The Role of the Gut Microbiome in the Social Stress Response

Megan Fairweather

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The candidate confirms that the work submitted is her own and that appropriate credit has been given where reference has been made to the work of others.

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Abstract

The gut microbiome has considerable influence over host health, and so alterations to its composition can result in disease. Research has demonstrated that environmental stress, including social contact, can influence lifespan. This is potentially dependent on changes in the microbiome, facilitated through gut-brain communication via the gut-brain axis. This thesis utilised a *Drosophila melanogaster* model to explore the interplay between the microbiome and responses to social stress. Previous research demonstrating the sensitivity of the male *Drosophila* microbiome to the social environment positioned it as a likely candidate for a modulator of various social responses. This thesis provides evidence for a causal pathway, implicating the microbiome in shaping the male longevity response to same-sex social contact, with antibiotic impact varying with the social environment. An involvement of the microbiome in the cognitive response, but not the mating response, was also established, alongside the expression of genes associated with cognitive development. There was some evidence for the role of the microbiome in behaviour, and for a slight protective effect of the age of the rival on socially-driven age-associated changes in behaviour. Additionally, KEGG predicted functional analysis was employed to infer microbial effects on host gene function in studies that observed a microbiome change, identifying pathways that may influence ageing. Subsequently, gene expression analysis determined one of these host pathways to be dependent on the microbiome. In conclusion, this thesis provides evidence that same-sex social contact in male *Drosophila* alters the microbiome in a way that reduces lifespan and triggers the cognitive response, which can be partially replicated in single flies by antibiotic perturbation. Furthermore, many responses were altered in an age-dependent way, interacting with senescence and possibly age-associated dysbiosis. These findings hold implications for leveraging the microbiome to develop targeted interventions against disease, particularly in the context of ageing.

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Abbreviations

ACTH	Adrenocorticotropic hormone
AIC	Akaike information criterion
AMP	Antimicrobial peptide
ANOVA	Analysis of deviance
ANS	Autonomic nervous system
ASD	Autism spectrum disorder
ASVs	Amplicon sequence variants
cnk	Connector enhancer of ksr
CNS	Central nervous system
Cq	Quantification cycle
CRF	Corticotropin-releasing factor
Dcp-1	Death caspase-1
Diap-1	Death-associated inhibitor of apoptosis 1
dILPs	*Drosophila* insulin-like peptides
Duox	Dual oxidase
dUSP36	Ubiquitin-specific protease 36
ECs	Absorptive enterocytes
ENS	Enteric nervous system
ERK	Extracellular signal-regulated kinase
EtOH	Ethanol
FAIR	Findability, Accessibility, Interoperability, and Reusability
FoxO	Forkhead box O

GABA	Gamma-aminobutyric acid
GBA	Gut-brain axis
GLM	Generaled linear model
GLMM	Generaled linear mixed model
HFD	High fat diet
hid	Head involution defective
HNF4α	Hepatocyte nuclear factor 4A
HPA	Hypothalamic pituitary adrenal
IBD	Intestinal bowl disease
IMD	Immune deficiency
ISC	Intestinal stem cell
JAK/STAT	Janus kinase/signal transducers and activators of transcription
JNK	c-Jun N-terminal Kinase
KDM5	Lysine demethylase 5
KEGG	Kyoto encyclopedia for genes and genomes
LM	Linear model
MAPK	Mitogen-activated protein kinase
MRS	Man, Rogosa, and Sharpe
NADPH	Nicotinamide adenine dinucleotide phosphate
NMDS	Non-Metric Multidimensional Scaling
Nox	NADPH oxidase
Nrx-1	Neurexin-1

NS	Non significant
p38c	p38c MAP kinase
PBS	Phosphate-buffered saline solution
PGRP	Peptidoglycan recognising protein
qPCR	Quantitative polymerase chain reaction
Ras85D	Ras oncogene at 85D
ROS	Reactive oxygen species
SCFA	Short-chain fatty acid
Sod2	Superoxide dismutase 2 (Mn)
SYA	Sugar-yeast agar
tBH	Tertiary-butyl hydroperoxide
TOR	Target of rapamycin
tra	Transformer
vn	Vein

Chapter 1
General Introduction

The microbiome encompasses the collection of microbial taxa and their genes present within a defined environment. The gut microbiome, made up of microbiota that occupy the digestive tract, has in particular become a subject of considerable scientific interest, as it is known to contribute significantly to the overall health and wellbeing of the host organism (Grenham et al., 2011). The gut microbiome (henceforth referred to as the microbiome) comprises of various microbial communities, including viruses, bacteria, archaea and fungi. The microbiome is sensitive to environmental factors that act to alter microbial composition through both extrinsic and intrinsic routes. For example, the social environment can affect the microbiome by the extrinsic transfer of microbiota between animals, or through a shared environment or diet (Chandler et al., 2011; Moeller et al., 2013). Alternatively, gut-brain communication through hormonal, immune and neural pathways that make up the gut-brain axis (GBA) mean that any environmental change that induces a physiological change in the host, including stress caused by the social environment, may result in changes in microbiota. Due to the importance of the microbiome in digestion and the delivery of nutrients and vitamins (Kang et al., 2012; Larsbrink et al., 2014), as well as other roles, including maintaining gut physiology (Zareie et al., 2006) and immune function (Ghaisas et al., 2016), changes in its composition can be detrimental to the health of the organism.

In many different taxa, the presence of conspecifics in an individual's environment and the interactions between individuals can have a substantial and

varying impact on their health. Competitive male-male contact can have especially adverse physiological effects, such as changes to stress hormones, the immune system, behaviour and lifespan (Creel et al., 2013). It is unclear how some external stressors result in these physiological effects, but one potential candidate linking them is the microbiome, due to its variability in different social environments (Bailey et al., 2011; Tung et al., 2015; Levin et al., 2016). This chapter explores the impacts of the microbiome on health, cognition and behaviour while also examining its susceptibility to modulation by stress and the social environment. Research on the fruit fly (*Drosophila melanogaster)* in particular highlights the implications certain social environments can have on health, and evokes questions regarding the potential mediation of these effects by the microbiome.

1.1 The Microbiome and Health

The microbiome consists of ‘transient’ and ‘core’ microbiota. Transient microbiota change with time, diet, environmental conditions, or interactions with other individuals. Although transient microbes may not have a substantial impact on the function of the microbiome, they can nonetheless exert influence over short-term microbial dynamics and host impacts. The core microbiome remains relatively constant, persisting over time and environmental conditions. Microbiota that are not pathogenic to the host, remain relatively stable, and do not trigger an exaggerated immune response are referred to as commensals. Commensals often perform beneficial functions within the gut.

Throughout the animal kingdom, commensal microbiota play a crucial role in maintaining the body, aiding digestion and the delivery of nutrients and vitamins

(Kang et al., 2012; Larsbrink et al., 2014). The products resulting from the breakdown of nutrients by microbiota include molecules that are bioactive in the host, capable of initiating signalling cascades and influencing various metabolic pathways (Abdul Rahim et al., 2019). Microbiota are also able to regulate and degrade metabolites within the host. For example, short chain fatty acids (SCFAs), fatty acids that contain less than six carbons and are made by the fermentation of dietary carbohydrates by gut bacteria, have many important roles within the host including providing energy and regulating inflammation and gut motility (Scheppach, 1994). Certain commensal bacteria are also able to influence gut physiology, for example altering the epithelial cell barrier (Zareie et al., 2006), and gut motility (Stappenbeck et al., 2002). Lastly, the microbiome also supports the immune system and regulates neurotransmitter release (Ghaisas et al., 2016).

As different species of microbiota have diverse metabolic activities, they are able to differentially influence host functions. Consequently, it is understandable that an individual's microbiota composition can influence their health, contributing to disease, ageing, and longevity (reviewed in Ghosh et al., 2022). Numerous diseases including inflammatory bowel disease (IBD), liver disease, and colorectal cancer, have been associated with microbial changes (Pasolli et al., 2016; Duvallet et al., 2017). Certain microbiota, often through the production of SCFAs, exhibit anti-inflammatory properties, thereby protecting against age-related diseases such as hypertension, diabetes, and cancer (Gassull, 2006; Freund et al., 2010). A study using mice demonstrated that the lifespan extension observed after treatment with the anti-diabetic drug, acarbose, was partially

attributed to changes in the microbiota that resulted in an increase in SCFAs (Smith et al., 2019).

Physiological alterations of the gut with age include increased inflammation, cellular senescence and dysfunction of the epithelial barrier (Funk et al., 2020). This can exert a substantial effect on the microbiome, and so it is unsurprising that ageing is characterised by a decline in commensal species which may protect against disease, along with a gain of pathobionts (DeJong et al., 2020). These shifts in the microbiome composition with age can significantly impact health outcomes and influence 'healthy ageing' in humans.

The link between microbial composition and host functions means the addition of certain bacteria can improve health in a multitude of ways, which has led to considerable interest in the use of probiotics to help fight disease. Ageing mice exhibited decreased inflammation after treatment with *Bifidobacterium lactis*, resulting in an improvement of colonic mucosal function and increased longevity (Matsumoto et al., 2011). Rats made free from microbiota (germ-free) had an improved morphology of the intestinal mucosa with addition of *Lactobacillus rhamnosus,* compared to conventional rats (Banasaz et al., 2002). Alternatively, some bacterial commensals may negatively impact the host, such as *Clostridium* which increased in abundance in response to stress in mice and is associated with gastrointestinal disease and inflammation (Libby & Bearman, 2009; Aguilera et al., 2013). Extensive research has demonstrated the successful application of probiotic use in humans to protect against infectious and inflammatory diseases (Reviewed in Isolauri, 2001). Furthermore, research demonstrates that polyphenol-rich food, prebiotics, and probiotics sometimes

exert more pronounced effects in older people (Cancello et al., 2019; Theou et al., 2019; Tran et al., 2019; Del Bo et al., 2021), suggesting a promising avenue for potential interventions to mitigate the effects of ageing.

1.2 The Microbiome and the Brain

As well as having a large impact on physical health, gut-brain communication through the mammalian GBA means that alterations in the microbiome may impact cognition, mood and behaviour. A considerable number of these effects can be attributed to the production of neurotransmitters in the gut. Many bacterial species are able to regulate tryptophan, the precursor of the neurotransmitter serotonin (Gao et al., 2018). Approximately 95% of the human body's serotonin is synthesized in the gut (Gershon, 2013), and both serotonin and tryptophan have been found at higher levels in germ-free mice compared to control mice (Clarke et al., 2013). Additionally, treatment of mice with *L. plantarum* increased dopamine and decrease serotonin in the mouse brain (Liu et al., 2016). SCFAs produced by gut bacteria altered serotonin release (Grider & Piland, 2007). Furthermore, a study exhibited that the SCFA acetate can move from the mouse colon to the brain and cross the blood-brain barrier to incorporate into Gamma-aminobutyric acid (GABA) to regulate appetite (Frost et al., 2014). Overall, the regulation of neurotransmitters by microbiota could be exerting an effect on the host brain through multiple avenues, potentially impacting cognition and behaviour.

1.2.1 Cognition and Behaviour

Research has shed light on a role for the microbiota in promoting brain health and influencing cognitive function. Research has shown that SCFAs produced by gut bacteria can alter memory (Vecsey et al., 2007; Garcez et al., 2018), and even protect against brain disorders such as Alzheimer's (Ho et al., 2018). In mice, the transplantation of microbiota from young donor mice into aged mice

reversed age-associated impairments in cognition such as memory and learning, as well as hippocampal metabolome and transcriptome changes (Boehme et al., 2021). In honeybees, *Lactobacillus* improved learning and memory via tryptophan metabolism (Zhang et al., 2022). These cognitive faculties are essential in social tasks including feeding and foraging (Laska et al., 1999). Taken with the finding that the microbiome varies among different castes exhibiting distinct social behaviours (Kapheim et al., 2015), this suggests that the microbiome may have a role in influencing sociality in honeybees.

As the microbiome allows for normal brain functioning, in mice, changes can impact behaviours including anxiety (Savignac et al., 2014) and sociability (Desbonnet et al., 2015). Antibiotic depletion of the microbiome caused deficits in social behaviour in rodents (Desbonnet et al., 2015), and the consumption of prebiotics increased prosocial behaviour in socially-stressed mice (Burokas et al., 2017). Reduced social interaction in diabetic mice was replicated in control mice through the transfer of the microbiome (Gacias et al., 2016). Additionally, microbiome dysbiosis associated with mouse maternal obesity affected the social behaviour of offspring, which could be reversed by treatment with a *Lactobacillus reuteri* (Buffington et al., 2016). These studies demonstrate that changes in the microbiome can have an impact on host behaviour.

1.2.2 The Stress Response

The composition of the microbiome can alter the physiological stress response and stress-related behaviours. For example, an exaggerated hypothalamic pituitary adrenal (HPA) stress response was demonstrated in germ-free mice (Sudo et al., 2004), and *Bifidobacteria* supplementation reduced anxiety and

depression-like behaviour in response to stress (Savignac et al., 2014). Another study demonstrated that antibiotics were able to reverse increases in circulating cytokines, an immunological response, driven by a social stress (Bailey et al., 2011). In another study, *Bifidobacteria* was present in social stress-resilient mice only, and addition of *Bifidobacteria* into the diet increased the number of resilient mice (Yang et al., 2017b). Studies found that *L. rhamnosus* consumption decreased stress-induced cortisol release in mice, as well as anxiety- and depression-like behaviour (Bravo et al., 2011; Bharwani et al., 2017). After faecal transfer, where a recipient, germ-free mouse was colonised by repeated oral gavage of faeces from control and depressed human subjects, the individuals that received microbiome samples from the depressed human subjects displayed increased compulsive anxiety-like behaviours (Kelly et al., 2016). Research is advancing into human clinical trials, with one study demonstrating that a formulation consisting of both *Lactobacilli* and *Bifidobacteria* can decrease psychological distress (Messaoudi et al., 2011).

The numerous and diverse impacts of the microbiome on health and behavioural effects emphasises the importance of understanding the external and internal drivers of microbiome community structure. Exploring these factors and the mechanisms that govern their influence on the microbiome represents a significant step towards developing potential interventions aimed at manipulating and optimising the microbiome for beneficial health outcomes.

1.3 The Gut-Brain Axis

The mammalian GBA allows for bidirectional communication between the cognitive centres of the brain and the gastrointestinal tract (Figure 1.1). One way this can occur is through neural signalling. Signalling molecules can directly interact with microbiota via neurotransmitter binding sites that are found on bacteria, resulting in metabolic and gene regulation (Hughes & Sperandio, 2008). Additionally, the brain can influence the gut, through the autonomic nervous system (ANS) including altering the intestinal barrier, gut motility and secretion of acid, bicarbonates and mucus, and consequently the microbiome (Macfarlane & Dillon, 2007; Mayer, 2011). For example, stress is known to increase rat colonic motility, which can in turn effect the transport of nutrients to microbiota (Gue et al., 1991). This effect is reciprocal as the microbiome can influence the gut environment, for example, probiotic species consumption in mice ensured intestinal epithelium tight junction barrier integrity (Ait-Belgnaoui et al., 2014). The enteric nervous system (ENS) innervates the gut and communicates with the ANS, allowing microbiota to interact with afferent sensory neurons by the microbial production of local neurotransmitters (Iyer et al., 2004). For example, in rats, *L. reuteri* was reported to inhibit the opening of enteric sensory neuron potassium channels, which may lead to a decrease in gut dysmotility and a reduction in pain perception (Kunze et al., 2009). Microbiota are also involved in the regulation of important neurotransmitters such as serotonin, dopamine and GABA (Tsavkelova et al., 2000; Barrett et al., 2012; Özoğul et al., 2012).

It has been proposed that the vagus nerve is an important neural pathway in the mammalian GBA, with vagal afferents forming synaptic connections with

enteroendocrine gut cells (Tsigos & Chrousos, 2002). Indeed, blockage of the vagus nerve reduced the effects of probiotic strains in rats (Bravo et al., 2011).

In addition to neural pathways, endocrine pathways also contribute to the GBA, and are particularly active in response to stress. Germ-free mice had an increased HPA response to stress, including increased plasma adrenocorticotropic hormone (ACTH) and corticosterone levels, and an altered expression of genes involved in HPA axis regulation (Sudo et al., 2004; Vodička et al., 2018). Bacterial by-products have been shown to induce enteroendocrine cell production of many neuropeptides, which can exert their effects locally to ENS neurons or extrinsically via secretion into the bloodstream (Holzer & Farzi, 2014a). For example, SCFAs produced by bacterial fermentation stimulate enteroendocrine cells to produce peptide YY, which acts as a satiety hormone, signalling to the brain to reduce food intake (Samuel et al., 2008).

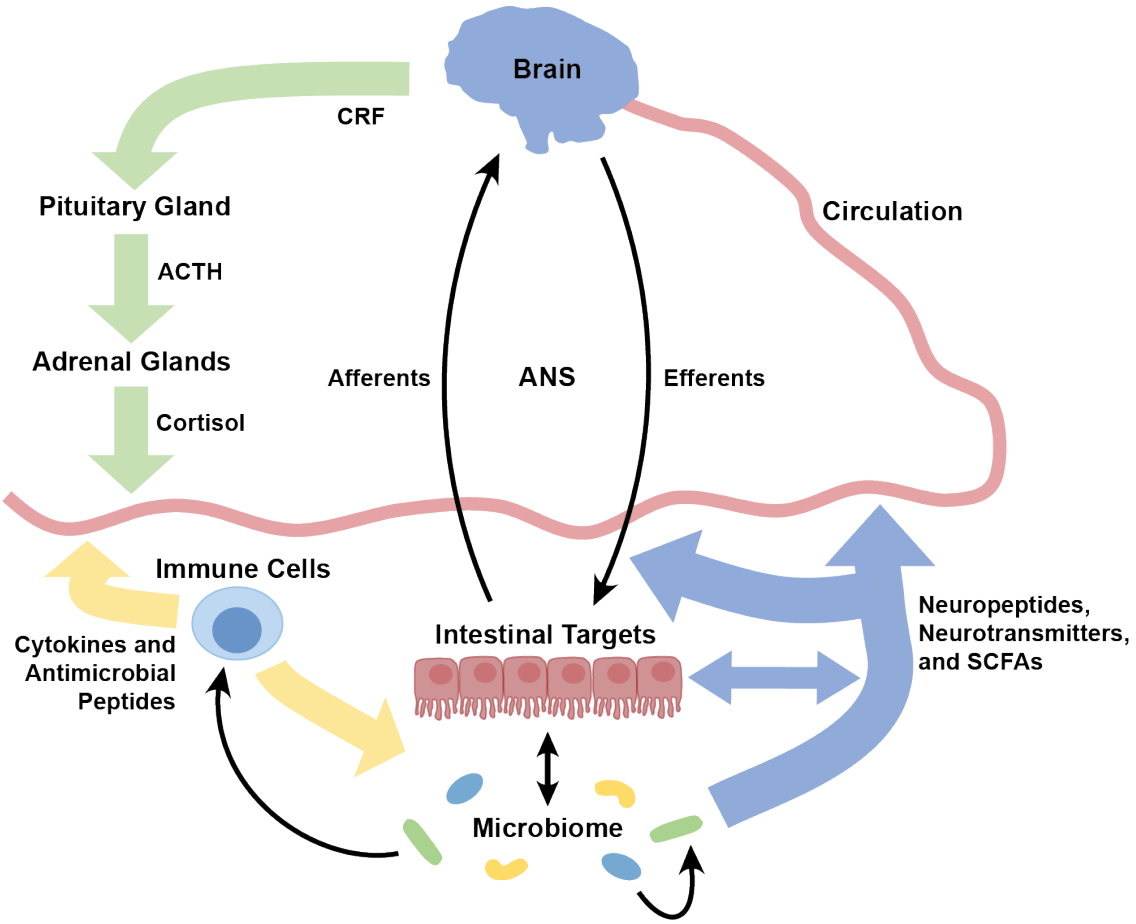

Figure 1.1 The Mammalian Gut-Brain Axis

Environmental stress, as well as cytokines, activate the HPA axis (green): the release of corticotropin-releasing factor (CFR) from the hypothalamus stimulates the release of adrenocorticotropic hormone (ACTH) from the pituitary gland, resulting in release of cortisol from the adrenal glands into the blood (Tsigos & Chrousos, 2002). The autonomic nervous system (ANS) comprises of afferents and efferents which transfer information back and forth between the brain and intestinal targets, such as epithelial cells and enteric muscles (Grenham et al., 2011). Changes in these intestinal targets go on to affect the microbiome and in turn the microbiome is also able to impact intestinal targets (Macfarlane & Dillon, 2007; Ait-Belgnaoui et al., 2014). Microbiota can impact other microbiota for example due to cross-feeding or interference competition (Martin et al., 2023). The microbiome is also able to regulate the immune system, causing immune cell release of cytokines and anti-microbial peptides which can affect the brain via the circulation (Macpherson & Uhr, 2002). Some microbiota and intestinal cells also produce neurotransmitters, neuropeptides and SCFAs which directly affect intestinal targets and the ANS as well as travelling to the brain (Iyer et al., 2004; Kimura et al., 2011; Holzer & Farzi, 2014b).

1.4 Using *Drosophila melanogaster* to Investigate Impacts of the Microbiome

D. melanogaster is frequently used as a model system due to its practicality, quick reproduction rate, ease of experimental manipulation, and lack of ethical restrictions regarding its use. Furthermore, there exists a considerable wealth of information currently available on *Drosophila*, such as the sequence of their genome (Adams et al., 2000). *Drosophila* has orthologs for approximately 75% of human disease-related genes (Reiter et al., 2001), and many homologous functions and pathways have been found between *Drosophila* and mammals, such as Notch signalling which controls the differentiation of intestinal stem cells (ISCs) into mature gut cells in both *Drosophila* and mammals (van Es et al., 2005). The human and *Drosophila* gut share anatomical similarities, for example, both are divided into three sections, and both have acidic regions. Additionally, both are lined with a monolayer of ISCs, absorptive enterocytes (ECs), secretory enteroendocrine cells and enteroblasts. Notably, ECs and ISCs in *Drosophila* and mammals possess microvilli to enhance the surface area within the gut (Shanbhag & Tripathi, 2009). Nevertheless, the microbiome of *Drosophila* is relatively simple compared to humans, with estimations of 5-30 bacterial species per individual (Chandler et al., 2011), compared to the estimate of hundreds or thousands of species in humans (Yatsunenko et al., 2012). However, it is worth noting that a more recent study has discovered a higher diversity of 150-200 bacterial species associated with individual flies, possibly due to more in-depth sequencing and alternative food, absent from bacteriostatic compounds (Dantoft et al., 2016).

While GBA mechanisms in *Drosophila* have not undergone as extensive research as in mammals, several fundamental features have been identified that facilitate communication between the gut and the brain. Hormonal signals and neuronal signals travel between the brain and the gut facilitated by the ANS or the circulation of haemolymph (Figure 1.2). *L. Plantarum* increased sleep in *D. melanogaster* via induction of a homolog of mammalian neuropeptide Y, neuropeptide F (Ko et al., 2022). In *Drosophila* larvae, serotonergic neurons that extend from the CNS to innervate the ENS are possibly functionally analogous to the vagus nerve in vertebrates (Schoofs et al., 2014). However, it remains uncertain whether this neural connection persists beyond metamorphosis and is present in the adult fly. It has been proposed that CChamide-2 cells in the gut sense food quality and alter feeding behaviour by releasing CCHamide-2 neuropeptide which is able to bind to brain receptors (Li et al., 2013).

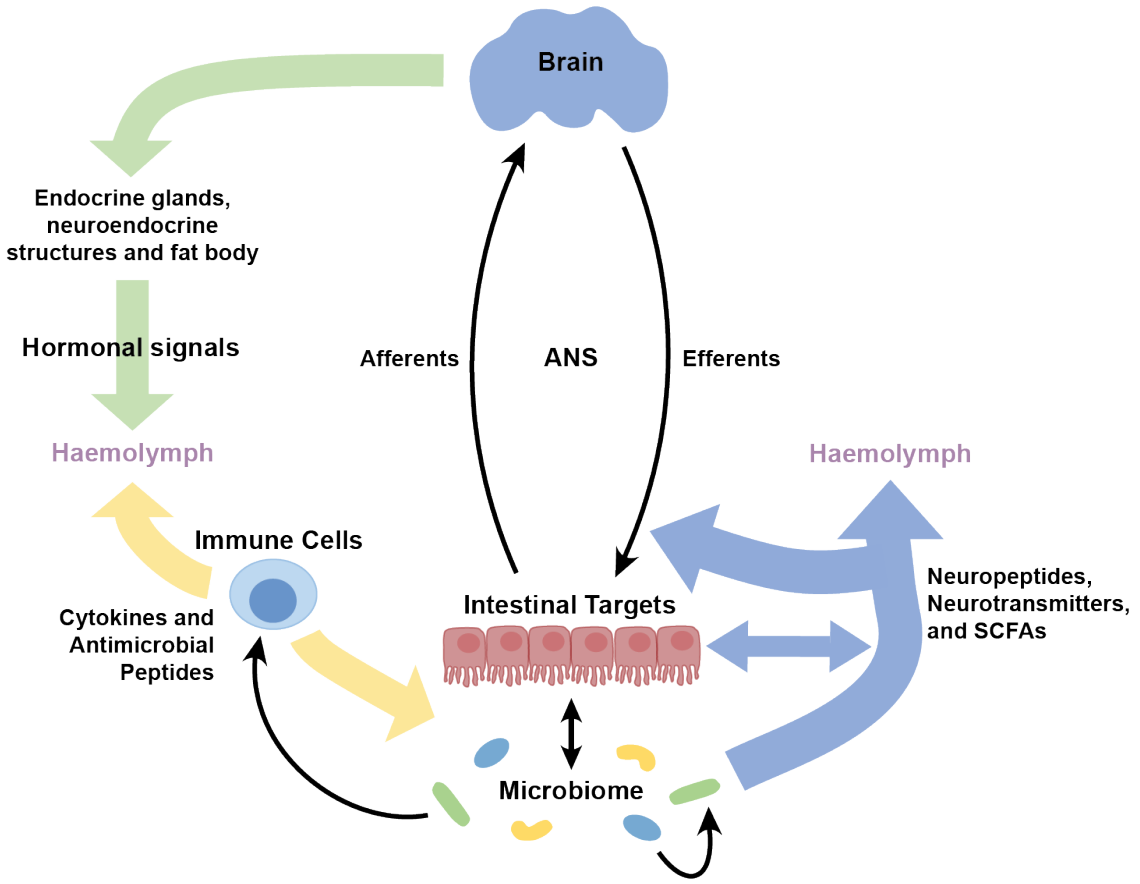

Figure 1.2 The *Drosophila* Gut-Brain Axis

Hormonal signals are produced by neuroendocrine structures, endocrine glands, or organs such as the fat body, and are released into the haemolymph. The autonomic nervous system (ANS) comprises of afferents and efferents which transfer information back and forth between the brain and intestinal targets, such as epithelial cells (Linneweber et al., 2014). Changes in these intestinal targets go on to affect the microbiome and in turn the microbiome is also able to impact intestinal targets (Li et al., 2016; Fast et al., 2018). Microbiota can impact other microbiota, for example due to cross-feeding or interference competition (Martin et al., 2023). The microbiome is also able to regulate the immune system, causing immune cell release of cytokines and anti-microbial peptides (Hoffmann, 2003; Buchon et al., 2009b). Some microbiota also produce neurotransmitters, neuropeptides and SCFAs which directly affect intestinal targets, the ANS as well as travelling to the brain (Koh et al., 2016; Ko et al., 2022). Intestinal targets can also produce neuropeptides such as CChamide that can affect behaviour via the brain (Li et al., 2013).

