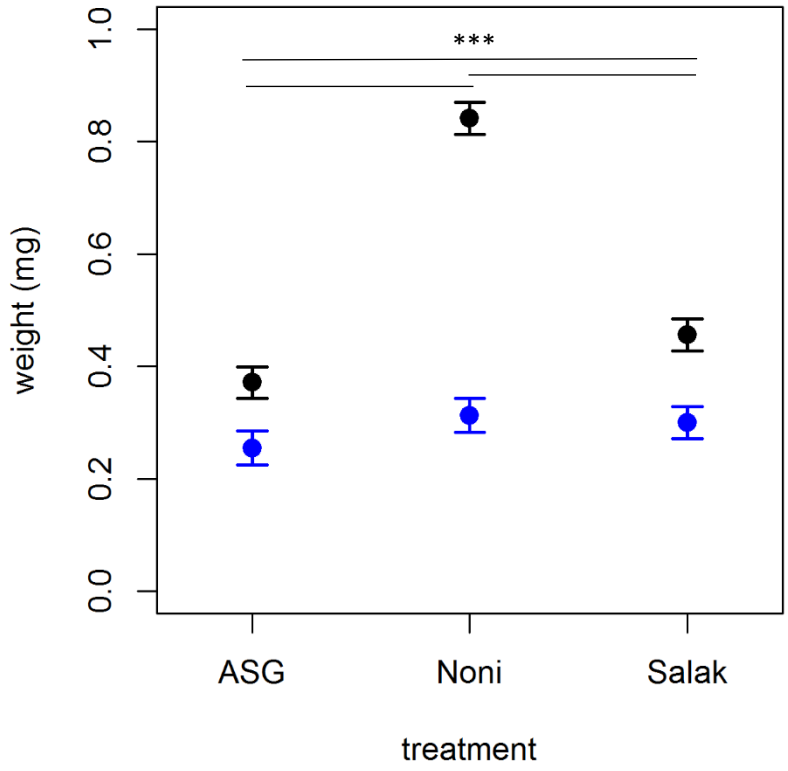

Figure 4. Weights (mg) of both male and female, newly emerged adult *D. sechellia* flies reared on one of three different diet types - either ASG, noni or salak. Newly emerged adults were collected at various time points and allowed two hours for wing inflation. Male flies are shown here using the blue plots, whilst females are depicted in the black plots. Significant results are marked with a *.

4.4.7 Mate choice assays: propensity to mate

The mating propensity of all pairs of flies was measured in order to determine if there are differences in the proportion of mated pairs, when pairs from different diets are placed into the mate choice assays. No

difference in mating propensity was observed between NxN flies and AxA (Z_5=0.299, P=0.765), NxA (Z_5=-1.465, P=0.142), or SxS (Z_5=-1.197, P=0.231), but a trend was observed between AxS, with AxS flies displaying lower mating propensity (Z_5=-1.896, P=0.056) (Figure 5). AxA flies had a significantly higher mating propensity than AxS flies (Z_5=-2.151, P=0.031). NxS flies had the lowest mating propensity and was significantly lower than SxS flies (Z_5=-2.425, P=0.015), AxA flies (Z_5=-3.768, P<0.001), NxN flies (Z_5=-3.572, P<0.001) and NxA (Z_5=2.206, P=0.027).

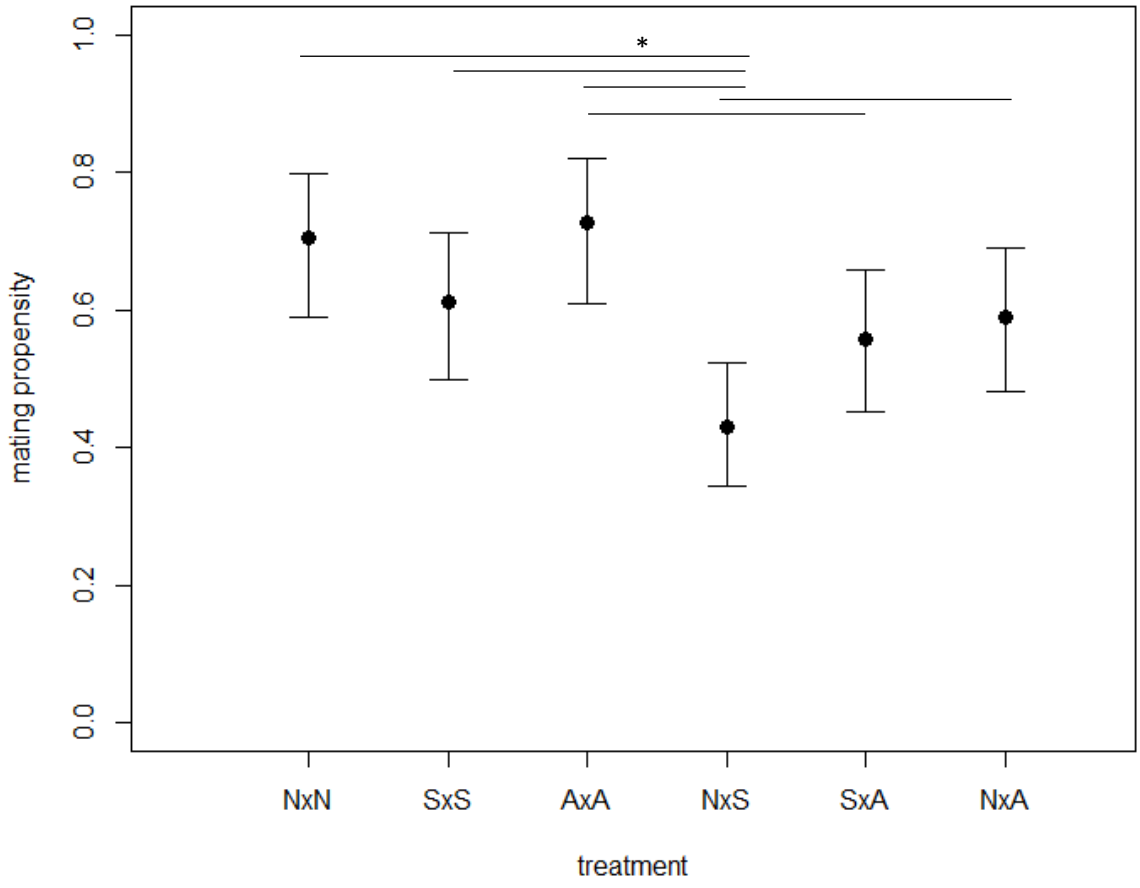

Figure 5. Mating propensity, measured as mating proportion, of pairs of seven-day-old, adult *D. sechellia* flies reared on either the same or different diets (ASG, noni and salak). Significant results are marked with a *.

4.4.8 Mate choice assays: mating latency

Only one significant difference was observed in the pairwise comparisons of mating latency across copulating pairs (Figure 6). Here, pairs of flies both reared on salak diet had a significantly longer mating latency than flies that were both reared on ASG (T_5=-2.293, P=0.022). All other pairwise comparisons were non-significant.

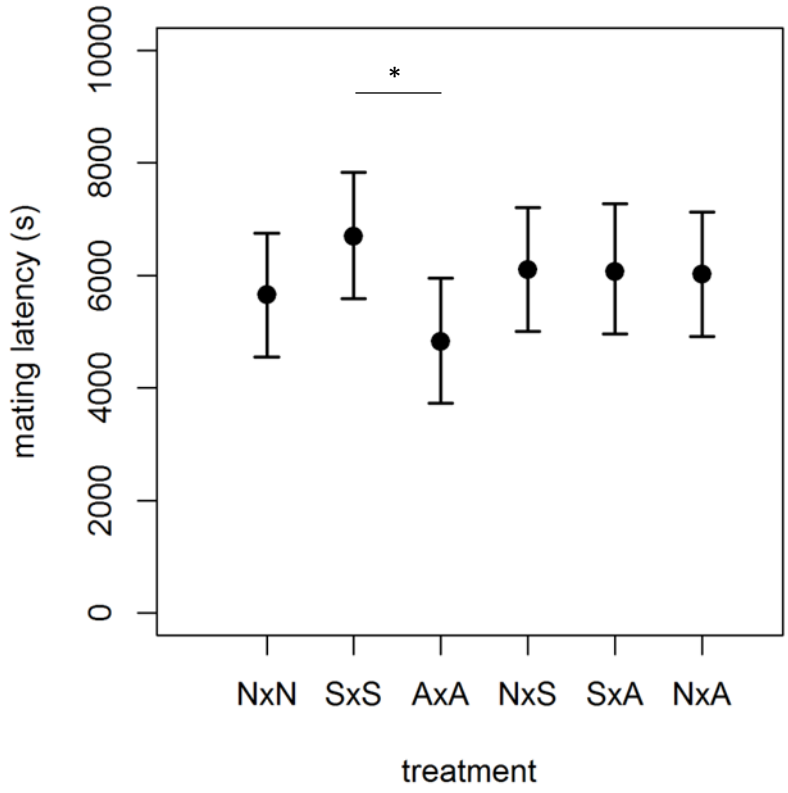

Figure 6. Mating latency (s) of pairs of seven-day-old, adult *D. sechellia* flies reared on either the same or different diets (ASG, noni and salak). Significant results are highlight with *.

4.4.9 Mate choice assays: copulation duration

Pairs of flies reared on salak were shown to mate for significantly longer than flies that were both reared on an ASG diet (T_5=-2.290, P=0.022) or on a noni diet (T_5=-2.177, P=0.030) (Figure 7). Flies that were both reared on salak

also mated for significantly longer than flies that were reared on different diets. Here, salak reared flies mated for longer than salak females mated with noni males (T_5=-1.986, P=0.048) and ASG females mated with salak males (T_5=-3.382, P<0.001). Interestingly, ASG females paired with salak males mated for significantly less time than noni females paired with ASG males (T_5=2.449, P=0.014). All other pairwise comparisons were non-significant.

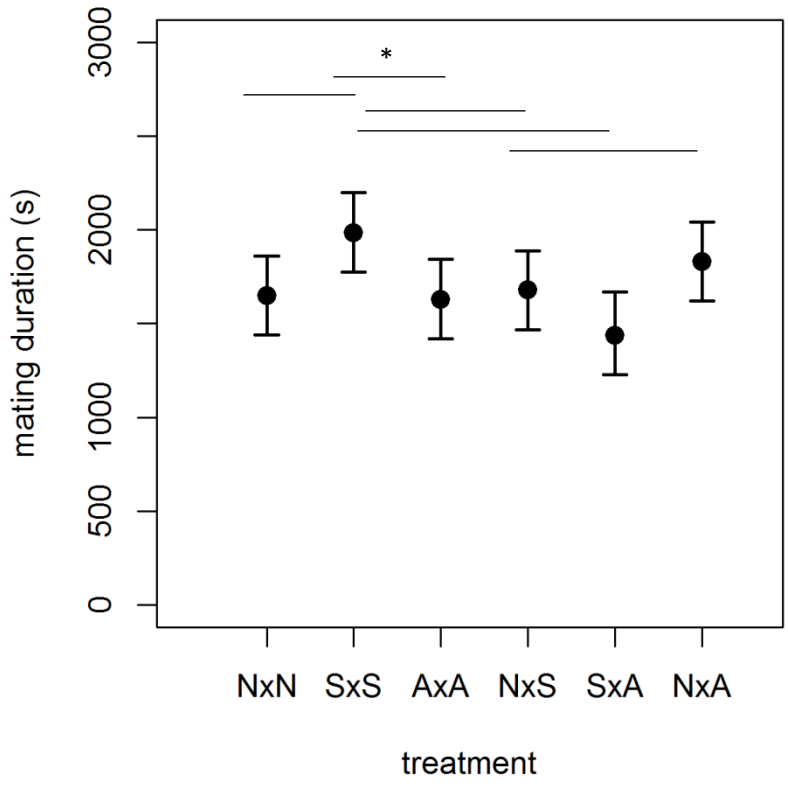

Figure 7. Mating duration (s) of pairs of seven-day-old adult *D. sechellia* flies reared on either the same or different diets (ASG, noni and salak). Significant results are marked with a *.

4.4.10 Fecundity

When accounting for female body weight, females from the AxA mated pairs laid significantly more eggs than females from both SxS treatments (T_5=-

2.068, P=0.039) and NxA treatments (T_5=-2.112, P=0.035) (Figure 8). Similarly, females from the NxS mated pairs laid significantly more eggs than females from NxA treatments (T_5=2.068, P=0.039). No significant differences were found between NxN mated females and any other treatment (Figure 8).

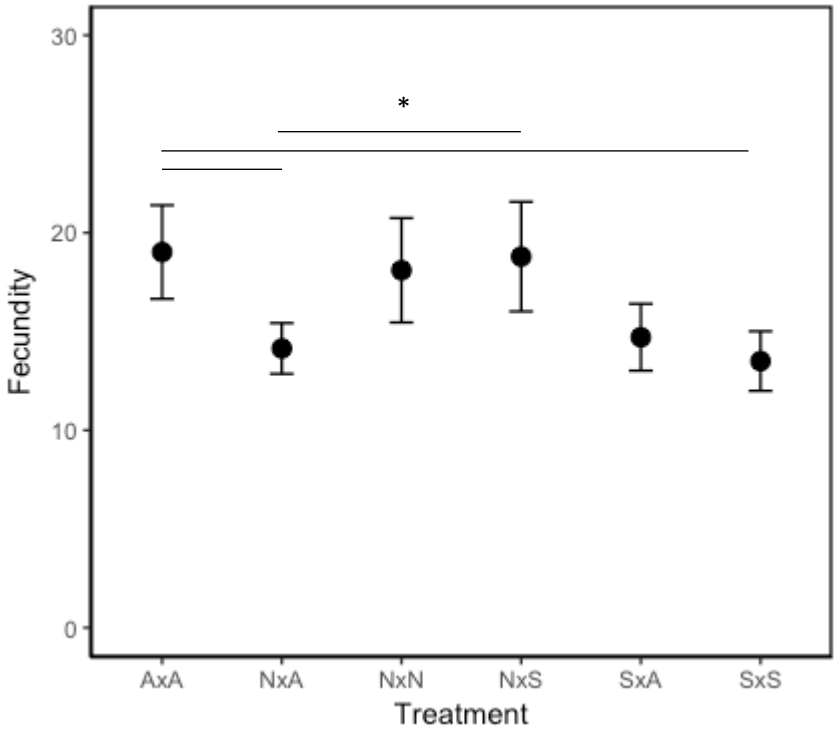

Figure 8. Fecundity (egg number) of female flies reared on a certain diet (ASG, noni and salak) and mated with a male from either the same or a different diet (ASG, noni or salak). Fecundity was measured as total number of eggs oviposited over a total of 72 hours post copulation. Significant results are marked with a *.

4.4.11 Bacterial analysis

Bacterial colony growth was observed in all treatments, with a greater diversity of bacteria found in the flies that were reared on the ASG diet (Table 3). In all flies that were reared on the noni and salak treatments, only

one kind of bacterial colony formed. Sanger sequencing data identified this as *L. plantarum.* In the ASG diet flies, *L. plantarum* was similarly observed, with *Paenibacillus sp*. and *Bacillus cereus* also found. Little difference was observed between the bacterial load of noni and salak reared flies, or between the different sexes.

Table 3. Number of bacterial colonies isolated from the midgut of adult flies that were reared on one of three diets – ASG, noni or salak. Males and females were quantified separately.

Diet	Replicate	Sex	*L. plantarum*	*Paenibacillus sp.*	*B. cereus*
ASG	1	F	2.52x10^2	1.30x10^1	0.20x10^1
	2	F	2.91x10^2	1.90x10^1	0
	1	M	1.89x10^2	2.30x10^1	0
	2	M	2.34x10^2	1.80x10^1	0.60x10^1
Noni	1	F	2.85x10^2	0	0
	2	F	3.19x10^2	0	0
	1	M	1.51x10^2	0	0
	2	M	1.99x10^2	0	0
Salak	1	F	1.31x10^2	0	0
	2	F	1.66x10^2	0	0
	1	M	2.12x10^2	0	0
	2	M	2.45x10^2	0	0

4.5 Discussion

Our results are the first to provide evidence that the gut microbiota may play a role in host specialisation in *Drosophila sechellia*. We characterised the gut microbiota of wild-type, laboratory reared *D. sechellia* and demonstrated the impact that a changing diet has on gut microbiota. We found that by rearing *D. sechellia* on a fruit diet similar in nutritional properties to its natural host plant but without the toxins, we observe a microbiota of very similar diversity. We then showed the effect that altering the gut microbiota via diet has on subsequent life history traits, larval, pupal and adult weight, with little

difference observed between larvae and pupae of all diet types. The only difference was that adults reared on the standard laboratory diet (ASG) weighed significantly less than noni reared flies. Noni flies had significantly higher development failure than salak flies, but they also weighed more at adult emergence, suggesting they have greater fitness. The combination of diet types had substantial effects on the mating behaviours of *D. sechellia*, with SxS paired flies mating for significantly longer than all other pairs, except NxA paired flies, but they also exhibited a high mating latency than AxA mating pairs. Mating propensity was lowest in all pairs that were not reared on the same diets (e.g. NxS flies). Interestingly, despite these differences in mating behaviour, little impact was found on female fecundity, with SxS females displaying the lowest fecundity of all mating pair combinations.

Little attention has been paid to the gut microbiota of *D. sechellia*, with the focus instead on genetic adaptation to its toxic host plant. Chandler et al. (2011) characterised the gut bacteria of wild *D. sechellia* found feeding on noni fruit and determined that the natural gut microbiota of this species is dominated by a single *Lactobacillales*. This is in stark contrast to other fruit-feeding, closely related species of *Drosophila* that exhibit considerably greater bacterial diversity, such as wild *D. melanogaster* which host a number of bacterial genera, including *Enterobacteriales*, *Burkholderiales* and *Pseudomonadales*. Here, we show that the gut microbiota of wild-type *D. sechellia* is diverse when individuals are kept under laboratory conditions on a formulated diet. We determined that both males and female guts contain *Paenibacillus sp.* and *Bacillus cereus.* Although numerous evidence has shown that the gut microbiota of laboratory reared species is considerably less diverse than their wild counterparts (Brummel et al., 2004; Roh et al., 2008), some studies do show deviation from the typically found bacterial genera of *Lactobacillus*, *Acetobacters* and *Enterobacter* in laboratory reared flies (e.g. Ren et al., 2007). It could therefore be argued that these genera of bacteria are present in wild populations of *D. sechellia*, but only thrive in great enough numbers for detection when placed onto a diet that encourages their growth. A bacterial pathogen, *Paenibacillus* species are known to be

present in honeybee larvae and are responsible for colony collapse by causing American Foulbrood (e.g. Genersch, 2010). Both *Paenibacillus nanensis* and *B. cereus* have been discovered in wild populations of *Drosophila ananassae* (Maji, Chakrabarti & Chatterjee, 2013), although their function or effect on the host is as yet unknown.

When individuals are then transferred onto the natural host noni fruit, the gut microbiota simplifies to a single species - *Lactobacillus plantarum* – as similarly shown in previous studies (Chandler et al., 2011). It could be suggested that colonies of *Lactobacillus plantarum* dominate when individuals are transferred onto noni, due to this bacterium acting as a detoxifying agent by metabolising the toxic compounds present in noni. In humans, *L. plantarum* is responsible for protecting the urogenital and intestinal tracts from infection from pathogenic bacteria (Reid and Burton, 2002). In *Drosophila*, *L. plantarum* has similarly been shown to protect against colonisation of pathogens in the gut (Ryu et al., 2008), by digesting sugars to produce lactic acid, which inhibits the growth of non-commensal organisms and promotes the growth of *Lactobacilli* that thrive in low pH conditions (e.g. Kleerebezem et al., 2003). It is also responsible for promoting larval growth when nutrients are scarce (Storelli et al., 2011), and plays a role in mating preferences (e.g. Sharon et al., 2010). Despite the clear role that *L. plantarum* plays on *D. sechellia* host physiology and likely role in digestion of toxic compounds, high levels of *L. plantarum* were also found when flies were reared on salak. Therefore, the dominance of *L. plantarum* may simply be due to the acidic conditions provided by both fruits. Further work is needed to elucidate the links between these two components.

Flies that were both reared on a salak diet were found to mate for significantly longer than salak flies that were paired with a partner from a different diet type, although they did also exhibit a significantly longer mating latency. Interestingly, pairs of flies both reared on ASG or both reared on noni mated for a similar duration as pairs that were reared on mixed diets. This finding is somewhat contrary to similar studies in other species. For example, studies have shown that individuals of three different species of

Drosophila with contrasting mating systems, *D. melanogaster*, *D. bifasciata* and *D. subobscura* (Lizé, McKay and Lewis, 2014), prefer to mate with partners reared on the same diet type. Although a preference was found for salak individuals to mate with other salak individuals, this preference did not translate into actual female mating investment, as females from this pairing displayed the lowest fecundity of all pairwise mating combinations. A number of studies have reported mixed responses in the resulting fecundity of mating preferences. For example, in *D. melanogaster*, no difference was observed in the number of eggs produced after a preferential mating (Heys et al., 2018a). Interestingly, although fecundity levels were high, we noted no significant increase in the egg production of females reared on noni fruit compared to other diets, despite the fact that noni provides *D. sechellia* with the dopamine precursor necessary for the progression of oogenesis by providing a critical chemical – octanoic acid (Lavista-Llanos et al., 2014). In fact, females reared on ASG and mated to a male also reared on ASG displayed a significant increase in egg production compared to females reared on noni and mated with an ASG male.

The weight of individuals at different life stages greatly varied depending on the diet on which they were reared. At the larval stage, no difference in weights was observed across any of the treatments, yet at the pupal stage, ASG pupae weighed significantly more than those reared on noni or salak. As such high abundances of *Lactobacillus plantarum* were found in the gut across all treatments; it could be that *L. plantarum*, which is known to promote larval growth under conditions where nutrients are scarce, is compensating for the host developing on this laboratory formulated diet. In contrast, at adulthood, both male and female ASG reared flies weighed significantly less than both noni and salak flies. One reason for this difference in weights at adulthood may be due to the presence of *B. cereus* and *Paenibacillus sp.* in the adult ASG flies that are not present in individuals reared on noni or salak. *B. cereus* and *Paenibacillus sp.* have been reported in wild populations of *D. ananassae* (Maji, Chakrabarti & Chatterjee, 2013), with some studies showing that the immune responses produced by *D. melanogaster* individuals in defence of the pathogen *B. cereus*, can have

detrimental effects on life span (Ma et al., 2012; Ma et al., 2013). Therefore, it could be that the immune responses elicited by *D. sechellia* when individuals are reared on the less–preferred diet of ASG override the beneficial effects of *L. plantarum* to negatively affect adult weight.

This is the first time the gut microbiota of *D. sechellia* has been examined in laboratory conditions under different dietary treatments. Our results are in-keeping with others that characterise the microbiota of wild-caught *D. sechellia* (Chandler et al., 2011) despite the fact that our population has been reared in the laboratory on a formulated diet for a number of years. Here we demonstrate that when *D. sechellia* are reared on its natural host at any time point, a shift in the gut microbiota can be seen. Our results are the first to show the direct change in gut microbiota when the same individuals are moved between vastly different diets – the natural host plant and a laboratory diet. Although we determine these differences in microbiota across dietary treatments, further work is needed to disentangle the effect of pH on the gut microbiota, from a shift in microbiota that enables specialisation within this species.

5. Chapter Five: Gut microbiota and octanoic acid detoxification in *Drosophila sechellia* and *Drosophila melanogaster*

5.1 Abstract

Adaptation to a novel food source can have significant evolutionary advantages. The fruit fly, *Drosophila sechellia*, is a specialist of the toxic plant, *Morinda citrifolia.* Little is known as to how *D. sechellia* has become resistant to the toxins in the fruit - comprised predominantly of octanoic acid - but behavioural preferences for the fruit have been documented due to the presence of two odorant-binding proteins (OBPs) that attract *D. sechellia* to *M. citrifolia*. We examine the potential role of the gut microbiota in adaptation to octanoic acid resistance in this species and its sister species, *Drosophila melanogaster*, to which the fruit is fatal. We use a combination of methods to analyse resistance to octanoic acid by conducting life history analysis, behavioural assays and bacterial analysis in both *D. sechellia* and *D. melanogaster*. We find that by creating experimental evolution lines of *D. melanogaster* supplemented with gut microbiota from *D. sechellia*, we can decrease aversion to octanoic acid, with flies even preferring to feed on food supplemented with the acid. We suggest this represents the first step in the evolutionary and ecological specialisation of *D. sechellia* to its toxic host plant, and that the gut microbiota, *Lactobacillus plantarum* in particular, is responsible for this.