1.4.1 Composition and Acquisition of the Microbiome

Although it must be considered that the *Drosophila* microbiome consists of various microbial communities including fungi, archaea and viruses, this thesis focusses on the dominant component, bacteria, due to the wealth of studies exploring host-bacterial interactions and their implications for *Drosophila* fitness. The majority of the *Drosophila* microbiome is represented by Proteobacteria and Firmicutes, with the Bacteroidetes often also making up a small proportion, in contrast to the human microbiome where the dominant phyla are Firmicutes and Bacteroidetes (Brummel et al., 2004; Corby-Harris et al., 2007; Ren et al., 2007; Ryu et al., 2008; Sharon et al., 2010; Chandler et al., 2011; Wong et al., 2011; Ridley et al., 2012; Blum et al., 2013; Staubach et al., 2013; Leftwich et al., 2017). Studies have found the *Drosophila* microbiome to be highly variable. One study investigated 21 individuals across 10 different *Drosophila* species and found not one bacterial species present in every fly (Wong et al., 2013). Like in many taxa, changes in microbial abundance and community structure have been observed throughout the different life stages of *Drosophila* (Bakula, 1969; Ren et al., 2007; Buchon et al., 2009a; Wong et al., 2011; Blum et al., 2013). The larval microbiome initially consists of a low abundance but high diversity of bacteria, before an increase in load likely caused by multiplication in the gut and ingestion of food (Wong et al., 2011). Metamorphosis leads to a decrease in microbial abundance during the pupal stage, associated with antimicrobial peptide (AMP) gene expression increase, possibly in order to eliminate bacteria released from the metamorphosing gut (Tryselius et al., 1992). Microbial abundance then increases dramatically with age during adulthood (Ren et al., 2007; Buchon et al., 2009a; Blum et al., 2013), and changes in dominant species occur, possibly as a side effect of changing conditions in the gut (Wong et al., 2011).

Diet plays a pivotal role in shaping the *Drosophila* microbiome. Differences in bacterial communities appear to be more related to food source than to *Drosophila* species (Chandler et al., 2011; Staubach et al., 2013). Both microbiome diversity and bacterial species dominance has shown to be dependent on food source in wild flies, with mushroom feeders having a higher bacterial diversity and higher proportions of Lactobacillales than flies feeding on other food sources, such as flowers (Chandler et al., 2011). This could stem from certain food sources enabling the growth of particular bacterial species, or from specific bacteria being associated with certain food sources. If the latter is true, it could have significant implications for research conducted across different laboratories as bacterial compositions may vary. Indeed, evidence suggests that most intestinal bacteria cannot persist in the gut and must be continuously replenished by the ingestion of food (Blum et al., 2013). Blum et al. (2013) observed that the increase in bacterial load over a fly's lifetime was augmented when the flies were transferred to new food less frequently. Bacterial load on the food also increased with less frequent changeover (Blum et al., 2013), suggesting that *Drosophila* develop their microbiome through the consumption of bacteria on their food, whilst simultaneously depositing bacteria through their faeces back onto the food. Such dependence on microbial ingestion and differences between laboratories could potentially account for contrasting results across studies. Possible effects of abiotic factors on the microbiome, such as climate and latitude, have also been investigated but do not appear to be a significant driver (Corby-Harris et al., 2007).

Evidence from Chandler et al. (2011), indicating a difference in bacterial composition between external and internal samples, implies that *Drosophila* are able to shape their microbiome through selective retention of specific microbiota. Furthermore, significant changes in microbiome composition occurring during larval development and ageing, despite diet remaining constant, suggest that host physiology works to regulate the microbiome (Ren et al., 2007; Buchon et al., 2009a; Wong et al., 2011). It is likely that the immune and physiological conditions of the gut play a crucial role in determining which bacteria can persist. It also must be considered that host behaviour may play a part, particularly in wild *Drosophila* which have more of a choice of substrate and diet.

Numerous host functional effects attributed to the microbiome are a result of by-products of microbial metabolism. Although this may occur passively, it must be considered that often, certain bacteria may actively produce these molecules for purpose of manipulating the host. Several studies have found microbial products to modulate feeding and egg laying behaviours in *Drosophila* (Venu et al., 2014; Farine et al., 2017; Fischer et al., 2017; Leitão-Gonçalves et al., 2017; Wong et al., 2017; Qiao et al., 2019). For example, Wong et al. (2017) demonstrated that flies exhibit an olfactory-mediated preference for a diet containing bacteria corresponding to their resident microbiome. Moreover, a preferred protein:carbohydrate ratio in a diet was dependent on resident bacteria, potentially reflecting the metabolic requirements of these bacteria. This underscores the ability of bacteria to manipulate host foraging behaviour to their advantage even if it does not necessarily align with the host's needs. Further research should focus on how bacteria change their behaviour within a host,

including direct comparisons of gene expression between their extra-host and intra-host states.

1.4.2 *Drosophila* Microbiome Regulation

Like that of mammals, *Drosophila* physiology includes regulatory systems to maintain homeostasis of the microbiome. One component of this regulation is the immune system, which also functions to protect against invading pathogens. Pathogenic and commensal bacteria activate two immune pathways; the humoral response which results in the production of antimicrobial peptides (Hoffmann, 2003) or an oxidative response via the production of reactive oxygen species (ROS) (Ha et al., 2005).

While ROS are produced by dual oxidase (Duox) in response to uracil from bacteria (Ha et al., 2005; Lee et al., 2013), they are also produced by NADPH oxidase (Nox) in response to lactate from bacteria such as *L. plantarum* (Jones et al., 2013) (Figure 1.3). Although these enzymes work together to control basal levels of commensal bacteria, when stimulated by pathogenic bacteria, Duox expression and activity is increased via the p38 mitogen-activated protein kinase (MAPK) pathway, stimulating a burst of ROS (Ha et al., 2009). In addition to eliminating potential pathogens, ROS can stimulate ISC proliferation in response to an immune challenge, prompting renewal and repair (Buchon et al., 2009b; Jiang et al., 2009; Hochmuth et al., 2011).

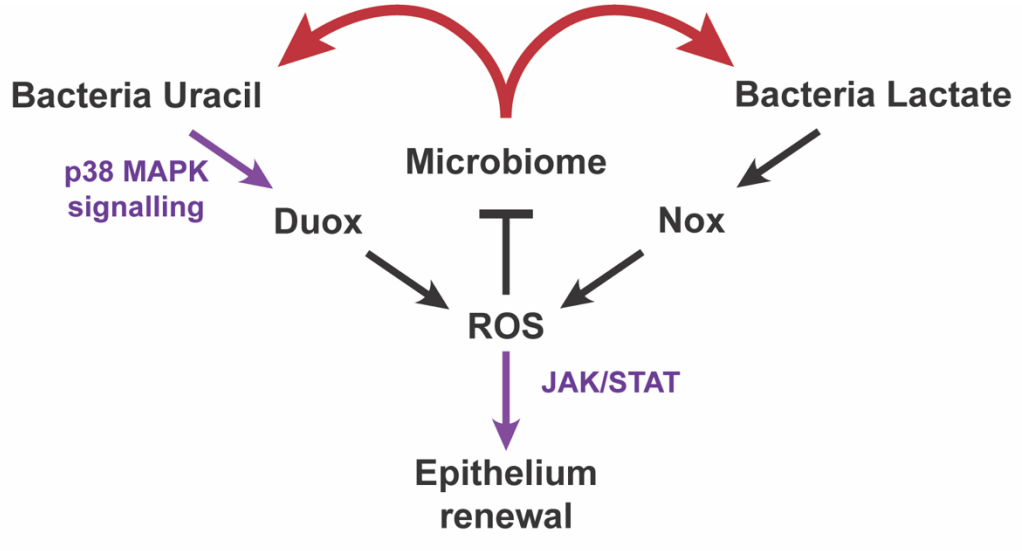

Figure 1.3 The *Drosophila* Oxidative Immune Response
Duox and Nox production of ROS in response to pathogenic and commensal bacteria. Red arrows represent negative feedback of the pathway by bacteria.

In insects, gram-positive bacteria and fungi mainly activate the Toll pathway, while gram-negative bacteria are mainly targeted by the immune deficiency (IMD) pathway. Induction of either pathway triggers the production of a range of AMPs, positively charged peptides that are secreted into the haemolymph and target the negatively charged membranes of bacteria (Imler & Bulet, 2005). The Toll pathway is not recognised as a major immune response pathway in the *Drosophila* gut, due to the incompatibility of proteolytic cascades, used in activation of the pathway, with the acidic conditions of the gut (Buchon et al., 2009a), however numerous studies have explored IMD signalling in *Drosophila*. The IMD pathway is activated via the recognition of peptidoglycan by peptidoglycan recognition proteins (PGRPs), such as PGRP-LC and PGRP-LE (Gottar et al., 2002; Takehana et al., 2002; Hoffmann, 2003) (Figure 1.4). The regulation of this pathway is essential to ensure its activation solely in response to invading pathogens, preventing constitutive activation by bacterial

commensals. Hence, several mechanisms are in place to prevent IMD activation in response to microbial stimuli (Kleino et al., 2008; Lhocine et al., 2008; Thevenon et al., 2009) (Figure 1.4). This ensures only pathogens will trigger an immune response, as they divide more rapidly than commensals, and so produce more peptidoglycan, which can translocate across the gut epithelium to activate the immune system (Gendrin et al., 2009). Multiple layers of negative regulation allow for a buffering of any oscillations in signalling activity and so results in less variation in microbiota (Aggarwal & Silverman, 2008).

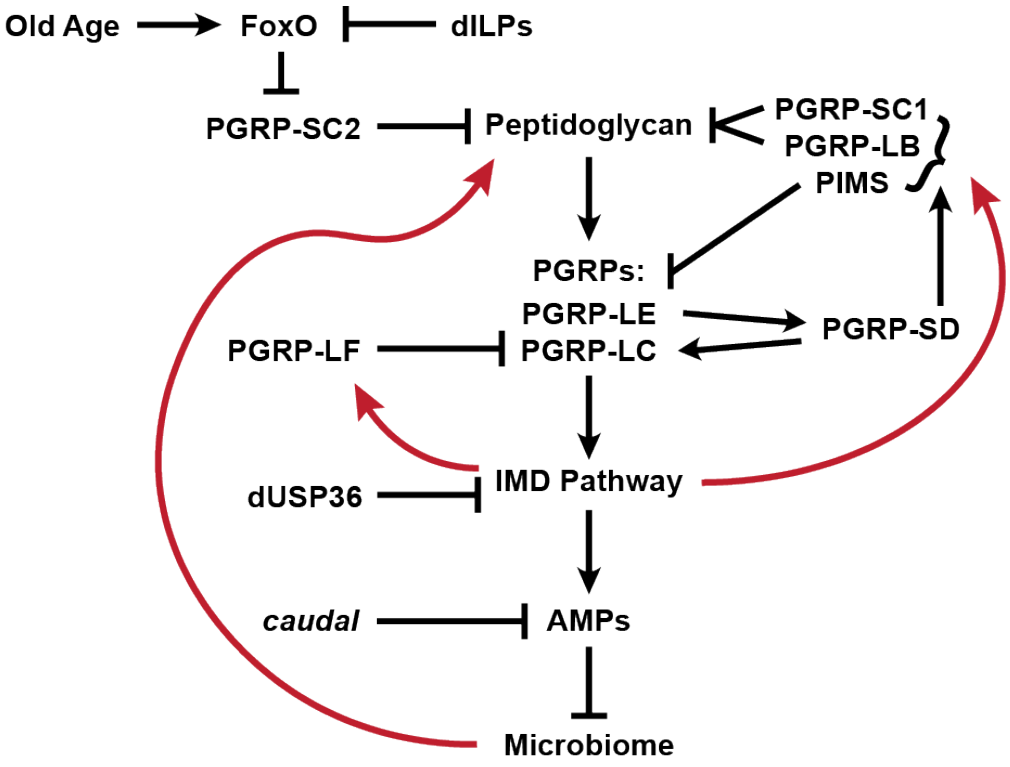

Figure 1.4 *Drosophila* AMP production via IMD signalling

Positive and negative regulation of the IMD pathway production of AMPs. Negative regulation can occur through PGRPs such as PGRP-SC1, PGRP-SC2 (Bischoff et al., 2006), and PGRP-LB (Zaidman-Rémy et al., 2006) that are able to remove peptides from the glycan strands in peptidoglycan so that it can no longer be detected by recognition PGRPs (Bischoff et al., 2006; Zaidman-Rémy et al., 2006; Royet & Dziarski, 2007). PGRP-SC1 and PGR-LB are activated by PGRP-SD (Iatsenko et al., 2018). Furthermore, PGRP-LC-interacting inhibitor of IMD signalling (PIMS) (Kleino et al., 2008; Lhocine et al., 2008) and PGRP-LF inhibits PGRP-LC (Maillet et al., 2008), and the ubiquitin-specific protease 36 (dUSP36) causes IMD protein degradation, all leading to the negative regulation of the IMD pathway (Thevenon et al., 2009). The intestinal homeobox genes *caudal* further inhibits AMP production (Ryu et al., 2008). *Lactobacillus plantarum* and *Acetobacter pomorum* can produce *Drosophila* insulin-like peptides (dILPs) which repress nuclear localisation of forkhead box class O (FoxO) transcription factor (Shin et al., 2011), a positive regulator of the IMD pathway that operates through inhibition of PGRP-SC2 (Guo et al., 2014). Red arrows represent negative feedback of the pathway by bacteria.

1.4.3 Experimental Removal of the Microbiome

Two primary techniques are employed to manipulate the *Drosophila* microbiome. One method involves supplementing fly food with antibiotics with the aim of removing a significant portion of the bacterial load or removing certain commensal bacterial species. An alternative method is the creation of germ-free (axenic) flies via egg dechorionation. During egg dechorionation, the protective egg chorion is stripped away by bleach, and the maternal faecal bacteria deposited during oviposition are consequently removed (Bakula, 1969). The egg chorion is typically consumed by the larvae upon hatching, facilitating the colonisation of bacteria within the larval gut (Wong et al., 2011); egg dechorionation ensures that this step cannot occur. The fly is subsequently kept on sterile or antibiotic food to maintain a germ-free environment.

Both approaches have their drawbacks and thus receive considerable criticism. In both methods, potential confounding effects may arise independently of bacterial loss. Antibiotics impact host enzymes and mitochondrial proteins (Brodersen et al., 2000), and are not completely effective at removing the whole microbiome (Ridley et al., 2013). While egg dechorionation is initially more effective at bacterial removal, it carries risk of inefficiency due to potential bacterial contamination, particularly during extended experiments that require frequent manipulation. Additionally, egg dechorionation is ineffective at removing the *Drosophila* endosymbiont, *Wolbachia*, as this bacterium is vertically transmitted within the egg cytoplasm (Ehrman, 1998). Furthermore, the process of egg dechorionation may also result in deleterious effects, as indicated by one study which demonstrated a reduction in longevity in first generation germ-free flies but not in the second or third generation (Lee et al., 2019).

1.5 The *Drosophila* Microbiome: Help or Hindrance?

Much like its role in mammalian hosts, the microbiome in *Drosophila* contributes significantly to many important processes, such as SCFA production (Shin et al., 2011), immune responses (Blum et al., 2013), and metabolism (Wong et al., 2014). Notably, it has demonstrated its protective capacity against infection and starvation (Glittenberg et al., 2011; Shin et al., 2011). Moreover, numerous studies have explored the impact of the microbiome on behaviour and cognition (Chen et al., 2019; DeNieu et al., 2019; Silva et al., 2021). However, instability of the microbiome can lead to disease, particularly in aged flies which exhibit intestinal dysplasia (Guo et al., 2014). This section summarises findings when studies experimentally remove the microbiome in *Drosophila* to assess its beneficial or detrimental effects on the host.

1.5.1 Measuring Longevity

Studies attempting to observe the effect of the microbiome on *Drosophila* lifespan have resulted in conflicting conclusions. One of the pioneering studies looking into microbiome-lifespan effects in *Drosophila* found that the loss the of microbiome decreased fly lifespan (Brummel et al., 2004). Other studies found no effect (Ren et al., 2007; Ridley et al., 2012; Li et al., 2016; Fast et al., 2018; Hanson & Lemaitre, 2022) or an increase in lifespan (Petkau et al., 2014; Clark et al., 2015; Dantoft et al., 2016; Galenza et al., 2016; Loch et al., 2017; Fast et al., 2018; Gould et al., 2018; Obata et al., 2018; Sannino et al., 2018; Lee et al., 2019). These contrasting results could be attributed to different microbiome manipulation techniques, such as egg dechorionation, different combinations of antibiotics and different methods of sterilising food, as well as different diets, all

possibly leading to a varying microbiome composition that could benefit or disadvantage the host. One study found that axenic flies had a reduced lifespan compared to conventional flies but second and third generation axenic flies had an increased lifespan, suggesting a detrimental effect of the egg dechorionation process (Lee et al., 2019). Therefore, the finding of a shortened or unaltered lifespan may attributed to the adverse effects of egg dechorionation (Brummel et al., 2004; Ren et al., 2007; Ridley et al., 2012). However, some of these studies have accounted for this by showing that flies from axenic eggs that were transferred to food containing bacteria after the dechorionation process, had a phenotype identical to conventional flies (Brummel et al., 2004; Ridley et al., 2012). Gould et al. (2018) found that although axenic flies lived for longer, they had a reduced reproductive fitness and so it is possible that the effect on lifespan may be a result of a trade-off.

Another reason for these differing microbial impacts may be the changes in numbers of certain beneficial or detrimental bacterial species after different antibiotic treatments. Gould et al. (2018) found a correlation between increased bacterial diversity and decreasing longevity. They also demonstrated that germ-free flies colonised only with *Lactobacillus brevis* or *Acetobacter pasteurianus* had an increased lifespan compared to conventional flies, or flies colonised with *L. plantarum, Acetobacter tropicalis* or *Acetobacter orientalis.* Fast et al. (2018) showed that a mono-association with *L. plantarum* decreased ISC renewal, thinned the intestinal epithelium and decreased lifespan compared to conventional flies. Obata et al. (2018) established that a reduction of *Acetobacter* species via antibiotics or oxidants extended lifespan. Westfall et al. (2018) demonstrated that addition of *L. plantarum*, *Lactobacillus fermentum* or *Bacillus*

longum to conventional flies increased longevity, potentially due to the restoration of age-driven changes in insulin-like signalling, oxidative stress and fatty acid metabolism (Westfall et al., 2018). Lastly, Lee et al. (2013) found that supplying axenic flies with commensal bacterial species that produce uracil, such as *Gluconobacter morbifer* or *Lactobacillus brevis*, induced ROS production and decreased lifespan in a uracil dependent way. These studies demonstrate that bacterial species differ in their functional impact, explaining why treatments, such as antibiotics, that are able to alter bacterial composition, can influence host health.

1.5.2 The Role of Dysbiosis During Old Age

Dysbiosis of the microbiome in aged flies may contribute to why the microbiome is often observed to have a negative effect on lifespan (Guo et al., 2014). Dysbiosis, the term for a microbial imbalance in the microbiome, occurs when its composition is altered in a negative way, often resulting in less diversity and a decrease in 'beneficial' bacterial species. In humans, it has also been linked to many diseases, including cardiovascular disease (Brown & Hazen, 2018), diabetes, obesity (Patterson et al., 2016) and colon cancer (Ian, 2009).

An example of microbiome dysbiosis in *D. melanogaster* is the proliferation of *G. morbifer* in response to IMD overactivation via caudal inhibition, associated with gut cell apoptosis and a decrease in lifespan (Roh et al., 2008; Ryu et al., 2008). Moreover, the introduction of *G. morbifer* induces this particular phenotype in germ-free flies, whereas it is not observed in conventional flies due to *G. morbifer* inability to proliferate (Ryu et al., 2008), signifying that it only persists in harmful numbers when other commensal species, more sensitive to AMPs, are

reduced. *Drosophila* dysbiosis occurs naturally with age due to an increase in forkhead box class O (FoxO), causing increased activation of the IMD pathway (Guo et al., 2014) (Figure 1.5). This dysbiosis stimulates Duox activity and overproduction of ROS, leading to enterocyte hyperproliferation and intestinal dysplasia (Hochmuth et al., 2011; Guo et al., 2014). This shortens lifespan, which can be reversed, along with dysbiosis and dysplasia, by the overexpression of PGRP-SC, a negative regulator of the IMD pathway (Guo et al., 2014). Reduced barrier function and increased metaplasia, which reduces mid-gut acidity, exacerbates dysbiosis, and in turn the inflammatory immune response (Li et al., 2016). This is concurrent with the finding that *Drosophila* AMP production (Buchon et al., 2009a; Guo et al., 2014; Clark et al., 2015), Janus kinase/signal transducers and activators of transcription (JAK/STAT) induction (Buchon et al., 2009a), IMD induction (Buchon et al., 2009a), and bacterial load (Guo et al., 2014; Clark et al., 2015; Lee et al., 2019) increases with age. Furthermore, germ-free flies have reduced ISC proliferation (Petkau et al., 2014), JAK-STAT induction (Buchon et al., 2009a), epithelial dysplasia (Guo et al., 2014; Petkau et al., 2014), and age-associated gene expression changes (Shukla et al., 2021), when compared to their conventionally raised counterparts.

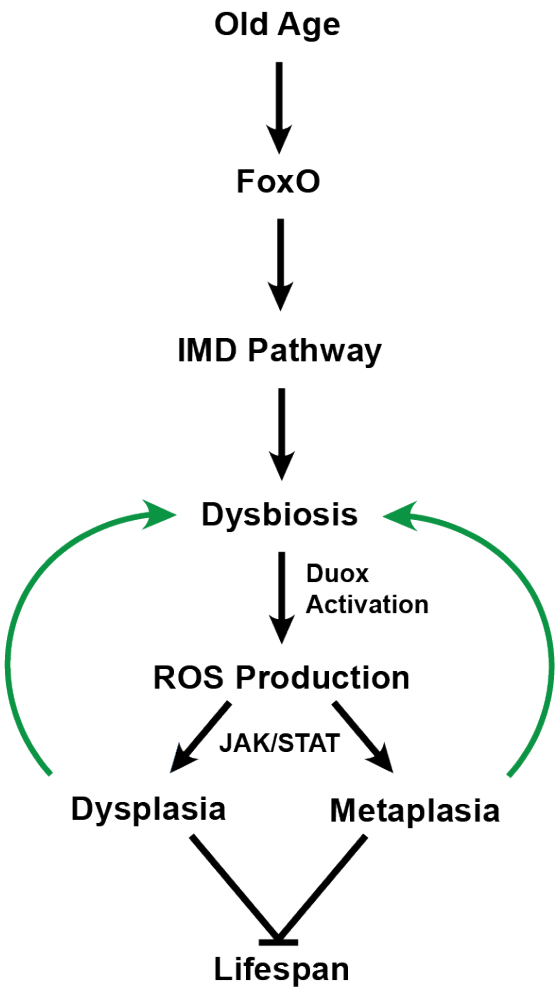

Figure 1.5 *Drosophila* Dysregulation of the IMD Pathway During Old Age
Old age leads to an increase of FoxO which overstimulates the IMD pathway, leading to dysbiosis and gut pathology. Green arrows represent positive feedback.

The observed detrimental effects of the ageing gut microbiome help elucidate findings from Brummel et al. (2004) who found that removal of the *Drosophila* microbiome from two weeks post eclosion increased lifespan, removal from eclosion decreased lifespan, and removal from four days had no effect. Additionally, microbiome supplementation of axenic flies at two days post eclosion resulted in a lifespan comparable to control flies but supplementation after four days resulted in a lifespan comparable to axenic flies (Brummel et al., 2004). Therefore, it seems as if the microbiome performs a beneficial function in

early life, perhaps due to developmental changes occurring at this time, such as the replacement of the larval fat body with the adult fat body, which is involved in the immune response. Subsequently, the microbiome becomes detrimental at an older age due to age-associated dysbiosis. Additional support for this hypothesis comes from a study by Lee et al. (2019), which demonstrated a decreased lifespan in flies receiving homogenates from 50-day old flies in comparison to those receiving homogenates from 10-day old flies. Moreover, when diluting 50-day old homogenates to the concentration of 10-day old homogenates the lifespan of flies resembled that of the 10 day homogenate-fed group, and the effect was reversed if 10-day old homogenates were concentrated, suggesting the lifespan effect was due to bacterial load, not composition (Lee et al., 2019).

1.5.3 Survival Under Suboptimal Conditions

Evidence from lifespan assays suggests early life microbiota may be important for *Drosophila* health (Brummel et al., 2004), and this theory gains further support from investigations into parameters assessed prior to flies experiencing later-life senescence. For example, the majority of *Drosophila* infection studies indicate that being in possession of a microbiome protected against pathogenic bacteria and increased survival (Glittenberg et al., 2011; Blum et al., 2013; Petkau et al., 2014; Leech et al., 2019). This may be through stimulation of the immune system so that it is primed for action against pathogenic microbiota (Clarke, 2014), or the direct competition for nutrients and production of antimicrobials by commensals. However, it is worth noting that one study found increased survival in germ-free flies after *Vibrio* infection (Galenza et al., 2016), and so the association between the commensal microbiota and infection survival appears to be complex. Yet another instance where the microbiome appears to be beneficial to the fly is under

nutrient limiting conditions. Germ-free larvae had a reduced survival on a limited-protein diet (Shin et al., 2011), and inoculation with certain bacterial species such as *Issatchenkia orientalis, Acetobacter indonesiensis* or *L. plantarum* extended axenic fly lifespan under these conditions (Yamada et al., 2015; Téfit & Leulier, 2017; Keebaugh et al., 2018). Another study found that axenic larvae were not able to survive without thiamine but survival could be restored by *A. pomorum* (Sannino et al., 2018).

1.5.4 Cognition and Behaviour

Similar to other taxa, numerous and diverse effects of the microbiome on cognition and behaviour have been recorded in flies, though consistency in these effects is lacking, prompting the need for further research. Dopamine, serotonin, octopamine and GABA, all produced by *Lactobacillus* species, have a role in *Drosophila* learning (Schwaerzel et al., 2003; Schroll et al., 2006; Kim et al., 2007; Liu & Davis, 2009; Monier et al., 2018; Pavlowsky et al., 2018). This may explain why studies observe axenic flies to have reduced learning and memory compared to flies with intact microbiomes (DeNieu et al., 2019; Silva et al., 2021). Moreover, supplementation with *Acetobacter* and *Lactobacillus* rescued this phenotype (DeNieu et al., 2019).

Research examining the impact of the microbiome on *D. melanogaster* behaviour has produced varied results. A loss of function mutation in lysine demethylase 5 (KDM5), a histone demethylase, found in patients with autism spectrum disorder (ASD), correlated with microbiome dysbiosis, an increase in serotonin, and changes in social behaviour, such as increased distance between individuals and decreased social contact (Chen et al., 2019). This was partially

rescued by antibiotics or probiotics, suggesting causation (Chen et al., 2019). Serotonin and octopamine have been shown to alter aggression in *Drosophila* (Johnson et al., 2009b; Alekseyenko et al., 2010; Certel et al., 2010b; Luo et al., 2014; Williams et al., 2014b), potentially explaining why many studies have investigated the link between aggressive behaviours and the microbiome. One study demonstrated decreased aggressive behaviour in antibiotic-fed or axenic flies, but this effect was only evident if the germ-free period occurred during the beginning of the larval stage (Jia et al., 2021). It was revealed that this response was dependent on octopamine signalling (Jia et al., 2021). In contrast, another study observed increased aggression in antibiotic-fed flies, though it is unclear whether antibiotic supplementation was started before or after development (Grinberg et al., 2022). Notably, both studies utilised different antibiotics. Additionally, Jia et al. (2021) assessed aggression between paired flies, whereas Grinberg et al. (2022) measured aggression in a group that included a decapitated female to provoke aggression.

In regards to activity, some studies observed an increase in germ-free flies (Schretter et al., 2018; Selkrig et al., 2018; Silva et al., 2021), again, rescuable by octopamine (Schretter et al., 2018), while other experiments demonstrated no effect (Selkrig et al., 2018; Jia et al., 2021). Although a few studies have observed a minor increase in anxiety-like behaviours in germ-free *Drosophila* (Selkrig et al., 2018; Silva et al., 2021), very little research has been carried out on how the microbiome impacts physiological stress responses. *Drosophila* must often contend with a multitude of biotic and abiotic stressors, and insights from vertebrate research have implicated the microbiome as a plausible candidate

responsible for orchestrating the adverse health and behavioural effects induced by these stressors.

1.6 The Effect of the Social Environment on Health

The presence of conspecifics in an individual's environment and the interactions with these conspecifics can have a substantial and varying impact on an individual's welfare. In many animal populations this may be due to increased perception of competition for a limited resource such as food or mates. A long-term stimulation of the stress response can lead to negative behavioural and physiological changes. For example, in groups of rats, subordinate males have higher corticosterone levels, altered serotonin and increased weight loss (Blanchard et al., 1993). Furthermore, cohabitation of male mice in already established territories increased depressive behaviours and decreased lifespan (Unno et al., 2011). This sex-dependent health impact can be observed in wild populations, for example, senescence can be augmented by increased population density in male but not female red deer (Mysterud et al., 2001). In humans, meta-analyses have established that stressful social relationships increase mortality risk, comparable to other factors including smoking (Holt-Lunstad et al., 2010). Moreover, research indicates that it is the quality, rather than frequency, of social contact that are predictors for healthy cognitive functioning in ageing individuals (DiNapoli et al., 2014).

It is becoming increasingly evident that the health effects of the social environment extend beyond what is typically considered to be a 'social' species. Research in *Drosophila* demonstrates that the social environment influences immune responses (Leech et al., 2017), gene expression (Mohorianu et al., 2017), and cancer progression (Dawson et al., 2018). In *D. melanogaster*, the social environment often appears to be stressful, with same-sex contact detrimentally affecting physiology, and ultimately, lifespan (Bretman et al., 2013).

However, same-sex pairing reduces lifespan more in males than females, and drives faster functional senescence in males, whereas it decreases the decline in females (Leech et al., 2017). Pairing of males, not females, reduced survival after oral infection of *Pseudomonas fluorescens* (Leech and McDowall et al. 2021). Furthermore, masculinization of the nervous system in females, by RNAi suppression of transformer (*tra)* gene expression, decreased lifespan in the presence of conspecifics to near that of a male fly, as well as triggered male courtship behaviours (Flintham et al., 2018). Moreover, feminization of the male nervous system, by expressing the female isoform of *tra*, reduced the difference in lifespan between single and grouped flies (Flintham et al., 2018).

These sex-dependent effects may arise from males serving as a cue for future mating competition to other males. Mating is competitive in *Drosophila* as females can mate with multiple males and males can displace or outcompete the previous males' sperm (Gromko et al., 1984). Therefore, males have evolved plastic responses, such as increasing their mating duration in response to future mating competition (Bretman et al., 2009; Wigby et al., 2009; Bretman et al., 2010; Bretman et al., 2011; Kim et al., 2012; Bretman et al., 2013; Bretman et al., 2016; Mason et al., 2016; Rouse & Bretman, 2016; Bretman et al., 2017; Hopkins et al., 2019), which benefits the transfer of seminal fluid proteins that are passed to the female after sperm transferal has finished (Wigby et al., 2009). These proteins have a handful of effects on the female, including increasing egg laying and reducing future sexual receptivity (Liu & Kubli, 2003). Indeed, males exposed to male rivals have more offspring compared to those kept alone (Bretman et al., 2013; Hopkins et al., 2019). However, evidence suggests that this future mating response is costly, even before mating occurs, as starved males are unable to

produce the response (Mason et al., 2016). This may explain why males exposed to rivals have a reduced longevity, irrespective of whether they get to mate (Bretman et al., 2013). However, other responses to male social contact, such as the increase in cognitive ability in paired flies, could also be responsible (Rouse et al., 2020).

This lifespan effect may be a result of a trade-off as investment into reproduction may result in decreased investment into other, possible life-lengthening assets such as intestinal barrier maintenance. Furthermore, this trade-off may favour only short-term fitness, as one study established that mating extension only occurs in the first two matings, and flies exposed to rivals produced fewer offspring than single flies after the fifth mating (Hopkins et al., 2019). Another study observed that flies exposed to rivals had fewer successful matings in later life (Bretman et al., 2013). Favouring early reproductive success over future success in both reproduction and longevity aligns with the concept that a relatively high risk of mortality in the wild would make investment in short-term assets beneficial.

The age of the rival fly seems to influence lifespan reduction in *Drosophila*. For example, short-lived males carrying a mutation in the *Sod* gene, involved in responses to oxidative stress, lived for longer if they were housed with wild-type flies compared to other *Sod* flies, but only if these flies were co-aged and not older (Ruan & Wu, 2008). The sensitivity of the *Sod* gene to social interactions may have implications for its association with age-dependent neurodegenerative disease (Noor et al., 2002). Other research found that wild-type males had a shorter lifespan when housed with older flies, than if housed with co-aged flies,

and a longer lifespan if housed with younger flies (Cho et al., 2021; Lin et al., 2022). Males housed with older flies had a decreased courtship duration compared to those housed with co-aged flies (Lin et al., 2022). Therefore, the detrimental lifespan effect appears to be independent of the future mating competition response. However, the beneficial effect of a young rival seems to occur at least partially through sensory inputs, as life-extension is reduced when flies were kept in constant darkness, rival flies were decapitated or when focal flies had olfaction, mechano-sensory or motor deficits (Ruan & Wu, 2008). The effect is also dependent on the rival flies' enzymes required for the biosynthesis of cuticular hydrocarbons, which cover the cuticle to prevent desiccation, and have an additional role in chemical communication between insects (Cho et al., 2021). This suggests that the effect is triggered by chemical signals from the rival fly, however it does not seem to be dependent on traces left on the food as swapping the food continuously between treatment groups did not alter the results (Ruan & Wu, 2008).

The negative effects of the social environment do not seem to be reflected in survival in response to certain stressors. Paired males exhibited increased survival in response to starvation stress compared to those kept singly, although the presence of a permeable opaque central divide between pairs raises the question of to what extent the removal of certain tactile cues, or element transfer between flies, contributed to this result (Moatt et al., 2013). Additionally, pairing increased survival after *Pseudomonas aeruginosa* infection via injection (Leech et al., 2019). Therefore, the response to rivals may involve redistributing resources to short-term survival, such as an investment into the immune system, allowing for better ability to cope with stressors such as infection, but having a

detrimental long-term effect on health which only becomes apparent if the fly manages to survive to an older age.

1.7 How the Microbiome is Influenced by the Social Environment

The social environment can be expected to have a large impact on the microbiome due to the extrinsic transfer of bacteria between organisms. Homogeneity between individual's microbiomes when living in close proximity is explained by similar diets, shared environments or transfer through direct contact. For example, humans cohabiting share similar microbiota to each other and even to their pet dogs (Song et al., 2013). Homogeneity has been observed between primates living in the same social groups and even between different species, with those living in the same location as heterospecifics sharing approximately 53% more bacterial phylotypes than with conspecifics living in isolation (Moeller et al., 2013).

However, due to the GBA, alterations to the microbiome can occur independently to the external transfer of bacteria. Stress, including that caused by the social environment, can alter the microbiome composition intrinsically. Few studies in humans have examined this link but one study found that reduced numbers of Lactobacilli were present in stool samples of college students partaking in exams when they were experiencing an increased perceived level of stress (Knowles et al., 2008). In rodent models, stress has been found to cause a decrease in the expression of tight junction protein RNA and an increase in colonic paracellular permeability (Kiliaan et al., 1998; Demaude et al., 2006). This allows for the transfer of bacteria and antigens across the epithelial barrier and may activate an immune response (Kiliaan et al., 1998). In mice, social defeat alters community structure of the microbiome, decreasing *Lactobacilli* numbers as well as reducing species diversity (Bailey et al., 2011; Galley et al., 2014; Bharwani et al., 2016; Bharwani et al., 2017; Yang et al., 2017c). Plus, individuals

most susceptible to social defeat that display larger behavioural changes also exhibit larger changes in the microbiome (Bharwani et al., 2016; Szyszkowicz et al., 2017). One study observed hamster microbiome composition to change after one stressful social encounter, including a decrease in Lactobacillales. (Partrick et al., 2018). In North American barn swallows, interactions altered corticosterone levels along with gut microbial diversity (Levin et al., 2016). Therefore, we know that socially-driven changes in host health and behaviours correlate with changes in the microbiome, but it is not always clear whether they are caused by them. Studies utilising antibiotics and germ-free models aid in this investigation. For example, Bailey et al. (2011) demonstrated an antibiotic reversal of the increases in circulating cytokines driven by a social stress in mice (Bailey et al., 2011)

The *Drosophila* stress response includes an increase in dopamine (Rauschenbach et al., 1993) and octopamine levels (Davenport & Evans, 1984), an upregulation of a whole host of genes as well as a decrease in glycogen, fatty acids and glucose (Sørensen et al., 2005); all of which may affect the gut and the microbiome. The costs associated with same-sex social exposure in males indicates this is a stressful environment (Bretman et al., 2013). Therefore, it is not surprising to find that the community structure and diversity of the microbiome is altered in grouped male *Drosophila* when compared to males kept singly (Leech and McDowall et al. 2021). This difference is larger in males and only becomes apparent at an older age, suggesting this occurs via a build-up of stress or senescence, rather than through the extrinsic transfer of bacteria. The age of the rivals also appears to be important, with flies exposed to young rivals having a more similar microbiome structure to single flies. Inferring the functional implications of this microbial change by referring to the Kyoto Encyclopedia of

Genes and Genomes (KEGG) database demonstrated that single flies were enriched for microbiota that were predicted to interact with ageing-associated pathways within the host, possibly explaining the extension in longevity (Leech and McDowall et al. 2021).

1.8 Research Aims of this Thesis

The overall objective of this thesis is to investigate the role of the microbiome in the social-stress response, utilising a *D. melanogaster* model. As the *D. melanogaster* microbiome has been identified as a significant contributor to various physiological and behavioural changes (Shin et al., 2011; Blum et al., 2013; Chen et al., 2019), and is susceptible to changes in the social environment (Leech and McDowall et al. 2021), the microbiome is a possible candidate for modulating male *Drosophila* responses to same-sex social contact, including the reduction in lifespan (Bretman et al., 2013), cognitive enhancement (Rouse et al., 2020), and increase in mating duration (Bretman et al., 2009) (Figure 1.6). A growing body of research highlights the importance of the microbiome in diverse animal systems, including humans (Scheppach, 1994; Gassull, 2006). Therefore, the discovery of a potential microbial involvement in *Drosophila* stress responses could hold substantial implications for addressing stress in both animal and human populations, where it has become a prevalent issue.

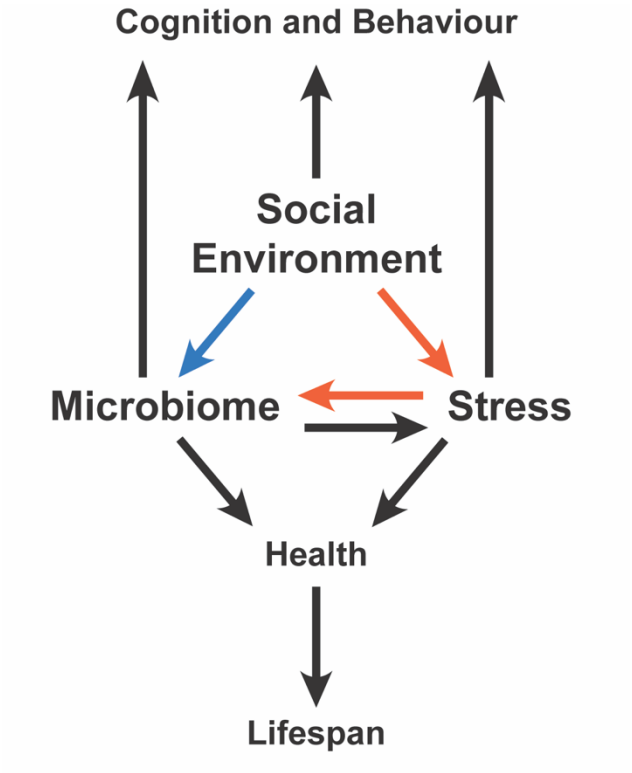

Figure 1.6 Interactions between the Social Environment, Microbiome and Stress in *Drosophila*

Interactions between the social environment, microbiome and stress, and the impacts they have. The blue arrow represent how the social environment can impact the microbiome extrinsically and the orange arrows represent the intrinsic effect.

In the third chapter, I use antibiotics to explore the role of the microbiome in the *Drosophila* lifespan response to future mating competition, in both the adult and larval environment. While numerous studies have independently examined the impact of the microbiome and the social environment on *Drosophila* lifespan (Brummel et al., 2004; Ren et al., 2007; Lee et al., 2019), no prior investigations have integrated these factors to elucidate a potential causal pathway. Furthermore, I explore how the age and novelty of the rival male alters lifespan effects under antibiotic treatment. This is to test the hypothesis that the

microbiome is responsible for the beneficial effect of a young rival which has been observed in previous studies (Ruan & Wu, 2008; Cho et al., 2021; Lin et al., 2022)

In the fourth chapter, I focus on the microbiome's effect on behavioural responses to social stress. Paired males have been shown to exhibit enhanced cognitive functions compared to their solitary counterparts (Rouse et al., 2020), so I hypothesised that the microbiome plays a role in this response, potentially explaining the lifespan reduction occurring via a trade-off. Next, to test the hypothesis that the increased investment in reproduction, which has been demonstrated in response to rivals (Bretman et al., 2009), is modulated by the microbiome, I determined whether this trade-off still occurred under my treatment conditions, when the microbiome is perturbed. Additionally, I undertook an assessment of the impact of my treatments on fly behaviour, such as activity, as previous research has yielded mixed results in this domain (Schretter et al., 2018; Selkrig et al., 2018; Jia et al., 2021; Silva et al., 2021).

The application of predicted functional analysis using the KEGG database enables the inference of potential impacts that alterations in the microbiome might have on host physiology. In my fifth chapter, I utilised this method to identify patterns in the general stress response, using data from previous studies conducted in insect and mammalian model systems. These findings guided my selection of pathways to focus on when examining the interplay between the microbiome, the social environment, and gene expression in this chapter.

Chapter 2
General Methods

Whilst data chapters have their own specific methods, this chapter outlines methods common to multiple chapters.

2.1 Fly Stocks and Maintenance

Drosophila melanogaster (wild-type Dahomey strain) were reared, and all experiments performed at 25°C and 50% humidity on a 12:12 light:dark cycle. They were fed on a standard sugar-yeast agar (SYA) medium containing 15 g of agar, 50 g of sugar, 100 g of brewer's yeast, 30 ml of Nipagin solution (100 g/l methyl 4-hydroxybenzoate in 95% ethanol) and 3 ml of propionic acid per litre of medium (Bass et al., 2007). For experiments, eggs were collected on purple grape juice agar plates (275 ml water, 12.5 g agar, 150 ml red grape juice, 10.5 ml Nipagin) placed in mass stock cages. After 24 hours, larvae were collected and put into vials containing 7 ml of SYA at a density of 100 per vial. After eclosion, virgin flies were sexed under ice anaesthesia and put into their experimental conditions. Non-focal flies were identified with wing clips as in previous studies (Bretman et al., 2011; Bretman et al., 2017; Leech and McDowall et al. 2021). All flies were moved to fresh vials, containing an excess of SYA, once a week. Throughout this thesis, age of flies refers to adult age only, and so is presented as days from eclosion.

Antibiotic food was made by supplementation with streptomycin, tetracycline and rifampicin so that they were present in the food at concentrations of 100 μg/ml, 50 μg/ml and 200 μg/ml respectively. This combination of antibiotics was

used as this has successfully reduced the microbiome in other studies (Sharon et al., 2010; Leftwich et al., 2017). Nevertheless, its effectiveness under the experimental conditions used in this thesis was confirmed (section 2.2).

2.2 Confirming Antibiotic Reduction of Microbiota

The effectiveness of the antibiotic treatment in removing the gut microbiome was verified by comparing culturable bacteria in flies fed control or antibiotic food for five days from eclosion. Individual flies were washed in 70% ethanol, rinsed with sterile H_2O, then sterile phosphate-buffered saline solution (PBS), before pulverisation with a sterile plastic pestle in 500 μl of PBS (Heys et al., 2018). The debris was left to settle and 100 μl of supernatant was plated on Man, Rogosa, and Sharpe (MRS) (Lactobacillus - selective) and Mannitol agar plates (*Acetobacter*, *Gluconobacter*, *Commensalibacter* - selective). MRS plates were incubated at 37°C for three days, and Mannitol plates at 30°C for 24 hours (Leitão-Gonçalves et al., 2017). No colonies were observed on antibiotic fly plates whereas control fly plates displayed many colonies (Figure 2.1).

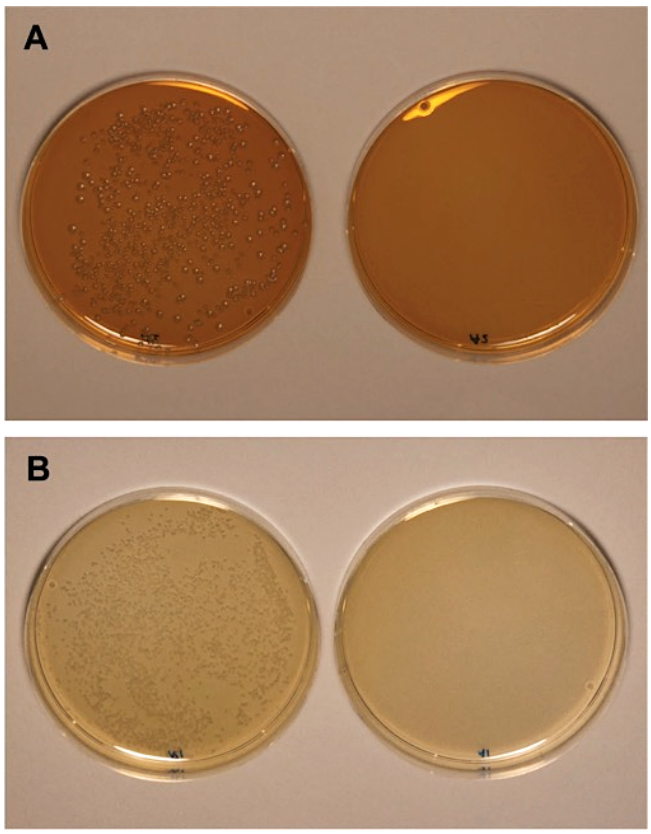

Figure 2.1 The Effect of Antibiotic Food on Culturable Bacteria in *Drosophila Melanogaster*

Plate cultures of homogenized flies on MRS agar (A) and Mannitol agar (B). Flies were fed control food (left) or food supplemented with a combination of antibiotics (100 μg/ml streptomycin, 50 μg/ml tetracycline and 200 μg/ml rifampicin) (right) for five days from eclosion.

2.3 qPCR

2.3.1 qPCR Protocol

qPCR primers were designed with a GC content between 20 and 80%. Primer efficiency was tested using a 10-fold serial dilution on whole body RNA and accepted if the efficiency was between 90 and 110%, and if the pipetting accuracy value (R^2) was above 0.98.

For each biological replicate, RNA was extracted from five whole body 10-day old males, or ten whole body 49-day old males using the Direct-zol™ RNA miniprep kit according to manufacturer's instructions. Concentration and purity was checked on a nanodrop, before 200 ng of RNA was used as a template for cDNA synthesis, using the Life Technologies First strand cDNA kit according to manufacturer's instructions.

Each biological replicate was run in triplicate, and a member of the triplicate was excluded if it varied by greater than 0.5. If more than one member differed by larger than 0.5, but less than 1, an average of all three was used. If more than one member differed by larger than 1, the biological replicate was excluded from analysis. Average quantification cycle (Cq) values were calculated for each biological replicate, and relative quantity was calculated using the ΔΔCT method (Hellemans et al., 2007). The Cq was subtracted from the lowest value across biological replicates, and the primer efficiency was raised to this number. Relative expression was calculated by dividing the relative quantity by the geometric mean of the reference genes, before being averaged across biological replicates to get a final relative expression value for each treatment group.

2.3.2 Housekeeping Gene Study

The stability of seven reference genes (Table 2.1) was determined using whole body RNA from 49 day flies kept singly or in groups of 10, either on control food or antibiotic food, (six biological repeats per treatment, 10 whole-body flies pooled per repeat). Stability was evaluated using GeNorm (Vandesompele et al., 2002), Bestkeeper (Pfaffl et al., 2004) and NormFinder (Andersen et al., 2004) software programmes (Table 2.2). Although not ranked highest in the study, *Act5C* and *RpL32* were chosen as reference genes as the differences in stability values between all primers but *αTubulin* were small.

Table 2.1 Forward and Reverse Oligonucleotides for Potential Reference Genes

Including efficiency and pipetting accuracy (R^2) value.

Gene	Forward	Reverse	Efficiency (%)	R^2
Act5C	CATCAGCCAGC AGTCGTCTA	GCAGCAACTTCT TCGTCACA	109.2	0.999
αTubulin	AAAAAGGTCCC GCATTAGA	GCCAATCTGGAT GGAGACTA	107.1%	0.992
eIF1A	GTCTGGAGGCA ATGTGCTTT	AATATGATGTCG CCCTGGTT	99.9%	0.996
GAPDH2	AACAATTTTTCG CCCGAGTT	TGATACCAATCT TCGACATGGTT	90.0%	0.997
RpL32	TACAGGCCCAA GATCGTGAAG	GACGCACTCTGT TGTCGATACC	105.1	0.999
Rpn2	CAAGTCGGAGG GAGACAAGT	CAACCTCGTTAA GGGAATCG	101.1%	0.997
Tbp	GGCAAAGAGTG AGGACGACT	GCCGACCATGTT TTGAATCT	97.8%	0.997

Table 2.2 Reference Gene Stability

Three different programmes were used to analyse Cq values of potential reference genes to produce a stability ranking.

Gene	geNorm (M)	geNorm Rank	bestKeeper (R value)	bestKeeper Rank	NormFinder	NormFinder Rank	Overall Rank
Act5C	0.366	5	0.924	6	0.194	5	5
αTubulin	0.840	7	0.680	7	0.822	7	7
EF1	0.330	2	0.936	2	0.170	3	2
GAPDH2	0.374	6	0.935	4	0.224	6	5
RpL32	0.344	4	0.936	2	0.142	1	3
Rpn2	0.313	1	0.945	1	0.142	1	1
Tbp	0.332	3	0.680	5	0.170	3	4

2.4 Statistical Analysis

Throughout this thesis, statistical analysis was performed in R (version 4.0.1) and RStudio (version 1.2.5019). All models were reduced to a minimal model and compared using analysis of deviance (ANOVA). The package 'lme4' (Bates et al., 2015) was used to make Generalised linear mixed models (GLMMs). Post-hoc tests were performed (emmeans with Tukey adjustment, Lenth (2023)) on the minimal model to determine where pairwise differences occurred. When models were able to be simplified to a null model, this was compared to the full model using ANOVA. Cohen's D (*d*) was calculated as a measure of effect size. For qPCR data, outliers were removed in each treatment group using inter-quartile ranges (remaining sample sizes used in analysis presented in Table 2.3).

Table 2.3 Gene Expression Assay Sample Sizes After Outlier Removal
In each experiment, six biological replicates, each made up of pools of five (10-day old) or 10 flies (49-day old), were run per treatment group. Outliers were removed using inter-quartile ranges, and analysis run on the remaining samples.

Gene	Age	Antibiotic Treatment	Social Environment	Sample Size
futsch	10 days	Control	Single	6
			Grouped	6
		Antibiotic	Single	6
			Grouped	6
	49 days	Control	Single	5
			Grouped	6
		Antibiotic	Single	5
			Grouped	6
	10 days (recovery)	Control	Single	6

			Grouped	6
		Temporary Antibiotic	Single	5
			Grouped	6
Nrx-1	10 days	Control	Single	6
			Grouped	6
		Antibiotic	Single	5
			Grouped	5
	49 days	Control	Single	6
			Grouped	5
		Antibiotic	Single	6
			Grouped	6
Dcp-1	10 days	Control	Single	6
			Grouped	5
		Antibiotic	Single	5
			Grouped	6
Diap-1	10 days	Control	Single	6
			Grouped	6
		Antibiotic	Single	6
			Grouped	6
hid	10 days	Control	Single	5
			Grouped	5
		Antibiotic	Single	6
			Grouped	6
cnk	10 days	Control	Single	6
			Grouped	6
		Antibiotic	Single	6
			Grouped	5
	49 days	Control	Single	6
			Grouped	5

		Antibiotic	Single	5
			Grouped	6
Duox	10 days	Control	Single	5
			Grouped	6
		Antibiotic	Single	6
			Grouped	5
	49 days	Control	Single	5
			Grouped	5
		Antibiotic	Single	6
			Grouped	5
p38c	10 days	Control	Single	6
			Grouped	5
		Antibiotic	Single	5
			Grouped	6
	49 days	Control	Single	6
			Grouped	5
		Antibiotic	Single	6
			Grouped	6
Ras85D	10 days	Control	Single	6
			Grouped	5
		Antibiotic	Single	6
			Grouped	6
	49 days	Control	Single	6
			Grouped	6
		Antibiotic	Single	5
			Grouped	5
Sod2	10 days	Control	Single	5
			Grouped	4
		Antibiotic	Single	5

			Grouped	3
	49 days	Control	Single	5
			Grouped	6
		Antibiotic	Single	5
			Grouped	6
vn	10 days	Control	Single	5
			Grouped	6
		Antibiotic	Single	6
			Grouped	6
	49 days	Control	Single	6
			Grouped	4
		Antibiotic	Single	5
			Grouped	6

Chapter 3
The Role of the Microbiome in Socially-Driven Changes in Lifespan

3.1 Introduction

3.1.1 Socially-Driven Impacts of the Microbiome on Lifespan

The microbiome is known to wield significant influence over host health (Grenham et al., 2011), due to its involvement in various processes including digestion (Kang et al., 2012; Larsbrink et al., 2014), gut health (Zareie et al., 2006) and SCFA production (Gassull, 2006; Freund et al., 2010). However, its susceptibility to environmental stress means that it can turn detrimental to the host (Bailey et al., 2011). The social environment, for example, can impact the microbiome through the direct transfer of microbes between hosts, which usually increases homogeneity between organisms that live in close contact with each other (Chandler et al., 2011; Moeller et al., 2013). However, the effect of the social environment extends beyond the exchange of microbiota, due to intrinsic effects facilitated via the gut-brain axis. For example, mice exposed to social defeat have an altered microbiome community (Bailey et al., 2011; Galley et al., 2014; Bharwani et al., 2016; Bharwani et al., 2017; Yang et al., 2017c). This may be one reason why antagonistic social environments in male rodents can cause adverse health effects, such as weight loss, a reduced immune response, an increase in depressive behaviours and a decrease in lifespan (Raab et al., 1986; Unno et al., 2011). Indeed, studies have demonstrated the efficacy of probiotics in mitigating stress-induced changes in cytokine and cortisol levels, along with behaviour (Bailey et al., 2011; Bravo et al., 2011; Savignac et al., 2014; Bharwani et al., 2017; Yang et al., 2017b). In order to address whether the socially-driven

microbiome changes are responsible for any cooccurring effects on health, it needs to be established whether these effects can be altered if the microbiome is manipulated.

Male *D. melanogaster* provide an excellent model for exploring this phenomenon. This model offers the flexibility to manipulate both the social environment and the microbiome, and has a relatively short lifespan, which can be easily measured. It exhibits sensitivity to a wide range of external stressors, including the social environment, evidenced by a reduction in male lifespan when exposed to rival males (Bretman et al., 2013; Zajitschek et al., 2013; Leech et al., 2017). As mating is competitive for males (Gromko et al., 1984), this lifespan reduction may be due to a trade-off between reproductive assets and other longevity-extending assets. It also may occur through a general stress response due to this high-competition environment. This stress response could be compounded by aggression between flies, which could also impact health through increased injury. Supporting this explanation, Hoffmann & Cacoyianni (1989) demonstrated that, when territories were established, male *Drosophila* selected for territoriality had a higher mortality than control males. Interestingly, Bretman et al. (2013) observed no difference in wing damage between single and paired flies, suggesting a lack of aggression or limited damage caused by aggression. However, Guo & Dukas (2020) found that aggression had an effect on health, independent of wing damage, as they observed no evidence of increased wing damage in hyperaggressive flies, but a lower starvation and desiccation survival compared to those in low aggression treatments.

All of these mechanisms that are able to explain socially-driven changes in lifespan have potential to occur through alterations to the microbiome. For example, increased investment into reproductive assets could lead to decreased investment in microbiome altering systems such as the immune system (Schwenke et al., 2016). Alternatively, the *Drosophila* stress response includes an increase in neurotransmitters (Davenport & Evans, 1984; Rauschenbach et al., 1993), upregulation of numerous genes and reductions in glycogen, fatty acids and glucose (Sørensen et al., 2005); all of which may affect the gut and its microbiome. Additionally, aggression may be modulated by changes in the microbiome. Serotonin and octopamine are known to regulate aggression in *Drosophila* (Johnson et al., 2009a; Alekseyenko et al., 2010; Certel et al., 2010a; Williams et al., 2014a). Jia et al. (2021) observed decreased aggression in antibiotic fed males, through octopamine signalling, however, antibiotics had to be fed during larval development for this effect to appear. Grinberg et al. (2022) observed the opposite pattern, with an increase in aggression in flies fed antibiotics. These contrasting results suggest that aggression may be induced or inhibited by the microbiome, depending on composition. Overall, the interplay between the microbiome and aggression in *Drosophila* remains poorly understood, but is likely complex.