5.2 Introduction

Many studies have examined the complex relationships between animals and plants (reviewed in Herrera and Pellmyr, 2009). The dynamic ecological and evolutionary interactions between an animal and its host plant can take many forms. For example, in many insect species, exploiting novel ecological niches involves the evolution of recognition systems to chemical cues, that

enable host-plant specificity. In some cases, insects have even become adapted to living on a toxic plant host, such as *Pierinae* butterflies that feed on toxic *Brassicales* (e.g. Edger et al., 2015). Developing resistance to a toxic host plant through specialisation can have a multitude of advantages, namely, the ability to exploit an otherwise unutilised resource and there are many ways that insects can overcome the toxins in the host plant. Such behavioural and physiological adaptations to exploit toxic resources can drive speciation of insects or other animals (Matsuo et al., 2007). However, it is argued that specialisation to a toxic host does not necessarily result in speciation itself, and therefore the role of ecological specialisation in speciation remains to be proven (Matsuo et al., 2007).

When plants are attacked, they often release harmful, secondary plant compounds in order to repel the attacker, also known as allochemicals (e.g. Gadamer, 1897; Erlich and Raven, 1964; Halkier and Gershenzon, 2006). Insects have evolved to cope with these harmful and often toxic compounds using an array of molecular and genetic adaptations (e.g. Matsuo et al. 2007). The participation of the gut microbiota in the transformation of plant chemicals is an important aspect to be considered when studying insect-plant interactions, and one that is gaining attention (e.g. Genta et al., 2006; Ceja-Navarro et al., 2015). Yet due to the complexity and diversity of gut microorganisms, there is little experimental evidence to support this idea. It was suggested by Douglas (1992) that a possible role of the midgut microbiota is in detoxification of toxic compounds and a study by Genta et al., (2006) in *Tenebrio molitor* highlighted the role of gut microbiota in detoxifying the cell walls of fungi and bacteria that typically inhabit their food source. Similarly, the coffee berry borer (*Hypothenemus hampei*), the most devastating insect pest of coffee crops worldwide, has adapted to metabolise caffeine – a toxic alkaloid present within coffee plants (Ceja-Navarro et al., 2015). Caffeine is shown to be degraded in the gut via *Pseudomonas* species, which subsist on caffeine as a sole source of carbon and nitrogen (Ceja-Navarro et al., 2015).

The majority of species within the *Drosophila melanogaster* species-complex are food generalists and saprophagous, meaning they feed on a variety of

decaying plant matter (Rohlfs and Kürschner, 2010). Studies have shown the importance of a diverse diet in creating and maintaining a diverse gut microbiota within *D. melanogaster*, with a diverse gut microbiota increasing survival and reducing development time (Rohlfs and Kürschner, 2010). In comparison, several species within the *D. melanogaster* species-complex have evolved some form of diet specialisation (e.g. Lachaise et al., 1988). One species within this group, *Drosophila sechellia*, is a specialist of ripe *Morinda citrifolia* fruit – a toxic fruit commonly known as the Tahitian Noni (Jones, 2005). *D. sechellia's* closely related species, *Drosophila simulans, Drosophila mauritiana* and *D. melanogaster* are notably repelled by the pungent scent of the fruit and even die upon contact (Legal, Chappe and Jallon, 1994; Legal, Moulin and Jallon, 1999). Resistance of *D. melanogaster* to the fruit is shown to be dependent on strain (Legal, David and Jallon, 1992).

Numerous studies have focussed on the underlying genetic and molecular mechanisms that have enabled *D. sechellia* to adapt to the toxins within noni. The ripe fruit is characterised by a large amount of carboxylic acids; notably, octanoic and hexanoic acids (Farine et al., 1996), with octanoic acid the primary reason for its high toxicity. Octanoic acid is a medium chain, fatty acid which *D. sechellia* has evolved resistance to and a preference for (Legal et al., 1994; Farine et al., 1996; Amlou et al., 1998), with *D. sechellia* being between five and six times more resistant than *D. melanogaster* to octanoic acid (Legal, Chappe and Jallon, 1994). The preference of *D. sechellia* to the noni fruit, and thus octanoic acid, is dependent on two genes (Matsuo et al., 2007). These genes encode odorant-binding proteins (OBPs) OBP57d and OBP57e which express on the gustatory sensilla on the legs. When *D. melanogaster* mutants for Obp57d and Obp57e encounter octanoic acid, they exhibit a behavioural shift and display a preference for the acid. This demonstrates that these genes are involved in the taste perception as an aversive signal of octanoic acid (Harada et al., 2008, 2012). The presence of both these OBPs in *D. sechellia* attracts flies to the fruit and is thought to be partly responsible for wild *D. sechellia* ovipositing exclusively on the noni fruit (Lachaise et al., 1988). In comparison, the presence of octanoic acid is

known to inhibit oviposition in *D. melanogaste*r (Legal, Moulin and Jallon, 1999). Although the genetic and molecular adaptations of *D. sechellia* to the noni fruit are well characterised, little attention has been paid to the potential role of gut microbiota in specialisation within this species.

In this study we investigated the effects of differing concentrations of the toxic compound octanoic acid, the main acidic constituent of *D. sechellia*'s natural host plant, *M. citrifolia*, on weight, development time, survival, bacterial load and diversity in *D. sechellia*. We then investigated the same effects in *D. sechellia's* sister species, *D. melanogaster*, to determine any differences between a fruit specialist and a fruit generalist species. We analysed both inbred and outbred lines in *D. melanogaster* in order to test the effect of strain on ability to withstand octanoic acid exposure. We predicted that *D. melanogaster* would have a reduced weight, development time and survival ability compared to *D. sechellia*, particularly in the inbred strain in which genetic diversity is low. We also predicted that we would observe differences in the diversity and abundance of the gut microbiota of *D. sechellia* compared to *D. melanogaster*, due to *D. sechellia* possessing a more specialised gut microbiota that enable them to withstand high concentrations of octanoic acid. We further examined the role of the gut microbiota in this specialisation by creating experimental evolution lines of *D. melanogaster* supplemented with *D. sechellia* gut microbes. As we predict the gut microbiota plays a key role in specialisation in *D. sechellia*, we predicted that after a number of generations *D. melanogaster* would be more resistant to exposure to octanoic acid.

5.3 Materials and methods

5.3.1 Fly maintenance

D. sechellia stocks were obtained from the National Drosophila Species Stock Centre located in San Diego. One line of outbred flies was utilised (line 0.08) that was collected on Cousin Island, Seychelles in 1980 and maintained in the laboratory ever since. Wild-type, *Wolbachia*-free *D. melanogaster* stocks were isolated from an outbred population collected in

Lyon. A *Wolbachia*-free isoline of *D. melanogaster* was obtained from the National Drosophila Species Stock Center located in San Diego. This line was originally collected in USA in 1980 and maintained in the laboratory ever since. All flies were reared at 25°C under a 12:12hour light:dark cycle. Flies were kept in standard 75x25mm *Drosophila* vials containing 25ml of standard *Drosophila* food composed of yeast/agar/maize/sugar. Flies were moved to new vials every 4 days.

5.3.2 Survival rate

To determine resistance to the toxic octanoic acid compound, the survival of the three different strains, outbred *D. melanogaster*, inbred *D. melanogaster* and *D. sechellia*, to exposure of differing concentrations of octanoic acid was measured. Newly emerged, virgin adults were isolated from the stock populations and transferred to fresh vials containing 25ml standard *Drosophila* media and left for 3 days to mature. During this time, ≥99% octanoic acid (purchased from Sigma-Aldrich) was diluted in distilled water to the following concentrations: 0% (distilled water), 1%, 5%, 10%, 25%, 50%. 30µl of the acid solute was pipetted onto the surface of a fresh vial containing 25ml standard *Drosophila* media. The vial was tipped to the side to ensure the acid covered the entire surface of the food media and left to dry for 2 hours. After this time, males and females were separated according to sex and placed at a standard density of 10 individuals per vial. The number of dead individuals was counted every 24hours for a total period of 168hours and the survival rate calculated ($N_{InbredDmel}$ = 50; $N_{OutbredDmel}$ = 50; $N_{Dsechellia}$ = 50 for each acid concentration).

5.3.3 Development time

Mated adults at a density of five females and five males were placed onto fresh vials containing the following concentrations of octanoic acid: 0% (distilled water), 1%, 5%, 10%, 25%, 50%, to determine the effects of different concentrations of octanoic acid on development time. The pairs

were left to oviposit. Development time was measured as the number of days from female oviposition to day of adult emergence. Vials were checked at three time points within each day – 9am, 12pm and 5pm – and the cumulative number of adults emerged from each time point was scored. Emergent adult flies from each time point were removed from the vial and placed into a fresh vial containing 25ml of standard *Drosophila* food (sample sizes are documented below (Table 1)).

Table 1. Sample sizes for the measurements of development time for each species and strain measured: *D. sechellia*, *D. melanogaster* inbred, *D. melanogaster* outbred.

Species	Treatment	Sample size
Inbred *D. melanogaster*	0%	279
Inbred *D. melanogaster*	1%	275
Inbred *D. melanogaster*	5%	202
Inbred *D. melanogaster*	10%	157
Inbred *D. melanogaster*	25%	117
Inbred *D. melanogaster*	50%	111
Outbred *D. melanogaster*	0%	235
Outbred *D. melanogaster*	1%	213
Outbred *D. melanogaster*	5%	185
Outbred *D. melanogaster*	10%	124
Outbred *D. melanogaster*	25%	121
Outbred *D. melanogaster*	50%	60
D. sechellia	0%	152
D. sechellia	1%	139
D. sechellia	5%	198
D. sechellia	10%	136
D. sechellia	25%	174
D. sechellia	50%	176

5.3.4 Offspring adult weight

We examined the effect of different concentrations of octanoic acid on *D. melanogaster* and *D. sechellia* adult emergence weight. Vials were checked daily at three time points – 9am, 12pm and 5pm – to check for any newly emerged individuals. The adult flies were isolated as virgins and separated

according to sex. Adults were placed into vials at a standard density of ten per vial and left for two hours to allow their wings to dry out and inflate. Two hours later, vials were placed into the freezer at -18°C and left overnight. Subsequently, the adults were removed and weighed using an Ohaus five place balance and their weight was recorded (in mg) to four decimal places. Male and female measurements for each treatment were recorded and analysed separately (sample sizes are documented below (Table 2)).

Table 2. Sample sizes for the measurements of adult emergence weight for each species, sex and strain measured: *D. sechellia*, *D. melanogaster* inbred, *D. melanogaster* outbred.

Species	Sex	Treatment	Sample size
Inbred *D. melanogaster*	Female	0%	200
Inbred *D. melanogaster*	Female	1%	275
Inbred *D. melanogaster*	Female	5%	46
Inbred *D. melanogaster*	Female	10%	45
Inbred *D. melanogaster*	Female	25%	50
Inbred *D. melanogaster*	Female	50%	44
Inbred *D. melanogaster*	Male	0%	235
Inbred *D. melanogaster*	Male	1%	50
Inbred *D. melanogaster*	Male	5%	50
Inbred *D. melanogaster*	Male	10%	50
Inbred *D. melanogaster*	Male	25%	50
Inbred *D. melanogaster*	Male	50%	43
Outbred *D. melanogaster*	Female	0%	59
Outbred *D. melanogaster*	Female	1%	52
Outbred *D. melanogaster*	Female	5%	69
Outbred *D. melanogaster*	Female	10%	50
Outbred *D. melanogaster*	Female	25%	50
Outbred *D. melanogaster*	Female	50%	50
Outbred *D. melanogaster*	Male	0%	51
Outbred *D. melanogaster*	Male	1%	49
Outbred *D. melanogaster*	Male	5%	56
Outbred *D. melanogaster*	Male	10%	45
Outbred *D. melanogaster*	Male	25%	50
Outbred *D. melanogaster*	Male	50%	53
D. sechellia	Female	0%	52
D. sechellia	Female	1%	51
D. sechellia	Female	5%	50
D. sechellia	Female	10%	51
D. sechellia	Female	25%	51

D. sechellia	Female	50%	52
D. sechellia	Male	0%	54
D. sechellia	Male	1%	51
D. sechellia	Male	5%	51
D. sechellia	Male	10%	53
D. sechellia	Male	25%	53
D. sechellia	Male	50%	50

5.3.5 Bacterial analysis

Flies were collected from vials containing each concentration of octanoic acid (0%, 1%, 5%, 10%, 25%, 50%) and were first surface sterilised in 70% ethanol, rinsed in distilled water and air dried. The head was then removed. Two guts were dissected into each Eppendorf containing 250µl of sterile LB (Lysogeny Broth) broth (Bertani, 2004). An equal number of males and females were used to ensure there were no sex-specific differences in the bacterial content. Gut tissue was homogenised with a sterile plastic pestle. 100µl of gut homogenate was pipetted onto BHI (Brain, Heart Infusion) agar (Atlas, 2004) and spread-plated using a sterile glass loop. BHI media was used as it was found to favour greater colony growth. Plates were left to air dry aseptically, before being closed and sealed with parafilm. Plates were incubated at 25°C for 72 hours, and bacterial load was quantified by performing CFU (Colony Forming Unit) counts.

Single colonies were isolated using a sterile 1µl loop and placed into an Eppendorf with 10µl sterile water. PCR amplification was performed in a 25µl reaction volume consisting of 10µl nuclease-free water, 13µl Taq green master mix, 0.5µl of forward primer 27F (5'- AGAGTTTGATCMTGGCTCAG-3') and reverse primer 1492R (5′-GGTTACCTTGTTACGACTT-3') and 1µl of template DNA. Thermal cycling was performed for 90 seconds at 95°C as initial denaturation, followed by 35 cycles of 30 sec at 95°C for denaturation, 30 sec at 55 °C as annealing, 90 sec at 72 °C for extension, and final extension at 72 °C for 5 min. 1500 bp 16S PCR products were purified with Ampure beads and subjected to Sanger sequencing. The resulting

sequences were identified using NCBI BLAST against the nt database (Altschul et al., 1990).

5.3.6 Experimental evolution of *D. melanogaster* lines exposed to *D. sechellia* gut microbiota

To determine whether it is the gut microbiota that enables *D. sechellia* to become attracted to and feed on a diet that contains high levels of the toxic compound, octanoic acid, we created a series of experimental evolution lines. Using the stock population of outbred *D. melanogaster*, we added *D. sechellia* gut solute to the dietary media over several generations. Here, stock population *D. sechellia* that were continually reared on a noni diet, were first surface sterilised in 70% ethanol, rinsed in distilled water and air dried. The head was then removed. Two guts were dissected into each Eppendorf containing 250µl of sterile LB (Lysogeny Broth) broth. An equal number of males and females were used to ensure there were no sex-specific differences in the bacterial content. Gut tissue was homogenised with a sterile plastic pestle. 30µl of gut isolate was then pipetted onto the surface of a vial containing 25ml standard *Drosophila* media. The vial was tipped to the side to ensure the solute covered the entire surface of the food media and left to dry for 20minutes. After this time, newly emerged, virgin males and females were isolated from the stock population of outbred *D. melanogaster* and placed at a standard density of 10 males and 10 females per vial. After pupae were seen in all vials, the adult flies were removed so the offspring and adults did not interbreed. Once the offspring had emerged as adults, a sub-sample of the first generation were isolated. Some of these individuals were harvested and their gut bacterial content and diversity analysed. The rest of the sub-sample of the first generation were placed into octanoic acid aversion trials (see protocol below). The remaining flies were placed onto a fresh vial similarly containing 25ml standard *Drosophila* media and 30µl of gut isolate. This process was repeated until the offspring reached the 10^{th} generation. Newly emerged adult offspring were harvested as before. The remaining adults were placed into octanoic acid aversion trials.

Thus, the aversion trials were conducted on unselected stock individuals for each species, and the first and tenth generations of the experimental evolution lines.

5.3.7 Octanoic acid aversion trials

In order to test whether experimental evolution of outbred *D. melanogaster* reared on a diet supplemented with *D. sechellia* gut microbiota reduces aversion to the toxic octanoic acid, aversion trials were performed, using a similar methodology to that utilised previously (e.g. Dekker et al., 2006). Newly emerged, virgin adults were isolated from the *D. sechellia* and outbred *D. melanogaster* stock populations, separated according to sex and placed into fresh vials containing 25ml standard *Drosophila* media. Similarly, newly emerged, virgin adults were isolated from the first generation (hereon known as Dmel 1) and 10th generation (hereon known as Dmel 10) experimental evolution lines described above, separated according to sex and placed into fresh vials containing 25ml standard *Drosophila* media. Flies were left to mature for 3 days before being placed into an aversion arena (Figure 1). The aversion arena consisted of a standard petri dish (measuring 100mm x 15mm) containing two pieces (10g) of standard *Drosophila* food located at opposite ends, with a marked line half-way across clearly showing the two separate sides. 10ul of concentrated octanoic acid was pipetted on to one of these pieces of food. An individual fly was gently aspirated into the centre of the arena and left to acclimatise for five minutes. After this time, the side on which the fly was located was scored as its preference, i.e., either the side with food containing octanoic acid, or without (female N_{Dmel} = 50, male N_{Dmel} = 50; female N_{Dsech} = 50, male N_{Dsech} = 50; female N_{Dmel1} = 51, male N_{Dmel1} = 50; female N_{Dmel10} = 49, N_{Dmel10} = 48).

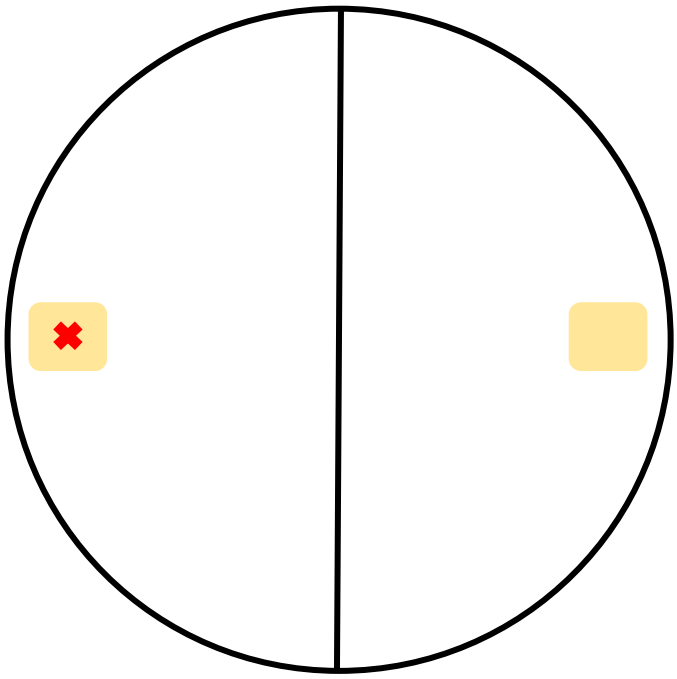

Figure 1. An example of the aversion arenas used to test how averse first and tenth generation *D. melanogaster* flies are to octanoic acid once they have undergone experimental evolution with *D. sechellia* gut microbiota. These flies were compared to stock population flies of *D. sechellia* and *D. melanogaster*, to see if their preferences initially differed. Sexes were tested separately. The red 'X' represents the addition of octanoic acid to the food.

5.3.8 Statistical analysis

All data were analysed using R (version 3.3.0; R Core Team, 2016). Adult weight was analysed using a General Linear Model (GLM), whereas the aversion data was analysed using a binomial GLM. Both adult weight and the aversion data were first analysed with both sexes grouped, before further analysis separated according to sex were performed. Variation in development time and survival response to octanoic acid was analysed via Cox Proportional-Hazard Regressions. Development failure of flies was used as the 'event' for the development time data and 'death' for the survival data. The *Survdiff* function was used to assess differences between two or more survival curves according to treatments. The *coxph* function was used to assess differences between treatments. This allowed treatments to be

compared in a pairwise fashion, to ascertain whether all treatments differed, or whether any significant differences observed were derived from a single treatment.

5.4 Results

5.4.1 Survival rate

D. sechellia females reared on 0% octanoic acid exhibited significantly higher survival than females reared on the 1% concentration (Z_5=3.340; P<0.001), the 5% concentration (Z_5=3.936; P<0.001), the 25% treatment (Z_5=4.740; P<0.001), or the 50% treatment (Z_5=4.464; P<0.001) (Figure 2). However, no significant difference was observed between 0% and 10% treatments (Z_5=1.664; P=0.096). Flies reared on the 10% treatment exhibited significantly higher survival than flies reared on the 25% treatment (Z_5=3.564; P<0.001) or the 50% (Z_5=3.448; P<0.001).

In female inbred *D. melanogaster*, no difference was observed in the survival ability of 0% treated and 1% treated flies (Figure 2) (Z_5=0.778; P=0.436), but 0% treated flies were significantly more able to survive than those reared on 5% (Z_5=4.020; P<0.001), 10% (Z_5=3.538; P<0.001), 25% (Z_5=9.943; P<0.001), or 50% (Z_5=13.075; P<0.001). Flies reared on the 1% treatment were significantly more able to survive than those at higher concentrations: 5% (Z_5=3.340; P<0.001), 10% (Z_5=2.836; P=0.004), 25% (Z_5=9.689; P<0.001), or 50% (Z_5=13.102; P<0.001). Females reared at the 50% octanoic acid concentration exhibited significantly higher mortality than those reared at 25% (Z_5=5.919; P<0.001).

In female outbred *D. melanogaster,* flies reared at a 25% concentration had a significantly higher mortality rate than flies reared on any other treatment: 0% (Z_5=-7.205; P<0.001), 1% (Z_5=-7.203; P<0.001), 5% (Z_5=-7.236; P<0.001), 10% (Z_5=-6.943; P<0.001), including the 50% concentration (Z_5=-2.789; P=0.005). Similarly, flies reared at 50% concentration had a significantly higher mortality rate than flies reared at 0% (Z_5=-5.825;

P<0.001), 1% (Z_5=-5.822; P<0.001), 5% (Z_5=-5.590; P<0.001) or 10% (Z_5=-5.816; P<0.001).

When comparing the proportion of survived females across species, there is little difference in survival rates in *D. sechellia* across all octanoic acid concentrations, compared to inbred *D. melanogaster* (Figure 2). Here, females reared at higher concentrations exhibit a higher mortality, with all individuals recorded as dead in the 25% and 50% treatments, unlike in *D. sechellia* in which approximately half the sample were still alive at the end of the trial. The proportion of survived individuals is around 0.3 in outbred *D. melanogaster* at higher concentrations (25% and 50%), with individuals reared at low concentrations (0%, 1%, 5% and 10%) exhibiting similar survival rates to *D. sechellia*.