Indeed, the *Drosophila* social environment has been shown to alter the microbiome in a sex and senescence dependent manner (Leech and McDowall et al. 2021), suggesting this is occurring through the intrinsic effects of experiencing the social environment rather than the extrinsic transfer of bacteria, which should have similar impacts to both males and females and should manifest earlier in life. Moreover, research has shown that alterations to the

Drosophila microbiome can also affect lifespan (Brummel et al., 2004; Clark et al., 2015; Fast et al., 2018), and so it is plausible that microbiome changes are at least part of the reason behind the observed reduction in male lifespan after exposure to rivals.

3.1.2 The Young Rival Effect

The age of the rival male has been shown to influence *Drosophila* lifespan during pairing. Males kept with young rivals have an increased lifespan compared to males kept with co-aged rivals (Cho et al., 2021; Lin et al., 2022), as well as decreased functional senescence and increased stress resistance (Cho et al., 2021). The presence of young rivals does not elevate longevity to a level equal to that of solitary flies (Bretman et al., 2013; Leech et al., 2017). Therefore, it seems to mitigate the decline in lifespan, perhaps by conferring a less detrimental effect on health than aged flies. Alternatively, it may be that exposure to a rival of any age negatively impacts lifespan, as it is acting as a cue for future mating competition, but young flies, and not old flies, also provide a slight benefit that can partially alleviate the reduction in lifespan. The mechanism behind this benefit is unclear but it seems to occur through sensing of the rival as it is dependent on olfaction and mechano-sensing (Ruan & Wu, 2008), as well as production of cuticular hydrocarbons by the rival fly (Cho et al., 2021).

One potential mechanism underlying the young rival effect is the microbiome. Bacterial species linked to host genetic pathways determining longevity were more enriched in younger flies than old (Leech and McDowall et al. 2021). Grouping with co-aged flies reduced these types of bacteria, but grouping with young flies did not. The ability of young companions to maintain

the microbiome at a state present in longer-lived single flies, and possibly more similar to young flies, could explain why previous studies have found a beneficial effect of younger companions (Ruan & Wu, 2008; Cho et al., 2021; Lin et al., 2022). If the young rival health effect is mediated by the microbiome, the microbiome is likely being altered intrinsically, rather than through the passing of the microbiota from the young fly to the aged fly, as the benefit is dependent on sensing of the young rival (Ruan & Wu, 2008; Cho et al., 2021).

3.1.3 The Larval Environment

The presence of adult males during larval development has been found to increase investment in reproductive tissues in larvae (Bretman et al., 2016), as well as decrease their lifespan (Leech and McDowall et al. 2021). Notably, this lifespan effect occurred in both sexes, with no interaction between the sex of the larvae and the presence of adult males, and so it seems likely to be driven by a mechanism distinct to the increased reproductive investment observed in males. One plausible candidate for this factor could be the extrinsic transfer of bacteria from adults to larvae as the presence of adult males during larval development also increased microbiome diversity in both male and female pupae (Leech and McDowall et al. 2021). Further research is needed to substantiate the role of the microbiome in this observed effect.

3.1.4 Chapter Aims

Many studies have discovered an effect of the same-sex social environment on male *D. melanogaster* health (Bretman et al., 2013; Zajitschek et al., 2013; Leech et al., 2017), and Leech and McDowall et al. (2021) recently found that these conditions also alter the microbiome. I sought to investigate whether these

microbial changes were responsible for the lifespan effect by manipulation of the microbiome, in combination with the social environment. Manipulation of the microbiome was achieved via antibiotics, which likely does not completely remove the microbiome, but reduces the bacterial load, and targets certain commensal bacteria (section 2.2). If the microbiome mediates the lifespan reduction in response to same-sex contact, then reducing the microbial difference between single and grouped flies will reduce the difference in lifespan.

Additionally, I explored the impact of the microbiome at specific ages. Drawing inspiration from the work of Cho et al. (2021), who identified a critical period of 40 days for young flies to convey a benefit, I aimed to pinpoint a similar critical period for the microbiome to have an effect by altering the age flies were exposed to antibiotics.

Initially experiments used males in groups of 10 in order to induce maximal stress, however, exposure to just one rival male can reduce lifespan in male *Drosophila* (Bretman et al., 2013; Leech et al., 2017). Therefore, I conducted two experiments with flies in pairs. Previous studies, observing a reduction in lifespan, changed rivals weekly to prevent acclimatisation (Bretman et al., 2013; Leech et al., 2017). However, it is unclear whether refreshing the rival is important in conferring the lifespan effect. Therefore, I investigated the effect of the novelty of the rival male on lifespan. Studies have found contradictory results on whether familiarity affects aggression (Hoffmann, 1990; Carazo et al., 2015; Le Page et al., 2017), which itself could impact lifespan, but the direct effect on familiarity on lifespan is yet to be established. Moreover, it is important to determine whether novelty may have served as a confounding factor in some experiments that

introduced new flies into one treatment group when attempting to manipulate an alternative factor. For example, Cho et al. (2021), and Leech and McDowall et al. (2021) compared flies kept with consistent, co-aged rivals with those kept with novel, young rivals to test for the effects of rival age. Furthermore, I compared lifespans between flies kept with co-aged constant rivals, co-aged rivals changed weekly, and young rivals changed weekly to separate age of rival from novelty. This comparison between co-aged and young rival treatments has not been explored before in paired *Drosophila*, only in larger groups (Ruan & Wu, 2008; Cho et al., 2021; Lin et al., 2022). I also utilised the experimental design of Cho et al. (2021) which kept males in co-aged groups until 40 days of age, when they were exposed to young rivals. Cho et al. (2021) found that this extended lifespan, and I used antibiotics to test whether the microbiome was responsible for this increase. Additionally, I attempted to establish whether the lifespan effects of adult presence during larvae development (Leech and McDowall et al. 2021) could be attributed to extrinsic microbiome changes.

Lastly, I looked into whether any socially-driven effects on lifespan could be explained by alterations in dopamine. Previous research has demonstrated a decline in dopamine levels in the ageing fly, alongside dopaminergic modulated behaviours such as courtship and learning (Neckameyer et al., 2000). The involvement of dopamine could occur via multiple pathways. As changes in dopamine levels can be triggered by the *Drosophila* stress response (Rauschenbach et al., 1993), perceiving environmental stress may alter dopamine levels which have an impact on gut physiology (El Kholy et al., 2021). Alternatively, the microbiome can modulate host dopamine signalling through the bacterial production of dopamine, as well as bacterial alteration of host dopamine

metabolism. For example, administration of *L. plantarum* lowered plasma tyrosine hydroxylase in a human trial (Chong et al., 2019). Therefore, dopamine produced by the altered microbiome may result in health effects, or may trigger other responses to the social environment, such as changes in cognitive ability or mating duration, ultimately resulting in the reduction in lifespan (Figure 3.1). Support for this lies in the finding that dysregulation of tyrosine hydroxylase can stimulate the human stress response (Chong et al., 2019). Therefore, in this chapter, the enzyme tyrosine hydroxylase was inhibited using 3-Iodo-L-tyrosine determine whether prevention of dopamine signalling would affect the lifespan response, perhaps in a similar way to microbial manipulation.

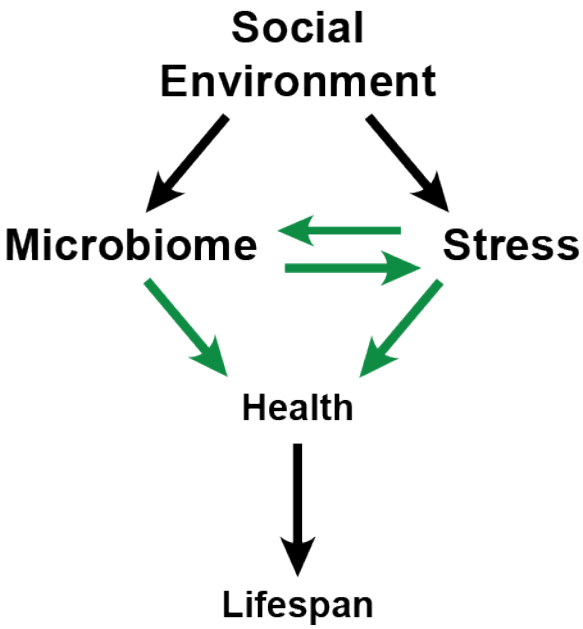

Figure 3.1 Possible Involvement of Dopamine in Socially-Driven Effects on Lifespan

Interactions between the social environment, microbiome and stress, and the impacts they have on lifespan. The green arrows represent effects that could be occurring through dopamine signalling.

3.2 Methods

3.2.1 Lifespan Assays

Flies were assigned to their treatment groups (Table 3.1) and checked daily for deaths. Focal fly deaths were recorded, and dead non-focal flies replaced with a fly of the same age, which had been maintained on the same food, unless stated otherwise.

Table 3.1 Lifespan Assays Presented in this Chapter
Treatment groups of experiments measuring lifespan in this chapter.

Experiment	Social Treatments	Focal Food Treatments
Antibiotic Microbiome Manipulation on the Grouping Response	• Single • Groups of 10	• Control • Antibiotic
The Critical Period for Microbiome Effects	• Single • Groups of 10	• Control Antibiotics from: • 30 days • 40 days • 50 days
The Effect of Rival Novelty	• Single • Pairs • ‘Novel’ pairs	Control
Antibiotic Microbiome Manipulation on the Pairing Response	• Single • Pairs • ‘Novel’ pairs • ‘Young’ pairs	• Control • Antibiotic
Antibiotic Microbiome Manipulation on the Effect of Rival Age	• Co-aged groups of 20 • Co-aged then 1:3 old:young at 40 days	• Control • Antibiotic
The Effect of Microbiome Homogenate During Development	Donor microbiome during development: • Single males • Grouped males • Single females • Grouped females • No flies (control)	Control
The Effect of Adult Males During Development with Antibiotic Manipulation of the Microbiome	During development: • Males absent • Antibiotic males absent Males present • Antibiotic males present	Control
Dopamine Manipulation on the Grouping Response	• Single • Groups of 10	• Control • Dopamine inhibitor

3.2.1.1 Antibiotic Microbiome Manipulation on the Grouping Response

To test for the influence of the microbiome on the lifespan response to grouping, flies were kept singly or in groups, either on control food or antibiotic food (n = 60 per treatment, made up of two blocks of 30). Groups consisted of a focal male, whose lifespan was to be measured, and nine non-focal males.

3.2.1.2 The Critical Period for Microbiome Effects

To test for a critical period for the microbiome to have an effect on lifespan, flies were kept either singly or in groups of 10, either on control food throughout life, or swapped to antibiotic food from 30, 40 or 50 days from eclosion (n = 50 per treatment).

3.2.1.3 The Effect of Rival Novelty

To test for the importance of the novelty of the rival, males were either kept singly, with a rival male, or a ‘novel’ rival male (n = 50 per treatment). The ‘novel’ non-focal was changed weekly with a co-aged male.

3.2.1.4 Antibiotic Microbiome Manipulation on the Pairing Response

To test for the influence of the microbiome on lifespan effects of pairing, males were either kept singly, with a rival male, a ‘novel’ rival, or a ‘young’ rival (n = 61 per treatment), and either on control or antibiotic food. The ‘novel’ non-focal was changed weekly with a co-aged male and the ‘young’ non-focal was changed weekly with a three-day old male.

3.2.1.5 Antibiotic Microbiome Manipulation on the Effect of Rival Age

To test for the influence of the microbiome on the lifespan effect of grouping with young rivals, males were either kept in co-aged groups for life or with young rivals from 40 days (as in Cho et al., 2021), and either on control or antibiotic food (n = 50 per treatment). 20 flies were kept per vial, and 40 days after eclosion, five were chosen at random to be the focal. In the co-aged group, the 15 non focal flies were wing-clipped, whereas in the young group they were removed and replaced with 15 one-day old wing-clipped flies.

3.2.1.6 The Effect of Microbiome Homogenate During Development

To test for the impact of microbiome transfer during larval development on lifespan, male or female donor flies were kept either singly or in groups of 10, before being homogenised at five days post eclosion, and added to standard food, which had been inoculated with 100 larvae immediately prior. Upon eclosion, five males and five females were collected from each vial, kept in these same-sex groups of five, and their lifespan recorded. This meant that the experimental design contained 10 groups (n = 50 per treatment, in 10 vials of five).

To make each homogenate, donor flies were snap-frozen, pooled into groups of 20, and washed, firstly with 70% ethanol and then dH_2O, before homogenisation in 80 μl PBS (Clark et al., 2015). Control solutions contained no flies. After centrifugation at 3075 × g for 1 min, glycerol was added to the supernatant to a final concentration of 20%. These homogenates were stored at -80°C and, when needed, were thawed and made up to 0.5 ml with dH_2O to allow the homogenate to spread out evenly on top of 7 ml of food in a vial.

3.2.1.7 The Effect of Adult Males During Development with Antibiotic Manipulation of the Microbiome

To test for the influence of the microbiome on the lifespan response to adult presence during larval development, larvae were raised in four different treatments: adults absent, antibiotic adults absent, adults present, and antibiotic adults present. The larvae in the two adult present treatments were raised along with a group of 10 five-day old flies, which had been fed on control or antibiotic food for five days from eclosion. The vials of food used to raise larvae in all treatments had an extra 0.5 ml of food dropped onto the 7 ml of food so it formed a thin layer over the top. In the antibiotic adults absent treatment, the 0.5 ml of food contained antibiotics to control for any antibiotics deposited onto the food by adult flies in the antibiotic adults present treatment.

Upon eclosion, five males and five females were collected from each vial, kept in these same-sex groups of five, and their lifespan recorded. This means the experimental design contained eight groups (n = 50 per treatment, in 10 vials of five).

3.2.1.8 Dopamine Manipulation on the Grouping Response

To test for the involvement of dopamine on lifespan effects of grouping, flies were kept singly or in groups, either on control food or food containing dopamine inhibitor (n = 50 per treatment, made up of two blocks of 25). Dopamine inhibitor food was made by supplementation with 3-Iodo-L-tyrosine to a final concentration of 1 mg/ml (Neckameyer, 1996). Groups consisted of a focal male, whose lifespan was to be measured, and nine co-aged non-focal males.

3.2.2 Statistical Analysis

All lifespan data violated the assumptions of a Cox regression, so the differences in lifespan between treatments were analysed using generalised linear models (GLMs). Nonetheless, survival curves were presented alongside boxplots to allow for visualisation of differences throughout time. To account for overdispersion, GLMs with quasi-poisson errors were used to explore the effect of rival novelty, or the effect on the pairing response. A GLMM with poisson errors was used to analyse critical period data so that a random factor could be included. GLMMs with negative binomial errors were used for the rest of the lifespan assays, to account for overdispersion and handle random factors (Table 3.2).

For the critical period data, to avoid loss of power due to multiple comparisons, the data was split by social environment and analysed separately using GLMMs with poisson errors, with antibiotic treatment as the only fixed factor. When exploring the effect of pairing and antibiotics, the data was split by antibiotic treatment and analysed separately using GLMs with quasi-poisson errors, with social environment as the only fixed factor.

Table 3.2 Factors Used in Models Measuring Effects on Lifespan

Fixed and random factors included in models exploring the effect of the social environment and the microbiome on lifespan.

Experiment	Fixed Factors	Random Factors
Antibiotic Microbiome Manipulation on the Grouping Response	• Social environment • Antibiotic treatment	• Block
The Critical Period for Microbiome Effects	• Social environment • Antibiotic treatment	• Block
The Effect of Rival Novelty	• Social environment	
Antibiotic Microbiome Manipulation on the Pairing Response	• Social environment • Antibiotic treatment	
Antibiotic Microbiome Manipulation on the Effect of Rival Age	• Social environment • Antibiotic treatment	• Vial ID
The Effect of Microbiome Homogenate During Development	• Sex • Donor fly	• Vial ID
The Effect of Adult Males During Development with Antibiotic Manipulation of the Microbiome	• Sex • Larval Environment	• Vial ID
Dopamine Manipulation on the Grouping Response	• Social environment • Inhibitor treatment	• Block

3.3 Results

3.3.1 Antibiotic Microbiome Manipulation on the Grouping Response

In order to test the effect of the social environment and the microbiome on lifespan, male flies were kept singly or in groups with nine other males, fed control food or food containing antibiotics, and had their longevity measured. There was a significant interaction between social environment and antibiotic treatment *(χ^2* = 8.414, *df* = 1, *p* = 0.004) (Figure 3.2), which was driven by a reduction in the effect of the social environment when flies were maintained on antibiotics. Pairwise comparisons revealed single flies lived significantly longer than grouped flies in the control treatment *(p* < 0.0001) and in the antibiotic treatment *(p* = 0.002). However, the effect size was larger in the control treatment (*d* = 1.940 compared to 0.619), with a median difference between single and grouped flies of 28 days, compared to a median difference of five days in the antibiotic treatment. This is because single flies on antibiotic food had a decreased lifespan compared to those on control food *(p* = 0.002, *d* = 0.669), whereas there was no significant difference between grouped flies fed on control or antibiotic food *(p* = 0.237).

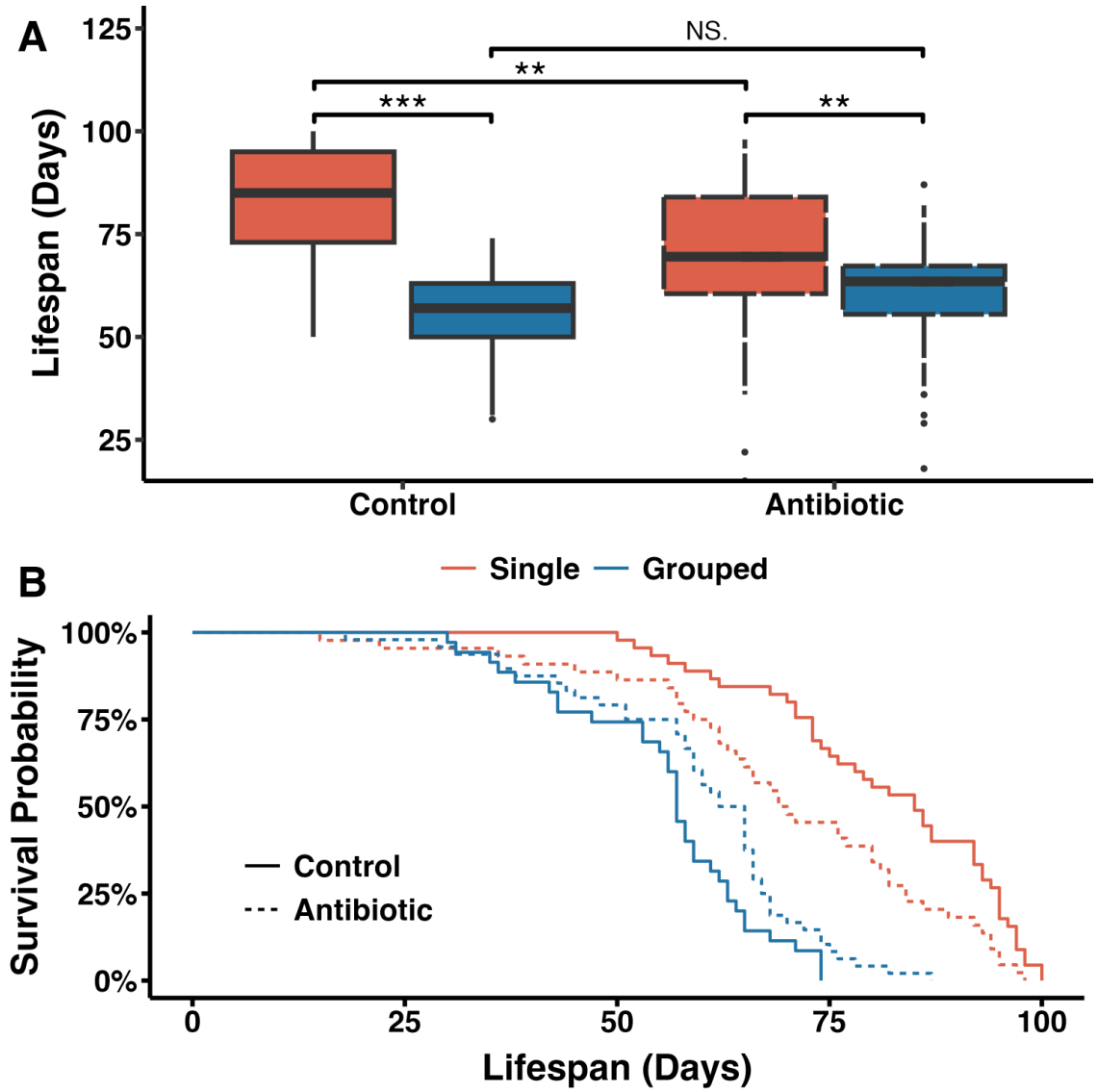

Figure 3.2 Effect of Antibiotics and Social Grouping on Lifespan

Males were kept singly on control food (n = 45), grouped on control food (n = 35), singly on antibiotic food (n = 44) or grouped on antibiotic food (n = 48). Lifespan is presented by A) a boxplot where significance levels are indicated for pairwise comparisons (NS. = non-significant, * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$), and B) a survival curve. Legends apply to both panels. Orange = single, blue = grouped. Solid line = control, dashed line = antibiotic.

3.3.2 The Critical Period for Microbiome Effects

To test for the existence of a critical period for the microbiome to have an effect on lifespan, single and grouped flies were kept on control food either life-long, or until 30, 40, or 50 days post eclosion when they were moved onto antibiotic food. There was no significant interaction between antibiotics start time and social group (χ^2 = 1.561, df = 3, p = 0.668) (Figure 3.3). There was an effect of social environment on lifespan (χ^2 = 138.31, df = 1, p < 0.0001; d = 0.953), with single flies living longer than grouped flies. There was also an effect of antibiotic start time (χ^2 = 9.278, df = 3, p = 0.026), and pairwise comparisons revealed that this was driven by a significant increase in lifespan displayed by flies fed antibiotics from 40 days when compared to 30 days (p = 0.031, d = 0.226).

To further explore the effects of antibiotic start time within social groups, the data was split by social environment. There was a significant effect of antibiotic start time on lifespan in the single flies (χ^2 = 9.220, df = 3, p = 0.027), driven by a significant increase in lifespan displayed by flies fed antibiotics from 40 days when compared to 30 days (p = 0.040, d = 0.435). However, there was no significant effect of antibiotic treatment in the grouped flies (χ^2 = 1.984, df = 3, p = 0.576).

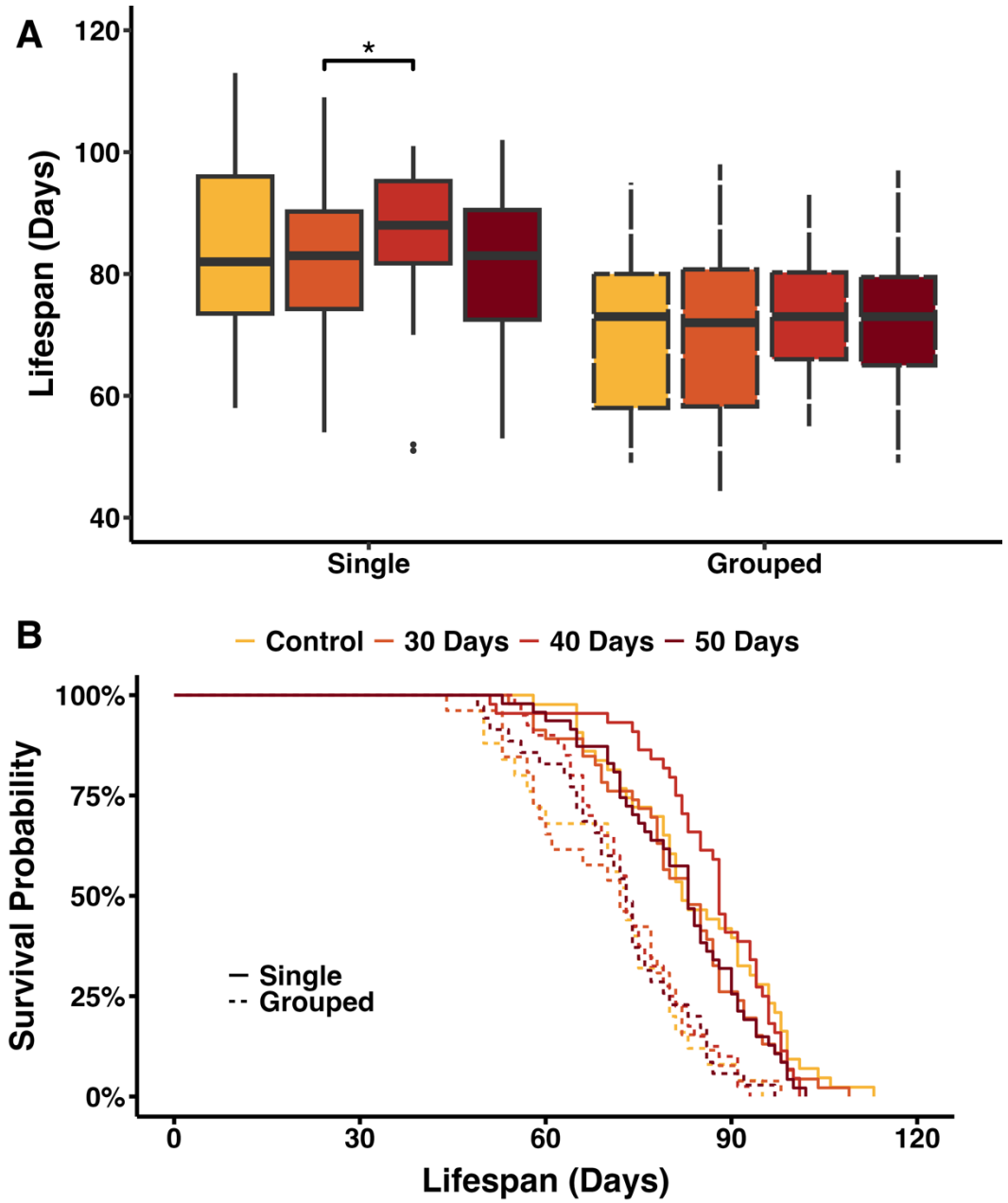

Figure 3.3 Effect of Antibiotics at Different Ages on the Lifespan Response to Grouping

Males were kept singly, or in groups of 10 and either kept on control food from eclosion till death (single: n = 43; grouped: n = 25), or changed onto antibiotic food at age 30 (single: n = 46; grouped: n = 26), 40 (single: n = 44; grouped: n = 40) or 50 days post eclosion (single: n = 47; grouped: n = 35). Lifespan is presented by A) a boxplot where significance levels are indicated for pairwise comparisons when data was split by social environment (NS. = non-significant, * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$), and B) a survival curve. Legends apply to both panels. Yellow = control, orange = 30 days, red = 40 days, dark red = 50 days. Solid line = single, dashed line = grouped.

3.3.3 The Effect of Rival Novelty

To test the impact of novelty on lifespan, male flies were kept singly or paired with a partner, who was either co-aged and unchanging or swapped weekly with a co-aged male. There was a significant effect of social environment on lifespan ($F_{2,139}$ = 5.753, p = 0.004) (Figure 3.4). Pairwise comparisons revealed single flies lived longer than either co-aged pairs *(*p = 0.011*,* d = 0.586), or novel pairs *(*p = 0.008, d = 0.614), but there was no difference between paired and novel treatments *(*p = 0.986).

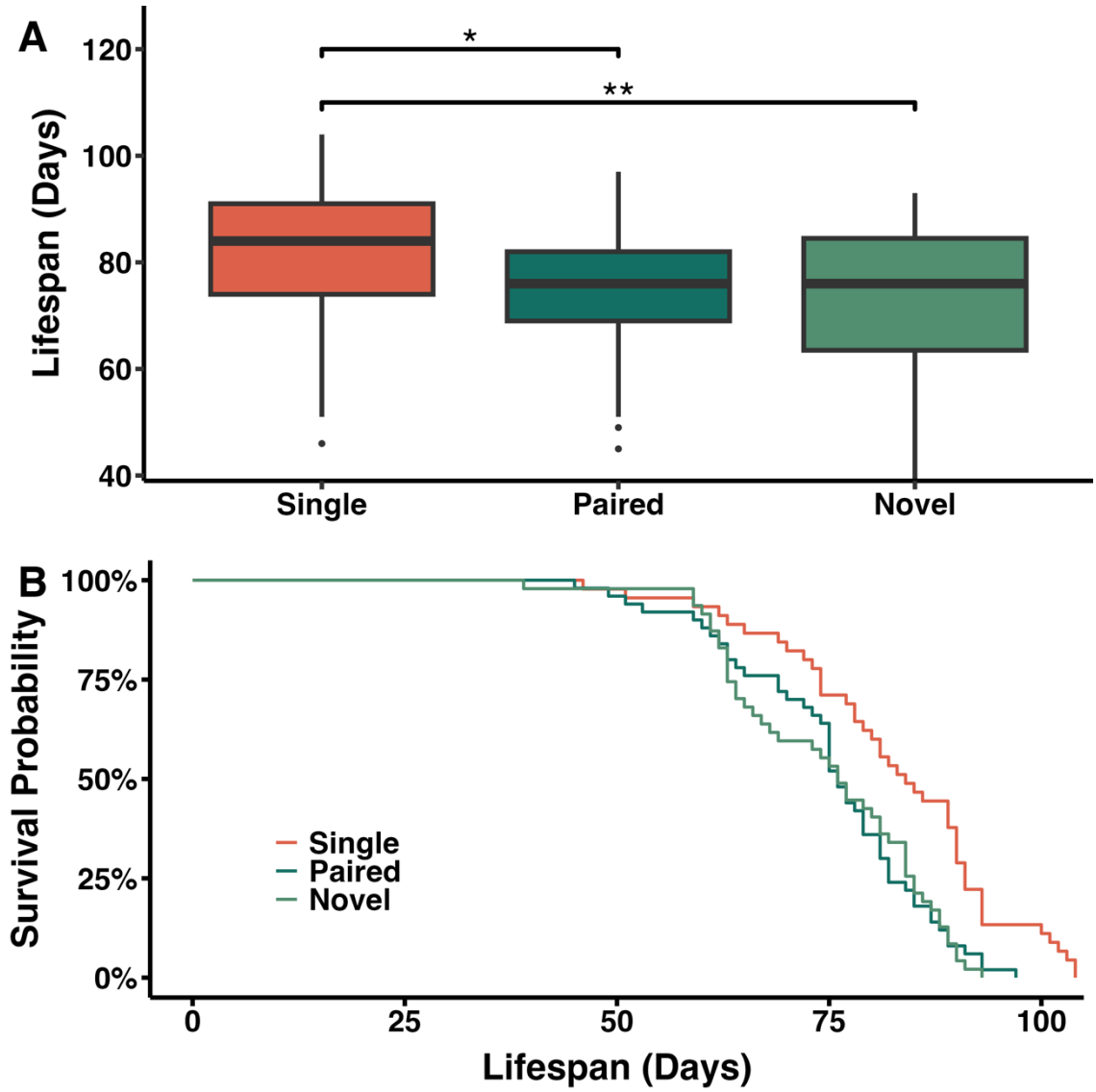

Figure 3.4 Effect of Social Pairing on Lifespan

Males were kept singly (n = 45), in a pair with a consistent rival male (paired, n = 50), or in a pair where the rival male was changed weekly with a co-aged male (novel, n = 47). Lifespan is presented by A) a boxplot where significance levels are indicated for pairwise comparisons (NS. = non-significant, * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$), and B) a survival curve. Orange = single, dark green = paired, green = novel.

3.3.4 Antibiotic Microbiome Manipulation on the Pairing Response

In order to test the effect of pairing and the microbiome on lifespan, male flies were either fed control food or food supplemented with antibiotics, kept singly or paired with a partner, who was either co-aged and unchanging, swapped weekly with a co-aged male or swapped weekly with a three-day old male. There was no significant interaction between social environment and antibiotic treatment *($F_{3,437}$* = 0.144, p = 0.934) (Figure 3.5). There was no significant effect of antibiotic treatment on lifespan ($F_{1,437}$ = 2.065, p = 0.151). There was a significant effect of social environment ($F_{3,441}$ = 5.526, p = 0.001). Pairwise comparisons revealed single flies lived longer than both novel flies *(p* = 0.001, d = 0.534), and young flies *(p* = 0.041, d = 0.468).

When split by antibiotic treatment, there was a significant effect of the social environment in control flies ($F_{3,222}$ = 3.471, p = 0.017), and pairwise comparisons revealed this was driven by single flies living longer than novel flies *(p* = 0.011; d = 0.589). In antibiotic treated flies there was no significant effect of the social environment ($F_{3,218}$ = 2.063, p = 0.106).

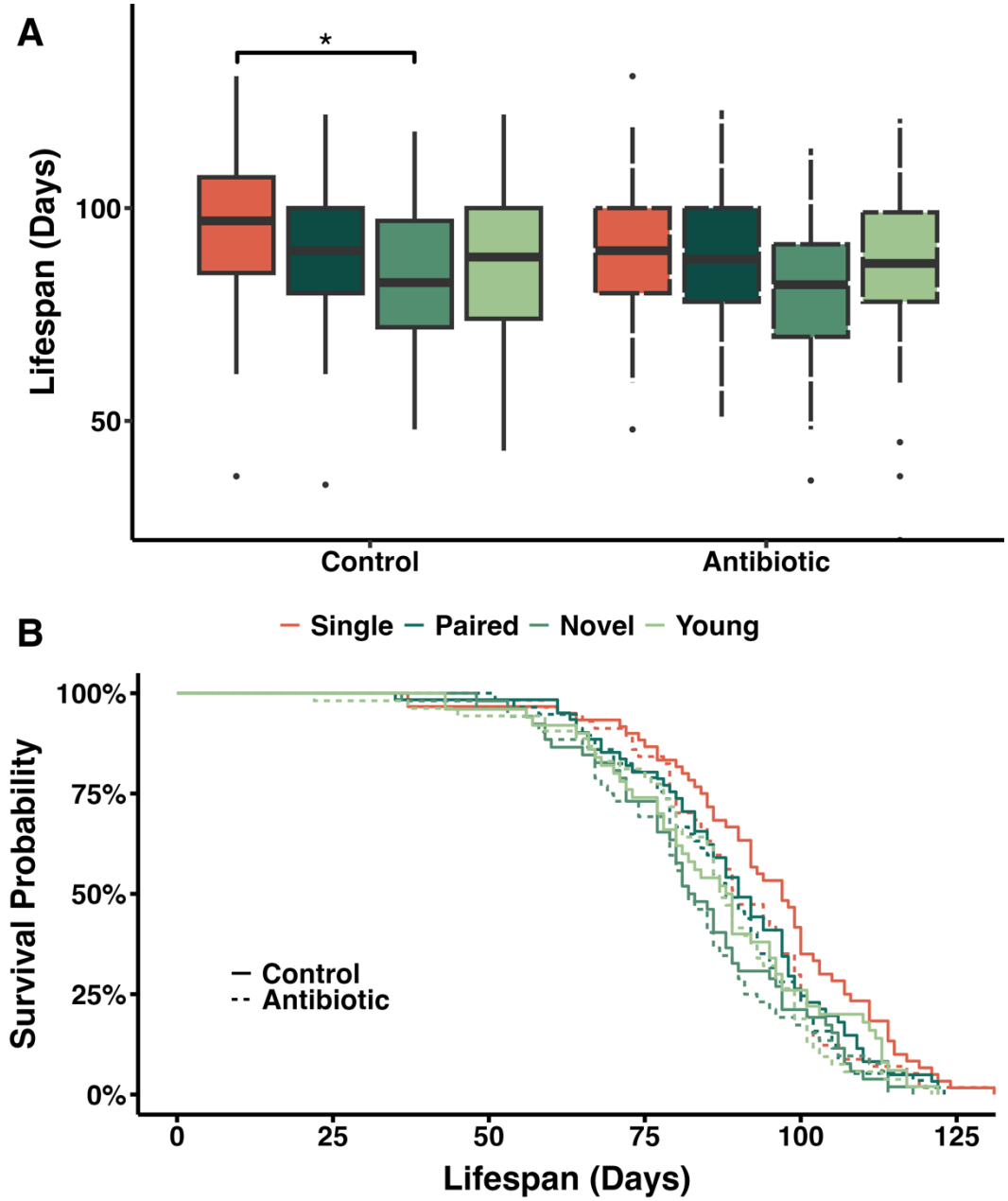

Figure 3.5 Effect of Antibiotics and Social Pairing on Lifespan

Males were kept on control food or antibiotic food, and either singly (single; control: n = 60; antibiotic: n = 57), or in a pair with a consistent rival male (paired; control: n = 61; antibiotic: n = 57), a weekly changing co-aged male (novel; control: n = 52; antibiotic: n = 52), or a weekly changing three-day old male (young; control: n = 50; antibiotic: n = 53). Lifespan is presented by A) a boxplot where significance levels are indicated for pairwise comparisons after data was split by treatment (NS. = non-significant, * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$), and B) a survival curve. Legends apply to both panels. Orange = single, dark green = paired, green = novel, pale green = young. Solid line = control, dashed line = antibiotic.

3.3.5 Antibiotic Microbiome Manipulation on the Effect of Young Rivals

In order to test the effect of young rivals and the microbiome on lifespan, males were fed control food or food containing antibiotics and kept in groups of 20 until 40 days post eclosion when they were either kept in their co-aged groups or kept with two-day old flies at a ratio of 1:3 (old:young). There was no significant interaction between antibiotic treatment and social environment (χ^2 = 0.203, df = 1, p = 0.652) (Figure 3.6). There was no effect of social environment (χ^2 =0.076, df = 1, p = 0.783), but there was an effect of antibiotic treatment (χ^2 = 12.778, df = 1, p = 3.51 x 10^{-4}; d = 0.622) on lifespan, with flies on antibiotics living longer than control flies.

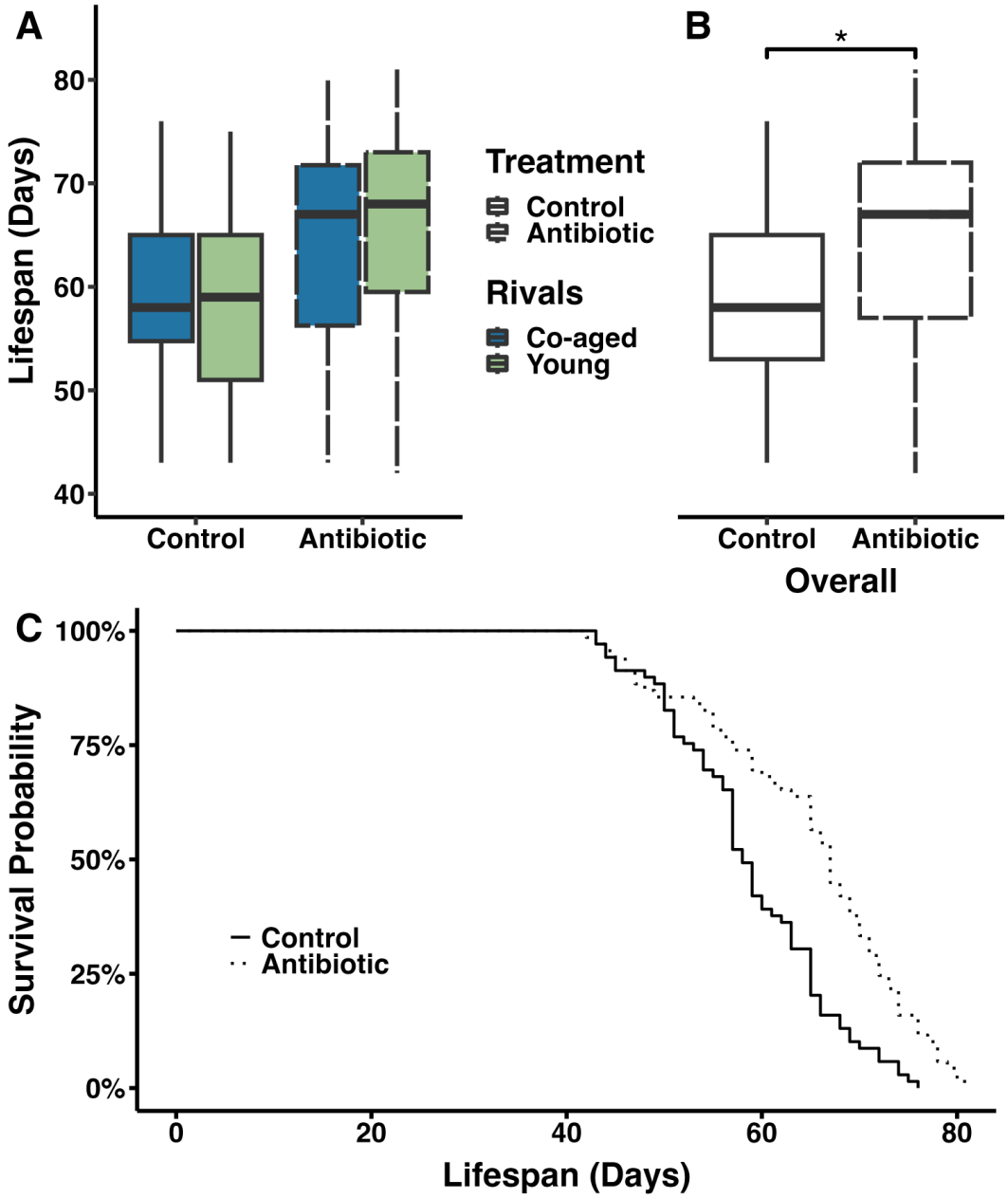

Figure 3.6 Effect of Young Rivals and Antibiotics on Lifespan

Males were kept on control food in co-aged groups (n = 36), on control food in 'young' groups (n = 33), on antibiotic food in co-aged groups (n = 43), or on antibiotic food in 'young' groups (n = 31). In all treatments, all flies were in groups of 20. In the 'young' treatments, at 40 days of age, 15 of each group were replaced with 15 two-day old flies. Lifespan is presented by A) a boxplot (blue = co-aged, pale green = young), B) a boxplot showing the overall effect of antibiotic treatment as this factor remained in the simplified model, with significance levels indicated (NS. = non-significant, * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$), and C) a survival curve showing the overall effect of antibiotic treatment. Solid line = control, dashed line = antibiotic.

3.3.6 The Effect of Microbiome Homogenate During Development

To test for the effects of adult fly microbiome transferal during development, larvae were added to vials which were inoculated with homogenates from flies that had been subjected to different social environments. After eclosion, they were collected, and their longevity measured. There was no significant interaction between focal fly sex and donor microbiome ($\chi^2 = 2.779$, $df = 4$, $p = 0.595$) (Figure 3.7). There was no significant effect of donor fly on lifespan ($\chi^2 = 1.185$, $df = 4$, $p = 0.881$), but a significant effect of focal fly sex, with females living for longer ($\chi^2 = 41.477$, $df = 1$, $p < 0.0001$; $d = 0.855$).

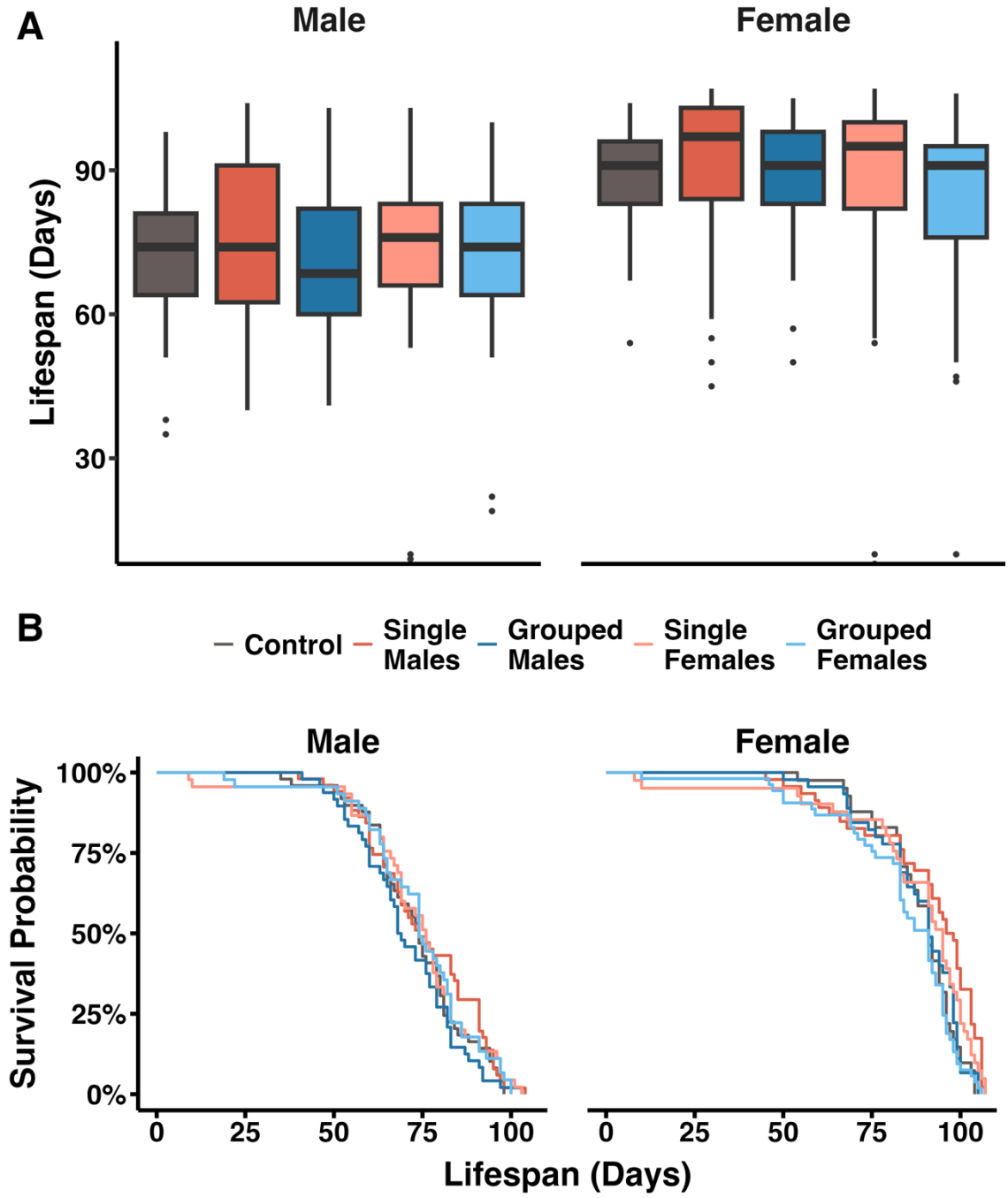

Figure 3.7 Effect of Microbiome Homogenate During Development on Lifespan

Males and females were collected from vials where they had developed alongside the homogenates of donor males kept singly (male: n = 51; female: n = 46), donor males kept in a group of 10 (male: n = 48; female: n = 45), donor females kept singly (male: n = 45; female: n = 41), or donor females kept in groups of 10 (male: n = 45; female: n = 53). A control solution with no flies was also included (male: n = 49; female: n = 41). Lifespan is presented by A) a boxplot, and B) a survival curve. Legend applies to both panels. Grey = control, orange = single males, blue = grouped males, peach = single females, pale blue = grouped females.

3.3.7 The Effect of Adult Males During Development with Antibiotic Manipulation of the Microbiome

To test for the effects of adult fly presence during development, larvae were reared with adult males present or absent. These males had been previously fed control food or antibiotic food, and to control for this a drop of antibiotic food was left on antibiotic adult absence vials. After larvae had developed, they were collected as adults, and their longevity measured. There was no significant interactions or effects (full vs. null model: $\chi^2 = 4.533$, $df = 7$, $p = 0.717$) (Figure 3.8).

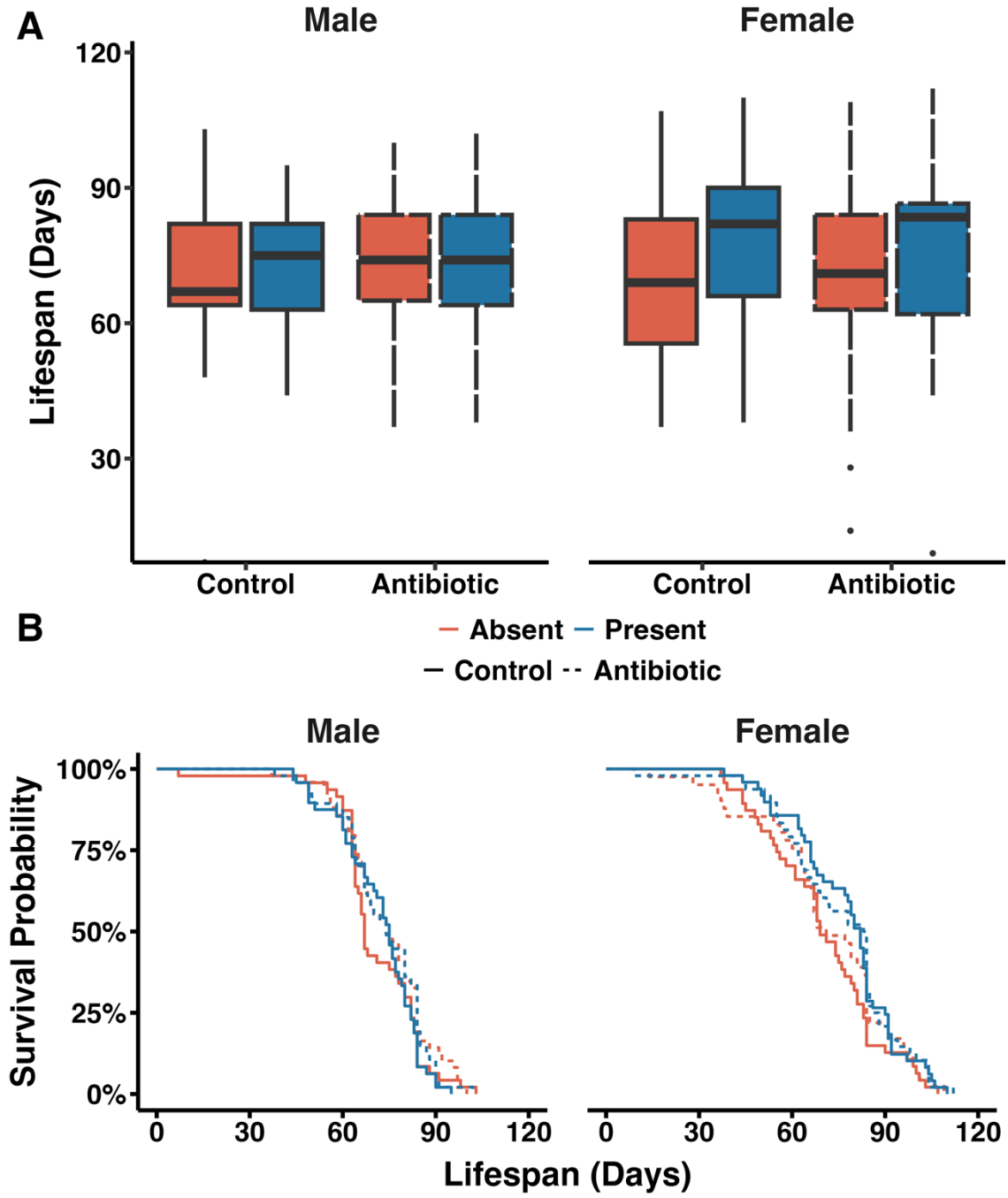

Figure 3.8 Effect of Adult Male Presence During Development on Lifespan

Males and females were collected from vials where they had developed alongside no adult males (absent control, male: n = 47; female: n = 47), 10 adult males (present control, male: n = 48; female: n = 49), no adult males with a drop of antibiotic food (absent antibiotic, male: n = 49; female: n = 41), or 10 adult males that had previously been on antibiotic food (present antibiotic, male: n = 47; female: n = 48). Lifespan is presented by A) a boxplot, and B) a survival curve. Legends apply to both panels. Orange = absent, blue = present. Solid line = control, dashed line = antibiotic.

3.3.8 Dopamine Manipulation on the Grouping Response

In order to test the effect of the social environment and dopamine inhibition on lifespan, male flies were kept singly or in groups with nine other males, on control food or food containing dopamine inhibitor, and their longevity measured. There was no significant interaction between social environment and dopamine inhibitor treatment ($\chi^2 = 0.542$, $df = 1$, $p = 0.462$) (Figure 3.9), and no significant effect of antibiotic treatment on lifespan ($\chi^2 = 0.453$, $df = 1$, $p = 0.501$). There was a significant effect of social environment ($\chi^2 = 25.23$, $df = 1$, $p < 0.0001$; $d = 0.815$).

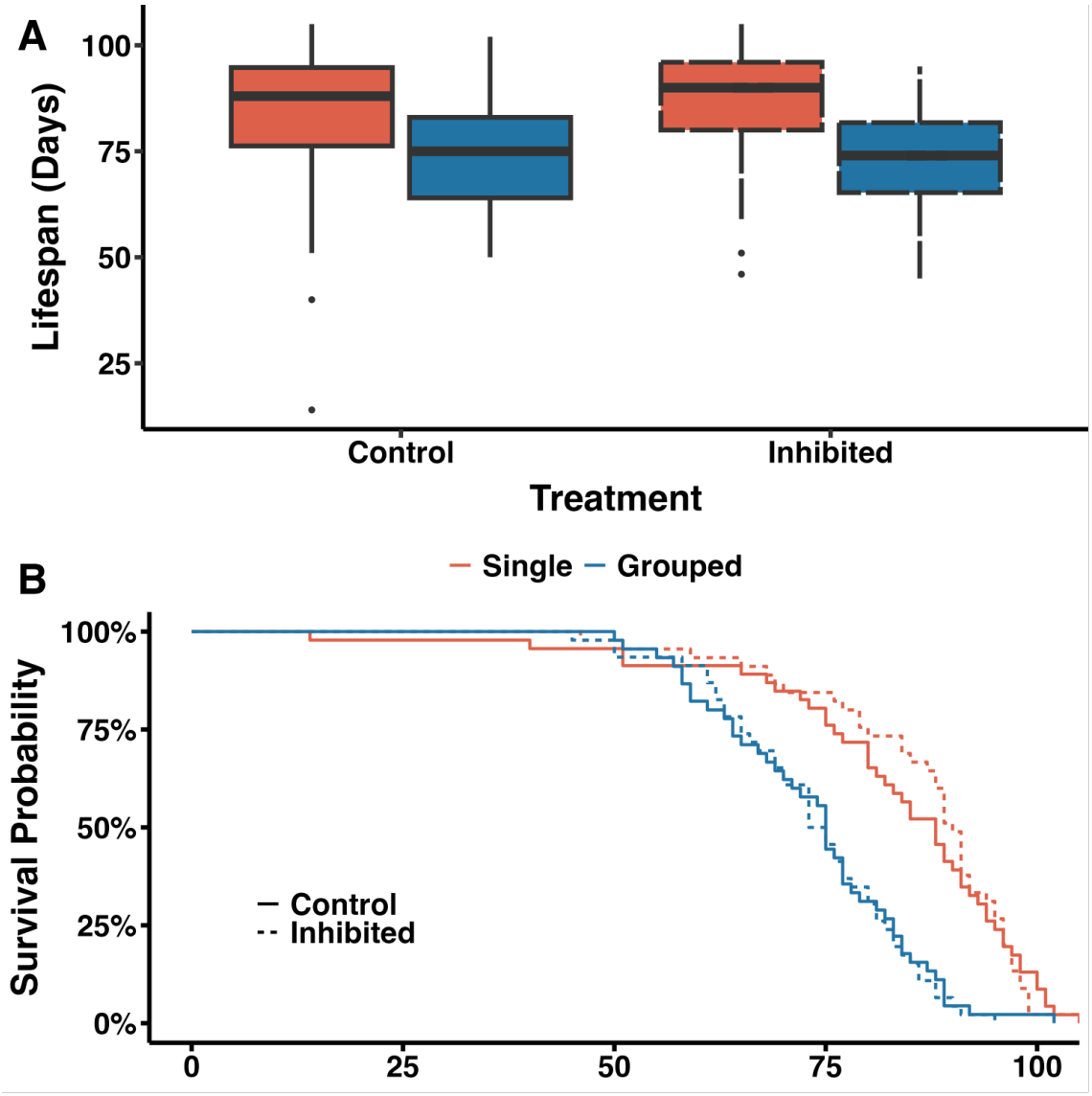

Figure 3.9 Effect of Dopamine Inhibition and Social Grouping on Lifespan
Males were kept singly on control food (n = 46), grouped on control food (n = 45), singly on dopamine inhibitor food (n = 45) and grouped on dopamine inhibitor food (n = 46). Lifespan is presented by A) a boxplot, and B) a survival curve. Legends apply to both panels. Orange = single, blue = grouped. Solid line = control, dashed line = antibiotic.