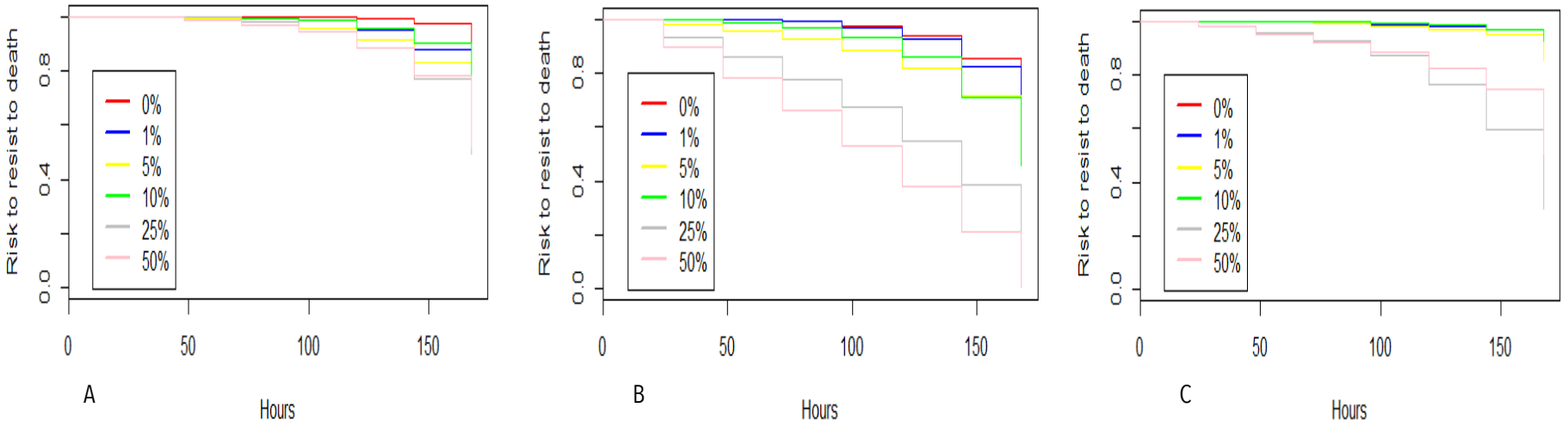

Figure 2. the proportion of survived female flies when exposed to standard *Drosophila* food containing differing concentrations of octanoic acid – 0%, 1%, 5%, 10%, 25%, 50%. The vials were checked every 24 hours with the last time point 168 hours, and the number of dead flies recorded. The graphs are arranged according to species: *D. sechellia* (A), inbred *D. melanogaster* (B) and outbred *D. melanogaster* (C).

In contrast to female survival rates, in male *D. sechellia*, flies reared on the 0% acid treatment had equal survival rates to all other treatments, with no difference found compared to 1% reared flies (Z_5=0.941; P=0.357), 5% (Z_5=1.341; P=0.180), 10% (Z_5=-1.448; P=0.148), 25% (Z_5=0.808; P=0.419) or 50% (Z_5=-0.045; P=0.964) (Figure 3). Similar to the female *D. sechellia*, male flies reared on the 10% treatment were significantly more able to survive than those reared on 1% treatment (Z_5=-2.239; P=0.019), the 5% treatment (Z_5=-2.691; P=0.007) and the 25% treatment (Z_5=2.207; P=0.027).

In male inbred *D. melanogaster* flies, 0% reared flies had a significantly higher survival ability than 25% flies (Z_5=7.830; P<0.001) and 50% flies (Z_5=12.660; P<0.001). Flies reared on the 1% acid concentration had a higher proportion survived than all other concentrations, including 0%: (Z_5=3.013; P=0.002), 5% (Z_5=3.405; P<0.001), 10% (Z_5=4.668; P<0.001), 25% (Z_5=9.556; P<0.001) and 50% (Z_5=13.244; P<0.001). A significantly higher mortality was observed in the 25% and 50% treatments compared to both the 5% (Z_5=7.481; P<0.001, Z_5=12.430; P<0.001, respectively) and the 10% treatment (Z_5=6.311; P<0.001, Z_5=11.670; P<0.001, respectively).

In male outbred *D. melanogaster*, no significant difference in survival ability is found between flies reared at the lower concentrations. Flies reared at 10% concentration had a significantly higher mortality than those reared at 0% (Z_5=-3.120; P=0.001), 1% (Z_5=-3.095; P=0.001) and 5% (Z_5=-2.618; P=0.008) but this was significantly lower than those reared at 25% and 50% (Z_5=5.770; P<0.001; Z_5=5.790; P<0.001, respectively). Similarly, flies reared at 25% and 50% had a significantly higher mortality than those reared at 0% (Z_5=-7.061; P<0.001, Z_5=-7.074; P<0.001), 1% (Z_5=-7.035; P<0.001, Z_5=-7.048; P<0.001) and 5% (Z_5=-7.026; P<0.001, Z_5=-7.040; P<0.001). All other pairwise comparisons for survival analysis are non-significant.

When comparing the proportions of survived males across species, there is a vast difference in the final proportion of survived *D. sechellia* males

compared to inbred *D. melanogaster* (Figure 3). Male inbred *D. melanogaster* are less able to survive than *D. sechellia* males, particularly when reared at high concentrations (e.g. 25% and 50%). The survival ability of outbred *D. melanogaster* flies is better than that of inbred flies with a higher proportion survived at the end of the trial, even when males were placed at high concentrations, but the proportion was less than that of *D. sechellia.*

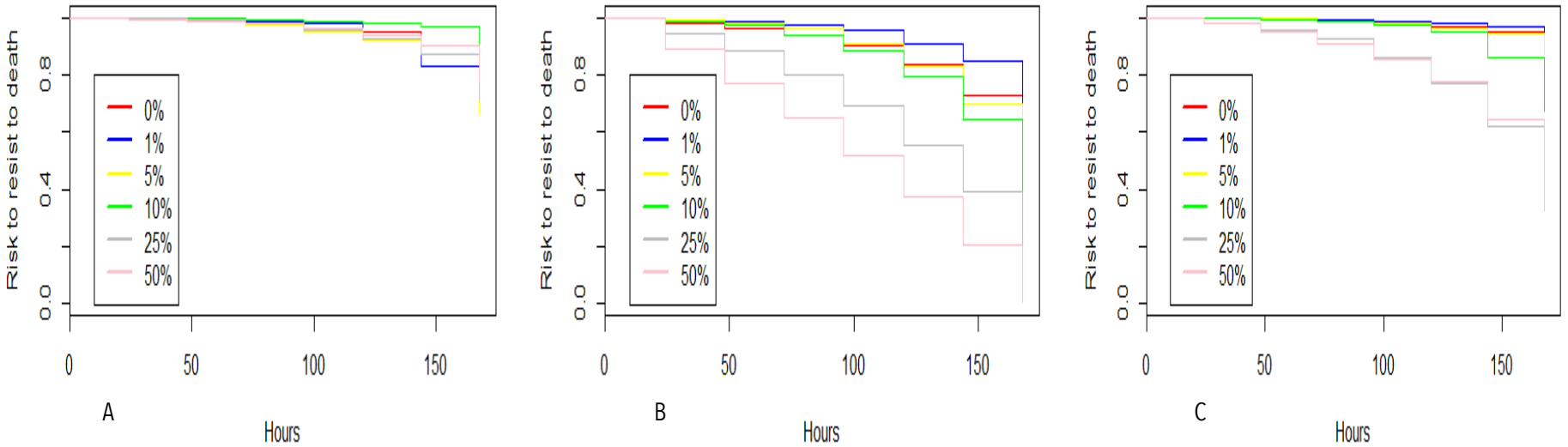

Figure 3. the proportion of survived male flies when exposed to standard *Drosophila* food containing differing concentrations of octanoic acid – 0%, 1%, 5%, 10%, 25%, 50%. The vials were checked every 24 hours with the last time point 168 hours, and the number of dead flies recorded. The graphs are arranged according to species: *D. sechellia* (A), inbred *D. melanogaster* (B) and outbred *D. melanogaster* (C).

5.4.2 Development time

In *D. sechellia*, no significant differences were found in the time taken to develop from egg to adulthood between any of the acid treatments (Figure 4). In particular, no significant difference was observed between the 0% and the 50% treatments (F_5=0.740; P=0.459). However, in the inbred line of *D. melanogaster*, significant differences were observed between all treatments with flies reared on higher concentrations of acid taking longer to develop than those reared on smaller concentrations (Figure 4). Similarly, variation was seen in the time taken for outbred *D. melanogaster* individuals to develop when reared on different concentrations of acid (Figure 4). Flies reared on 0% acid concentration took significantly less time to develop than those reared on 1% treatment (F_5=-7.930; P<0.001), 5% treatment (F_5=-4.647; P<0.001), 10% treatment (F_5=-8.679; P<0.001), 25% (F_5=-7.808; P<0.001) or 50% (F_5=-4.900; P<0.001). Similar to the inbred *D. melanogaster*, no significant differences were observed between 10% and 25% reared flies (F_5=0.759; P=0.447),10% and 50% flies (F_5=1.733; P=0.083), 25% and 50% flies (F_5=1.098; P=0.272). All other pairwise comparisons are non-significant. A full summary of all pairwise comparisons is provided in the appendix.

When comparing the development times across species, there is little variation in the time taken to develop on different concentrations of octanoic acid in *D. sechellia* (Figure 4). In comparison, inbred *D. melanogaster* take the shortest time to develop when reared at low concentrations, with time increasing as concentration increases. In outbred *D. melanogaster*, flies reared at low concentrations (e.g. 0% and 1%) take the shortest time to develop, similar to inbred *D. melanogaster*. However, outbred *D. melanogaster* flies reared at the higher concentrations (e.g. 25% and 50%) take less time than those reared at middling concentrations, such as 5% and 10%.

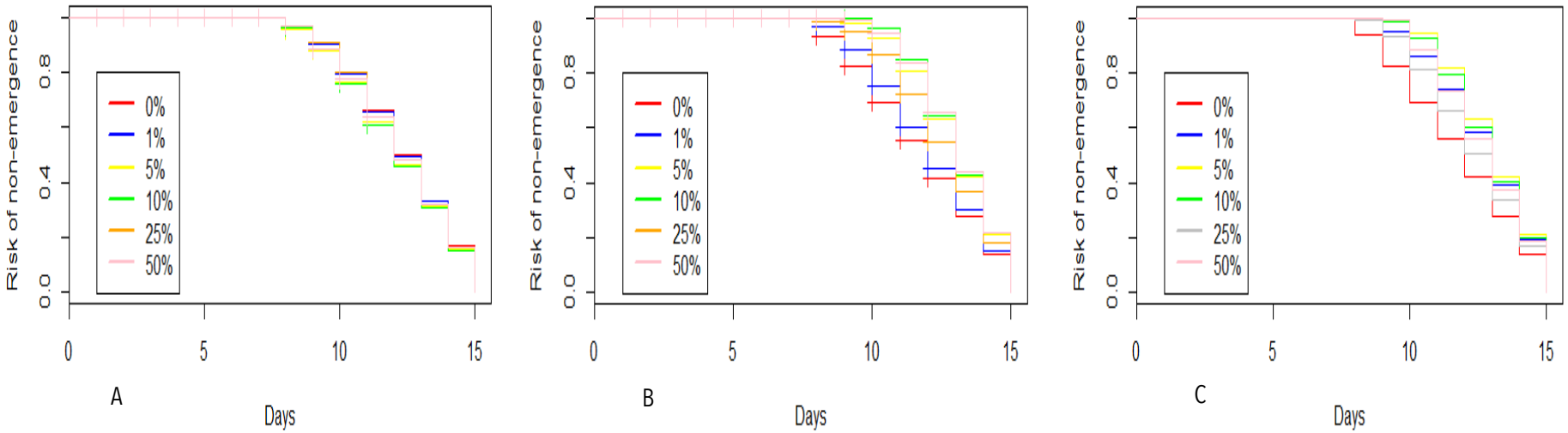

Figure 4. Development time failure measured in days, as the risk to die before adulthood. Eggs were reared on standard *Drosophila* media that was supplemented with differing concentrations of octanoic acid – 0%, 1%, 5%, 10%, 25%, 50%. The graphs are arranged according to species: *D. sechellia* (A), inbred *D. melanogaster* (B) and outbred *D. melanogaster* (C).

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5.4.3 Female offspring adult weight

In *D. sechellia*, female offspring reared on a 0% acid concentration weighed significantly more than females reared on 1% (T_5=-4.244; P<0.001), 10% (T_5=-2.581; P=0.010) or 25% (T_5=-2.294; P=0.022), but not 5% (T_5=-1.806; P=0.071) or 50% (T_5=1.109; P=0.2684) (Figure 5). Females reared on 50% acid concentration were significantly heavier than flies reared on all other concentrations, except 0%.

In inbred *D. melanogaster* offspring, females reared on 0% acid concentration weighed significantly more than all other treatments, except 5% (T_5=-1.265; P=0.207): 1% (T_5=-7.877; P<0.001), 10% (T_5=-10.339; P<0.001), 25% (T_5=-6.760; P<0.001) and 50% (T_5=-19.368; P<0.001). Flies reared on 50% acid concentration weighed significantly less than all other treatments: 1% (T_5=9.573; P<0.001), 5% (T_5=14.628; P<0.001), 10% (T_5=7.163; P<0.001), 25% (T_5=10.603; P<0.001).

Female offspring of outbred *D. melanogaster* reared on 1% acid concentration weighed significantly more than all other concentrations: 0% (T_5=-4.853; P<0.001), 5% (T_5=--9.595; P<0.001), 10% (T_5=-14.142; P<0.001), 25% (T_5=-13.217; P<0.001) and 50% (T_5=-12.149; P<0.001). Females reared on 0% acid concentration weighed significantly more than 5% flies (T_5=-4.704; P<0.001), 10% (T_5=-9.727; P<0.001), 25% (T_5=-8.777; P<0.001) and 50% (T_5=-7.661; P<0.001).

Female weight has highly variable depending on species (Figure 5). Overall, female *D. sechellia* weighed less than inbred *D. melanogaster* flies, but there was less difference in weight across octanoic acid concentration. Female weight was more variable in both inbred and outbred *D. melanogaster* strains, with flies generally weighing more at low concentrations compared to high.

5.4.4 Male offspring adult weight

In male *D. sechellia* offspring, 0% flies weighed significantly less than 5% flies (T_5=2.346; P=0.19), 10% flies (T_5=3.401; P<0.001) and 25% flies

(T_5=2.630; P=0.008) (Figure 5). Male offspring reared at 1% concentration weighed significantly less than 5% (T_5=2.797; P<0.005), 10% (T_5=3.841; P<0.001) and 25% flies (T_5=3.081; P=0.002).

In male inbred *D. melanogaster* offspring, unlike female flies, 0% flies weighed significantly more than all other treatments: 1% (T_5=-9.574; P<0.001), 5% (T_5=-6.607; P<0.001), 10% (T_5=-16.772; P<0.001), 25% (T_5=-7.971; P<0.001) and 50% (T_5=-19.016; P<0.001). Flies reared on 1% acid concentration weighed significantly more than 10% flies (T_5=-5.603; P<0.001) and 50% flies (T_5=-7.993; P<0.001) but weighed significantly less than flies reared on 5% acid concentration (T_5=2.516; P=0.012).

In comparison, male outbred *D. melanogaster* offspring reared at 0% weighed significantly more than flies reared on 1% acid concentration (T_5=-7.564; P<0.001), 5% (T_5=-5.830; P<0.001), 10% (T_5=-13.483; P<0.001), 25% (T_5=-11.467; P<0.001) and 50% (T_5=-12.639; P<0.001). Males reared on 1% and 5% acid concentrations weighed significantly more than flies reared on 10% (T_5=-6.028; P<0.001, T_5=-8.137; P<0.001, respectively), 25% (T_5=-3.826; P<0.001, T_5=-5.929; P<0.001, respectively) and 50% (T_5=-4.840; P<0.001, T_5=-7.022; P<0.001, respectively).

Similar to females, male weight was also highly variable across species (Figure 5). Male *D. sechellia* weight varied less than both inbred and outbred *D. melanogaster* males, with *D. sechellia* males always weighing more than both inbred and outbred *D. melanogaster* males at high concentrations, such as 50%.

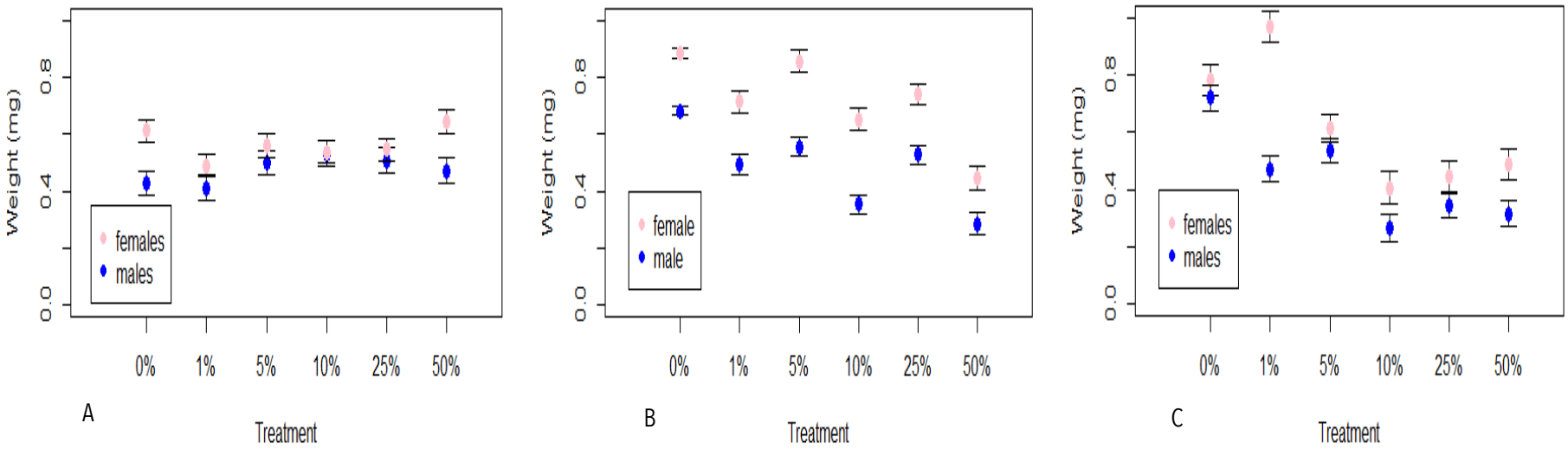

Figure 5. Weight of adult offspring (mg) of newly emerged adults, when their parents were reared on different concentrations of octanoic acid – 0%, 1%, 5%, 10%, 25%, 50%. The graphs are arranged according to species: *D. sechellia* (A), inbred *D. melanogaster* (B) and outbred *D. melanogaster* (C). Females are shown here by the pink dots and males represented by the blue.

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5.4.5 Bacterial analysis

Bacterial colony growth was observed in all treatments, with both greater diversity and greater abundance of bacteria found in the *D. melanogaster* inbred and outbred flies (Table 1). Sanger sequencing data identifies colony 1 as *Lactobacillus plantarum*; colony 2 as *Paenibacillus sp*. and colony 3 as *Bacillus cereus*. In all the *D. sechellia* adult flies and almost all the outbred *D. melanogaster* flies, only *L. plantarum* growth was observed. This is in comparison to the adult inbred *D. melanogaster* flies, which exhibited substantial growth of both *L. plantarum*, *Paenibacillus sp*. and *B. cereus* bacteria. *Paenibacillus sp*. and *B. cereus* colonies appear to be present in higher numbers when the *D. melanogaster* strains are reared at higher concentrations of the octanoic acid. The number of colonies identified of each bacterial species, from each *Drosophila* species, is included within the appendix (Table 2).

Table 1. Presence or absence of bacterial species detected in the midgut of adult *D. sechellia* and both outbred and inbred *D. melanogaster* flies, when reared on diets containing different concentrations of octanoic acid. Presence of a certain bacterial species is denoted with a tick (✓) and absence is with a cross (×).

Species	Concentration	*L. plantarum*	*Paenibacillus sp.*	*B. cereus*
D. sechellia	0%	✓	×	×
D. sechellia	1%	✓	×	×
D. sechellia	5%	✓	×	×
D. sechellia	10%	✓	×	×
D. sechellia	25%	✓	×	×
D. sechellia	50%	✓	×	×
Outbred *D. melanogaster*	0%	✓	×	×
Outbred *D. melanogaster*	1%	✓	×	×
Outbred *D. melanogaster*	5%	✓	×	×
Outbred *D. melanogaster*	10%	✓	×	×
Outbred *D. melanogaster*	25%	✓	×	×
Outbred *D. melanogaster*	50%	✓	✓	×
Inbred *D. melanogaster*	0%	✓	×	×
Inbred *D. melanogaster*	1%	✓	×	×
Inbred *D. melanogaster*	5%	✓	✓	✓
Inbred *D. melanogaster*	10%	✓	✓	✓
Inbred *D. melanogaster*	25%	✓	✓	✓
Inbred *D. melanogaster*	50%	✓	✓	✓

5.4.6 Octanoic acid aversion trials

Males and females were tested separately to determine if there was a difference in aversion rate according to sex. No difference was observed so subsequent analysis was performed with both sexes grouped together and separated according to species. Unselected *D. sechellia* (hereon known as Dsech ST) were found to prefer the food containing octanoic acid, in comparison to the unselected *D. melanogaster* stock population (hereon known as Dmel ST), which were significantly more averse (F_5=-2.124; P=0.027). First generation *D. melanogaster* (Dmel 1) flies that had been reared on a diet supplemented with *D. sechellia* gut microbiota, were significantly more averse to the food containing octanoic acid than Dsech ST flies (F_5=-2.541; P=0.011). There was no difference in aversion of octanoic acid found between Dsech ST and tenth generation *D. melanogaster* (Dmel 10) flies that had been reared on a diet supplemented with *D. sechellia* gut microbiota (F_5=1.371; P=0.170). Dmel 10 flies were also found to be significantly less averse to octanoic acid than Dmel ST (F_5=2.889; P=0.003); with no significant difference shown between Dmel ST and Dmel 1 flies (F_5=-0.973; P=0.330). Notably, Dmel 1 were found to be significantly more averse to the food containing octanoic acid than Dmel 10 flies (F_5=3.774; P<0.001).

Table 2. Proportions of times flies from each treatment group – *D. sechellia, D. melanogaster, D. melanogaster* 1st generation and *D. melanogaster* 10th generation – chose either the diet with octanoic acid present, or the diet without, when placed in the aversion assays.

	Choice	
Species	**Proportion with octanoic acid**	**Proportion without**
D. sechellia	0.60	0.40
D. melanogaster	0.35	0.65
D. melanogaster 1st gen	0.29	0.71
D. melanogaster 10th gen	0.69	0.31

5.5 Discussion

As predicted, differences in survival ability, development time and resulting offspring adult weight were shown across the three different study species – *D. sechellia*, inbred *D. melanogaster* and outbred *D. melanogaster* – when exposed to increasing concentrations of toxic, octanoic acid. *D. sechellia* generally exhibited higher survival rates in both males and females, when exposed to higher concentrations of octanoic acid. Inbred and outbred *D. melanogaster* exhibited similar survival ability, with higher mortality seen at higher acid concentrations. The development time of offspring whose parents were reared on differing concentrations differed according to species, with *D. sechellia* and outbred *D. melanogaster* exhibiting a similar development time, with more variance seen in inbred *D. melanogaster*. Offspring weight was also more variable in inbred and outbred *D. melanogaster* compared to *D. sechellia*, with both strains of *D. melanogaster* weighing more than *D. sechellia* at lower concentrations, but this effect was reversed at higher concentrations. Similar to previous results (Chandler et al., 2011), when reared on a standard *Drosophila* diet supplemented with octanoic acid, *D. sechellia* gut microbiota was found to be consistent to those isolated from the natural host plant, *M. citrifolia*. The bacteria isolated from *D. sechellia* was characterised as *L. plantarum*. This was also present in the gut of both inbred and outbred *D. melanogaster*, with *B. cereus* and *Paenibacillus sp.* also identified.

As predicted, both female and male *D. sechellia* were able to survive on a diet containing the highest concentration of octanoic acid (50%) with around 50% of individuals still alive at the end of the assay. Little differences were observed between any of the different acid concentrations, with the main difference that males reared on diets supplemented with 10% acid displayed a higher survival than those reared on a diet containing 0% octanoic acid. This suggests that addition of octanoic acid to *D. sechellia* is beneficial to survival, at least at lower concentrations. The ability of *D. sechellia* to survive at high concentrations of octanoic acid is somewhat to be expected as *M. citrifolia's,* main toxic constituent is octanoic acid, although there is some

variation in the natural concentrations found, with some studies reporting 58% (Farine et al., 1996) and others 70% (Pino et al., 2010).