3.4 Discussion

3.4.1 The Microbiome Contributes to the Difference in Lifespan Between Flies in Differing Social Environments

The findings presented in this chapter indicate that the microbiome has a role in the male *D. melanogaster* lifespan response to rivals. When differences in the microbiome between single flies and flies kept in pairs or groups were reduced by antibiotics, lifespan differences were reduced. This occurred through single flies having a reduced lifespan in the antibiotic treatment, so that their lifespan was more similar to that of a paired or grouped fly. This may occur through antibiotics perturbating the microbiome in a single fly in a way similar to the stress of the social environment.

Previous research has observed that grouped flies have an altered microbiome when compared to single flies (Leech and McDowall et al. 2021). Commensal dysbiosis can occur naturally in aged *Drosophila*, which can lead to intestinal stem cell hyperproliferation, epithelial dysplasia and a shortened lifespan (Guo et al., 2014). This could occur through an increase in certain bacterial species that have a negative impact on health, a decrease in certain bacterial species that have a beneficial effect on health, or just an imbalance of certain groups. Considering that grouping has been shown to alter microbial predicted effect on gene function in a manner similar to ageing (Leech and McDowall et al. 2021), it is conceivable that a similar dysbiosis is occurring in grouped flies too. Dysbiosis in grouped flies could occur through the extensive impact stress can exert on the microbiome via neurotransmitter changes (Davenport & Evans, 1984; Rauschenbach et al., 1993), gene upregulation and decreases in glycogen, fatty acids and glucose (Sørensen et al., 2005).

Alternatively, it may also signify a trade-off scenario in which resources that could influence the microbiome are allocated towards increased investment in reproductive assets. Regarding the research in this chapter, antibiotic fed single flies may exhibit a lifespan more similar to grouped flies because antibiotics perturbed the microbiome in a way similar to the stress of grouping.

It is worth noting that antibiotic treatment did not completely abolish the lifespan difference between single and grouped flies. This may be due to limitations in the methodology. Antibiotics likely do not completely abolish microbial differences between single and grouped flies. In other words, antibiotics may not perfectly replicate the effects of grouping on the single fly microbiome. Antibiotics and grouping may cause different levels of dysbiosis, resulting in differing impacts on lifespan (Figure 3.10a). Alternatively, the remaining difference between single and grouped flies after antibiotics may signify that the microbiome is only partially involved in this response and may share responsibility with other mechanisms, such as costs linked to increasing mating duration (Figure 3.10b). The use of germ-free flies would help to determine which of these is true as this would completely eliminate the microbial component.

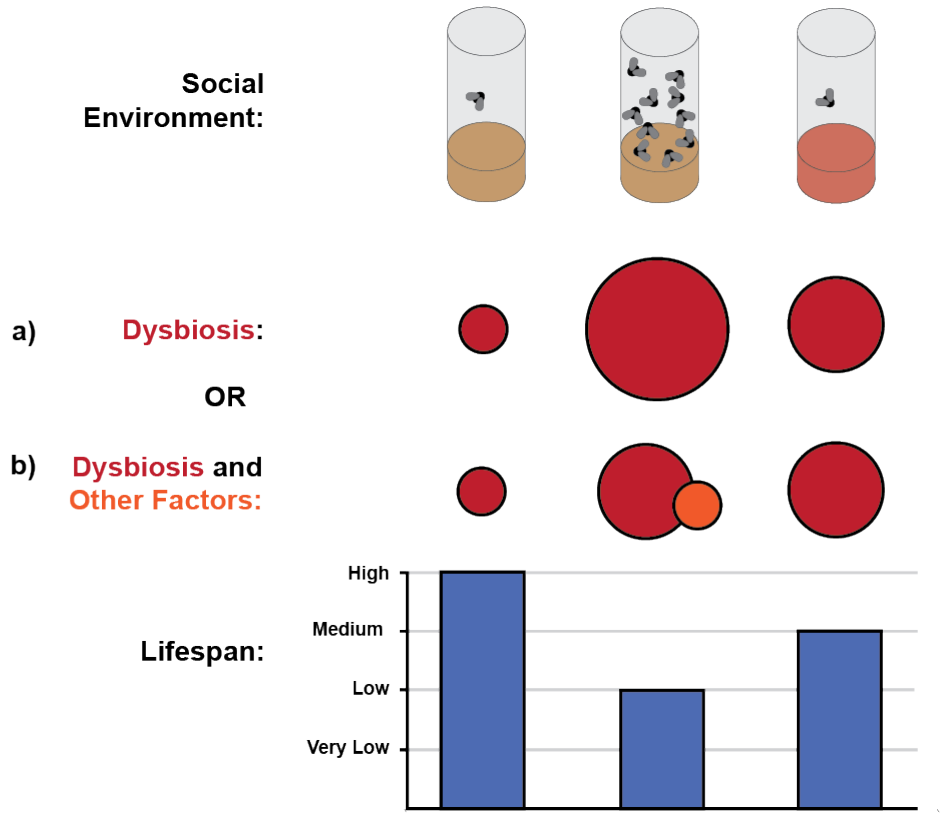

Figure 3.10 Possible Explanations Behind Lifespan Differences

The possible effect of grouping and antibiotic food (shown by red food) on the extent of dysbiosis (shown by red circles) and how this is predicted to translate to lifespan. An alternative explanation is that other factors are involved concomitantly with dysbiosis (orange circle show effects that cannot be influenced by antibiotics, such as trade-off effects of the future mating competition response). Circles size is relative to the strength of the effect. The differences between the size of factor effects and lifespan levels are not linear in reality, but are displayed linearly to simplify the diagram.

Previous research exclusively used grouped flies when testing for the effects of antibiotics, and found contrasting effects (Brummel et al., 2004; Ren et al., 2007; Ridley et al., 2012; Petkau et al., 2014; Clark et al., 2015; Dantoft et al., 2016; Galenza et al., 2016; Li et al., 2016; Loch et al., 2017; Fast et al., 2018; Gould et al., 2018; Obata et al., 2018; Sannino et al., 2018; Lee et al., 2019; Hanson & Lemaitre, 2022). The work in this chapter concluded that the combination of antibiotics used had no effect on the lifespan of flies kept in groups

of 10. However, a novel adverse effect of antibiotics on single fly lifespan was identified.

Results suggest that the lifespan difference between single flies and those exposed to rivals were not exclusively due to aggressive interactions, as single flies do not experience these and yet their lifespan was still changeable by antibiotics. However, the lifespan difference that still remained in antibiotic groups may be due to the aggression experienced in flies exposed to rivals. Whether aggression was modulated by antibiotics, as in other studies (Jia et al., 2021; Grinberg et al., 2022), is yet to be determined.

In some animals, social isolation acts as a stressor, reducing lifespan, rather than increasing it (reviewed in Cacioppo et al., 2011). It is worth noting, even though the results presented in this chapter suggest no negative effect of isolation in *Drosophila*, one may still be present, masked by other effects that are beneficial to lifespan, including those caused by the microbiome. A beneficial effect of being with rivals may also be overshadowed by negative effects such as those caused by perception of future mating competition. Studies present some support for a negative effect of isolation in *Drosophila*. In one study isolated flies slept more and demonstrated transcriptomic similarities to a starving fly (Li et al., 2021). In another study, single flies had a decreased survival in response to starvation stress when compared to flies kept in pairs that were separated by a permeable divide (Moatt et al., 2013).

3.4.2 The More Rivals, the More Detrimental the Microbiome

It seems that the impact of the microbiome is less discernible when flies are paired, possibly as pairing does not appear to decrease lifespan to the same extent as grouping, which is evident when comparing effect sizes. The effect sizes of pairing in flies on control food in two assays (pairing assay: 0.586; antibiotic pairing assay: 0.276) were smaller than those observed for grouping in three assays (antibiotic grouping assay: 1.940; critical period assay: 1.056; dopamine inhibition assay: 0.632). Notably, these effect sizes exhibit considerable variability between experiments even when using the same conditions, suggesting the influence of additional factors. One such factor could be the initial microbiome composition of the flies, or the introduction of potentially pathogenic bacteria at any point in an experiment, both which may lead to alternative effects of socially-driven dysbiosis.

Additionally, while testing for the effects of young rivals in a group, a positive effect of antibiotics on lifespan was observed, in contrast to the previous findings that observed no effect on grouped flies. This is likely because, in order to remain consistent with the methodology used by Cho et al. (2021), flies in this assay were kept in groups of 20, not 10. This would explain why many previous studies that have looked at flies in groups of 20 or more have observed an increase in lifespan when the microbiome was removed (Dantoft et al., 2016; Loch et al., 2017; Fast et al., 2018; Obata et al., 2018; Sannino et al., 2018; Lee et al., 2019).

The reduction in lifespan with increasing group size could be attributed to the usual increase in bacterial load with age (Guo et al., 2014; Clark et al., 2015;

Lee et al., 2019) being exacerbated by increased density. Increased social density may also be more stressful and so alter other factors like stress hormones or immune gene expression. In multiple animal studies, increased population density has been shown to correlate with glucocorticoid levels (Boonstra & Boag, 1992; Novikov & Moshkin, 1998; Harper, 2009; Bian et al., 2011; Werner et al., 2015), yet it is unclear whether this is caused by social stress as it is confounded with resource competition and increased predation. Both increased bacterial load and a stress response may increase the chance of dysbiosis, leading to associated health implications, such as gut dysplasia (Hochmuth et al., 2011; Guo et al., 2014) (Figure 3.11a). Previous studies determined that increasing group size did not affect mating duration (Bretman et al., 2009; Bretman et al., 2010), and so the group-size effect may be independent of the future mating competition effect. However, another study found increased progeny if males were kept in groups compared to in pairs (Hopkins et al., 2019), so an increase in reproductive investment may still be occurring and may be responsible for any group-size effect (Figure 3.11b). A higher bacterial load may also result in increased pathogenic bacteria leading to increased mortality by disease (Figure 3.11c). Furthermore, the explanation may not be as simple as just one of these options. For example, disease may be dependent on a certain level of dysbiosis, only reached during higher densities, as pathogenic bacteria perpetuated by increased fly densities may only be able to proliferate if the protective commensal microbiota balance is disturbed. Research shows that this effect may occur at smaller fly densities when disease is induced. Pairing resulted in a reduced survival following infection when the pathogen was introduced orally (Leech and McDowall et al. 2021), but not when via injection, which bypasses the microbiome (Leech et al., 2019), implying that reduced survival in paired flies might be

attributed to changes in the microbiome. It seems likely that the single fly microbiome helps protect against infection, while dysbiosis occurring in paired flies hinders this protective effect, which may become detrimental in flies in larger groups that have a higher chance of disease.

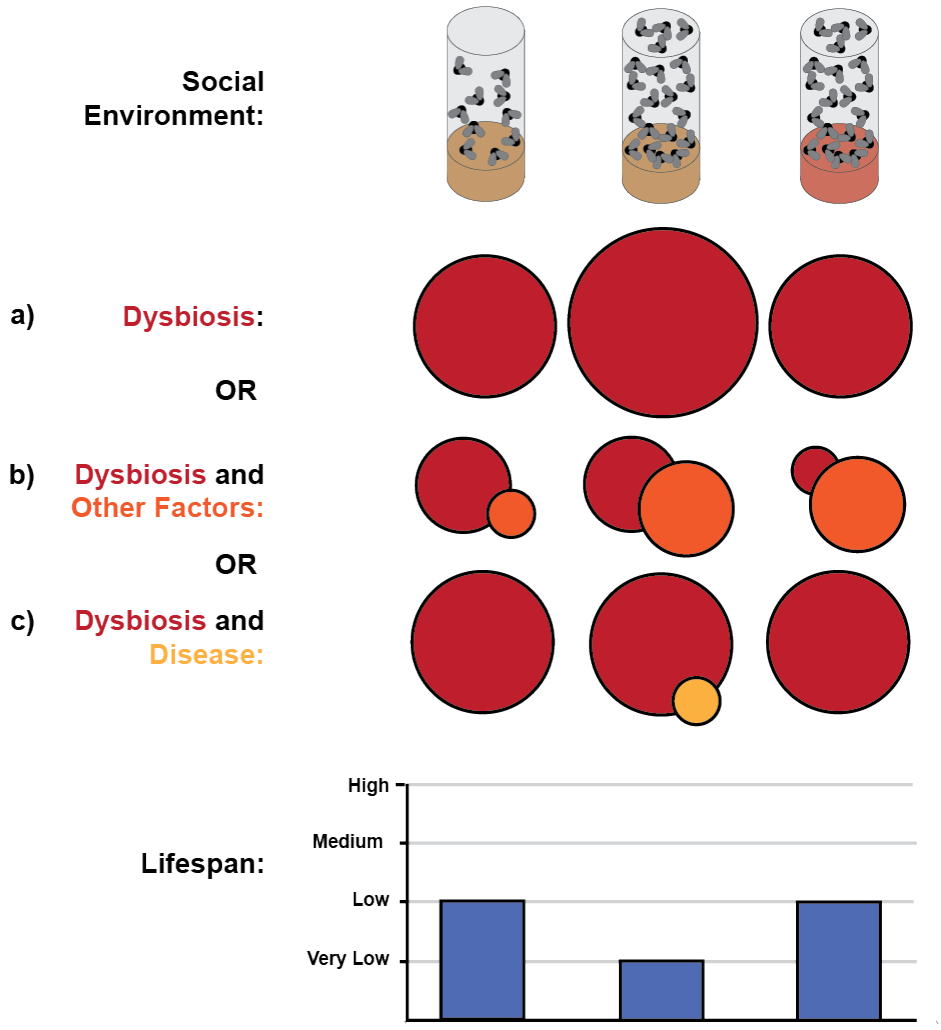

Figure 3.11 Possible Explanations Behind Lifespan Differences in Larger Groups

The possible effect of groups of 10 or groups of 20, and antibiotic food (shown by red food), on the extent of dysbiosis (shown by red circles), and how this is predicted to translate to lifespan. An alternative explanation is that other factors are involved concomitantly with dysbiosis (orange circles show effects that cannot be influenced by antibiotics, such as trade-off effects of the future mating competition response; yellow circles show effects such as disease that can influenced by antibiotics). Circles size is relative to the strength of the effect. The differences between the size of factor effects and lifespan levels are not linear in reality, but are displayed linearly to simplify the diagram.

3.4.3 The Microbiome Becomes More Detrimental as Flies Senesce

Leech and McDowall et al. (2021) only observed a change in the microbiome with social environment at 49 days and not at 11 days. Furthermore, Cho et al. (2021) identified a critical period of 40 days for the social environment to benefit lifespan. By exposing flies to antibiotics at various stages in later life, it was hoped that any critical period during which the microbiome played a role in extending single fly lifespan would be determined. However, when antibiotics were given to single flies at older ages, they did not exhibit the expected reduction in lifespan observed when given from eclosion. This dependence on the age at which antibiotics were given was also observed in a previous study, where antibiotics from two weeks increased lifespan in grouped flies, but decreased lifespan if given from eclosion (Brummel et al., 2004). This outcome suggests the microbiome may exert a positive effect in early but not later life, meaning removal in later life does not result in the same loss of beneficial effects as removal from eclosion. This is concurrent with the antagonistic pleiotropy theory of ageing, which suggests that certain traits beneficial for an organism's fitness in early life can become detrimental with age (Williams, 1957). In the young fly, the microbiome is beneficial, aiding in digestion and protecting against disease (Glittenberg et al., 2011) and starvation (Shin et al., 2011). However, as the fly ages, an altered microbiome can lead to dysbiosis, meaning the microbiome which once provided immune protection may exacerbate inflammation and cause immune dysregulation in older age. Additionally, gut barrier integrity which is maintained and strengthened by the microbiome in early life, may be compromised by the dysbiotic microbiome, leading to increased intestinal permeability and inflammation. Furthermore, the method by which antibiotics reduce lifespan when given from eclosion may be by quickening the onset of dysbiosis that usually

occurs with old age (Guo et al., 2014). Consequently, antibiotic exposure at a later age, when dysbiosis is already underway, will not have as detrimental an effect.

Though no significant interaction was found between social environment and start of antibiotic treatment, splitting the data by social environment revealed a significant effect of start of antibiotic treatment in single but not in grouped flies. This was driven by a slight increase in lifespan in single flies that were exposed to antibiotics from 40 days of age. This is of interest as Cho et al. (2021) also identified a critical period of 40 days for young rivals to have a positive effect on males. It is possible that 40 days is the age where senescence has a pronounced impact making any factors that extend lifespan particularly advantageous at this stage. Cho et al. (2021) conjectured that the sensory systems responsible for identifying the social environment may be particularly responsive at this age. Nonetheless, given the modest nature of the effect of antibiotics at 40 days, this warrants further investigation to confirm its significance.

3.4.4 Novel Rivals Induce the Same Reduction in Lifespan as Consistent Rivals

Previous studies testing a lifespan effect of pairing, changed rivals weekly to prevent acclimatisation (Bretman et al., 2013; Leech et al., 2017). Additionally, some studies introduce novel flies into experiments to change another variable, such as age (Cho et al., 2021; Leech and McDowall et al. 2021) and so it needed to be established whether novelty was an important factor in determining lifespan. No difference in lifespan was observed between flies paired with a consistent male and flies paired with a weekly changing co-aged male. However, in the

assay where antibiotics were used, novel pairs appeared to have the shortest lifespan in the control group, with a significant difference between single and novel flies, but no other pairing treatments. Therefore, although the novelty of rivals does not confer a large effect relative to other drivers of lifespan, it should still be considered and kept constant between treatments in future experiments. Additionally, novelty was only measured in pairs and not groups, and the impact of novelty might be more pronounced when the fly is exposed to more than one novel fly.

A potential confounding factor in the experiments exploring novelty in this chapter is the possibility that the novel rivals may not actually be novel to the focal fly. This is because of the possibility that they were placed in the same vial as larvae and so reared together. Furthermore, they may have been related as all eggs were collected from the same stock cage of flies. Carazo et al. (2015) found that related and co-reared males were less aggressive with each other than if they were unrelated and reared apart, although this result could not be replicated by Le Page et al. (2017). Therefore, it would be valuable for future experiments to manipulate larval relatedness and familiarity, and determine if these affect adult longevity.

3.4.5 Young Rivals do not Always Induce a Positive Lifespan Effect

Previous studies have found the age of the rival to be important in modulating lifespan (Ruan & Wu, 2008; Cho et al., 2021; Lin et al., 2022). However, experiments described in this chapter did not detect this effect. When pairing males, a young rival resulted in the same lifespan reduction as a co-aged rival. This differing result may be due to the weekly rotation of young flies in this assay

to maintain their young age, whereas in other studies, young flies were only added when focal flies reached a certain older age (21 – 40 days) and were not rotated after this (Cho et al., 2021; Lin et al., 2022). Swapping the rival fly weekly may have negated any positive effect that a young rival incurred. Indeed, in flies kept on control food, a significant difference in lifespan was only observed between single flies and novel pairs, suggesting swapping co-aged rivals weekly is the most detrimental to lifespan. Therefore, a slight negative effect of the novelty of the rivals in the young pairings may have been enough to conceal any small beneficial young rival effect. Alternatively, if the effects are occurring through perception of a young rival, it may take a while for the focal male to detect the age of its rival. Or if the lifespan extension is caused by positive social connections, these may take time to form. Replacing the rival would reset these processes. Indeed, Cho et al. (2021) observed that when young flies were rotated every seven days, the increase in lifespan was still present but was reduced.

Additionally, flies in previous studies have been kept in groups whereas, in the first study exploring rival age in this chapter, flies were kept in pairs. It may be that one young fly may not have been enough to confer a benefit. Cho et al. (2021) found that a group of focals and young rivals at a ratio of 1:1 did not result in a positive effect, which was the ratio used in the pairing assay. However, the second assay measuring the effect of young rivals in this chapter was not able to replicate results from Cho et al. (2021) when using their method and ratio; no increase in lifespan was observed when swapping co-aged rivals with young rivals at age 40 compared to keeping them co-aged. Cho et al. (2021) found that if young flies were added when the focal flies were even 10 days younger the effect was abolished, and so it may be that the flies used in the experiment

presented in this chapter had a slightly different critical period for young flies to have a beneficial effect. This could be attributed to differences between laboratory environments, methods and fly stocks, as these factors may interact with the mechanisms behind the beneficial effect. For example, the Dahomey fly strain was used in this study whereas Cho et al. (2021) used *Canton-S, Oregon-R* and w^{1118}. Differences in fly maintenance such as fly food could also contribute to this inconsistency. This highlights that future studies should first predetermine the particular critical period for this beneficial effect within a stock of flies before testing the involvement of other factors, such as the microbiome.

3.4.6 No Influence of Microbiome Transfer During Larval Development on Lifespan was Observed

Leech and McDowall et al. (2021) found a significant negative effect of adult exposure during larval development on lifespan. Although there was no significant effect of donor fly microbiome on lifespan, Figure 3.7 suggests a slight non-significant decline in lifespan when males were reared with the microbiome of grouped males. The effect may be too small to be identified with the power of this experiment and so would have to be confirmed by an assay with a larger sample size. The small effect could be attributed to the experimental process as this may not transfer the microbiome in the same way as adult presence does. Alternatively, the effect on lifespan may be driven by alternative cues from adult flies, such as cuticular hydrocarbons or mechano-sensory cues, which have been shown to be important in conferring lifespan responses in the adult environment (Ruan & Wu, 2008; Cho et al., 2021).

Consequently, an alternative way of testing for the microbiome's influence on this response was adopted, where adult flies were present in the larval environment, as well as antibiotics. However, the previously recorded decrease in lifespan with adult presence (McDowall, 2019) was still not observed.

In this assay, no overall lifespan effect of antibiotic treatment was detected. This may be as only a small amount of antibiotics was transferred for a small amount of time, either deposited on the food from the adults that had previously ingested antibiotics, or from a small drop of antibiotic food on the top of the control food.

3.4.7 The Lifespan Response Does Not Seem to be Driven by Dopamine Changes

The inhibition of dopamine did not alter the effect of grouping on lifespan. However, further tests are needed to determine whether 3-Iodo-L-tyrosine successfully inhibited dopamine biosynthesis within the host. HPLC could be utilised to measure dopamine levels in treated flies, as well as other neurotransmitters that may be of interest. Exploring other neurotransmitters such as serotonin, GABA or octopamine, also produced by microbiota (Tsavkelova et al., 2000; Barrett et al., 2012; Özoğul et al., 2012), holds promise for future studies. Furthermore, even if 3-Iodo-L-tyrosine was successful in inhibiting host tyrosine hydroxylase, dopamine may be produced by members of the microbiome (Özoğul et al., 2012), and it is unclear whether feeding of 3-Iodo-L-tyrosine to the fly in this manner inhibits the enzyme within bacteria.

SCFAs produced by microbiota also exert various effects on *Drosophila* biology, such as developmental rate and body-size (Shin et al., 2011). These have been shown to have anti-inflammatory properties and protect against disease in vertebrates (Gassull, 2006; Freund et al., 2010), presenting another promising candidate for future investigation.

3.4.8 Application to Other Taxa and Contexts

While insects and mammals exhibit numerous differences, the interactions occurring between the social environment, the microbiome and health seem to be present in both. Administration of testosterone, a hormone triggered by reproductive competition in many mammals (Blanchard et al., 1993; Barrett et al., 2002; Ostner et al., 2008), had a larger impact on the microbiome of female rats than switching to a high fat diet did (Moreno-Indias et al., 2016). Thus, although mechanisms may differ from insects, it seems that the microbiome's sensitivity to stress also exists in mammals. The link between dysbiosis and many diseases, including cardiovascular disease (Brown & Hazen, 2018), diabetes, obesity (Patterson et al., 2016) and colon cancer (Ian, 2009), demonstrates the mammalian link between the microbiome and health. Finally, the health effects of stress are numerous and well-studied in humans (reviewed in Dougall & Baum, 2001). All this evidence raises the possibility that the causal pathway linking stress, the microbiome, and health observed in *Drosophila* may be a universal phenomenon extending to other species. Confirming the existence of this causal pathway in other species could position *Drosophila* as an accessible model for probing these interactions, and elucidating potential underlying mechanisms.

While this study primarily examined the microbiome's role in response to increased social exposure, it is possible that in other animals, other social conditions could trigger a stress response. Social isolation, for instance, has been recognised as a stressor in some animals, associated with immune defects, increased stress hormones, and reduced longevity (reviewed in Cacioppo et al., 2011). These effects could potentially be explained by mechanisms associated with microbiome changes. Furthermore, responses to other stressors, independent of the social environment, could likewise be microbiome dependent.

The human microbiome can be readily altered through interventions like pre- and probiotics. For example, *Lactobacillus* or *Bifidobacterium* probiotics were shown to reduce infections and inflammation in elderly subjects as well as increase blood phagocyte activity (Matsumoto & Benno, 2006; Fukushima et al., 2007; Moro-García et al., 2013). Extending the findings from this chapter to human health presents a promising means for discovering treatments for stress-induced conditions. To harness this potential, we must first gain a comprehensive understanding of the effects of social stress on the microbiome and the subsequent implications for overall health. *Drosophila* offers an accessible model for initial exploration of these effects.

The results of this research could be useful in the context of livestock production. There is already evidence for interplay between stress, the microbiome and animal health. For example, in dairy cows, heat stress decreases productivity (Ray et al., 1992) and has been shown to alter the microbiome along with cytokine levels (Chen et al., 2018). Dysbiosis is a common occurrence in various livestock diseases (reviewed in Khalil et al., 2022). The research in this

chapter suggests that the impact of stress on the microbiome may offer an explanation for some health effects, and, therefore, warrants further attention. Moreover, antibiotics are routinely used in livestock farming to prevent infections, and increase growth. The rise of antibiotic resistance (Zalewska et al., 2021) has raised concerns about their usage, and recent research has shed light on other associated adverse health effects, occurring through dysbiosis. Antibiotic-induced dysbiosis led to a disease-related microbiome in calves (Dobrzanska et al., 2020), as well as exacerbated disease in goats, via inducing inflammatory immune responses (Tong et al., 2020). Exploring these connections further can contribute to better-informed practices and strategies in the livestock industry, ultimately benefitting animal well-being and productivity.

3.4.9 Conclusion

The findings presented in this chapter offer valuable insights into the interplay between social environments, microbiota and lifespan in *D. melanogaster*. Whereas previous research focusses on the antibiotic impacts on grouped flies, this chapter reveals a previously undiscovered adverse consequence of microbiome disruption on single fly lifespan. This underscores that the microbiome can impact the lifespan response to the social environment, with implications for the role of the microbiome in other taxa susceptible to similar stressors.

Furthermore, single flies seem to benefit from a healthy microbiome only at an early age as perturbation of the microbiome at a later age was not detrimental to single fly lifespan. Additionally, the novelty and age of the rival do not seem to have a large impact on lifespan, although this observation is subject

to significant variability and context dependency. This study revealed the importance of group size when investigating microbiome impacts. Antibiotics led to a decreased lifespan in single flies, had no effect on flies in groups of 10, but extended lifespan for flies in groups of 20. This discovery holds significance, as it underscores the need for careful consideration when selecting group size in future studies, given its potential to significantly influence lifespan outcomes.

Chapter 4
The Role of the Microbiome in Socially-Driven Changes in Behaviour

4.1 Introduction

Due to connections between the brain and the microbiome via the gut-brain axis (Figure 1.1), changes in the microbiome contribute to brain health and function, potentially leading to changes in behaviour (Bravo et al., 2011; Gareau et al., 2011; Bharwani et al., 2017; Boehme et al., 2021). Transferring the microbiome of young mice to ageing mice attenuated the age-associated decline in cognitive behaviour, involving memory and learning, along with the prevention of age-associated brain metabolome and transcriptome changes (Boehme et al., 2021). Germ-free mice had an impaired memory compared to conventional mice (Gareau et al., 2011). These effects could occur through the generation of microbial products, such as sodium butyrate, which has been observed to reverse age-associated impairments in memory and reduce DNA damage in rats (Garcez et al., 2018).