Males and females from both the inbred and outbred lines of *D. melanogaster* were able to survive when placed onto a diet containing all concentrations of octanoic acid (Legal, Chappe & Jallon, 1994; Legal, Moulin & Jallon, 1999), however survival at high concentrations was lower than *D. sechellia* flies. This result is somewhat surprising as previous studies have noted that *D. melanogaster* dies upon contact with *M. citrifolia*, with most doing so within one hour (Legal, Chappe & Jallon, 1994; Legal, Moulin & Jallon, 1999). Survival of both sexes in the inbred *D. melanogaster* strain was significantly reduced at higher concentrations compared to a non-acidic diet, with nearly all individuals recorded dead at the end of the time period. The survival ability of outbred *D. melanogaster* was substantially better than the inbred strain, with only around 50% of females and 30% of males recorded as dead when reared at 50% acid concentration, at the end of the study. The outbred *D. melanogaster* strain may have a better ability to survive on the octanoic acid due to it being a wild-type strain and maintaining genetic diversity. Further, as *D. sechellia* is a sister species of *D. melanogaster*, the outbred or wild-type strain are more likely to share more genetic information than the inbred strain, including genes that underpin resistance to *M. citrifolia*. Animals with increased genetic diversity are also known to adapt to stress better than those with reduced genetic diversity (e.g. Bell, 2013).

The development time of offspring that emerged from adults that were reared on different concentrations of octanoic acid was measured. In *D. sechellia*, no differences were observed in the development time of offspring across any of the octanoic acid concentrations. Similarly, the outbred strain of *D. melanogaster* also exhibits little variation in development time between all octanoic acid concentrations, but flies reared at 0% took the shortest time to develop. This is in comparison to the inbred *D. melanogaster*, in which flies reared at the 0% acid concentration took significantly longer to develop than flies reared at higher concentrations. It is surprising that outbred *D. melanogaster* flies displayed a similar development time to *D. sechellia*. This

is likely due to the conserved genetic diversity in the outbred line. Similar studies have shown that inbred *D. melanogaster* lines have a significantly reduced lifespan when exposed to dietary-restrictions, compared to that of outbred lines such as Dahomey (Grandison et al., 2009).

No difference was observed in adult weight of *D. sechellia* offspring, in parent flies reared on both 0% and 50% octanoic acid concentrations, with little variation observed in both males and females overall. In comparison, outbred *D. melanogaster* flies reared at higher concentrations, between 10% and 50%, exhibited a significantly reduced weight in both sexes, with flies reared at 1% in females, and 0% in males weighing the most. Similar to the outbred line, male and female inbred *D. melanogaster* had a more variable weight range, with flies reared at 0% acid concentration weighing significantly more than flies reared at higher concentrations. As the concentration of octanoic acid in noni is between 58% (Farine et al., 1996) and 70% (Pino et al., 2010), the results for *D. sechellia* are contrary to expected. It could be predicted that *D. sechellia* reared at 0% concentration of octanoic acid would weigh less as this does not mimic its natural host plant. However, as this strain, although they are outbred, have been maintained in the laboratory since 1980, they may have become better adapted to the laboratory diet over time (e.g. Telonis-Scott, Guthridge and Hoffmann, 2006). Variation between the weights of inbred and outbred strain flies is likely due to the conserved genetic diversity in the outbred line. Similar studies have shown that inbred *D. melanogaster* lines have a significantly reduced lifespan when exposed to dietary-restrictions, then that of outbred lines such as Dahomey (Grandison et al., 2009).

Drosophila melanogaster is known to die upon contact with the natural host plant of its sister species, *D. sechellia*, and as such has evolved mechanisms to detect and avoid this fruit (Legal, Chappe and Jallon, 1994; Legal, Moulin and Jallon, 1999). Using a series of aversion assays, we highlighted the differences in behavioural response to the presence of octanoic acid – the main chemical component of noni and the chemical responsible for its pungent scent and toxic nature. Similar to previous results, stock population *D. melanogaster* were shown to be significantly more averse to octanoic acid

than *D. sechellia* (Legal et al., 1999; Dekker et al., 2006). This is undoubtedly due to the presence of the OBPs (OBP57d and OBP57e) present in both sexes of *D. sechellia* that attract the flies to the octanoic acid within the fruit. Detection of the octanoic acid scent increases oviposition in females and so females seek the fruit in order to lay (Legal, Moulin and Jallon, 1999).

A significant difference was found between *D. sechellia* and first-generation *D. melanogaster* flies that had been supplemented with *D. sechellia* gut microbiota. Here, first-generation *D. melanogaster* flies were significantly more averse to the food containing octanoic acid, and thus resembled the behaviour of standard stock population *D. melanogaster* (Legal et al., 1999; Dekker et al., 2006). However, no difference was found in preference for tenth-generation *D. melanogaster* flies compared with *D. sechellia*, showing that the aversion response was significantly reduced between first-generation and tenth-generation *D. melanogaster* individuals. This result potentially illustrates the first step in an organism specialising to a novel and toxic host. The genetic basis for aversion in *D. melanogaster* is due to the absence of the odorant-binding proteins (OBPs) OBP57d and OBP57e that are present on the gustatory sensilla on the legs of *D. sechellia* (e.g. Amlou et al., 1998; Jones, 2005; Matsuo et al., 2007; Dworkin and Jones, 2009). These enable the flies to detect the odour from up to 150m away (R'Kha et al., 1991). However, no attention has been paid to the role of the gut microbiota in evolutionary preference and ability to synthesis toxic octanoic acid. We suggest that the gut microbiota can interact with the genetic mechanisms within the fly to override the natural aversion response, and thus contribute to the role of specialisation in this insect.

Our analysis of the *D. sechellia* gut showed high prevalence of only one species of bacteria: *L. plantarum*. Similarly, the gut microbiota of wild-caught *D. sechellia* has previously been sequenced and discovered to be dominated by a single *Lactobacillales* (Chandler et al., 2011). *L. plantarum* is known to protect against pathogens in the gut in *Drosophila* (Ryu et al., 2008) and to promote larval growth when under nutrient scarcity (Storelli et al., 2011). In both inbred and outbred strains of *D. melanogaster*, high CFU counts were obtained for *L. plantarum*, with these generally increasing with adult flies that

were reared on the higher concentrations of octanoic acid. In outbred *D. melanogaster* adults, no additional colony growth was detected. However, in the larval and pupal stages, *Paenibacillus sp.* and *Bacillus cereus* were present, with *B. cereus* occurring in higher numbers in the pupal stage but remaining fairly consistent across octanoic acid concentrations. This is in comparison to the inbred strain of *D. melanogaster* in which *Paenibacillus sp.* and *Bacillus cereus* were detected across all life stages. In the inbred strain however, a trend can be observed of both *Paenibacillus sp.* and *Bacillus cereus* increasing in colony number at higher concentrations.

A bacterial pathogen, *Paenibacillus* species are known to be present in honeybee larvae and are responsible for colony collapse by causing American Foulbrood (e.g. Genersch, 2010). Similarly, *B. cereus* is a pathogen that is thought to have detrimental effects on life span in *Drosophila* (Ma et al., 2012; Ma et al., 2013). Presence of these pathogens in both strains on *D. melanogaster* may be due to the natural inability and avoidance of *D. melanogaster* to tolerate the octanoic acid. Although, previous studies have reported death on contact with either the noni fruit or octanoic acid (R'Kha et al., 1991; Dekker et al., 2006), our study shows *D. melanogaster* are able to survive on moderate to high concentrations of the acid for a considerable period. This may be due to the strains of *D. melanogaster* used. Susceptibility to bacterial pathogens may be unwanted side effect of this survival ability, due to a weakened immune system. This may particularly be the case for the inbred strain of *D. melanogaster*, where a lack of genetic diversity may render individuals more susceptible to pathogen colonisation (e.g. Alarco et al., 2004).

The gut microbiota of *D. melanogaster* is diverse but highly dependent on a number of factors, including diet (e.g. Sharon et al., 2010), age (Zerofsky et al., 2005), or strain (e.g. discussed in Heys et al., 2018b). When reared on a standard *Drosophila* diet (0% acid concentration), our study determined that *L. plantarum* is present within both the *D. melanogaster* and *D. sechellia* gut. The results of the experimental evolution line of *D. melanogaster* supplemented with *D. sechellia* gut microbiota that we created, supports our argument that *L. plantarum* acts a detoxifying agent within the *Drosophila*

gut. Octanoic acid is responsible for the majority of toxicity within the fruit (Farine et al., 1996; Jones, 1998). We predict the gut is able to withstand the toxicity of both the octanoic and hexanoic acids, via metabolising the toxic compounds into less harmful products which are able to be digested. Previous studies have shown that pH can determine the gut microbial composition (see Overend et al., 2016) and it could therefore be argued that sole presence of *L. plantarum* within the *D. sechellia* gut is due to pH alone. By determining the difference found between the first and tenth generation experimental evolution lines in aversion to octanoic acid, we can dispute the idea that the high colony numbers of *L. plantarum* is simply due to the increased pH. For example, similar scenarios can be viewed in the mealworm, *Tenebrio molitor,* which detoxifies the cell walls of fungi and bacteria within its diet (Genta et al., 2006), and the coffee berry borer, *Hypothenemus hampei*, a specialist of coffee plants where *Pseudomonas* species within the gut microbiota metabolise caffeine - a toxic alkaloid (Ceja-Navarro et al., 2015).

The present study provides evidence that the gut microbiota is responsible for specialisation in *D. sechellia*. *D. melanogaster* is known to be highly averse to the scent profile of octanoic acid, in comparison to *D. sechellia* in which it is a chemoattractant (Louis and David, 1986; R'Kha et al., 1991; Higa and Fuyama, 1993; Amlou et al., 1998; Legal et al., 1999). By creating experimental evolution lines of outbred *D. melanogaster* that are supplemented with *D. sechellia* gut microbiota, we have significantly reduced aversion of *D. melanogaster* to octanoic acid after only ten generations. In particular we suggest that *Lactobacillus plantarum*, the main bacterial constituent of the *D. sechellia* gut, acts as a detoxifying agent to metabolise the toxic octanoic acid compound – the main chemical present in the natural host plant, M. *citrifolia*. Little is known as to the origins of speciation of *D. sechellia* from its sister species, however, our results suggest that shifts in the gut microbiota may have led to ecological divergence, and later speciation, within this species. We have demonstrated an evolutionary shift in preference to food containing octanoic acid in *D. melanogaster*, to which it is naturally averse. Reducing aversion to a novel host plant could be the first

step in ecological and evolutionary divergence. Further work is needed to understand how *L. plantarum* metabolises octanoic acid into presumably harmless components that can be utilised by the host.

6. Chapter Six: General conclusions

This thesis aimed to investigate some of the relationships between the gut microbiota and animal behaviour. Using a series of different approaches in three species of *Drosophila* with varied ecologies, *D. melanogaster, D. pseudoobscura* and *D. sechellia*, I have explored the role of the gut microbiota in fundamental behavioural processes within these three species. I evaluated current methods of eliminating the gut microbiota in *D. melanogaster*, investigated age-based mating cues in *D. pseudoobscura*, and finally examined the role of the gut microbiota in dietary specialisation in *D. sechellia* in comparison to food generalist, *D. melanogaster*.

6.1 Conclusions from Chapter Two: The effect of gut microbiota elimination in *Drosophila melanogaster*: A how-to guide for host-microbiota studies

Due to the rapid surge in interest in studying host-microbiota relationships, determining the most effective method for eliminating the gut microbiota is essential. There are a number of current methods used to eliminate the gut microbiota in *Drosophila*, with the most popular being egg dechorionation (e.g. Ridley et al., 2013). In order to provide a solid foundation for further experiments conducted in this thesis, examining not only the effectiveness but also any additional consequences to the host physiology of the fly, was imperative. Using a fully factorial design, I analysed the efficacy of egg dechorionation, axenic diets and the addition of low-dose streptomycin to the dietary media in the model organism, *D. melanogaster*. Unsurprisingly, manipulating the microbiota was found to have a detrimental effect on the overall health and development of the fly, as weight and development time were shown to be negatively affected when the gut microbiota was manipulated across all treatments. *D. melanogaster* with an intact microbiota

are known to possess bacteria such as *L. plantarum* (reviewed in Broderick and Lemaitre, 2012), which promotes larval growth rate (Storelli et al., 2011), and *A. pomorum*, which regulates homeostatic signalling of physiological processes such as development (Sannino et al., 2018). I demonstrate that the addition of streptomycin to the dietary media both effectively eliminates the resident gut microbiota in *D. melanogaster*, whilst resulting in the fewest non-specific, deleterious effects.

This research has been published in the peer-reviewed journal, Ecology and Evolution (Heys et al., 2018b). Although this study does effectively evaluate the current methods of gut microbiota elimination in *Drosophila*, it does have some limitations. One key limitation is that as the *Drosophila* gut microbiota is known to vary depending on laboratory and strain (e.g. Chandler et al., 2011), I cannot conclude that the addition of low-dose streptomycin is the best method for all *Drosophila* researchers, as was the case in our study. As such, I can only conclude that this is the best method in our research and encourage other host-microbiota researchers to perform basic life history and efficacy assays, before conducting a further body of research on host-microbiota interactions. Further, I conclude that this is the best method for studying the behavioural outcomes of host-microbiota research, due to the ease of manipulating individuals without using carbon dioxide gas anaesthesia resulting in the negative impacts found on mating behaviours (e.g. Verspoor et al., 2015), but primarily due to the reduced potential for contamination with external bacteria. I hope that the publication of this study will encourage others to consider the methods they use when conducting host-microbiota research, to ensure a robust experimental design.

6.2 Conclusions from Chapter Three: *Drosophila* sexual attractiveness in older males is mediated by their microbiota

The role of the gut microbiota in mate choice was also examined. Females of the species *D. pseudoobscura* are known to discriminate against younger males, by preferring to mate with older males (Avent et al., 2008). When the

gut microbiota is removed via the addition of streptomycin to the dietary media, this female preference disappears, therefore suggesting that an intact microbiota is a key component of attractiveness in older males. This suggests that the gut microbiota communicates an honest signal used by females to assess male age, as old age is thought to be an indicator of genetic superiority or a more reliable signal of quality by older males. By removing the gut microbiota in this way, the gut microbiota can confidently be implicated in conveying this information to females. Although the precise mechanism is unknown, this likely occurs via changes in the CHC profile of older males compared to younger males. A limitation of this study and something to consider for future research, would be to characterise the CHC profiles of older and younger males in this species, as conducted in Heys et al. (2018a). This would enable the pinpointing of specific CHCs or blend of CHCs that are altered according to the gut microbiota and age.

6.3 Conclusions from Chapter Four: A potential role for the gut microbiota in the specialisation of *Drosophila sechellia* to its toxic host, *Morinda citrifolia*

The final chapters of this thesis investigated the role of the gut microbiota in specialisation in *D. sechellia* to its toxic host plant, *M. citrifolia*. Adapting to an unused ecological niche can have profound benefits, such as less competition for food resources, but is often not without cost. *M. citrifolia*, also known as Tahitian noni (Jones, 2005), is highly toxic to all other *Drosophila* species, but *D. sechellia* has evolved resistance to the toxic octanoic and hexanoic acids. The gut microbiota of *D. sechellia* when reared on noni is very simple with only one bacterial colony identified – *L. plantarum*. Yet when *D. sechellia* is reared on a standard laboratory diet (known as ASG), the bacterial diversity increases with *L. plantarum, B. cereus* and *Paenibacillus sp*. identified. *D. sechellia* are widely kept in the laboratory on a formulated diet with little attention paid to the effect this may have on the gut microbiota and thus, any subsequent behavioural changes. Both *B. cereus* and

Paenibacillus sp. are bacterial pathogens in other species (Genersch, 2010), but their role in *Drosophila* is unknown.

This dietary shift from the wild noni fruit diet, to a laboratory formulated diet is also accompanied by a change in pH. Noni has an acidic pH at 3.86 whereas ASG is pH 5.97. By comparing flies reared on noni fruit, ASG and an additional diet, salak fruit, with similar acidity (pH 3.86) as noni but without containing the toxic compounds, I could determine whether this simplification of the gut microbiota is caused by pH or the gut microbiota responding to the presence of octanoic acid within the fruit. When reared on salak, a similar gut bacterial diversity was observed to flies reared on noni. By examining changes in the gut microbiota caused by diet, I could determine the effects this may have on subsequent life history assays and mate choice. Noni reared flies were found to weigh more than salak and ASG flies but took longer to develop. This effect did not translate into an effect on mate choice, as salak reared flies mated for significantly longer than all other flies, but with a higher mating latency. However, although differences in the gut microbiota can be determined when flies are reared on different diets, in order to determine if the gut microbiota is responsible for host specialisation, further work is needed to disentangle the effect of pH on the gut microbiota composition.

6.4 Conclusions from Chapter Five: Gut microbiota and octanoic acid detoxification in *Drosophila sechellia* and *Drosophila melanogaster*

In order to disentangle the effects of pH on determining the gut microbiota from the coevolution of the gut microbiota and adaptation to its toxic host, I determined the effect of octanoic acid on fruit specialist *D. sechellia* and its sister species, the fruit generalist, *D. melanogaster*. Octanoic acid is the main toxic constituent of noni and is known to attract *D. sechellia* and increase oviposition in females (Lachaise et al., 1988), but is highly toxic to *D. melanogaster* (Legal, Chappe and Jallon, 1994; Legal, Moulin and Jallon, 1999). By rearing both species, including inbred and outbred strains of *D.*

melanogaster, on a formulated laboratory diet but with the addition of octanoic acid at varying concentrations, I could determine whether shifts in the gut microbiota are coupled with the detection of octanoic acid and thus represent a role in specialisation, rather than a by-product of acidity within the fruit. This also allowed me to determine the sensitivity of each species to varying concentrations of octanoic acid in response to development time, adult weight and survival ability. Similar to when *D. sechellia* are only reared on noni fruit, the gut microbiota of *D. sechellia* simplifies to only *L. plantarum*, whereas *D. melano*gaster were found to possess *L. plantarum, B. cereus* and *Paenibacillus sp.* As such, it could be suggested that *L. plantarum* acts as a detoxifying agent in *D. sechellia* and metabolises toxic compounds into less harmful compounds.

The key evidence from this study that suggests that the gut microbiota is responsible for host specialisation in this species was found via the creation of experimental evolution lines. When *D. melanogaster* were reared on food that was supplemented with *D. sechellia* gut microbiota for ten generations, they were found to be significantly less averse to the scent of octanoic acid than first generation flies. Such a rapid shift in behaviour is indicative that the gut microbiota has enabled *D. sechellia* to transition from an ancestral food generalist, to exploit an unused food resource and become a specialist. The reduction of aversion in *D. melanogaster* likely represents the first step in ecological and evolutionary divergence. A potential limitation of this study and something to examine at a later date, would be to determine the mechanism of how the gut microbiota can utilise octanoic acid and metabolise it into less harmful compounds.

6.5 Implications and future directions

The topics that are studied in this thesis could have the potential for a number of applications for wider research. Firstly, as noted above, by evaluating the current methods used to eliminate the gut microbiota in *D. melanogaster*, I encourage others to determine the efficacy and the

physiological implications that these methods may have on their host system. Using an evaluation of methods in this way could also benefit the research focussing on other insect systems. This could be of particular importance in species such as in the desert locust, *Schistocerca gregaria*, where the gut microbiota is known to play an essential role not only in digestion, but is also responsible for producing aggregation pheromones, communicating essential information to conspecifics about food resource availability (Dillon & Charnley, 2002). In this thesis, gut microbiota has also been implicated in cues that confer information regarding age in *D. pseudoobscura.* As age-based mate choice preferences are determined within *D. pseudoobscura*, it is likely that similar mechanisms are also present in other *Drosophila* species, and in other insect systems. The gut microbiota has now been implicated in age-based cues and in kin recognition mechanisms (Lizé et al., 2013; Heys et al., 2018a), showing the key roles in the gut microbiota plays in sexual signalling and thus sexual selection, within *Drosophila*. It is likely that the gut microbiota also acts on other mechanisms that underlie sexual selection in *Drosophila* and these should further be explored.

Discovering more about the role the gut microbiota plays on a wealth of behaviours within *Drosophila* could also be used in insect pest control systems. For example, the crop pest *Drosophila suzukii* originating from Japan is rapidly spreading across the USA, South America and Europe (Walsh et al., 2011). *D. suzukii* is similar to *D. sechellia*, in that it exploits an ecological food niche that is not used by other species of *Drosophila*. This is because the *Drosophila* diet relies on the large proportion of yeasts found within this nutrient-rich, decaying fruit, as yeasts are known to be an essential component of the *Drosophila* microbiota (Becher et al., 2012). *D. suzukii* feeds on any ripening, soft-skinned fruit and as such has become a major concern to fruit producers worldwide (Walsh et al., 2011). As it is spreading rapidly across continents there are few methods for controlling this species in the field. This thesis has determined the potential role that the gut microbiota plays in specialisation within *D. sechellia*, and thus it can be suggested that the gut microbiota of *D. suzukii* has also evolved to utilise this nutrient-poor diet of ripening, rather than rotting fruit. Characterising the gut

microbiota of *D. suzukii* and determining any similarities between it and *D. sechellia*, and how the gut microbiota mitigates the effects of this nutrient-poor diet, may lead to potential ways of disrupting the gut microbiota and limiting the spread of this invasive crop pest.

Understanding the dynamics of host-microbiota interactions can help to answer a number of important questions in ecological and evolutionary processes. This thesis examined three different species of *Drosophila* and determined the role of the gut microbiota in essential host processes within each species. I identify the potential role of the gut microbiota in dietary specialisation in *D. sechellia*, evaluate methods of eliminating the gut microbiota in *D. melanogaster*, and finally assess the cues produced by *D. pseudoobscura* that underlie female preference. These chapters when studied independently or combined, provide further evidence of the importance of host-microbiota interactions, and how they underpin the ecology and evolution of behaviour within *Drosophila*.

7. Reference List

Drosophila melanogaster photograph on front cover: https://www.um.edu.mt/think/tag/drosophila-melanogaster/

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8. Appendix

8.1 Supporting publications: The effect of gut microbiota elimination in *Drosophila melanogaster*: A how-to guide for host-microbiota studies

This study forms Chapter Two of my thesis and was accepted for publication in Ecology and Evolution, in March 2018. The full manuscript is presented below.

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ORIGINAL RESEARCH

WILEY Ecology and Evolution

The effect of gut microbiota elimination in *Drosophila melanogaster*: A how-to guide for host–microbiota studies

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Abstract

In recent years, there has been a surge in interest in the effects of the microbiota on the host. Increasingly, we are coming to understand the importance of the gut microbiota in modulating host physiology, ecology, behavior, and evolution. One method utilized to evaluate the effect of the microbiota is to suppress or eliminate it, and compare the effect on the host with that of untreated individuals. In this study, we evaluate some of these commonly used methods in the model organism, *Drosophila melanogaster*. We test the efficacy of a low-dose streptomycin diet, egg dechorionation, and an axenic or sterile diet, in the removal of gut bacteria within this species in a fully factorial design. We further determine potential side effects of these methods on host physiology by performing a series of standard physiological assays. Our results showed that individuals from all treatments took significantly longer to develop, and weighed less, compared to normal flies. Males and females that had undergone egg dechorionation weighed significantly less than streptomycin reared individuals. Similarly, axenic female flies, but not males, were much less active when analyzed in a locomotion assay. All methods decreased the egg to adult survival, with egg dechorionation inducing significantly higher mortality. We conclude that low-dose streptomycin added to the dietary media is more effective at removing the gut bacteria than egg dechorionation and has somewhat less detrimental effects to host physiology. More importantly, this method is the most practical and reliable for use in behavioral research. Our study raises the important issue that the efficacy of and impacts on the host of these methods require investigation in a case-by-case manner, rather than assuming homogeneity across species and laboratories.

KEYWORDS
Drosophila, life-history traits, microbiota, physiology

1 | INTRODUCTION

In the past few years, there has been an explosion of interest in the gut microbiota and the myriad ways in which it affects host processes from modulating immune responses (Round & Mazmanian, 2009) to mate selection (Lizé, McKay, & Lewis, 2014). To date, using a Web of Science search, there have been 4,617 articles published on the gut microbiota, across diverse species (search terms: gut microbio* under TITLE). Of that number, 3,281 (71%) of these were published in the last 4 years. However, there is little consensus regarding the

most effective method for eliminating the gut microbiota, despite its importance for our understanding of the effects the gut microbiota may have on the host.