Studies demonstrated that consumption of prebiotics increased prosocial behaviour in mice (Burokas et al., 2017) and consumption of *Lactobacillus rhamnosu* reduced stress-induced cortisol release, as well as anxiety- and depression-linked behaviour (Bravo et al., 2011; Bharwani et al., 2017). Furthermore, in other studies using mice, antibiotics reduced anxiety-linked behaviour (Desbonnet et al., 2015; Mosaferi et al., 2021), and loss of the microbiome increased motor activity (Diaz Heijtz et al., 2011). The histone demethylase KDM5 loss of function *Drosophila* mutant had an altered

microbiome, gut barrier dysfunction, and impaired social behaviour (Chen et al., 2019). It was proposed that loss of KDM5 led to IMD pathway upregulation, triggering a dysbiotic microbiome, which could both, along with social impairments, be partially reversed by antibiotics or probiotics. Serotonin levels were also observed to be higher in KDM5 loss of function flies, providing a potential mechanism explaining how the altered microbiota were able to influence behaviour (Chen et al., 2019). Neurotransmitters such as serotonin and dopamine, have been associated with microbiome changes in multiple host species, due to their production by microbiota (Clarke et al., 2013; Gershon, 2013; Liu et al., 2016; Gao et al., 2018).

In many animals, the social environment plays a pivotal role in determining brain health and functioning. The social brain hypothesis theorises that increasing social group complexity drives the evolution of enhanced cognition (Dunbar, 2009). In honeybees, social isolation can inhibit growth in the olfactory learning centres of the brain (Maleszka et al., 2009). In humans, studies have linked social isolation with poorer overall cognitive functioning (DiNapoli et al., 2014; Hajek et al., 2020). Social environment effects can extend to behaviour, with social isolation triggering anxious behaviours in chickens in the open field (Suarez & Gallup, 1983), and affecting locomotor activity and anxiety-like behaviour in rodents (Reviewed in Fone & Porkess, 2008). On the other hand, social interactions, if antagonistic, can act as a stress. For example, aggressive social interactions can induce depressive and anxiety-like behaviours in rodents (Keeney & Hogg, 1999). As evidence also suggests that the microbiome is sensitive to the social environment (Bailey et al., 2011; Galley et al., 2014; Bharwani et al., 2016; Bharwani et al., 2017; Yang et al., 2017c), it is possible

that socially-driven behavioural responses are occurring due to changes in the microbiome.

4.1.1 Behavioural and Cognitive Responses to the Social Environment in *Drosophila*

An example of a behavioural response to the social environment is the increase in cognitive performance in *D. melanogaster* in response to rival males (Rouse et al., 2020). Rouse et al. (2020) observed that paired males exhibited improved performance in virgin-finding and olfactory assays, which tested learning and memory. This may be a response to increased mating competition, as improved cognitive abilities may act as an advantage in a competitive setting. *Drosophila* also display other behavioural and physiological changes when exposed to rival males, such as increasing mating duration and paternity share (Bretman et al., 2009). Therefore, it may be that sensing and reacting to the social environment to carry out these responses is cognitively challenging, and so stimulates cognitive improvement. This is supported by the lack of a cognitive improvement observed when pairs were female or heterospecific (Rouse et al., 2020), suggesting a link to the reproductive response, which would be unnecessary with other species and is absent in females. Additionally, it has been determined that the mating duration response depends on processes involved in learning and memory (Rouse et al., 2018). Moreover, research suggests fluctuating conditions stimulate increased cognitive investment (Sol, 2009), and social context can fluctuate considerably both spatially and temporally (Kasumovic et al., 2008). Other research has revealed that social grouping in *Drosophila* induces lateral ventral neuron synapse growth (Donlea et al., 2014), and increased larval density increases the size of the olfactory learning centres of the brain (Heisenberg et al.,

1995). Rouse et al. (2020) observed that paired flies display an increase in expression of cognition-related genes, *futsch* and *Neurexin-1* (*Nrx-1*). *futsch* is involved in the growth of synapses (Roos et al., 2000), whilst *Nrx-1* is involved in synaptic plasticity (Choi et al., 2011), with overexpression linked to an increase in sleep, which is important in developing synaptic circuitry (Larkin et al., 2015).

There is some research exploring the impact of the microbiome on cognition in *Drosophila*. Germ-free flies appear to have a reduced memory and learning ability (DeNieu et al., 2019; Silva et al., 2021), which can be rescued by *Acetobacter* and *Lactobacillus* (DeNieu et al., 2019). Additionally, another study demonstrated that *L. acidophilus* and *L. rhamnosus* supplementation improved *Drosophila* memory functions (Ho et al., 2024). This evidence gives support for a potential microbial involvement in the *Drosophila* socially-driven cognitive response.

Another example of a *D. melanogaster* behavioural response to the social environment is its mating duration extension (Bretman et al., 2009). Since mating is more competitive for males than it is for females in this species (Gromko et al., 1984), an extended mating duration is beneficial as it increases the transfer of seminal fluid proteins that are passed to the female after sperm transferal has finished (Wigby et al., 2009). Mating duration extension has been observed to result in a higher reproductive success, demonstrated by an increased paternity share (Bretman et al., 2009; Wigby et al., 2009; Bretman et al., 2013).

Additionally, there is some evidence that reproductive investment can be influenced by the microbiome. Gould et al. (2018) found that germ-free flies had

a reduced reproductive fitness. Morimoto et al. (2017) found an increase in offspring production when male *Drosophila* were monoassociated with *L. plantarum* compared to *A. pomorum*. They theorised that *A. pomorum* may cause a trade-off between the immune system and reproduction as it induces haemocyte circulation (Munier et al., 2002).

4.1.2 Linking Behavioural Responses to Lifespan Effects in *Drosophila*

Results from chapter three suggest that social stress in male *Drosophila* shapes a specific microbiome which determines a lifespan decrease, that can be replicated in single flies with antibiotics. However, perhaps this lifespan response does not occur through the direct effects of a detrimental microbiome. Instead, the response could be attributed to an increased allocation of resources into other assets, which is triggered by the microbiome. For example, could the microbiome be the reason behind the improvement in cognitive ability in response to rivals?

An association between the cognitive response and the lifespan response could exist in multiple ways, possibly through the involvement of the microbiome. Firstly, social exposure could stimulate cognitive pathways leading to increased investment into cognitive assets (Figure 4.1a). This increased investment may lead to a decreased investment into other life-lengthening assets, such as gut maintenance or the immune system. As well as directly affecting lifespan, decreased investment into these assets could also affect the microbiome, which could impact lifespan. If this were to be the case, using antibiotics to eliminate differences between single and grouped fly microbiomes, would affect the

lifespan response, but not the cognitive response, as this would occur upstream of the microbiome response.

Alternatively, the stress of the social environment may influence the microbiome via an increase in neurotransmitters (Davenport & Evans, 1984; Rauschenbach et al., 1993), numerous gene upregulation and reductions in glycogen, fatty acids and glucose (Sørensen et al., 2005) (Figure 4.1b). This change in the microbiome may be what triggers the cognitive response, which then results in a change in lifespan, as a trade-off leads to a decreased investment into life-lengthening assets. Studies showing how changes in the microbiome impact cognition (Schwaerzel et al., 2003; Schroll et al., 2006; Kim et al., 2007; Liu & Davis, 2009; Monier et al., 2018; Pavlowsky et al., 2018; DeNieu et al., 2019; Silva et al., 2021; Ho et al., 2024) support this explanation. If this were to be the case, using antibiotics to eliminate differences between single and grouped fly microbiome would affect both the lifespan and cognitive responses. Of course, it is also possible that these processes occur completely independently from each other (Figure 4.1c). Exposure to rivals may influence the microbiome and cognition, which both alter lifespan independently. If this were the case, removing any differences between the single and grouped fly microbiome would only affect the lifespan response. If antibiotics affect the cognitive response, but do not completely abolish the difference in cognitive ability between single and grouped flies, it may be that a mixture of both pathways in Figure 4.1a and b are occurring.

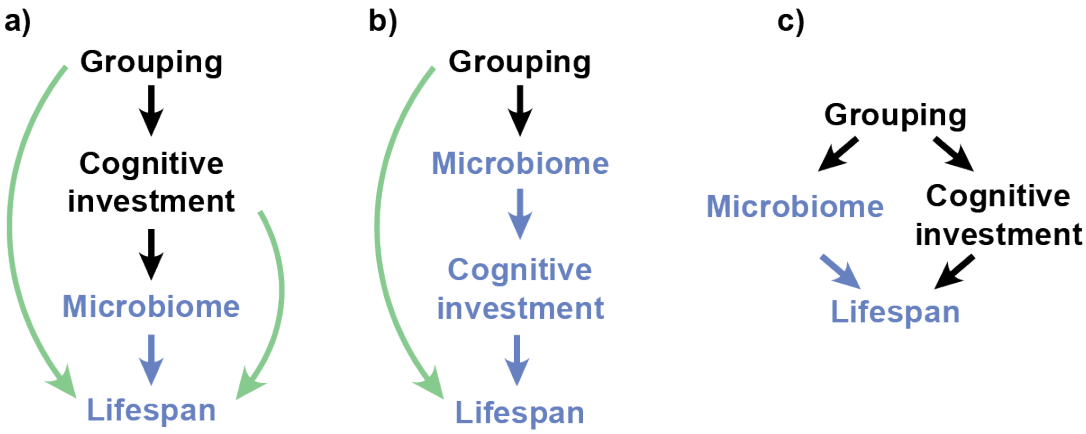

Figure 4.1 Potential Mechanisms Incorporating the Involvement of the Cognitive Response in the Causal Pathway Linking the Social Environment, Microbiome and Lifespan

Pathways explaining the cognitive response to the social environment and how it interacts with the microbiome and lifespan. In a) the cognitive response is triggered by the social environment independently of the microbiome but is responsible for alterations in the microbiome. In b) the cognitive response is triggered by socially-driven changes in the microbiome. In c) the microbiome and cognitive response alter lifespan independently. Green arrows represent other effects that could be simultaneously taking place. Factors in blue would be expected to be altered if antibiotics were applied.

These three mechanisms can also be applied to explain how other responses to the social environment could be linked to the microbiome and lifespan. For example, the *D. melanogaster* increase in mating duration in response to rival males (Bretman et al., 2009). This extension could trigger or be triggered by changes in the microbiome. There is evidence that this response is costly, as starved males lack it (Mason et al., 2016), and so it would seem likely that increased reproductive investment would lead to decreased investment in other assets. Moreover, a study has provided evidence suggesting that the association between reproduction and lifespan may be attributed to the microbiome, as microbiota from female flies, selected for late reproduction and

an extended lifespan, was able to lengthen the lifespan of control flies (Yakovleva et al., 2023). Therefore, it seems likely that the mating duration response trades with other assets which effect lifespan through the microbiome (Figure 4.1a) or more directly (Figure 4.1b). Research conducted by Morimoto et al. (2017) suggests that *A. pomorum* monoassociation can lead to reduced offspring production, potentially attributable to a trade-off between the immune system and reproduction, thus supporting the latter theory (Figure 4.1b).

4.1.3 Chapter Aims

To investigate the hypothesis that the microbiome is involved in triggering the *D. melanogaster* cognitive response to rival males (Rouse et al., 2020), I used antibiotics to alter the microbiome, in combination with manipulating the social environment. If the microbiome mediates the cognitive response, then antibiotic supplementation would remove the difference in cognitive ability between single and grouped flies, in a similar way as it did to longevity in Chapter 3. I measured the cognitive ability of learning and memory by testing the ability of males to find and court a virgin when presented with a choice of virgin or mated females (Hollis & Kawecki, 2014). As the transfer of seminal fluid proteins make mated female flies less receptive to courtship (Wolfner, 1997), and male flies can sense female mating status by their cuticular hydrocarbons (Friberg, 2006), males should learn and remember which females are mated, and focus courtship attempts on virgin females. I also tested whether the microbiome influenced the change in cognitive gene expression also observed by Rouse et al. (2020), establising whether this could explain any cognitive changes. To determine if the effects observed were due to the removal of the microbiome, and not to any side effects caused by the

antibiotics, I ascertained whether the antibiotic effects remained when the microbiome was allowed to recover. This was done in a previous study, which observed a reversal of antibiotic-driven gene expression changes if the antibiotics were stopped and the microbiome was restored by exposure to *Drosophila* microbial flora (Shukla et al., 2021).

The same treatment groups were also used to test the hypothesis that the microbiome is involved in triggering the mating extension displayed by flies exposed to rivals (Bretman et al., 2009). If the microbiome mediates the mating duration response, then antibiotic supplementation would remove the difference in mating duration between single and grouped flies. To see if any microbiome effect on mating duration was reflected in reproductive success, I also tested progeny produced by these treatment groups.

Leech et al. (2017) observed increased functional senescence in paired male *Drosophila* by measuring climbing ability. Rouse et al. (2020) observed less activity in single flies whereas Leech et al. (2017) observed a marginal increase in activity, though both did not explore aged flies. Additionally, activity may be a marker of sleep, and Li et al. (2021) found sleep to be increased in grouped flies. I hypothesised that paired flies would display an overall decrease in activity that was exacerbated with age. To test this, I measured activity and sitting on food, at several ages to see if social environment interacted with senescence. As activity may be a marker of the cost of increased investment into other assets, and if the microbiome is triggering this increased investment (Figure 4.1b), manipulating the microbiome may alter activity levels. Therefore, I also tested the effects of antibiotics on activity, in which other studies have presented contrasting results,

with antibiotics either increasing (Schretter et al., 2018; Silva et al., 2021) or having no effect (Selkrig et al., 2018; Jia et al., 2021; Grinberg et al., 2022).

As previous studies have observed mixed results when measuring the effect of familiarity of a rival on aggression (Hoffmann, 1990; Carazo et al., 2015; Le Page et al., 2017), I wanted to explore whether aggression differed between flies paired with a consistent rivals or a new rival. I also wanted to determine if any differences in activity between pairings could be attributed to a change in aggression. As aggression has been shown to be influenced by changes in the microbiome (Jia et al., 2021; Grinberg et al., 2022), it was of interest to explore whether antibiotics were able to alter aggressive interactions in these social groups. I also measured time spent in close proximity to a rival to test for the effect of the treatments on sociability.

To summarise, I measured the occurrence of activity, aggression, sitting on food and close proximity to a rival at different ages displayed by single flies, flies paired with a co-aged consistent rival, flies paired with a co-aged rival changed weekly, or flies paired with a young rival changed weekly, either fed control or antibiotic food. Overall, findings in this chapter will help determine why socially-driven lifespan changes are influenced by the microbiome, potentially implicating it as a modulator of trade-offs or stress responses within animals systems.

4.2 Methods

4.2.1 Virgin-Finding Learning Assays

To measure learning and memory, a virgin finding assay was used, which tests for the ability of males to find and court a virgin when presented with a choice of virgin or mated females (Hollis & Kawecki, 2014). Flies were kept singly or in groups of 10. To measure the effect of the microbiome, they were kept on control or antibiotic food and tested after 10 or 49 days (n = 80 per treatment, made up of four blocks of 20).

In this assay, each male fly was put into a vial with one virgin female and five females that had been mated within the last 24 hours. Behaviour was observed for five seconds every minute for 20 mins or until they began mating. For every scan, courtship behaviour towards a mated or virgin female was recorded as present or absent. If a mating occurred, this was recorded along with the female's previous mating status. A wing clip was given 24 hours previously to virgin females in half the trials and mated females in the other half. Trials were conducted on 12 vials at a time (three per treatment), where observation of vials were shared by two experimenters. Males and virgin females were replaced after every trial, and mated females every other trial. The order of vials in which the males and virgin females were added to alternated between trials.

4.2.2 *futsch* and *Nrx-1* Gene Expression

Flies were kept singly or in groups of 10, either on control food or food containing antibiotics, and flash frozen at either 10 (six biological repeats per treatment, each consisting of five pooled whole-body flies) or 49 days from eclosion (six biological

repeats per treatment, each consisting of 10 pooled whole-body flies). Cognitive genes of interest were chosen as they had previously been shown to be upregulated in males held with rival males (Rouse et al., 2020) (Table 4.1) (see section 2.3 for methods regarding qPCR analysis).

Table 4.1 Forward and Reverse Oligonucleotides for Cognitive Genes Including efficiency and pipetting accuracy (R^2) value.

Gene	Forward	Reverse	Efficiency (%)	R^2
futsch	ATTCCTCTCCT GGGACGTTT	GCATACTGGA TGAGCCGTTC	110.0	0.981
Nrx-1	GGAATATCAG TGCCCCAGTG	AACTCTGCTTG CCAGTTTCC	104.5	0.990

4.2.3 Gene Expression After Microbiome Recovery

Flies were kept singly or groups of 10, on control food or antibiotic food (six biological repeats per treatment, each consisting of five pooled whole-body flies). After three days, they were transferred to control food for three more days. Then, they were moved to food that had been contaminated for three days by flies of the same group size. Flies were snap frozen after four more days, when they were 10 days old. Gene expression of *futsch* (Table 4.1) was measured using RT-qPCR, to examine whether expression of antibiotic flies could be restored to control levels if flies recovered their microbiome.

4.2.4 Mating Duration

Flies were kept singly or in groups of 10, on control or antibiotic food and tested for mating duration extension after five days (n = 30 per treatment), using a common protocol (Bretman et al., 2009). Starting at 9 am, male flies were put into vials with a single five-day old virgin female and were checked every minute for two hours or until they finish mating, recording latency to mate and mating duration.

4.2.5 Progeny Counts

For the first experiment, flies were kept singly or in groups of 10 and on control or antibiotic food (n = 50 per treatment) and mated after five days. For the second experiment, only the effect of antibiotics was tested, but for 10 days, so flies were kept singly and on control or antibiotic food (n = 50 per treatment). To ensure mating, males were isolated with a single five-day old virgin female and were observed until mating occurred. Males were removed after mating and females removed after 24 hours. Progeny was frozen after two weeks to allow for counting.

4.2.6 Behavioural Assays

To measure the effect of social pairing and antibiotics on behaviour, males were either kept singly, with a rival male, a ‘novel’ rival, or a ‘young’ rival, and either on control or antibiotic food (n = 60 per treatment, made up of six blocks of 10). The ‘novel’ non-focal was changed weekly with a co-aged male and the ‘young’ non-focal was changed weekly with a three-day old male. Behavioural assays were

carried out in the vials the flies were housed in, in their social conditions, at an average of 3, 22, 50 and 80 days after eclosion.

Each assay started at 9 am, when vials were placed on a viewing platform and fly behaviour was scanned once every 10 minutes for 10 seconds for a total of eight scans and an hour and 20 minutes. For each scan, pre-defined behaviours were noted as present or absent. These behaviours were activity, being stationary on the food at the bottom of a vial, aggression (wing flicking or fencing with their partner (Chen et al., 2002)), or being in close proximity to their partner (within a body length, without aggressive interactions). Aggressive interactions were noted as aggression and activity. Flies could be active and in close proximity to their partner.

4.2.7 Statistical Analysis

4.2.7.1 Cognitive Assays

For cognitive data, courtship presence and absence counts were summed across all scans for each fly, so that the response variable used the cbind function in R to combine the number of scans where courtship was observed and the number of scans where courtship was not observed.

For the graph of overall courtship, percentage time spent courting was calculated by dividing the number of scans a male was observed courting with total number of scans, not including scans where males were mating.

To observe the effect on overall virgin courtship, the number of scans where virgin courtship was observed was compared against number of scans where it was not (males were courting mated females or not courting). For the graph, percentage time spent courting the virgin was calculated by dividing the number of scans a male was observed courting the virgin with total number of scans, not including scans where males were mating.

To observe the effect on accuracy of courtship, the number of scans where virgin courtship was observed was compared against number of scans where non-virgin courtship was observed. For the graph, percentage time spent courting accurately was calculated by dividing the number of scans a male was observed courting the virgin with number of scans a male was observed courting overall, not including scans where males were mating.

The cbind responses were used in GLMMs with binomial distributions, where age of fly, social environment and antibiotic treatment were used as fixed factors and time of day and block number were used as random factors. Models were run with and without random factors and Akaike information criterion (AIC) identified that for the effect on accuracy of courtship, the model with the best fit only included time of day as a random factor.

Mating was a binomial response (mated/not-mated) so a GLMM with a binomial distribution was used for the mating data from the virgin-finding assays. For overall mating, the response variable was whether the fly had mated or not. For both virgin mating measurements, the response variable was whether the fly had mated with a virgin or not, but for accuracy of matings, only flies that had

mated were used in analysis. Age of fly, social environment and antibiotic treatment were used as fixed factors. Models were run with and without random factors and AIC identified the models with the best fit only included time of day as a random factor. For the overall mating and virgin mating graphs, percentage of males mating was calculated by dividing the number of males mating (either with any female or with a virgin female), within the 20 minutes, by the total number of males. For the accuracy of mating graphs, the percentage of males mating a virgin female was divided by the total number of matings.

4.2.7.2 Gene Expression

Linear models (LMs) were used to explore gene expression differences between treatments, as model residuals were normal. Social environment and antibiotic treatment were used as fixed factors.

4.2.7.3 Reproductive Assays

A LM was used to explore mating duration differences between treatments. A GLM with an inverse-gaussian distribution was used to explore differences in mating latency between treatments. Antibiotic treatment and social environment were used as fixed factors. GLMs with quasipoisson errors were used to model progeny count data to account for overdispersion, with antibiotic treatment and social environment as fixed factors.

4.2.7.4 Behavioural Assays

Behavioural presence and absence counts were summed across all scans for each fly, so that the response variable used the cbind function in R to combine

the number of scans where a certain behaviour was observed and the number of scans where it was not. This was used in GLMMs using the glmmTMB package (Brooks et al., 2017) to account for zero inflation. Binomial errors were used for sitting on the food, aggression or proximity to a rival, but for activity, binomial structures were unstable and would not converge, so beta-binomial errors were fitted. Age of fly, social environment and antibiotic treatment were used as fixed factors, and vial ID and block number as random factors. As behaviour was measured within the flies' social environment, single flies could not display aggression or close proximity to rival and so these were excluded from analysis of these behaviours. For behavioural graphs, raw data means and standard errors were plotted for ease of viewing. The Spearman's correlation was calculated between aggression and activity and proximity to a rival and aggression.

4.3 Results

4.3.1 Virgin-Finding Assay

To test for the effect of the social environment and antibiotic treatment on cognitive ability, male flies were kept singly or in groups of 10, fed control food or food containing antibiotics, and their ability to identify, court, and mate a virgin female over four other mated females was tested.

4.3.1.1 Courtship

For overall courtship (any courtship, independent of female mating status), there was no significant three-way interaction between age, social environment and antibiotic treatment (χ^2 = 0.043, *df* = 1, *p* = 0.837) (Figure 4.2), and no significant interaction between antibiotic treatment and age of fly (χ^2 = 0.059, *df* = 1, *p* = 0.809). There was a significant interaction between age and social environment (χ^2 = 4.488, *df* = 1, *p* = 0.034), and a significant interaction between antibiotic treatment and social environment (χ^2 = 11.635, *df* = 1, *p* = 0.001).

Pairwise comparisons revealed that for 10-day old flies, social environment had no effect on overall courtship in control flies *(p* = 0.759), but single flies courted more than grouped flies in the antibiotic group *(p* < 0.0001, *d* = 0.245). For 49 days, single flies courted more than grouped flies in both control and antibiotic treatments, but the effect size in control flies was smaller (control: *p* = 0.005, *d* = 0.306; antibiotic: *p* < 0.0001, *d* = 0.638). In both ages, there was an increase in overall courtship for single flies on antibiotics compared to control food (10 days: *p* < 0.0001, *d* = 0.295; 49 days: *p* < 0.0001, *d* = 0.449), whereas

there was no effect of antibiotics on grouped flies (10 days: $p = 0.814$; 49 days: $p = 0.814$).

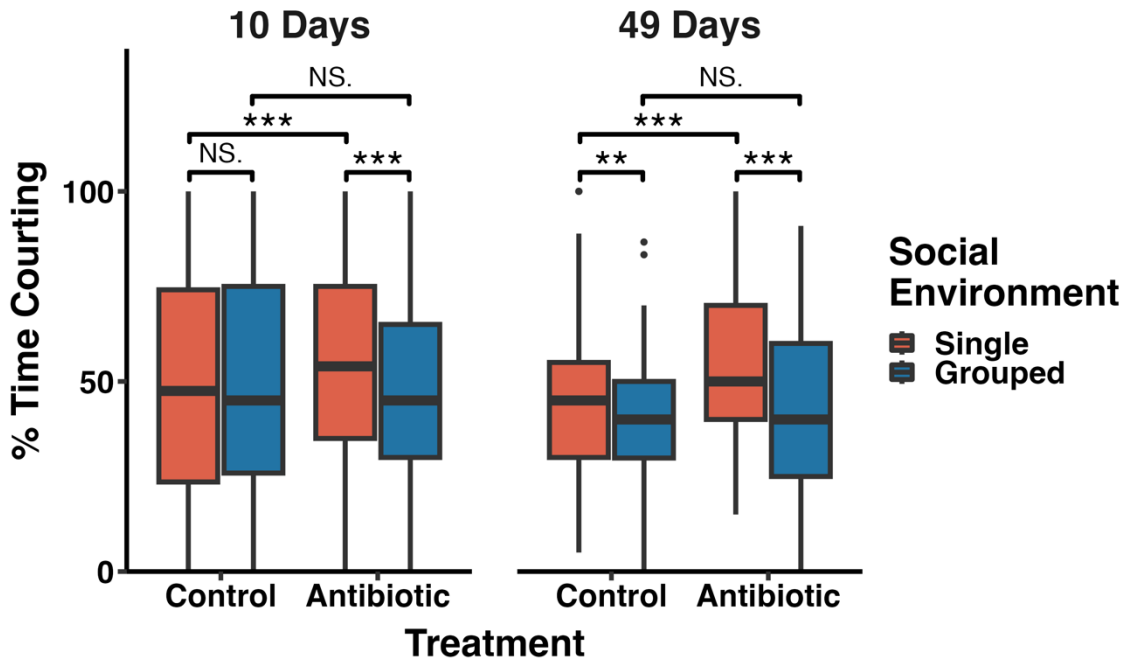

Figure 4.2 Effect of Antibiotics and Social Grouping on Overall Courtship
Males were kept on control food singly (10 day: n = 82; 49 day: n = 79), on control food in groups of 10 (10 day: n = 82; 49 day: n = 80), on antibiotic food singly (10 day: n = 84; 49 day: n = 77), or on antibiotic food in groups of 10 (10 day: n = 81; 49 day: n = 73). At 10 or 49 days they were tested in a virgin-finding assay, for their ability to identify and court a virgin female over four other mated females. This graph displays the percentage of total scans where a male is courting a female, irrespective of her previous mating status. Significance levels are indicated for pairwise comparisons (NS. = non-significant, * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$).

For proportion of virgin courtship out of overall scans, there was a significant three-way interaction between age, social environment, and antibiotic treatment ($\chi^2 = 10.798$, $df = 1$, $p = 0.001$) (Figure 4.3). In 10-day old flies, in both control and antibiotic treatments, the grouped flies courted the virgin more than the single flies (control: $p < 0.0001$, $d = 0.471$; antibiotic: $p < 0.0001$, $d = 0.304$). Both single and grouped flies increased virgin courting on antibiotic food

compared to control food (single: $p = 0.030$, $d = 0.348$; grouped: $p = 0.007$, $d = 0.256$). At 49 days old, flies showed the same pattern of grouped flies performing better than single flies in both control and antibiotic flies but the effect size was reduced in antibiotic flies (control: $p < 0.0001$, $d = 0.813$, antibiotic: $p = 0.046$, $d = 0.087$). This was likely driven by a change in the behaviour of single flies, which when on antibiotics, increased virgin courtship compared to the control flies ($p < 0.0001$, $d = 0.807$), whereas grouped flies did not ($p = 0.057$).