Drosophilid species, particularly *Drosophila melanogaster*, have become an important model for examining how changes to, or differences in, the gut microbiota affect the host, for example, by regulating intestinal regeneration (Buchon, Broderick, & Lemaitre, 2013), or through driving mating preferences (Sharon et al., 2010). For such studies to be considered reliable, effective methods of altering the gut microbiota must be utilized in concordance with a given study system.

In *Drosophila*, there are two particularly common methods of altering gut bacterial communities: supplementing dietary media with antibiotics or creating sterile or axenic flies using egg dechorionation. The protective outer layer of the egg, the chorion, is coated with highly diverse bacteria transmitted largely from fecal deposits from the mother during oviposition (Wong, Ng, & Douglas, 2011). Emerging larvae then ingest the chorion and the bacteria coating it, forming the basis of their microbial community (Bakula, 1967). Removal of this embryonic chorion using bleach creates axenic, or microbe-free, adults. Supplementing the dietary media with antibiotics is a considerably simpler method. Here, a broad-spectrum antibiotic such as streptomycin or tetracycline is added to the diet; some studies use a combination of antibiotics in order to remove the microbiota (Sharon, Segal, Zilber-Rosenberg, & Rosenberg, 2011; Sharon et al., 2010).

Both the use of antibiotics and dechorionation of the egg are widely applied and widely criticized. Therefore, evaluating the efficacy of current methods and how they impact the study organism is vital for the investigation of host–microbiota relationships. Some recent publications have favored the use of antibiotics (Sharon et al., 2010, 2011). Yet while broad-spectrum antibiotics are active against a wide range of bacterial species, they also act on host enzymes and mitochondrial proteins by inhibiting synthesis, and/or nucleic acid metabolism and repair (Brodersen et al., 2000). In pseudoscorpions, this has been shown to reduce sperm viability, and the effect can be passed down generations (Zeh, Bonilla, Adrian, Mesfin, & Zeh, 2012). The repeated use of broad-spectrum antibiotics also has severe consequences in other organisms. For example, in humans, long-term antibiotic use is thought to correlate, directly or indirectly, with diseases such as type 2 diabetes and early-life obesity (Blaser & Falkow, 2009). Egg dechorionation in egg-laying animals is thought to be a less hazardous method of eliminating gut bacteria. However, studies comparing this with antibiotic treatment have only ever used harsher antibiotics such as chlortetracycline or rifampicin and in high concentrations (Ridley, Wong, Westmiller, & Douglas, 2012). The impacts on the host of tetracycline use have been fairly well documented (e.g. O'Shea & Singh, 2015; Zeh et al., 2012), yet to date, little attention has focused on low-dose streptomycin.

In this study, we analyzed the efficacy and the physiological effects on the flies, of the most common methods used to eliminate the resident host gut microbiota in *D. melanogaster*. We compared flies reared via a range of methods, in a factorial design: those reared on streptomycin, egg-dechorionated individuals, and flies reared on an axenic diet (Figure 1). Parallel to bacterial analyses determining the effectiveness of the techniques in eliminating the gut microbiota, we conducted a series of standard physiological assays in order to test the effect of each treatment on the overall health and fitness of the fly host. We measured development time from egg to adulthood (Tantway & El-Helw, 1970), adult weight (Partridge & Fowler, 1993), egg to adult survival, and how adults responded to stress. In the natural environment, the ability of *D. melanogaster* to develop more quickly on the limited food source of rotting fruit is beneficial to survival, as it ensures an individual can achieve pupation before the food source is exhausted (Nunney, 1996). This pressure is also increased if multiple females lay eggs on the same fruit. Thus, measuring development time is a fundamental assay of an individual's physiological fitness. Similarly, size directly correlates with mating success in *Drosophila*, with larger males being more successful (Partridge & Farquhar, 1983). Starvation assays measure how long a fly can survive when deprived of nutrition (Service, Hutchinson, Mackinley, & Rose, 1985), while locomotion assays such as the rapid iterative negative geotaxis (RING) assay (Gargano, Martin, Bhandari, & Grotewiel, 2005) measure the innate escape response, where individuals ascend the walls of a container after being knocked to the bottom. From these results, we suggest addition of antibiotics to the diet is the most effective method for eliminating the gut microbiota in our *Drosophila* system, with the least deleterious effects for the host. We note that this method is both more practical and reliable when conducting behavioral experiments, as, when using axenic

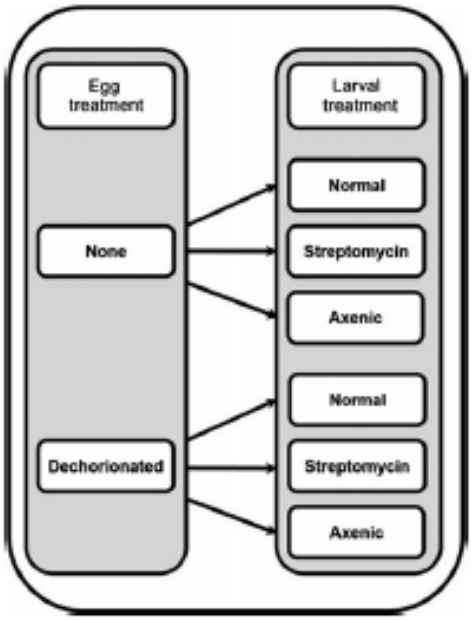

FIGURE 1 Schematic representation of our 2 × 3 factorial design of egg and larval treatments. Physiological assays were conducted on flies from each treatment type

TABLE 1 Treatment abbreviations used throughout and sample sizes for each

Treatments	Abbreviations	Egg to adult survival/ Development time	Weight		Starvation		Response to stress (rapid iterative negative geotaxis assay)	
			Males	Females	Males	Females	Males	Females
Eggs reared normally Larvae reared normally	N-Norm	1,100/438	68	87	97	104	35	25
Eggs reared normally Larvae reared on streptomycin food	N-Strep	1,100/353	62	62	90	95	25	25
Eggs reared normally Larvae reared on axenic food	N-Ax	1,400/188	53	59	60	51	25	25
Eggs dechorionated Larvae reared normally	D-Norm	1,050/152	80	67	54	52	30	25
Eggs dechorionated Larvae reared on streptomycin food	D-Strep	1,100/127	68	56	53	54	25	25
Eggs dechorionated Larvae reared on axenic food	D-Ax	1,450/82	50	50	51	53	25	25

individuals, there is a high likelihood of introducing external bacteria through the very nature of manipulating the study organisms. Our results demonstrate the importance of considering the potential impacts of each method with respect to the host organism studied, and the target research area.

2 | MATERIALS AND METHODS

2.1 | Fly stocks

Wild-type, *Wolbachia*-free *D. melanogaster* stocks were isolated from an outbred population collected in Lyon. Flies were reared at 25°C under a 12:12-hour light:dark cycle. Recently mated females were placed into vials containing 25 ml standard yeast–cornmeal diet (for 1 L of water: 85 g of sugar, 60 g of corn, 20 g of yeast, 10 g of agar, and 25 ml of nipagin) and left to lay eggs for 24 hr. The following day, the females were removed and eggs were collected using a fine paintbrush. The eggs and their successive developing larvae were then assigned to one of the six treatments (Figure 1). Henceforth, we abbreviate our treatments as outlined in Table 1.

Once eggs had been harvested and a treatment assigned (e.g. dechorionated or not), they were placed into vials at a standard density of fifty per vial. Eggs that were not subjected to the dechorionation were still physically manipulated in the same way, but without the chemical treatment. Thus, we controlled for any potential effects of physically manipulating the eggs, across all treatments. Eggs were then left to hatch, and the emergent larvae left to develop. At eclosion, newly emerged adults were isolated using an aspirator and separated according to sex. Males and females were stored separately in groups of 10 in vials containing 25 ml of the diet on which they were reared as larvae.

2.2 | Experimental treatments

2.2.1 | Normal diet

Eggs assigned to a normal diet treatment were transferred into vials containing 25 ml standard yeast–cornmeal diet at 25°C and left to develop.

2.2.2 | Diet containing streptomycin

Once harvested from the stock vials, eggs were then transferred into vials containing 25 ml standard yeast–cornmeal diet that had been supplemented with streptomycin at a concentration of 400 μg/ml, as is common in the literature (Lizé et al., 2014; Sharon et al., 2010). Upon cooling, 4 ml of a solution composed of 10 g of streptomycin in 100 ml of ethanol was added per liter of food. Food was then dispensed into vials with 25 ml in each.

2.2.3 | Axenic diet

An axenic diet was produced by autoclaving vials of standard yeast–cornmeal diet, without the addition of nipagin, for 20 min at 120°C. Nipagin was added once the media had cooled to 65°C. Any manipulation of the axenic diet was conducted under a laminar flow cabinet to ensure sterility. Twenty-five milliliter of the media was then dispensed into sterile vials.

2.2.4 | Egg dechorionation

Eggs were gently harvested using a sterile paintbrush and placed onto a piece of fine cloth mesh. They were then placed into a strainer and washed with sterile, deionized water once. They were then immersed in a 10% sodium hypochlorite solution for 5 min (Ridley et al., 2012). The eggs were washed three more times with sterile, deionized water and then carefully removed using a sterile paintbrush and placed onto the desired food treatment. All work was conducted under a laminar flow cabinet to ensure sterility. Eggs from all treatments were subjected to the physical manipulation utilized during the egg dechorionation treatment, but without the addition of bleach, in order to control for any deleterious effects of the action.

2.3 | Physiological assays

2.3.1 | Development time

Once treated, eggs were placed into the development time assay and the number of days for these eggs to emerge as newly eclosed adults was counted. Vials were checked at three time points within each day—9 a.m., 12 p.m., and 5 p.m.—and the cumulative number of adults emerged from each time point was scored. Emergent adult flies from each time point were removed from the vial and placed into a fresh vial of their corresponding diet treatment.

2.3.2 | Egg to adult survival

Each vial was set up to contain fifty eggs so that the number of flies that reach adulthood could be counted. Vials were checked at three time points within each day—9 a.m., 12 p.m., and 5 p.m.—and the cumulative number of alive, newly eclosed adult flies was counted. Emergent flies were then removed from the vial and placed into a fresh vial of their corresponding diet treatment. This was repeated daily until there were no live larvae or pupae left in the vial. The mortality rate was then calculated from the number of flies that had reached adulthood compared to the number of eggs set up.

2.3.3 | Adult weight

Vials were checked daily at three time points—9 a.m., 12 p.m., and 5 p.m.—and any newly emerged, virgin adults were isolated and separated according to sex. They were placed into vials at a standard density of 10 per vial and left for 2 hr to allow their wings to dry out and inflate. Flies reared in the egg dechorionation treatments and the axenic larval treatments were always manipulated within the laminar flow cabinet in order to prevent contamination. Two hours later, vials were placed into the freezer at −18°C and left overnight. The following morning, individuals were collected from the freezer using a Kahn balance and their weight was recorded (in mg) to four decimal places. Male and female measurements for each treatment were recorded and analyzed separately.

2.3.4 | Starvation resistance

Newly emerged, virgin adults were isolated and separated according to sex. Flies reared in the egg dechorionation treatments, and the axenic larval treatments were always manipulated within the laminar flow cabinet in order to prevent contamination. Flies were placed into vials at a standard density of 10 per vial and left to mature for 2 days. After this time, they were transferred to a fresh vial containing 10 ml of non-nutritional agar in order to prevent desiccation. Fresh agar was used to prevent microorganismal growth—no bacterial and fungal growth was observed during the course of the experiment. Flies were left in these vials to acclimatize for 24 hr, and then, the starvation assay was started. The time to starvation death was measured by monitoring the flies every 8 hr—at 8 a.m., 4 p.m., and 12 a.m.. Here, the number of dead flies was counted and the starvation assay continued until there were no more living flies. This assay was conducted at 25°C. Male and female measurements for each treatment were recorded and analyzed separately.

2.3.5 | Locomotion—RING (rapid iterative negative geotaxis)

Newly emerged, virgin adults were isolated and separated according to sex. Flies reared in the egg dechorionation treatments, and the axenic larval treatments were always manipulated within a laminar flow cabinet in order to prevent contamination. Flies were placed into vials at a standard density of 10 per vial and left to mature for 2 days. After this time, flies were placed into fresh vials containing 10 ml of the diet type on which they were reared. Five vials were then placed into an apparatus similar to that described by Gargano et al. (2005) and Nichols, Becnel, and Pandey (2012), and flies were left to acclimatize for 30 min. After this time, the apparatus was rapped sharply on the work surface three times in rapid succession in order to initiate the negative geotaxis response. After a 3-s rest, a photograph was taken of the vials, recording the flies' position within the vial and thus their negative geotaxis response to the stimulus. After a 1-min rest, the procedure was repeated. This procedure was repeated five times in total for each set of flies, resulting in five digital images for each vial. This assay was performed at 25°C. Male and female measurements for each treatment were recorded and analyzed separately.

Digital images were later analyzed manually by measuring the distance each fly had travelled following the tapping stimulus. All 10 flies in each vial were measured across the five digital images generated.

An average distance travelled value was then created for each vial and statistical analysis performed.

2.4 | Bacterial analysis

In order to quantify the bacterial load within flies reared on each treatment, and therefore the efficacy of each treatment, we cultured the bacteria present in both the whole gut and the whole fly. Newly

emerged, virgin adults were isolated and separated according to sex. Flies reared in the egg dechorionation treatments and the axenic larval treatments were always manipulated within a laminar flow cabinet in order to prevent contamination. Flies were placed into vials at a standard density of 10 per vial and left to mature for 2 days.

2.4.1 | Gut bacterial analysis

Following maturation, adults were isolated using gas anesthesia and surface-sterilized in 70% ethanol, rinsed in distilled water, and air-dried. The head was then removed. Three guts were dissected into each Eppendorf containing 500 µl of sterile PBS (phosphate-buffered saline solution). An equal number of males and females were used in order to ensure there were no sex-specific differences in the bacterial content. Gut tissue was homogenized with a sterile plastic pestle. One hundred microliter of gut homogenate was pipetted onto MRS (de Man, Rogosa and Sharpe) agar and spread-plated using a sterile glass loop. Plates were left to air-dry aseptically, before being closed and sealed with parafilm. Plates were incubated at 25°C for 72 hr, and bacterial load was quantified by performing colony-forming unit (CFU) counts.

2.4.2 | Whole-fly bacterial analysis

Following maturation, flies were isolated using gas anesthesia and placed into a sterile Eppendorf containing 500 µl sterile PBS. Three flies were added into each Eppendorf. An equal number of males and females were used in order to ensure there were no sex-specific differences in the bacterial content. The whole-fly solute was then homogenized using a sterile, plastic pestle. One hundred microliter of the whole-fly solute was pipetted into the center of a petri dish containing MRS media and spread across the plate using a sterile glass loop. The plate was left to dry close to the flame before being closed and sealed using parafilm. Plates were incubated at 25°C for 72 hr and then checked for bacterial growth. Bacterial load was quantified by performing CFU counts.

Single colonies were isolated using a sterile 1 µl loop and placed into an Eppendorf with 10 µl sterile water. PCR amplification was performed in a 25 µl reaction volume consisting of 10 µl nuclease-free water, 13 µl Taq green master mix, 0.5 µl of forward primer 27F (5′-AGAGTTTGATCMTGGCTCAG-3′) and reverse primer 1492R (5′-GGTTACCTTGTTACGACTT-3′), and 1 µl of template DNA. Thermal cycling was performed for 90 s at 95°C as initial denaturation, followed by 35 cycles of 30 s at 95°C for denaturation, 30 s at 55°C as annealing, 90 s at 72°C for extension, and final extension at 72°C for 5 min. One thousand five hundred base pair 16S PCR products were purified with Ampure beads and subjected to Sanger sequencing. The resulting sequences were identified using NCBI BLAST against the nt database (Altschul, Gish, Miller, Myers, & Lipman, 1990).

2.5 | Statistical analysis

Sample sizes are given in Table 1. All analyses were performed in R (3.1.3) (Ihaka & Gentleman, 1996), and the effects of egg (dechorionation or not) and larval treatments (normal, axenic, and streptomycin) were studied in addition to their interactions. Egg to adult survival, weight, and response to stress (RING assay) were analyzed by fitting a General Linear Model with binomial, Gaussian, and Gaussian distributions, respectively. Weight data were Box–Cox-transformed to improve normality of the GLM residuals. All GLMs were followed by an ANOVA to test for global effects, and post hoc multiple comparisons between treatments were conducted using Tukey's HSD tests. Following these general GLMs, sexes were studied separately for weight and response to stress (starvation and RING assay).

Cox proportional hazard regressions for survival were used to assess variation in development time and survival under starvation. Survival analysis involves the modeling of time to event data, with death being considered the "event." Death and development failure of flies was used as the "event" for survival data and development time data, respectively. The *Survdiff* function was used to assess differences between two or more survival curves according to egg and larval treatments. The *coxph* function was used to assess differences between treatments. This allowed treatments to be compared in a pairwise fashion, to ascertain whether all treatments differed or whether any significant differences observed were derived from a single treatment.

3 | RESULTS

3.1 | Development time

Globally, egg dechorionation (*Survdiff*, $\chi^2_1 = 473$, $p < .001$) and larval treatments (*Survdiff*, $\chi^2_2 = 726$, $p < .001$) altered fly development time (Figure 2). When compared to N-Norm flies, egg dechorionation (*Coxph*, $\beta \pm SE = 0.068 \pm 0.101$, $Z = -26.305$, $p_z < .001$) and larval treatments (*Coxph*, Ax, $\beta \pm SE = 0.091 \pm 0.093$, $Z = -25.603$, $p_z < .001$, Strep, $\beta \pm SE = 0.089 \pm 0.077$, $Z = -31.110$, $p < .001$) increased development time of flies. Moreover, egg dechorionation and larval treatment effects interacted with each other (*Coxph*, D-Ax, $\beta \pm SE = 1.461 \pm 0.173$, $Z = 2.187$, $p_z = .028$, D-Strep, $\beta \pm SE = 8.406 \pm 0.143$, $Z = 14.875$, $p_z < .001$). Thus, removing or altering the microbiota increased development time.

3.2 | Egg to adult survival

Globally, across all treatments, dechorionation ($p < .001$) and larval treatments ($p < .001$) affected egg to adult survival both as factors and via interaction ($p = .024$; Figure 3). More specifically, larval treatments (Ax and Strep) significantly increased mortality during development compared to Norm when eggs were intact (N-Norm–N-Ax: $p < .001$, N-Norm–N-Strep: $p < .001$, N-Ax–N-Strep: $p < .001$). In dechorionated eggs, only the Ax treatment increased mortality during development compared to Norm and Strep (D-Norm–D-Ax: $p < .001$, D-Norm–D-Strep: $p = .434$, D-Ax–D-Strep: $p = .011$). Furthermore, egg dechorionation also increased mortality within larval treatments (N-Norm–D-Norm: $p < .001$, N-Strep–D-Strep: $p < .001$, and N-Ax–D-Ax: $p < .001$).

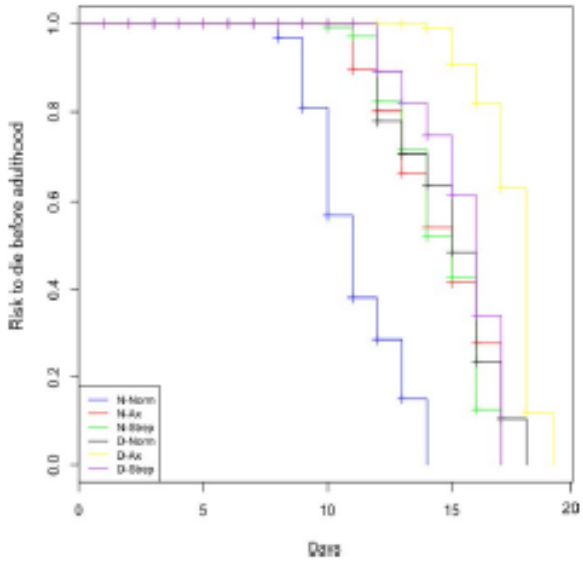

FIGURE 2 Development time failure measured as the risk to die before adulthood over time in days when eggs were dechorionated (D), or not (N), and when larvae were reared in a conventional diet (Norm), an axenic diet (Ax), or an antibiotic-supplemented diet (Strep)

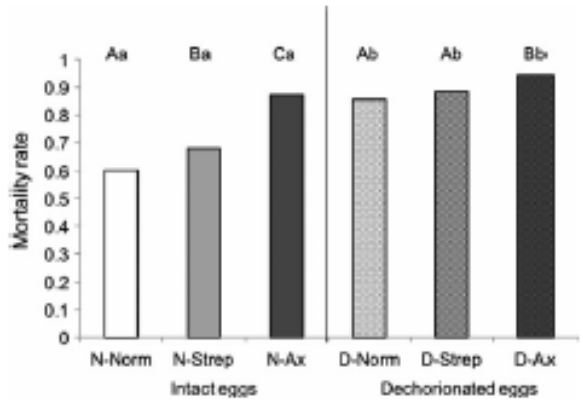

FIGURE 3 Egg to adult survival measured as mortality rate when eggs were dechorionated (D) or not (N), and when larvae were reared conventionally (Norm), or with the antibiotic streptomycin (Strep), or with axenic media (Ax). Different uppercase letters represent significant differences between larval treatments within egg treatment, while different lowercase letters represent significant differences within larval treatment between egg treatments

In this assay, it should be noted that egg to adult survival for nondechorionated eggs and conventionally reared larvae is quite low (mortality rate of 60%) compared to previous studies where egg to adult viability is approximately 100% (Kristensen et al., 2015). However, as nondechorionated eggs were manipulated the same way as dechorionated eggs, but without the chemical agents to remove the chorion, we are confident that the results are comparable.

3.3 | Weight

Unsurprisingly, adult males were always found to weigh less than adult females across all treatments ($p < .001$). When males and females are treated separately, dechorionation ($p < .001$) and larval treatments ($p < .001$) affected male adult weight both as factors and via interaction ($p = .024$; Figure 4). In intact eggs, Ax and Strep larval treatments significantly decreased male adult weight compared to Norm (N-Norm–N-Ax: $p < .001$, N-Norm–N-Strep: $p < .001$, N-Ax–N-Strep: $p = .011$). By contrast, in dechorionated eggs, Ax treatment increased male adult weight when compared to Norm (D-Ax–D-Norm: $p = .011$) and Strep (D-Ax–D-Strep: $p < .001$), while Strep decreased male adult weight when compared to Norm (D-Strep–D-Norm: $p < .001$). Furthermore, egg dechorionation also decreased male adult weight within larval treatments (N-Norm–D-Norm: $p < .001$, N-Strep–D-Strep: $p = .020$), except for Ax (N-Ax–D-Ax: $p = .928$).

In females, dechorionation ($p < .001$) and larval treatments ($p < .001$) affected female adult weight both as factors and via interaction ($p < .001$; Figure 4). In intact eggs, Ax and Strep larval treatments significantly decreased female adult weight compared to Norm (N-Norm–N-Ax: $p < .001$, N-Norm–N-Strep: $p < .001$), while Ax had no effect on female adult weight compared to Strep (N-Ax–N-Strep: $p = .372$). In dechorionated eggs, only the Strep larval treatment significantly decreased female adult weight compared to Norm (D-Norm–D-Strep: $p = .019$) or Ax (D-Ax–D-Strep: $p = .009$), while Ax larval treatment had no significant impact on female adult weight (D-Norm–D-Ax: $p = .997$). Furthermore, egg dechorionation decreased female adult weight within the Norm treatment (N-Norm–D-Norm: $p < .001$), while increasing it within the Ax treatment (N-Ax–D-Ax: $p = .006$), but egg dechorionation had no effect within the Strep treatment (N-Strep–D-Strep: $p = .448$).

3.4 | Starvation

As expected, males and females did not react the same way to starvation stress, with males dying more quickly than females (*Coxph*, $\beta \pm SE = 0.424 \pm 0.193$, $Z = -4.431$, $p_z < .001$). Thus, males and females were analyzed separately.

In females, egg dechorionation (*Survdiff*, $\chi^2_1 = 117$, $p < .001$) as well as larval treatments (*Survdiff*, $\chi^2_3 = 90.6$, $p < .001$) affected female survival (Figure 5a). Egg dechorionation increased female resistance to starvation (*Coxph*, $\beta \pm SE = 0.508 \pm 0.172$, $Z = -3.918$, $p_z < .001$). Axenic rearing of the larvae had no significant impact on female resistance to starvation when compared to conventionally reared larvae (*Coxph*, $\beta \pm SE = 1.379 \pm 0.172$, $Z = 1.864$, $p_z = .062$). In contrast, antibiotic rearing of the larvae decreased female resistance to starvation when compared with conventionally reared larvae (*Coxph*, $\beta \pm SE = 2.092 \pm 0.144$, $Z = 5.122$, $p_z < .001$).

In males, egg dechorionation had no significant impact on male resistance to starvation (*Survdiff*, $\chi^2_1 = 1.1$, $p = .291$; Figure 5b). In contrast, larval treatments affected male resistance to starvation (*Survdiff*, $\chi^2_3 = 450$, $p < .001$), with axenic rearing of the larvae (*Coxph*,

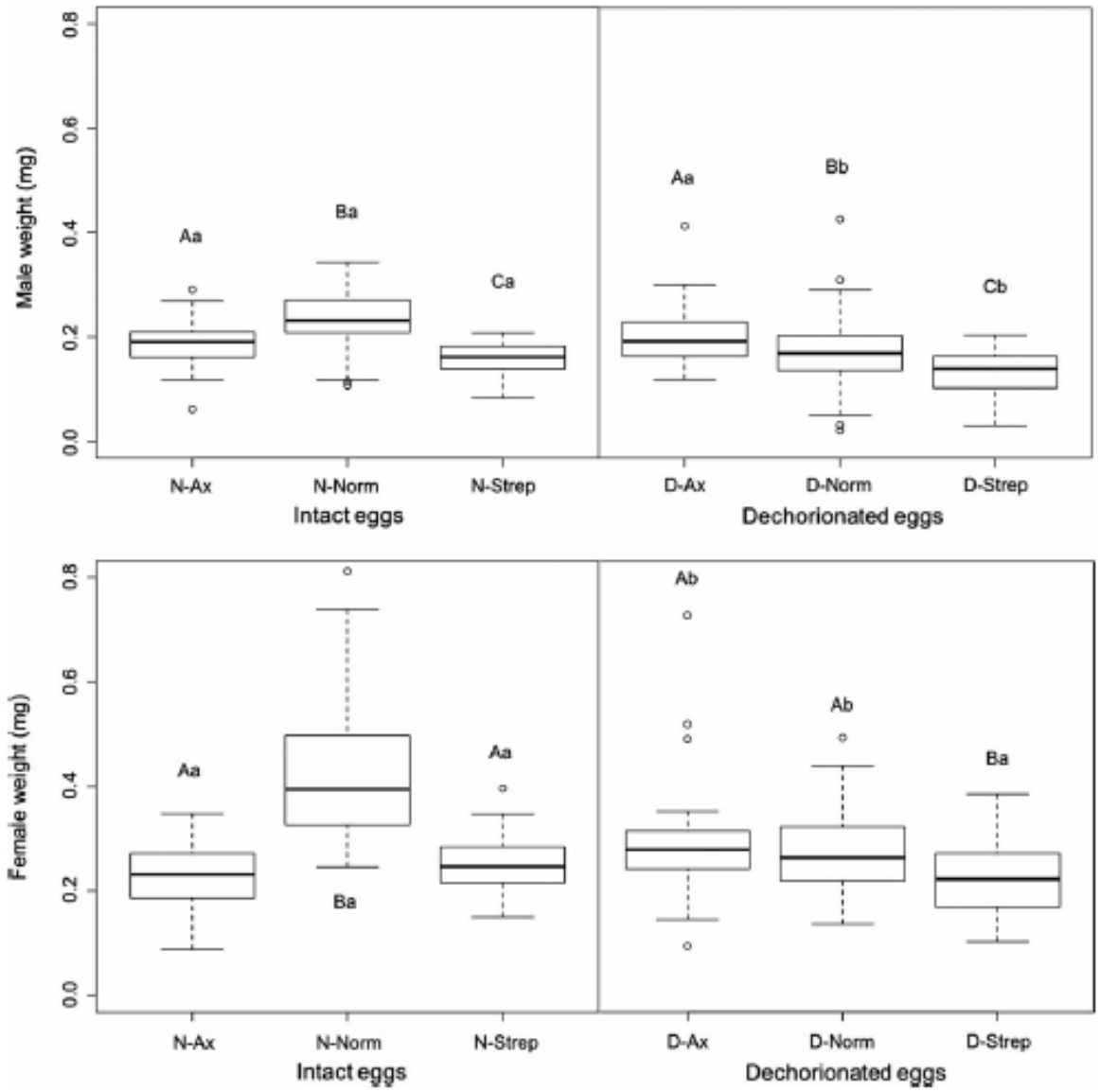

FIGURE 4 Boxplot of adult male and female weight according to egg treatments (dechorionated [D] or not [N]), and larval treatments (conventionally reared [Norm], axenic medium [Ax], or antibiotic-supplemented medium [Strep]). Different uppercase letters represent significant differences between larval treatments within egg treatment, while different lowercase letters represent significant differences within larval treatment between egg treatments

$\beta \pm SE = 2.191 \pm 0.257$, $Z = 3.050$, $p_z = .002$) in addition to antibiotic rearing of the larvae (*Coxph*, $\beta \pm SE = 2.162 \pm 0.245$, $Z = 3.146$, $p_z = .001$), increasing male resistance to starvation when compared to conventionally reared larvae.

3.5 | Response to stress (RING assay)

Global effects show that sex ($p = .311$) had no significant effect on fly locomotion. However, sex interacted significantly with larval treatments ($p < .001$) in determining fly locomotion. Therefore, we treated males and females separately.

In males, larval treatments ($p = .001$) affected their locomotion as a factor and via an interaction with egg treatments ($p = .001$), while egg treatment as a factor had no significant effect on male locomotion ($p = .988$; Figure 6). In intact eggs, Ax and Strep larval treatments had no significant effect on male locomotion (N-Norm–N-Ax: $p = .913$, N-Norm–N-Strep: $p = .051$, N-Strep–N-Ax: $p = .518$). By contrast, in dechorionated eggs, Ax significantly reduced male

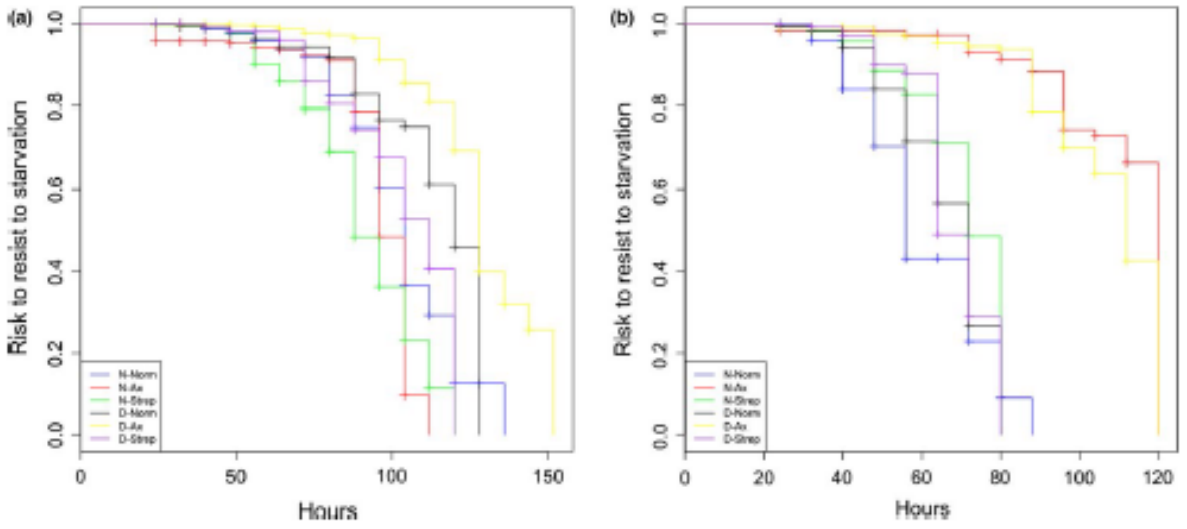

FIGURE 5 Female (a) and male (b) survival curves representing the risk to resist starvation over time in hours when eggs were dechorionated (D), or not (D), and reared as larvae in a conventional diet (Norm), an axenic diet (Ax), or an antibiotic-supplemented diet (Strep)

locomotion compared to Norm (D-Ax–D-Norm: $p = .001$) or Strep (D-Ax–D-Strep: $p = .006$), while no significant effects on male locomotion was found for Strep when compared to Norm (D-Norm–D-Strep: $p = .999$). Furthermore, egg dechorionation had no effect on male locomotion within larval treatments (N-Norm–D-Norm: $p = .090$, N-Ax–D-Ax: $p = .153$, N-Strep–D-Strep: $p = .990$).

In females, larval treatments ($p = .001$) affected their locomotion as a factor and via an interaction with egg treatments ($p = .004$), while egg treatment as a factor had no significant effect on female locomotion ($p = .139$; Figure 6). In intact eggs, Ax larval treatment significantly reduced female locomotion when compared to Norm (N-Norm–N-Ax: $p < .001$) and Strep (N-Strep–N-Ax: $p = .001$), while Strep had no effect on female locomotion when compared to Norm (N-Norm–N-Strep: $p = .999$). In dechorionated eggs, both Ax and Strep larval treatments significantly reduced female locomotion compared to Norm (D-Ax–D-Norm: $p < .001$, D-Strep–D-Norm: $p < .001$, D-Ax–D-Strep: $p < .001$). Furthermore, egg dechorionation had no effect within Norm and Ax larval treatments (N-Norm–D-Norm: $p = .545$, N-Ax–D-Ax: $p = .829$), while it significantly decreased female locomotion within the Strep larval treatment (N-Strep–D-Strep: $p = .032$).

3.6 | Bacterial analysis

In order to assess the efficacy of each treatment in eliminating the gut microbiota, we dissected the midgut of adult *D. melanogaster* and used spread plates on to MRS media to determine the contents. We analyzed the bacterial content of the midgut as this is one of the only larval structures that stays intact during pupation. It is known that a sharp decrease in bacterial density occurs 24 hr after pupation, only increasing again after 48 hr (Storelli et al., 2011), but the midgut is contained and develops within a transient pupal epithelium (Takashima, Younossi-Hartenstein, Ortiz, & Hartenstein, 2011). As the midgut remains unchanged during pupation while almost all other structures are histolyzed, the midgut is an accurate representative of the gut bacterial content and diversity within an adult *Drosophila*. We also analyzed the bacterial content of the whole fly in a similar manner in order to determine whether our treatments affected the whole host–microbiota. We used colony-forming unit (CFU) counts to measure the bacterial load of flies from each treatment in triplicate by taking the average, which is a standard measure of estimating bacterial load (Nadkani, Martin, Jacques, & Hunter, 2002).

We discovered bacterial colony growth on all plates from all treatments, except those from flies reared on the streptomycin diet alone. In the case of the latter, there were zero colonies present on all spread plates containing the dissected midgut. For D-Strep flies, only one of three replicate midgut plates contained any colony growth (Table 2), with the other two replicates containing zero colonies. This is likely an anomaly due to potential contamination of the media during spread plating, or transfer of bacteria from other parts of the fly during midgut dissection.

The results for the midgut contrast with the results of the whole-fly spread plates, in which colony growth occurs on all replicates for both the N-Strep and the D-Strep flies (Table 2), although it can be noted that these results are considerably lower compared to all other treatments. Considerably more colonies were found for the whole-fly spread plates for each treatment in comparison with the midgut contents. The highest number of colonies was found on the normal treatment, which is to be expected (Table 2). Yet similar numbers of bacterial colonies were found for the whole-fly plates from the axenic and the combined egg dechorionation and axenic treatment.

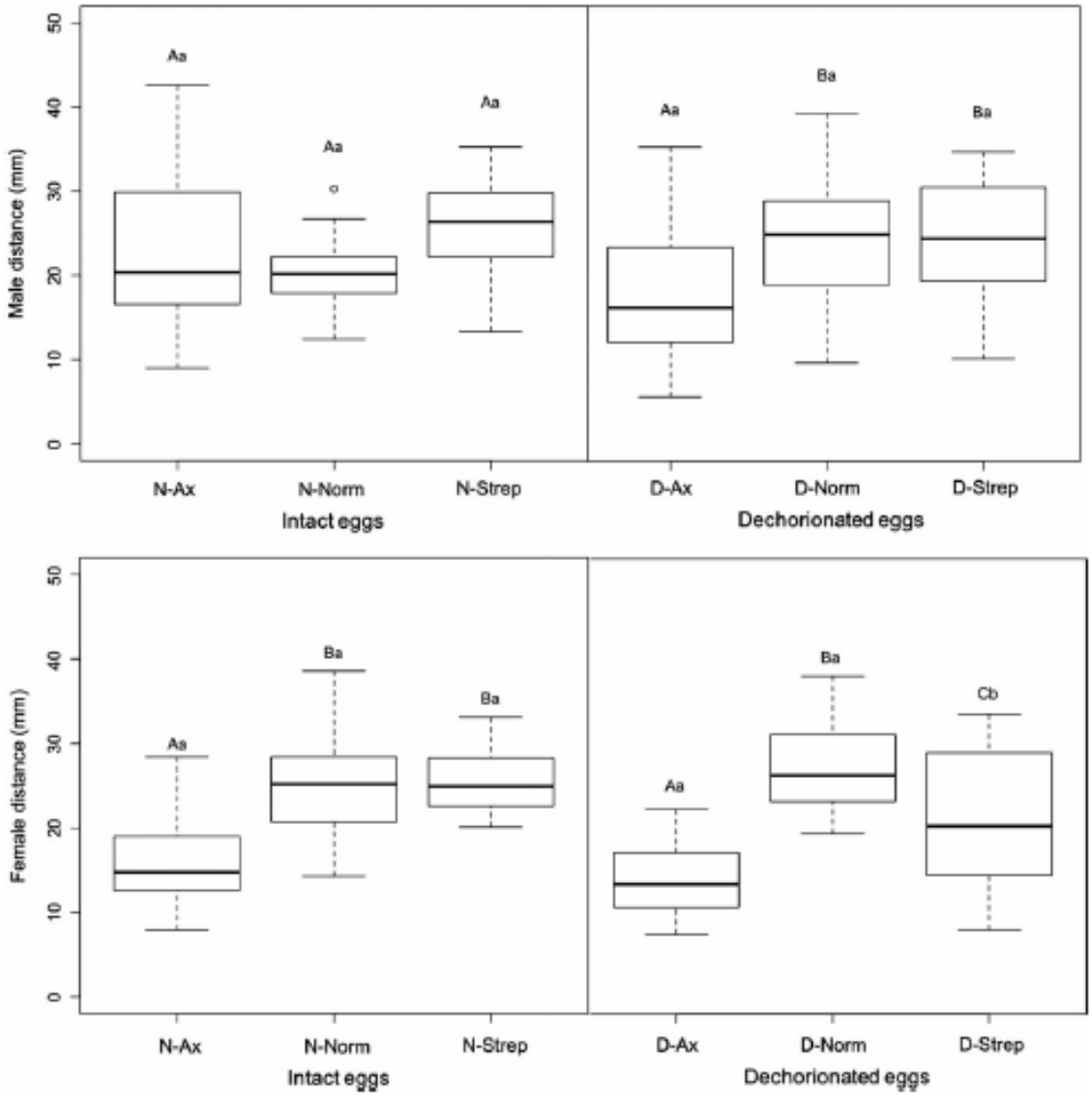

FIGURE 6 Boxplot of male and female locomotion, measured as distance travelled (RING) according to egg treatments (dechorionated (D) or not (N), and larval treatments (conventionally reared [Norm], axenic medium [Ax], or antibiotic-supplemented media [Strep]). Different uppercase letters represent significant differences between larval treatments within egg treatment, while different lowercase letters represent significant differences within larval treatment between egg treatments

4 | DISCUSSION

Effectively eliminating the resident gut microbiota is essential to the study of host–microbiota interactions, through which we can gain a greater understanding of a species' fundamental ecology. From the array of physiological assays conducted, it is clear that manipulating the microbiota has a profound effect on the overall health of the host. This is particularly true for development time and adult weight; individuals from all treatments took significantly longer to develop, and weighed less, compared to normal flies. This is hardly surprising considering the gut microbiota is known to affect a wealth of host developmental and physiological processes (Sommer & Backhed, 2013). In *D. melanogaster*, a symbiotic relationship exists between the fly and its gut microbe, *Acetobacter pomorum* (Shin et al., 2011). Acetic acid produced by the alcohol dehydrogenase of *A. pomorum* initiates insulin signaling and thereby tunes the homeostatic signaling of the fly, controlling a variety of factors included developmental rate and body size.

TABLE 2 Number of bacterial colonies

Treatments	Origin of bacteria	Average number of bacterial cells per gut in each replicate
N-Norm	Gut	3.1×10^1
	Gut	5.9×10^1
	Gut	6.2×10^1
	Whole fly	4.5×10^2
	Whole fly	3.8×10^2
	Whole fly	6.3×10^2
N-Strep	Gut	0
	Gut	0
	Gut	0
	Whole fly	2.1×10^1
	Whole fly	2.8×10^1
	Whole fly	1.0×10^2
N-Ax	Gut	1.8×10^2
	Gut	3.3×10^2
	Gut	2.0×10^2
	Whole fly	5.5×10^2
	Whole fly	5.7×10^2
	Whole fly	4.4×10^2
D-Norm	Gut	5.5×10^1
	Gut	2.8×10^1
	Gut	8.3×10^1
	Whole fly	1.4×10^2
	Whole fly	7.3×10^1
	Whole fly	6.4×10^2
D-Strep	Gut	0
	Gut	0
	Gut	0.4×10^1
	Whole fly	0
	Whole fly	4.5×10^1
	Whole fly	2.1×10^1
D-Ax	Gut	0.04×10^1
	Gut	1.0×10^1
	Gut	7.6×10^1
	Whole fly	6.2×10^2
	Whole fly	5.3×10^2
	Whole fly	4.5×10^2

In terms of mortality rate of individuals, considerably fewer flies survived to adulthood when reared on axenic and streptomycin diets compared with normal flies. Sterilization of the diet by rendering it axenic had the most profound effect on egg to adult survival. Removal of the egg chorion also increased mortality rate in all larval treatments (Norm, Strep, and Ax). Dechorionation involved the use of bleach and alcohol to remove the chorion, which acts as a barrier to the environment, and protects against dehydration in insects such as coleopterans (Biémont, Chauvin, & Hamon, 1981) and dipterans (Klowden, 2013). Thus, dechorionation in itself (i.e. the absence of the barrier) might explain the higher mortality rate observed. Sterilization or antibiotic supplementation of the diet kills all or part of the bacteria present in the diet that are ingested by the flies. These bacteria could be used as a food source by the flies and/or help the flies in digesting complex carbohydrates present in the diet, as shown by previous studies (Storelli et al., 2011; Wong et al., 2015). Some of the treated flies could thus have died due to poor nutrition and/or inability to develop through their life cycle. Our findings contrast to previous studies that found that dechorionation had no effect on survivorship from egg to adulthood (Ridley, Wong, & Douglas, 2013). The stark differences in these results highlight the importance for individual laboratories to evaluate the impacts of the methods employed to remove or alter the microbiota in their experiments. Such differences in results are likely due to the ability of different strains of *D. melanogaster*, for example, wild-type compared to laboratory strains, to cope with environmental stressors.

Fly responses to starvation were sexually dimorphic. Males exhibited higher resistance to starvation and thus survival when reared in a diet free of or with reduced bacterial load (the axenic, antibiotic treatments). Egg dechorionation had no effect on male resistance to starvation. In contrast, females exhibited increased resistance to starvation when their eggs were dechorionated; being reared in an axenic diet had no effect, and an antibiotic-supplemented diet decreased female resistance to stress. From these results, it is clear that antibiotic has some deleterious effects on females when they are faced with starvation, and some beneficial effects on male resistance to starvation. Thus, there is a contradictory effect of antibiotic according to sex. Egg dechorionation and axenic rearing of the larvae increased resistance to starvation in females and males, respectively. However, depending on sex, removal of bacteria could be beneficial when starving. Different scenarios possibly explain this. Bacteria residing in the guts need to feed in order to develop, and may compete with the host for nutritional resources. An alternative explanation is that some bacteria may have deleterious effects on the host, and in their absence, the flies are healthier.

The presence/absence of bacteria in the diet during development of the fly also altered locomotion in relation to sex, while egg dechorionation had no impact. Females showed a decrease in their level of activity when reared in an axenic and/or antibiotic-supplemented medium. This result demonstrates that bacterial feeding by females during development is essential for activity levels. Males are less affected by the absence of bacteria during development. Potentially females' needs are higher than males due to egg production; bacteria may participate in this process either through the digestion of nutrients, or through the hormonal pathway. Indeed, *Lactobacillus plantarum* is known to control hormonal growth signaling (Storelli et al., 2011). It could be that the symbiosis between the fly and their gut microbiota is tighter in females than males, rendering females more susceptible to the absence of bacteria during development.

In addition to determining deleterious effects of treatments on the overall health and physiology of the fly, a key part of this study

was confirmation of the effectiveness of each treatment. Our results showed that flies reared on a streptomycin diet had their gut bacteria completely eliminated; no bacteria were present on the plates. This result remained fairly consistent for the egg dechorionation, streptomycin treatment, in which two of the replicates were devoid of bacteria. One of these replicates, however, did contain some bacteria, though at low titer, and is likely to have resulted from contamination from another part of the *Drosophila* during dissection. The treatments containing streptomycin did, however, still possess a substantial amount of bacteria when the whole fly was analyzed, although less than the normal flies. This is to be expected, as adding streptomycin to the dietary media was designed to specifically eliminate the gut microbiota, rather than the entire *Drosophila* microbiota. Both treatments reared on axenic media contained similar numbers of colonies to the normal flies. Across all treatments, we identified the bacteria present as *Lactobacillus brevis*, a bacterium that has been previously found to dominate in flies with reduced bacterial diversity, as a result of being reared on a sterile diet (Broderick, Buchon, & Lemaitre, 2014).

An essential aspect of behavioral experiments relies on the ability to easily manipulate individuals when conducting an experimental design. In *Drosophila*, and other insect research, aspirators are commonly used to move individuals between treatments, as it allows for individuals to be manipulated without the use of carbon dioxide anesthesia, which has been shown to negatively impact on mating behavior in some species (e.g. Verspoor, Heys, & Price, 2015). Producing axenic or egg-dechorionated individuals inhibits this ability to aspirate flies directly, in order to prevent external bacteria being transmitted onto the fly or their immediate environment, which could potentially confound experimental results. Therefore, we propose that the purpose of the experiment be an integral factor when considering which gut microbiota elimination method to choose; based on our results, we would suggest that the addition of streptomycin to the dietary media is the most favorable for behavioral research.

Drosophila melanogaster is one of the most useful and powerful models to study host–microbiota interactions. The fly harbors differing levels of bacterial diversity depending on rearing condition (e.g. natural vs. laboratory), but overall this diversity is disproportionately lower than in mammals. Thus, the fly is a highly convenient model for evaluating interactions between bacteria, and between bacteria and the host, and how these interactions affect the host. To date, most studies of the interactions of *D. melanogaster* with its microbiota have focused on the molecular dialog between them (Lhocine et al., 2008; Storelli et al., 2011); Buchon, Broderick, Chakrabarti, & Lemaitre, 2009). Our study highlights the need to take into account not only the molecular dialog, but also the final phenotypic effects of the interaction between the host and its microbiota, in terms of host fitness traits, as these could have strong evolutionary implications for host populations. It also demonstrates that the addition of streptomycin to the larval growth media effectively eliminates the resident bacteria within the *D. melanogaster* gut while resulting in the fewest non-specific, deleterious effects in our host organism. However, it is important to consider that microbiota even within the same species/strains can differ between laboratories, so evaluating individual methods is necessary for a robust experimental design. Of equal importance is the consideration of the type of experiment performed. Adding low-dose streptomycin to the dietary media is the most reliable and practical method of eliminating the gut bacteria, while still allowing easily manipulation of the host for behavioral experiments, and without introducing external bacteria. This method has the potential for widespread use for elucidating the understanding of host–microbiota systems, not only in *Drosophila*, but across all other insect systems.

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CONFLICT OF INTEREST

The authors declare no competing financial interests. Data has been deposited in Dryad

AUTHOR CONTRIBUTIONS

CH, AL, AD, and ZL designed the experiment, and wrote the first draft of the manuscript. CH, FB, and LW conducted the experiment. CH, FB, and AL analyzed the data. All read and edited drafts of the manuscript.

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8.2 Supporting publications: Evidence that the microbiota counteracts male outbreeding strategy by inhibiting sexual signaling in females

This publication entitled “Evidence that the microbiota counteracts male outbreeding strategy by inhibiting sexual signaling in females”, is presented on the following page. Whilst this does not form part of my thesis, its content is highly relevant and referred to throughout this thesis.

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Evidence That the Microbiota Counteracts Male Outbreeding Strategy by Inhibiting Sexual Signaling in Females

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The microbiota is increasingly being recognized as having important impacts on many host biological processes. However, evidence of its effects on animal communication and breeding strategy is lacking. In this three-factorial study, we show that females were more willing to mate with related males, with relatedness likely being assessed through the microbiota. By contrast, male mating investment is concurrently determined by both the relatedness and microbiota status of the female. When the microbiota in female *Drosophila melanogaster* is altered by an antibiotic, male investment in sperm number increased when mating with unrelated females compared to related ones. Contrastingly, the presence of an intact microbiota in females canceled this male outbreeding strategy. As a consequence, the microbiota, when intact, decreased the fitness of the mating couple. Furthermore, we showed that female sexual signaling (cuticular hydrocarbons), with regards to kin recognition, significantly interacts with microbiota. Interestingly, the interaction is significant for hydrocarbons expressed by both sexes, but not for female-specific compounds. Taken together, our results suggest that microbiota can influence kin recognition by disfavoring male outbreeding strategies, likely by inhibiting key olfactory sexual signaling. This represents the first evidence of a host outbreeding strategy counteracted by their microbiota.

Keywords: microbiota, sexual signaling, chemical communication, kin recognition, mating behavior, outbreeding strategy

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INTRODUCTION

The host microbiota is increasingly being shown to have important effects on host developmental, physiological, behavioral, and evolutionary processes. In particular, horizontally transmitted endosymbionts are well documented in their ability to manipulate their hosts to increase their own transmission (Hughes et al., 2012). In the 1970s it was suggested that commensal bacteria could alter the scent of the host, thereby potentially affecting animal communication, such as kin recognition and mate choice (Gorman, 1976). However, over the decades since this was proposed, the role of commensal bacteria in influencing host behaviors used to communicate/interact with conspecifics has largely been ignored (Lizé et al., 2013).

The studies that do address the link between microbiota and animal behavior have primarily been conducted on *Drosophila melanogaster*. For example, it has been shown that virgin *D. melanogaster* females prefer to mate with unfamiliar flies (Ödeen and Moray, 2008). This behavior may occur to avoid multiple matings with individuals of similar genotypes (see Eakley and Houde, 2004) and potentially microbiomes. In contrast, some studies have shown that both wild and laboratory-reared females prefer to mate with related males over unrelated ones (Loyau et al., 2012; Robinson et al., 2012), with inbred flies preferring to mate with individuals reared on the same diet (Sharon et al., 2010; Najarro et al., 2015). However, as this effect disappears when the diet is supplemented with antibiotics, it has been suggested that these preferences in mate choice are mediated by the commensal gut microbiota (Sharon et al., 2010; but see Leftwich et al., 2017).

The core composition of the *D. melanogaster* gut microbiota is cultivable, relatively simple—between 1-30 taxa—and exhibits considerably lower bacterial diversity than observed in vertebrates (Broderick and Lemaitre, 2012). *Acetobacter*, *Gluconobacter*, *Enterococcus*, and *Lactobacillus* are all associated with *Drosophila* (Brummel et al., 2004; Corby-Harris et al., 2007; Cox and Gilmore, 2007; Ren et al., 2007; Roh et al., 2008; Wong et al., 2011), but specific composition is known to change across diet type and laboratory. For example, flies reared on starch medium were dominated by *L. plantarum*, whereas flies reared on CMY (cornmeal, molasses, yeast) media exhibited a greater bacterial diversity including *Bacillus firmus* and *Enterococcus faecalis* (Sharon et al., 2010). Recent work has shown that the microbiota in *D. melanogaster* inhibits kin recognition. In fact, males with an intact microbiota, were unable to alter their mating investment (copulation duration) according to their mate relatedness level, while males were able to when their microbiota was altered (Lizé et al., 2014). Further, a higher mating propensity was observed in related pairs, yet increased mating investment (in terms of copulation duration) was observed in unrelated pairs. Thus, in this species, the microbiota may inhibit outbreeding through masking the host sexual signal of relatedness. This may be because the gut bacteria are driving their own preference and exhibiting kin recognition (e.g., Strassman et al., 2011; Wall, 2016), although the potential benefits to the bacteria are unknown. There is the potential for the modulation of female recognition impacting on microbiota fitness, as the microbiota is vertically transmitted by female *D. melanogaster* to their offspring via egg-smearing (Bakula, 1969). Therefore successful host reproduction will in turn increase microbiota fitness. However, to date the microbiota has never been implicated in inbreeding/outbreeding strategies of an organism.

In the current study, we examined the effect of relatedness, diet, and alteration of the microbiota, on female's mating propensity and male's mating investment, and resultant consequences on the fitness of the mating couple; we measured mating propensity as whether females mated or not, and male mating investment as male sperm transfer. We measured female mating propensity, male sperm transfer, and female resulting egg production in no choice mating tests, when mating occurred with a related (sister, brother) or an unrelated partner, that developed on the same or different food, and whose microbiota was intact or altered via streptomycin, in a fully factorial design (**Figure 1**). We hypothesized that any difference in behavior due to diet or microbiota manipulations might result from changes in the CHC (cuticular hydrocarbon) profile through olfactory cues that are used during mating in many insects, including *D. melanogaster* (Ferveur, 2005). Using two subsamples of virgin females reared under each treatment, we performed gas chromatography-mass spectrometry (GC/MS) to examine whether relatedness and diet, in the presence of an intact or altered microbiota, affected CHC profiles.

MATERIALS AND METHODS

Wild-type *D. melanogaster* stocks were isolated from a *Wolbachia*-free population collected from Dahomey (Benin) in 1970 and maintained in the laboratory since. Flies were reared at 25°C under a 12:12 h light:dark cycle. Recently mated females were placed into laying cages containing grape juice supplemented with a yeast-water paste and allowed to oviposit. Emerging first instar larvae were isolated and placed at a standard density of 10 per vial on one of two diets: 15 ml of either standard corn-based medium (for 1 l of water: 85 g of sugar, 60 g of corn, 20 g of deactivated yeast, 10 g of agar and 25 ml of nipagin), or banana-based medium (for 1 l of water: 137.5 g of banana, 47.5 g of sugar syrup, 30 g of malt, 27.5 g of deactivated yeast, 6.5 g of agar, and 2 g of mold inhibitor). To test whether microbiota altered sperm transfer and/or egg production, half of the vials of both banana and corn-based media were supplemented with the antibiotic streptomycin at a concentration of 0.04% (4 ml of 10 g streptomycin/100 ml ethanol solution per liter melted into the growth medium). Streptomycin is a generalist antibiotic naturally produced by *Streptomyces sp* bacteria living in the soil (Schatz et al., 1944). Streptomycin destroys mainly Gram-negative bacteria, and some Gram-positive bacteria, but is mostly ineffective against anaerobic bacteria, fungi and viruses (Schatz et al., 1944; Courvalin, 1994). It has already been used in several *Drosophila* studies in order to disturb their microbiota (Sharon et al., 2010; Lizé et al., 2014; Leftwich et al., 2017), and has been shown to have no deleterious effects on mortality, longevity and growth development when used at concentrations below 0.4% (Graf and Benz, 1970). Streptomycin is also known to have no effect on *Wolbachia*, an endosymbiont generally found in insects (Fenollar et al., 2003).

Mating Trials

At emergence, virgin flies were collected and isolated under CO_2 anesthesia. Emergents were placed at standard densities of 10 females per vial and males kept individually, in vials containing their larval growth medium. At sexual maturity (3 days old), flies were aspirated into a mating vial containing 15ml of neutral food medium (comprising only sugar, yeast and agar—where corn or banana is absent). A single female was added first and allowed a short period of rest before one male was added. The tested individuals always had an intact microbiota, while its mating partner was either antibiotically treated or not (intact). Female propensity at mating was recorded for a total of

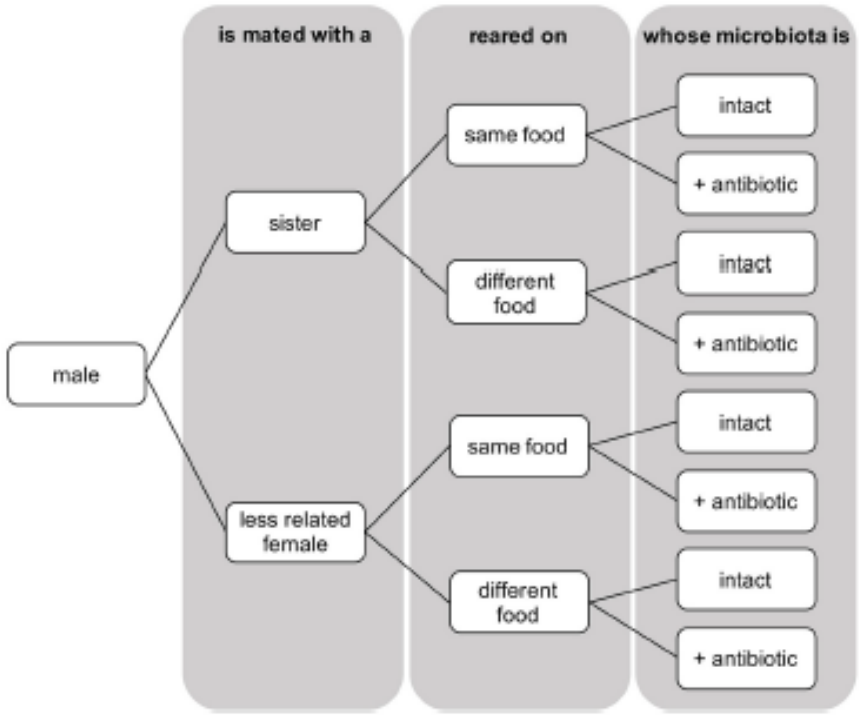

FIGURE 1 | Schematic representation of the three-factorial design showing the eight treatments across which numbers of sperm transferred and eggs laid by females were measured in *D. melanogaster*.

257 mating assays, and measured as the proportion of mating occurring within 3 h of observation, providing ample time for copulation to take place as 74.2% of mating occurred within 1 h. When mating occurred, mating pairs ($N_{total} = 221$) were assigned according to whether the sexual partner was either related ($N_{without\ streptomycin} = 58$; $N_{streptomycin} = 50$) or not ($N_{without\ streptomycin} = 58$; $N_{streptomycin} = 55$), reared on the same type of food ($N_{without\ streptomycin} = 49$; $N_{streptomycin} = 51$) or a different type of food ($N_{without\ streptomycin} = 67$; $N_{streptomycin} = 54$), making eight possible treatments in the three-factorial design (**Figure 1**).

Following copulation, mated females were then transferred to one of two scenarios. Females assigned for egg production were transferred shortly after mating to a vial containing 15 ml of neutral food medium (i.e., where banana or corn is absent) supplemented with two grains of yeast, before being placed at 25°C. Eggs laid by females ($N_{without\ streptomycin} = 39$; $N_{streptomycin} = 48$) were counted every 24 h for a total of 72 h, with the female transferred to a new vial of neutral food medium every 24 h. Females assigned for analysis of sperm transfer ($N_{without\ streptomycin} = 58$; $N_{streptomycin} = 59$) were immediately dissected according to standard protocol (Price et al., 2008), and a subsample of the transferred ejaculate was counted providing an estimate of the relative number of sperm transferred to each female. Sperm subsamples were measured as four lots of 5 µl of the original 50 µl sperm solution transferred to a siliconized glass slide. The number of sperm per slide was measured as the average of the four droplets of sperm solution multiplied by the dilution factor. The number of sperm transferred to a female during copulation is a standard measure of male investment (e.g., Friberg, 2006). Similarly, the number of eggs produced by a female is commonly used as a measure of female fitness (e.g., Dhole and Pfennig, 2014). Egg or sperm counts could not be assessed for seven females.

GC-MS Analysis of CHC Profiles

On emergence, pairs of sisters from four different parental origins (i.e., families) ($N_A = 11$, $N_B = 14$, $N_C = 13$, $N_D = 17$) reared on the two diets ($N_{same\ diet} = 38$, $N_{different} = 17$), and with ($N_{GC-MS,\ streptomycin} = 35$) or without streptomycin ($N_{GC-MS,\ without\ streptomycin} = 20$), as described above, were also frozen for subsequent GC-MS analysis. Two females from the same vial were placed in a 2 ml screw top clear glass vial (Supelco), which was then filled with 50 µl of hexane (Sigma-Aldrich) containing 40 ng/µl hexacosane (Fluka) as internal standard. The vials were shaken gently for 5 min at room temperature to allow any volatiles to enter the hexane. A 2 µl aliquot of each extract was taken for GC-MS analysis. Samples

were run in random order and in technical duplicate. The analytical column used was a 30 m BPX5, 0.25 mm i.d., 0.25 µm film (S.G.E), installed in a Thermo Scientific Polaris GC-MS instrument. The carrier gas was helium at a flow rate of 0.8 ml/min. The split-less injection mode was employed and the injection temperature was 230°C. The column oven temperature program was: initial temperature 50°C held for 2 min, then 25°C/min to 150°C, followed by 10°C/min to 290°C with a final time of 14 min. The transfer line temperature was 250°C and the mass spectrometer source temperature was 200°C. The mass range scanned was 40–600 Daltons. GC-MS TIC peak areas were determined using the peak area measuring tool in the Thermo Excalibur software.

Statistical Analysis

All statistical analyses were performed using R 3.1.0 (R core development team, 2008). Propensity at mating was analyzed by a generalized linear model assuming a Binomial distribution with a logit link function (McCullagh and Nelder, 1989), followed by *post-hoc* Tukey HSD tests to determine differences between treatments. Numbers of sperm transferred by males, and eggs laid by females were transformed to normality using a Box-Cox procedure (Box and Cox, 1964). Transformed data were analyzed using two generalized linear models assuming a Gaussian distribution with an identity link function (McCullagh and Nelder, 1989); these were followed by *post-hoc* Tukey HSD tests to determine differences between treatments. Relatedness, antibiotic treatment, diet and the interactions were used as explanatory variables and the numbers of sperm transferred and eggs laid by females as dependent variables.

Overall the all set of CHC extracted, 14 CHC compounds were quantified by integrating the area under each chromatograph peak for each fly. CHC scores were normalized by calculating the quantity relative to that of an internal standard peak within each sample, which corresponded to a fixed 40 ng/µl concentration of hexacosane. As CHC scores were not normally distributed, they were Log transformed. We used the "MCMCglmm" package in R (Hadfield, 2010) to analyze CHC profiles using multivariate mixed models with Bayesian inference. Due to the high dimensionality of the CHC data (14 traits) and the complexity of the model needed, the models were over-parameterized when ran with all 14 CHCs, so we analyzed three separate groups of CHC: (1) Female-specific dienes (tricosidiene, pentacosadiene, heptacosadiene, and nonacosadiene); (2) Alkanes and monoenes found in both sexes (docosane, tricosene, tricosane, pentacosene, and pentacosane); and (3) Methyl-branched alkanes found in both sexes (methyldocosane, methyl-tetracosane, methyl-hexacosane, methyl-octacosane, and methyltriacontane). For each set of CHCs, we compared two models. The first model included antibiotic treatment and diet (and their interaction) as fixed effects, and family and family × antibiotic treatment interaction as random effects. The second model had the same fixed effect structure, and only family as a random effect. We tested the significance of the genotype-by environment interaction across families and antibiotic treatments by comparing the full model with a model where the family × antibiotic interaction term had been removed. For all models, Markov chains were run for 2,000,000 iterations, with a burn-in of 100,000 and a thinning interval of 50. Each model used a proper prior distribution, and trait-specific variances and co-variances were estimated with the "us" (unstructured) variance structure in MCMCglmm. We compared the models with and without the interaction between family and antibiotic for each subset of CHCs using the model deviance information criterion (DIC) (Spiegelhalter et al., 2002), and estimated the support for each model by calculating the model posterior probability. We then used the posterior distribution of the models to calculate the genetic correlation for expression of each CHC across antibiotic treatments, as a between-environment genetic correlation (Lynch and Walsh, 1998). These models therefore allowed a more detailed analysis of genetic variation that might underlie CHC production and the response to the environmental treatments.

In order to visualize differences in CHC profile between families, we carried out linear discriminant analysis (LDA) with all 14 CHCs modeled against "family" as a four-level factor. This analysis was done twice: first on the subset of data without streptomycin, and second on the subset of data with streptomycin. This allowed us to explore the antibiotic x family interaction by examining the separation between families in overall CHC profile, compared across the two antibiotic treatments.

RESULTS

Probability of Mating With an Unrelated Male (Figure 2)

When modeled as fixed effects, the microbiota status (intact/altered) (GLM, $\chi^2 = 0.232$, 254 df, $p = 0.629$) as well as diet (GLM, $\chi^2 = 3.664$, 254 df, $p = 0.055$) had no significant effect on female mating propensity. By contrast, relatedness significantly affected female's propensity to mate (GLM, $\chi^2 = 5.704$, 254 df, $p = 0.016$), with female mating propensity reduced when the male was unrelated to her (**Figure 2**). Pairwise comparisons of different combinations of factors showed that female mating propensity was reduced with unrelated males, compared to related (Tukey HSD, $p = 0.016$), developing on the same diet as the female. This effect disappeared when males developed on a different diet (Tukey HSD, $p = 0.972$). In the same way, female propensity to mate with unrelated males decreased when unrelated males developed on the same diet as the female compared to unrelated males that developed on a different diet (Tukey HSD, $p = 0.026$). This is not observed for related males (Tukey HSD, $p = 0.999$). Moreover, diet and the microbiota interacted significantly, with female propensity to mate reduced with males reared on the same diet as the female, when the microbiota was intact, compared to males reared on a different diet (Tukey HSD, $p = 0.013$). This effect disappeared when the male microbiota was altered (Tukey HSD, $p = 0.834$) (**Figure 2**).

Number of Sperm Transferred (Figure 3)

Numbers of sperm transferred to females (our measure of male investment in copulation) were not altered by diet [GLM, $F_{(3, 116)}$

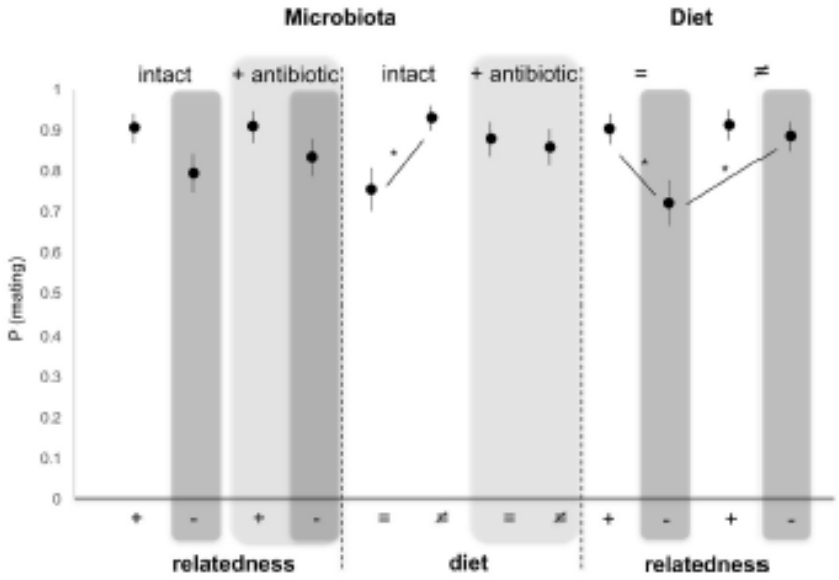

FIGURE 2 | Variation in female propensity to mate when exposed to a single related (brother) (+) or unrelated (–, dark gray) male, reared on the same (=) or different (≠) diet, and whose microbiota was intact or antibiotically-treated (light gray). *Depicts $P < 0.05$.

$= 0.140$, $p = 0.708$], nor by the microbiota status [GLM, $F_{(3, 116)} = 2.214$, $p = 0.139$] as fixed effects. By contrast, numbers of sperm transferred were significantly altered by relatedness of the female partner [GLM, $F_{(3, 116)} = 7.850$, $p = 0.006$], with males transferring more sperm to unrelated females compared to sisters (**Figure 3**). Pairwise comparisons of different combinations of factors showed that males mating with females reared on different diets transferred more sperm to unrelated females than to related one (Tukey HSD, $p = 0.014$). This effect disappeared when females developed on the same diet as their male partners (Tukey HSD, $p = 0.802$). In the same way, sperm number was determined by a significant interaction between relatedness and microbiota. Indeed, numbers of sperm transferred increased when males mated with antibiotically-treated unrelated females compared to antibiotically-treated sisters (Tukey HSD, $p = 0.035$), while this effect disappeared when males mated with untreated unrelated females compared to untreated sisters (Tukey HSD, $p = 0.615$). This was not a side effect of the altered microbiota (or the use of antibiotic) as there were no significant differences in numbers of sperm transferred when males mated with untreated- or antibiotically-treated unrelated females (Tukey HSD, $p = 0.313$), as well as when males mated with untreated- or antibiotically-treated sisters (Tukey HSD, $p = 0.995$) (**Figure 3**).

Numbers of Eggs Laid by Females (Figure 4)

Numbers of eggs laid by females (our measure of fitness consequences) were not altered by diet [GLM, $F_{(3, 86)} = 1.946$, $p = 0.166$] as a fixed effect. By contrast, numbers of eggs laid were significantly decreased when the microbiota was altered [GLM, $F_{(3, 86)} = 7.315$, $p = 0.008$], and increased when males mated with unrelated females compared to sisters [GLM, $F_{(3, 86)} = 4.895$, $p = 0.029$] as fixed effects (**Figure 4**). Pairwise comparisons of different combinations of factors showed that numbers of eggs laid by females reared on a different food than their male partner decreased significantly when females were antibiotically treated compared to untreated females (Tukey HSD, $p = 0.038$), while this effect disappeared when females were reared on the same diet as their male partner (Tukey HSD, $p = 0.750$) (**Figure 4**).

Variation in CHC Profile in Relation to Relatedness and Diet in the Presence or Absence of Gut Bacteria (Figure 5)

CHCs were extracted from pairs of female sisters reared on different diets, and whose microbiota was intact or antibiotically-treated, and analyzed by GC-MS. Of these CHCs, we analyzed 14 of them using multivariate mixed models with Bayesian inference (Markov Chain Monte Carlo method) (Supplementary Data Sheet 1). Due to the high dimensionality of the CHC data (14 traits) and the complexity of the model needed, three separate groups of CHC were analyzed: (1) Female-specific dienes (tricosidiene, pentacosadiene, heptacosadiene, and nonacosadiene); (2) Alkanes and monoenes found in both sexes (docosane, tricosene, tricosane, pentacosene, and pentacosane); and (3) Methyl-branched alkanes found in both sexes (methyldocosane, methyl-tetracosane, methyl-hexacosane, methyl-octacosane and methyltriacontane). For each set of CHCs, we compared two models. The first model included antibiotic treatment and diet (and their interaction) as fixed effects, and family and family × antibiotic treatment interaction as random effects. The second model had the same fixed effect

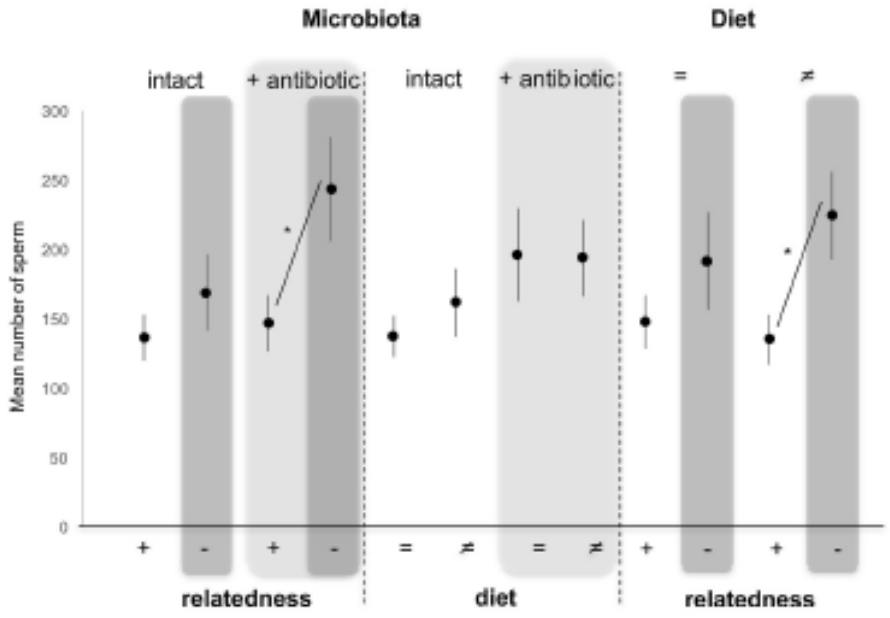

FIGURE 3 | Variation in mean sperm (±s.e.) numbers counted in females after mating with a related (brother) (+) or an unrelated (–, dark gray) male, reared on the same (=) or different (≠) diet, and whose microbiota was intact or antibiotically-treated (light gray). *Depicts $P < 0.05$.

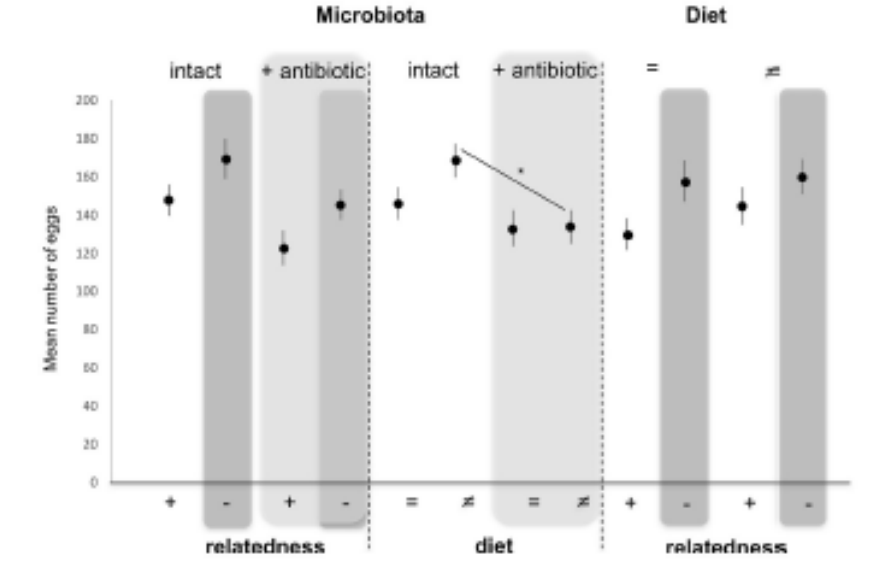

FIGURE 4 | Variation in mean egg (±s.e.) numbers counted in females after mating with a related (brother) (+) or an unrelated (–, dark gray) male, reared on the same (=) or different (≠) diet, and whose microbiota was intact or antibiotically-treated (light gray). *Depicts $P < 0.05$.

structure, and only family as a random effect. By excluding the family × antibiotic interaction when compared to the first model, comparisons of these two models tested the significance of the genotype-by environment interaction across families and antibiotic treatment.

Overall, the fixed effects of diet, antibiotic treatment and diet x antibiotic were not significant for any CHC (results not shown). The best model for the expression of female-specific CHCs did not include the interaction between relatedness and antibiotic treatment, but the best models for the two groups of CHCs

TABLE 1 | Summary of the model results showing the model DIC (deviance information criterion) and the model posterior probability (in brackets) for the models with and without the family × antibiotic interaction for each subset of CHCs.

	Subset of CHCs	Model with interaction	Model without interaction
(1)	Female-specific CHCs	589.73 (0.07)	**584.42 (0.93)**
(2)	Alkanes and monoenes expressed by both sexes	**675.62 (0.69)**	677.25 (0.31)
(3)	Methyl-branched alkanes expressed by both sexes	**710.10 (0.80)**	712.91 (0.20)

The best model for each set of CHCs is in bold.

TABLE 2 | Genetic correlation of each CHC across antibiotic treatments (r_X), calculated as a cross-environment genetic correlation following (Lynch and Walsh, 1998), with 95% credible interval (CI) around each estimate.

CHCs	r_X (95% CI)
(1) FEMALE-SPECIFIC CHCs – DIENES	
Tricosadiene	0.21 (−0.57 to 0.92)
Pentacosadiene	0.06 (−0.70 to 0.84)
Nonacosadiene	0.18 (−0.061 to 0.90)
Heptacosadiene	0.31 (−0.54 to 0.76)
(2) ALKANES AND MONOENES EXPRESSED BY BOTH SEXES	
Docosane	0.05 (−0.73 to 0.82)
Tricosene	0.00 (−0.78 to 0.78)
Tricosane	0.07 (−0.71 to 0.83)
Pentacosene	0.14 (−0.65 to 0.86)
Pentacosane	0.30 (−0.49 to 0.94)
(3) METHYL-BRANCHED ALKANES EXPRESSED BY BOTH SEXES	
Methyl-docosane	−0.07 (−0.84 to 0.72)
Methyl-tricosane	0.09 (−0.69 to 0.85)
Methyl-hexacosane	0.02 (−0.75 to 0.79)
Methyl-octacosane	0.25 (−0.54 to 0.92)
Methyl-triacontane	0.05 (−0.72 to 0.82)

expressed by both sexes did include the relatedness × antibiotic interaction (**Table 1**). This interaction term suggests that there is genetic variation between these families in terms of how CHC production responds to the intact/altered microbiota.

For each CHC individually, the genetic correlation across antibiotic treatments was generally low, although 95% credible intervals were wide and overlapping in each case due to the relatively small number of families used in the calculations (**Table 2**). Consistent with the overall results of the models, where the family × antibiotic interaction was not significant for female-specific CHCs, correlations for these CHCs tended to be higher than for the CHCs expressed by both sexes.

Further analysis of CHCs used linear discriminant analysis (LDA) to visualize overall differences in CHC profiles among family groups (Supplementary Data Sheet 1). This analysis used all 14 CHC traits as the explanatory variables, and analyzed separation between the four families. This was done separately for the data with and without streptomycin treatment, in order to explore the antibiotic × family interaction identified for some CHCs in the linear modeling above. The results show that although families group together fairly consistently when the microbiota is intact or antibiotically treated (**Figure 5**), this grouping differs according to the microbiota status. Unexpectedly, separation appears to be clearer when microbiota is intact (without antibiotic), although the separation on the first discriminant vector (LD1) depends heavily on two families with low sample size (colored red and blue in **Figure 5**). Separation between families with antibiotic is clear as an interaction between the variation described by the first two discriminant vectors (**Figure 5**).

DISCUSSION

In females, we found the major criterion determining female propensity to mate is the relatedness of the male they mate with, with mating being less probable with unrelated males compared to related ones (brothers). Interestingly, relatedness interacted significantly with diet, and diet interacted significantly with the microbiota the male harbored (intact/altered). Indeed, females were able to detect male relatedness only when these males developed on the same diet as the female. Moreover, the female ability to detect that a male developed on the same diet as she did, is dependent on the microbial status of the male. Therefore, although female mating propensity is determined by relatedness of their male partners, assessment of male relatedness is based on the diet the male developed on, but only when the male microbiota was intact. Female *D. melanogaster* relied on microbiota-depend olfactory signals of males to detect whether they developed on same diet as the female, and males developing on a different diet to the female are potentially viewed as being more likely to be unrelated to her than males developing on the same diet as she did. Some of our results are contrary to those of Sharon et al. (2010), who found that flies preferentially mate with individuals reared on the same media, but mate randomly when the microbiota is suppressed (but see Leftwich et al., 2017). This difference is likely due to the strain of flies used (inbred versus outbred), *Wolbachia* infection status (*Wolbachia* positive vs. negative) and the additional measures used in our current study (relatedness of mating pairs).

Whilst we have not quantified the microbiome in this study, previous studies have demonstrated the effect of antibiotics and of diet on the resident microbiota in *D. melanogaster* (e.g., Sharon et al., 2010; Chandler et al., 2011; Ridley et al., 2013; Wong et al., 2013; Broderick et al., 2014; Leftwich et al., 2017). For example, Ridley et al. (2013) determined the microbiota for *D. melanogaster* reared on a conventional glucose-agar-yeast diet and the same diet supplemented with chlortetracycline (CT) (50 $\mu g\ ml^{-1}$). They found that the addition of CT to the dietary media caused an overall reduction in the bacteria present but the occurrence of small numbers of some species, e.g., *Lactobacillus brevis* and *L. plantarum* were only present in the CT reared flies. Similarly, Broderick

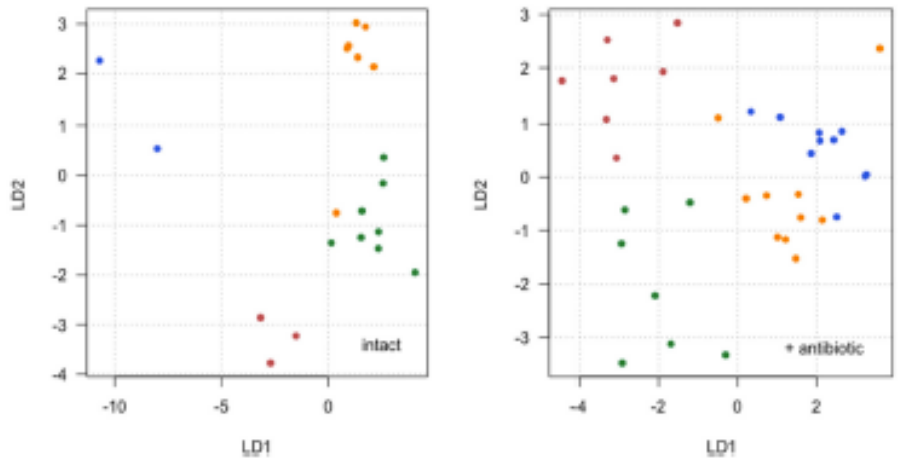

FIGURE 5 | Individual female CHC scores along the first linear discriminant vector (LD1) plotted against scores along the second LD vector (LD2) for two separate LDA: intact microbiota **(Left)** and with antibiotic added **(Right)**. Families are identified by point color with each color representing a different family. In the LDA without antibiotic **(Left)**, 71 and 22% of variation between families is described by LD1 and LD2 respectively, and in the LDA with antibiotic **(Right)**, 6 and 25% of variation between families is described by LD1 and LD2 respectively.

et al. (2014) found that shifting flies onto an autoclaved diet limited gut bacterial diversity to only *L. plantarum*, *A. pasteurianus*, and *L. brevis*. Further, sampling of wild populations of *D. melanogaster* living on varying diet of decaying vegetation and apples, demonstrated drastically different bacteria, with none of the same species detected in each population (Wong et al., 2013).

In males, we found that the major factor determining male sperm investment is relatedness, which is only detected by the male when the female developed on a different diet to the male, providing that the female microbiota was altered. In fact, males transferred more sperm to unrelated females than to sisters when females developed on a different diet than the male, or when female microbiota had been altered via antibiotics. This translated into significantly higher numbers of eggs laid by inseminated females. This indicates that males increase their investment when mating with unrelated females only when the female developed on a different diet that the male, or when the microbiota is altered. Our results suggest that males are not able to fine tune their sperm investment when females developed on the same diet as them, because, either directly or via metabolite production, the microbiota mask female olfactory cues allowing kin recognition to occur in *D. melanogaster*. This has fitness consequences for mating partners for at least 3 days after mating, as females mated to an unrelated male will lay more eggs over this period. The previous suggestion that ecology determines kin recognition is supported here (Lizé et al., 2014). *D. melanogaster* is a generalist, polyandrous species that incurs little cost when mating with a related partner. If kin recognition is of secondary importance within this species, it is probable that other aspects of its ecology, such as familiarity or environment, provide more important mate choice cues. Therefore, this study suggests that an intact microbiota in females could disfavor male outbreeding strategies in laboratory *D. melanogaster*.

Our analysis of CHC profiles suggested that variation occur in females according to family, and that the microbiota only impacts on the CHCs that can be expressed by both sexes (a significant family × microbiota interaction for alkanes, monoenes, and methyl-branched alkanes). Therefore, any modification of the expression of these compounds in one sex are susceptible to also occur in the other sex in a common environment, which would maintain male-female recognition as partners. Maintaining male-female recognition may have fitness consequences for the microbiota, as this is vertically transmitted by female *D. melanogaster* to their offspring via egg-smearing (Bakula, 1969), so successful host reproduction will increase microbiota fitness. This family × microbiota interaction was non-significant for the dienes we analyzed, which are only expressed in females in this species (Dallerac et al., 2000; Chertemps et al., 2006). It is therefore likely that quantitative variation in expression of CHCs that are shared between males and females (alkanes, monoenes, and methyl-branched alkanes) could be used as kin recognition cues by males to determine whether a female is related, but that this sexual signal of relatedness is masked by the microbiota at least in females. *D. melanogaster* also acquire microbiota via horizontal transfer between individuals. For example, when females deposit eggs onto a food source, attractant pheromones are also released encouraging additional females to aggregate (Bartelt et al., 1985; Wertheim et al., 2005). It may be that the resulting transfer of microbiota among females and emerging larvae is responsible for masking this sexual signal of relatedness.

Although females *D. melanogaster* mate preferentially with their brothers, males invest more sperm in unrelated females with impaired microbiota, which results in more

eggs being laid by these unrelated females. Three hypotheses (detailed below) may explain these contradictory strategies developed by males and females of this species: (1) there is a male trade-off between searching costs and kin recognition benefits in nature (Kokko and Ots, 2006), (2) females take advantage of the microbiota in the context of sexual conflict, (3) the microbiota manipulates the host outbreeding strategy.

(1) Male trade-off between searching costs and kin recognition benefits in nature

In males, a trade-off might exist between distinguishing related females in nature, whose microbiota is much more diverse (Chandler et al., 2011), and the energy allocated by males to find females. If the energy allocated to find a female in nature is higher than the fitness reward gained by distinguishing a related female, then males should not differentiate females based on relatedness. Context-dependent kin recognition is widely used by amphibian (Blaustein and Waldman, 1992; Hokit et al., 1996; Nichols, 2017), and hymenopteran species (Hepper, 1991; Starks et al., 1998; Buczkowski and Silverman, 2005) and this could explain why kin recognition is observed only when the microbiota is altered in the laboratory in *D. melanogaster*.

(2) Females take advantage of the microbiota in the context of sexual conflict

Our data showed that female mating propensity was influenced by male relatedness. Other studies have shown that *D. melanogaster* females prefer to mate with related males, and that they are therefore more prone to inbreeding (Loyau et al., 2012; Robinson et al., 2012). Our data with regards to male investment demonstrated the opposite, with males promoting outbreeding but only when female microbiota was altered. This sheds light on the sexual conflict that has been described in this species (Chapman et al., 2003), but also highlights that female may use their microbiota to take advantage of this conflict, thereby rendering a male outbreeding strategy impossible.

(3) The microbiota manipulates the host breeding strategy

Finally, another alternative would be that outbreeding in *D. melanogaster* is costly for the microbiota. Currently, there is no data describing this phenomenon and further studies are needed to demonstrate it. However, host outbreeding strategies increase the genetic diversity in their offspring, which is known to increase immune defense (van Houte et al., 2016). Increasing immune defense might be costly for the microbiota (Bolnick et al., 2014).

In this study, female propensity for mating was greater for related males, with relatedness being assessed through the diet the male developed in, and most probably through the microbiota induced by diets. By contrast, males invested more sperm in unrelated females, whose microbiota was antibiotically treated, and this influences subsequent egg production. *D. melanogaster* is a generalist species, thus the alteration of the composition of the microbiota community as a result of feeding on different food sources is likely to have important ecological consequences for mating behavior, and in turn divergence between populations. To our knowledge, this is the first evidence that an intact microbiota, via its effects on the host chemical communication, can alter the host breeding strategy by disfavoring outbreeding.

ETHICS STATEMENT

No ethical approval or specific permit was needed for rearing and experimental use of *Drosophila melanogaster*.

AUTHOR CONTRIBUTIONS

AL, TP, and ZL designed the experiments. CH, HC, FI, MP, TP, and AL performed the experiments. ZL, FI, CH, and AL wrote the manuscript and all authors amended it.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fevo.2018.00029/full#supplementary-material

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Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

8.3 Supplementary materials

Chapter Five: Gut microbiota and adaptation to octanoic acid in *Drosophila sechellia* and *Drosophila melanogaster*

Table 1. All pairwise comparisons of the test statistics and P values given from the Cox Proportion-Hazard Regressions of the development time data

Species	Treatment	Comparison	Test statistic	P value	Significance
D. sechellia	0%	1%	0.169	0.866	
D. sechellia	0%	5%	0.545	0.586	
D. sechellia	0%	10%	1.304	0.192	
D. sechellia	0%	25%	1.608	0.108	
D. sechellia	0%	50%	0.740	0.459	
D. sechellia	1%	5%	0.351	0.725	
D. sechellia	1%	10%	1.110	0.267	
D. sechellia	1%	25%	1.394	0.163	
D. sechellia	1%	50%	0.547	0.584	
D. sechellia	5%	10%	0.855	0.392	
D. sechellia	5%	25%	1.157	0.247	
D. sechellia	5%	50%	0.224	0.822	
D. sechellia	10%	25%	0.218	0.827	
D. sechellia	10%	50%	-0.630	0.529	
D. sechellia	25%	50%	-0.905	0.365	
Inbred *D. mel*	0%	1%	-2.463	0.013	*
Inbred *D. mel*	0%	5%	-7.033	<0.001	***
Inbred *D. mel*	0%	10%	-9.552	<0.001	***
Inbred *D. mel*	0%	25%	-9.323	<0.001	***
Inbred *D. mel*	0%	50%	-9.187	<0.001	***
Inbred *D. mel*	1%	5%	-4.721	<0.001	***
Inbred *D. mel*	1%	10%	-7.479	<0.001	***
Inbred *D. mel*	1%	25%	-7.499	<0.001	***
Inbred *D. mel*	1%	50%	-7.413	<0.001	***
Inbred *D. mel*	5%	10%	-3.076	<0.001	***
Inbred *D. mel*	5%	25%	-3.564	<0.001	***
Inbred *D. mel*	5%	50%	-3.576	<0.001	***
Inbred *D. mel*	10%	25%	-0.727	0.467	
Inbred *D. mel*	10%	50%	-0.795	0.426	
Inbred *D. mel*	25%	50%	-0.076	0.939	
Outbred *D. mel*	0%	1%	-7.930	<0.001	***
Outbred *D. mel*	0%	5%	-4.647	<0.001	***
Outbred *D. mel*	0%	10%	-8.679	<0.001	***

Outbred *D. mel*	0%	25%	-7808.000	<0.001	***
Outbred *D. mel*	0%	50%	-4.900	<0.001	***
Outbred *D. mel*	1%	5%	2.952	0.003	**
Outbred *D. mel*	1%	10%	-2.139	0.032	*
Outbred *D. mel*	1%	25%	-1.281	0.200	
Outbred *D. mel*	1%	50%	0.170	0.865	
Outbred *D. mel*	5%	10%	-4.590	<0.001	***
Outbred *D. mel*	5%	25%	-3.749	<0.001	***
Outbred *D. mel*	5%	50%	-1.825	0.067	
Outbred *D. mel*	10%	25%	0.759	0.447	
Outbred *D. mel*	10%	50%	1.733	0.083	
Outbred *D. mel*	25%	50%	1.098	0.272	

Table 2. Species and number of bacterial colonies isolated from *D. sechellia* and both outbred and inbred *D. melanogaster* flies when reared on diets containing different concentrations of octanoic acid. The bacterial load of adult flies is quantified from the midgut.

Species	**Life stage**	**Concentration**	***L. plantarum***	***Paenibacillus sp.***	***B. cereus***
D. sechellia	Adult	0%	1.51x10^4	0	0
D. sechellia	Adult	0%	1.51x10^4	0	0
D. sechellia	Adult	1%	5.25x10^3	0	0
D. sechellia	Adult	1%	5.56x10^3	0	0
D. sechellia	Adult	5%	4.16x10^2	0	0
D. sechellia	Adult	5%	5.20x10^2	0	0
D. sechellia	Adult	10%	9.93x10^3	0	0
D. sechellia	Adult	10%	1.04x10^4	0	0
D. sechellia	Adult	25%	1.8x10^1	0	0
D. sechellia	Adult	25%	6.7x10^1	0	0
D. sechellia	Adult	50%	2.23x10^2	0	0
D. sechellia	Adult	50%	1.24x10^2	0	0
Outbred *D. melanogaster*	Adult	0%	1.57x10^4	0	0
Outbred *D. melanogaster*	Adult	0%	1.53x10^4	0	0
Outbred *D. melanogaster*	Adult	1%	1.46x10^4	0	0
Outbred *D. melanogaster*	Adult	1%	1.45x10^4	0	0
Outbred *D. melanogaster*	Adult	5%	3.32x10^3	0	0
Outbred *D. melanogaster*	Adult	5%	4.48x10^3	0	0
Outbred *D. melanogaster*	Adult	10%	1.30x10^3	0	0
Outbred *D. melanogaster*	Adult	10%	2.34x10^3	0	0
Outbred *D. melanogaster*	Adult	25%	5.25x10^3	0	0
Outbred *D. melanogaster*	Adult	25%	7.96x10^3	0	0
Outbred *D. melanogaster*	Adult	50%	7.02x10^3	0.10x10^1	0

Outbred *D. melanogaster*	Adult	50%	8.20x10^3	0	0
Inbred *D. melanogaster*	Adult	0%	1.42x10^4	0	0
Inbred *D. melanogaster*	Adult	0%	1.12x10^4	0	0
Inbred *D. melanogaster*	Adult	1%	8.76x10^3	0	0
Inbred *D. melanogaster*	Adult	1%	7.45x10^3	0	0
Inbred *D. melanogaster*	Adult	5%	3.24x10^3	0.30x10^1	0
Inbred *D. melanogaster*	Adult	5%	3.81x10^3	0.50x10^1	0.10x10^1
Inbred *D. melanogaster*	Adult	10%	1.25x10^3	0.10x10^1	0.20x10^1
Inbred *D. melanogaster*	Adult	10%	2.01x10^3	0.40x10^1	0.10x10^1
Inbred *D. melanogaster*	Adult	25%	4.58x10^3	0.80x10^1	0.30x10^1
Inbred *D. melanogaster*	Adult	25%	5.23x10^3	1.00x10^1	0.80x10^1
Inbred *D. melanogaster*	Adult	50%	8.98x10^3	1.30x10^1	0.40x10^1
Inbred *D. melanogaster*	Adult	50%	9.03x10^3	1.60x10^1	0.60x10^1