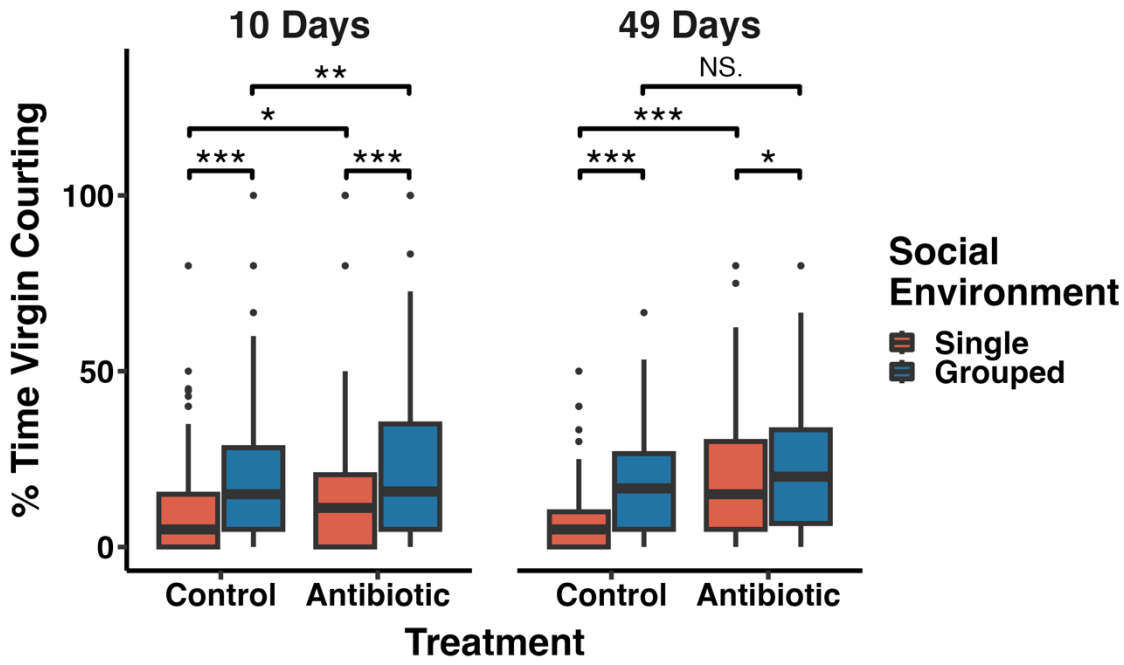

Figure 4.3 Effect of Antibiotics and Social Grouping on Courtship With a Virgin Female

Males were kept on control food singly (10 day: n = 82; 49 day: n = 79), on control food in groups of 10 (10 day: n = 82; 49 day: n = 80), on antibiotic food singly (10 day: n = 84; 49 day: n = 77), or on antibiotic food in groups of 10 (10 day: n = 81; 49 day: n = 73). At 10 or 49 days they were tested in a virgin-finding assay, for their ability to identify and court a virgin female over four other mated females. This graph displays the percentage of total scans where courtship is directed towards a virgin female. Significance levels are indicated for pairwise comparisons (NS. = non-significant, * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$).

For percentage of accurate courting, measured by proportion of virgin courtship out of overall courtship, there was a significant three-way interaction between age, social environment, and antibiotic treatment (χ^2 = 9.738, *df* = 1, *p* = 0.002) (Figure 4.4). For both ages and for both control and antibiotic treatments, grouped flies showed more courtship accuracy than single flies (all $p < 0.0001$), so effect sizes were compared. In 10-day old flies, the effect size of the social environment was similar in both control and antibiotic treatments (*d* control: 0.561; antibiotic: 0.623). In grouped flies, courtship accuracy was increased in antibiotic flies compared to control flies *(*$p < 0.004$, $d = 0.205$). But in single flies, there was no difference between control and antibiotic flies *(*$p = 0.410$, $d = 0.252$), however the effect size was larger. In 49-day old flies however, the effect size of the social environment was smaller in antibiotic flies than control flies (*d* = control: 1.000; antibiotic: 0.400). This difference in effect size at 49 days is likely driven by a larger effect of antibiotics on single fly courtship accuracy compared to grouped flies (single: $p < 0.0001$, $d = 0.805$; grouped: $p = 0.015$, $d = 0.140$).

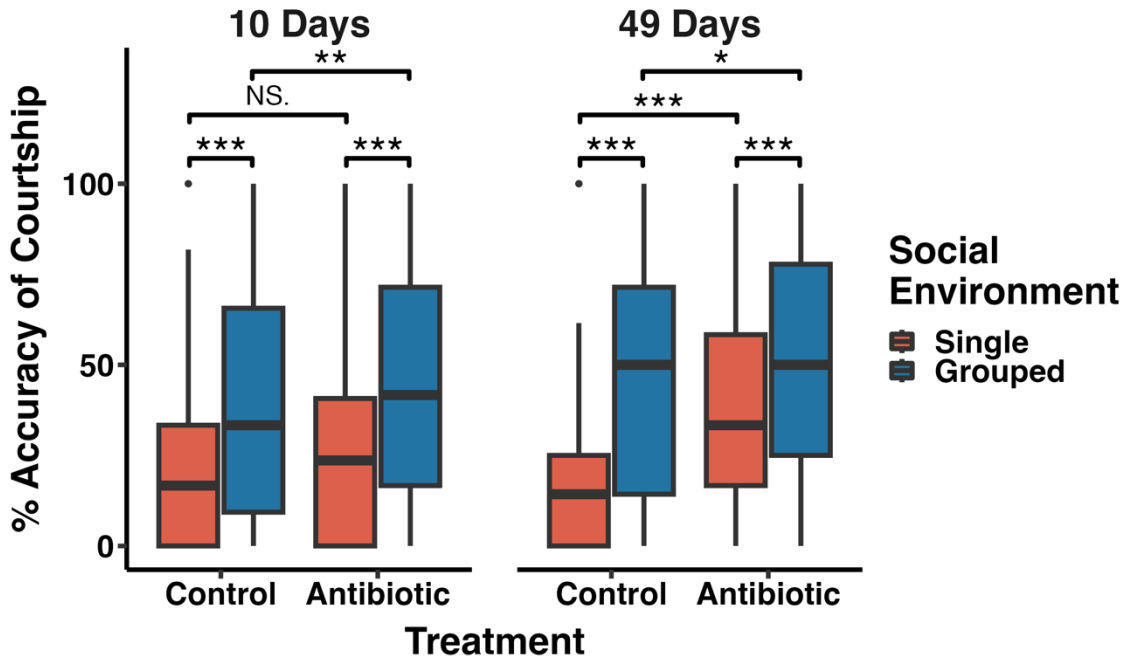

Figure 4.4 Effect of Antibiotics and Social Grouping on Accuracy of Courtship

Males were kept on control food singly (10 day: n = 82; 49 day: n = 79), on control food in groups of 10 (10 day: n = 82; 49 day: n = 80), on antibiotic food singly (10 day: n = 84; 49 day: n = 77), or on antibiotic food in groups of 10 (10 day: n = 81; 49 day: n = 73). At 10 or 49 days they were tested in a virgin-finding assay, for their ability to identify and court a virgin female over four other mated females. This graph displays the percentage of courtship directed towards a virgin female, out of total courtship. Significance levels are indicated for pairwise comparisons (NS. = non-significant, * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$).

4.3.1.2 Mating Success

For overall mating success, there were no significant interactions or effect of age (Table 4.2; Figure 4.5). There was an effect of social environment on overall mating success ($\chi^2 = 5.404$, df = 1, $p = 0.020$), with single flies mating more. There was also an effect of antibiotic treatment ($\chi^2 = 5.990$, $df = 1$, $p = 0.014$), with antibiotic treated flies mating more.

Table 4.2 Interactions and Effect Statistics for Overall Mating

Interactions and effects	χ^2 value	*df*	*p* value
Antibiotic treatment : Age : Social environment	1.276	2	0.528
Antibiotic treatment : Age	0.400	1	0.527
Age : Social environment	0.532	1	0.466
Antibiotic treatment : Social environment	1.633	1	0.201
Age	0.831	1	0.362
Social environment	5.404	1	0.020*
Antibiotic treatment	5.990	1	0.014*

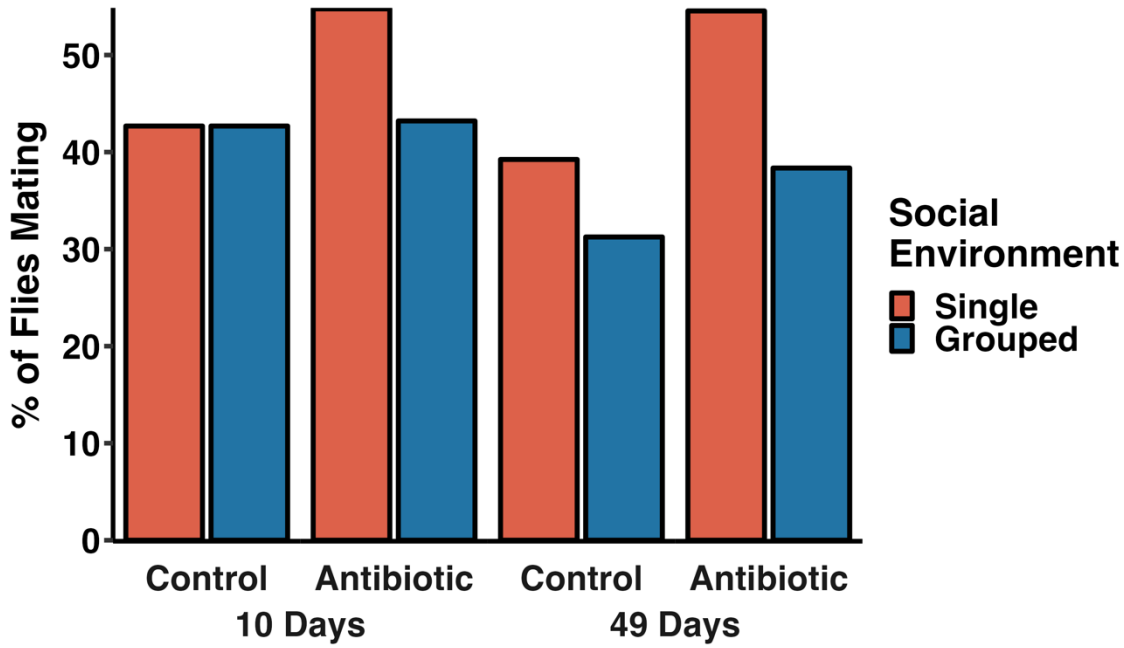

Figure 4.5 Effect of Antibiotics and Social Grouping on Overall Mating
Males were kept on control food singly (10 day: n = 82; 49 day: n = 79), on control food in groups of 10 (10 day: n = 82; 49 day: n = 80), on antibiotic food singly (10 day: n = 84; 49 day: n = 77), or on antibiotic food in groups of 10 (10 day: n = 81; 49 day: n = 73). At 10 or 49 days they were tested in a virgin-finding assay, for their ability to identify and court a virgin female over four other mated females. This displays the percentage of flies that mated within the 20 minute assay.

For proportion of flies virgin mating, there were no significant interactions or effect of age or social environment (Table 4.3; Figure 4.6). There was an effect of antibiotic treatment (χ^2 = 5.730, df = 1, p = 0.017), with antibiotic flies mating with virgin females more than control flies.

Table 4.3 Interactions and Effect Statistics for Virgin Mating

Interactions and effects	χ^2 value	*df*	*p* value
Antibiotic treatment : Age : Social environment	0.001	1	0.978
Antibiotic treatment : Age	0.132	1	0.716
Age : Social environment	0.590	1	0.443
Antibiotic Treatment : Social environment	1.674	1	0.196
Social environment	0.755	1	0.185
Age	2.129	1	0.145
Antibiotic treatment	5.730	1	0.017*

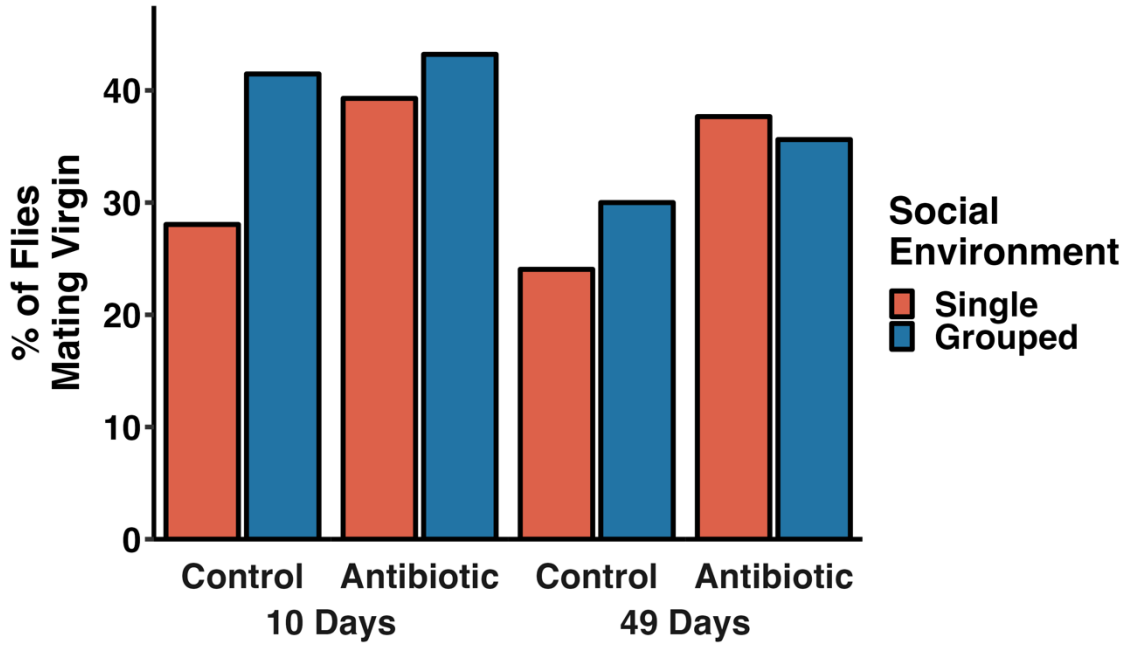

Figure 4.6 Effect of Antibiotics and Social Grouping on Mating with a Virgin Female

Males were kept on control food singly (10 day: n = 82; 49 day: n = 79), on control food in groups of 10 (10 day: n = 82; 49 day: n = 80), on antibiotic food singly (10 day: n = 84; 49 day: n = 77), or on antibiotic food in groups of 10 (10 day: n = 81; 49 day: n = 73). At 10 or 49 days they were tested in a virgin-finding assay, for their ability to identify and court a virgin female over four other mated females. This graph displays the percentage of flies that mated with a virgin female within the 20 minute assay.

For proportion of matings that were with virgin females, there were no significant interactions or effect of age or antibiotic treatment (Table 2.1; Figure 4.7). There was an effect of social environment ($\chi^2 = 43.875$, $df = 1$, $p < 0.0001$), with grouped fly virgin matings occurring more often than in single flies.

Table 4.4 Interactions and Effect Statistics for Virgin Mating

Interactions and effects	χ^2 value	*df*	*p* value
Antibiotic treatment : Age : Social environment	1.597	1	0.206
Antibiotic treatment : Social environment	0.028	1	0.868
Age : Antibiotic treatment	0.069	1	0.794
Age : Social environment	1.241	1	0.264
Age	0.724	1	0.395
Antibiotic treatment	0.741	1	0.389
Social environment	43.875	1	< 0.0001*

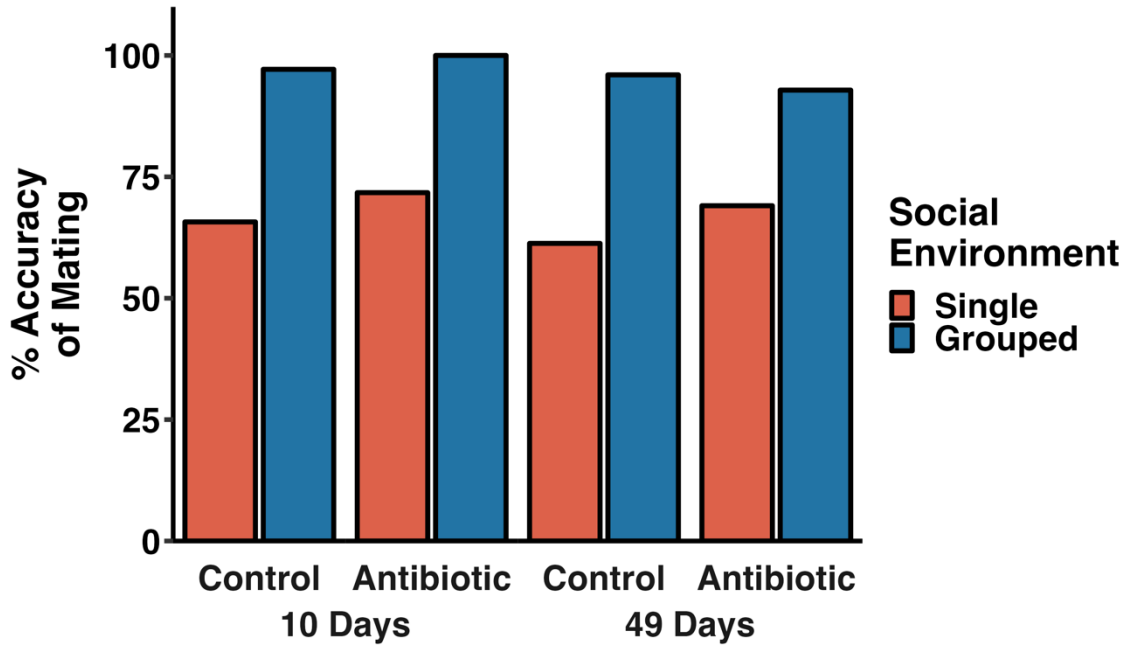

Figure 4.7 Effect of Antibiotics and Social Grouping on Accuracy of Mating

Males were kept on control food singly (10 day: n = 82; 49 day: n = 79), on control food in groups of 10 (10 day: n = 82; 49 day: n = 80), on antibiotic food singly (10 day: n = 84; 49 day: n = 77), or on antibiotic food in groups of 10 (10 day: n = 81; 49 day: n = 73). At 10 or 49 days they were tested in a virgin-finding assay, for their ability to identify and court a virgin female over four other mated females. This displays the percentage of matings that were with a virgin female within the 20 minute assay.

4.3.2 Socially-Dependent Gene Expression

In order to test for the effect of antibiotic treatment on socially-dependent gene expression, males were kept singly or in groups of 10, either on control food or food containing antibiotics, and gene expression measured at 10 or 49 days from eclosion.

4.3.2.1 futsch

For *futsch* expression at 10 days, there was a significant interaction between antibiotic treatment and social environment ($F_{1,21}$ = 4.852, p = 0.040) (Figure 4.8). Pairwise comparisons revealed that in the control group, grouped flies had a higher expression than single flies *(*p = 0.007, d = 1.955) whereas in the antibiotic group, there was no significant difference between social treatments *(*p = 0.913). Moreover, antibiotic treatment had no effect on *futsch* expression in both social treatments per se, however the effect sizes were large, with Figure 4.8 showing a non-significant increase in single fly expression when on antibiotics (p = 0.157, d = 0.975), and a non-significant decrease in grouped fly expression when on antibiotics (p = 0.116, d = 0.608).

For *futsch* expression at 49 days, there was a marginally non-significant interaction between antibiotic treatment and social environment ($F_{1,19}$ = 4.063, p = 0.059) (Figure 4.8). There was no significant effect of antibiotic treatment ($F_{1,19}$ = 0.501, p = 0.488), or social environment ($F_{1,20}$ = 0.834, p = 0.372) on gene expression.

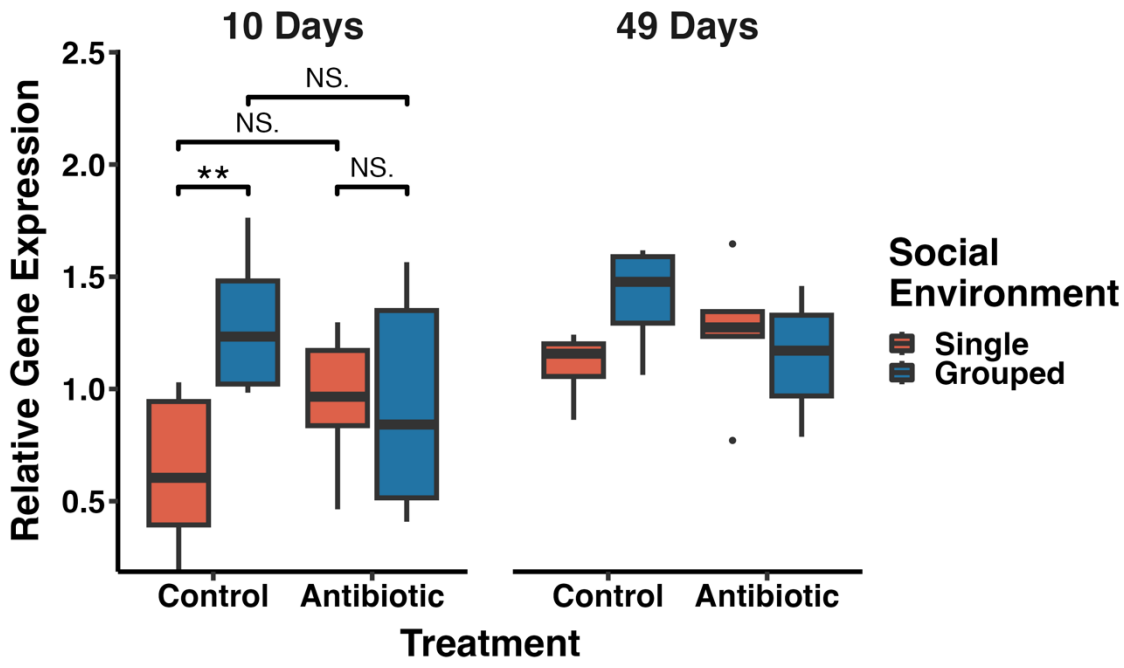

Figure 4.8 Effect of Antibiotics and Social Grouping on *futsch* Gene Expression

Relative expression of *futsch* in males kept singly or in groups of 10, on control food or food containing antibiotics, and snap-frozen at 10 or 49 days of age (see Table 2.3 for sample sizes after outlier removal). Significance levels are indicated for pairwise comparisons (NS. = non-significant, * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$).

4.3.2.2 Nrx-1

For *Nrx-1* expression at 10 days, there was no significant interaction between antibiotic treatment and social environment ($F_{1,19} = 0.651$, $p = 0.431$) (Figure 4.9). There was no significant effect of antibiotic treatment ($F_{1,19} = 0.462$, $p = 0.505$), or social environment ($F_{1,20} = 2.376$, $p = 0.139$) on gene expression.

For *Nrx-1* expression at 49 days, there was a significant interaction between antibiotic treatment and social environment ($F_{1,20} = 4.834$, $p = 0.041$) (Figure 4.9). Pairwise comparisons revealed that in the control group, grouped flies had a higher expression than single flies ($p = 0.024$, $d = 1.866$) whereas in

the antibiotic group, there was no significant difference between social treatments (*p* = 0.547). Moreover, antibiotic treatment had an effect on *Nrx-1* expression in grouped flies (*p* = 0.005, *d* = 2.981) but not in single flies (*p* = 0.915)

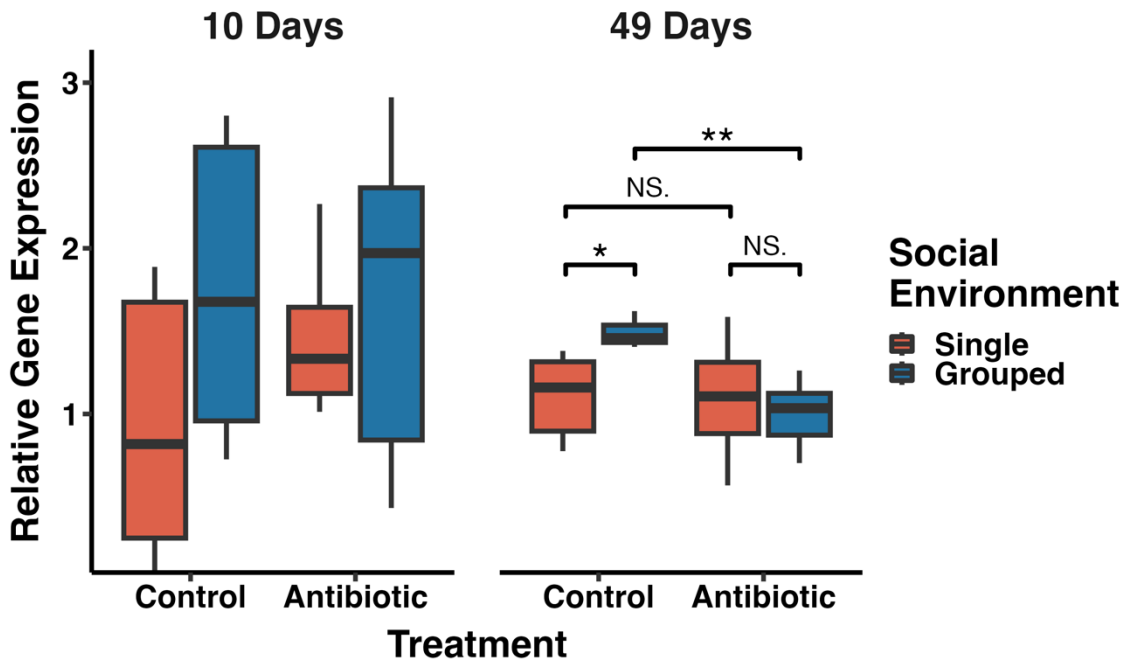

Figure 4.9 Effect of Antibiotics and Social Grouping on *Nrx-1* Gene Expression

Relative expression of *Nrx-1* in males kept singly or in groups of 10, on control food or food containing antibiotics and snap-frozen at 10 or 49 days of age (see Table 2.3 for sample sizes after outlier removal). Significance levels are indicated for pairwise comparisons (NS. = non-significant, * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$).

4.3.3 Gene Expression After Microbiome Recovery

In order to test if *futsch* gene expression changes were reversed when antibiotics were removed and the microbiome was allowed to recover, flies were first kept on antibiotic food and then moved to food that had been contaminated with other fly microbial flora. There was no significant interactions or effects on gene expression (full vs. null model: $F_{3,22} = 0.467$, $p = 0.709$) (Figure 4.10).

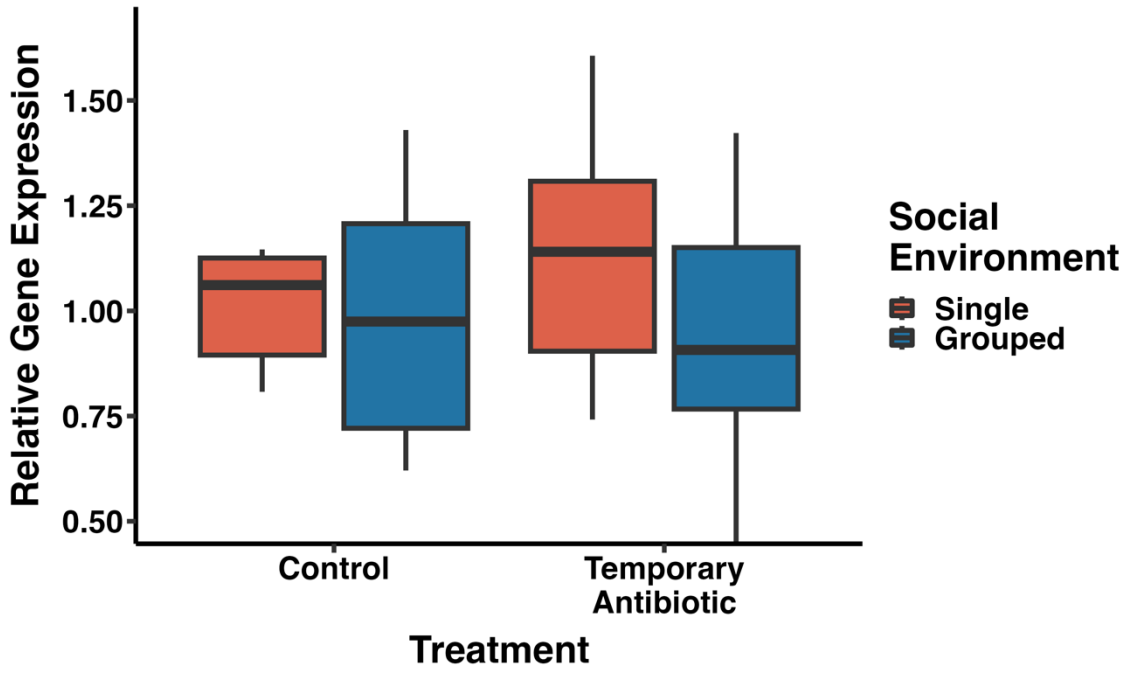

Figure 4.10 Effect of Microbiome Recovery on *futsch* Gene Expression
Relative expression of *futsch* in 10-day old males kept singly or in groups of 10. Flies were kept either on control food or food containing antibiotics (temporary antibiotic) for three days, moved to control food for three days, then moved to food that had been contaminated by control flies for four more days (see Table 2.3 for sample sizes after outlier removal).

4.3.4 Mating Duration

To test for the effect of antibiotic treatment on extended mating duration, male flies were kept singly or in groups with of 10, fed control food or food containing antibiotics, and their mating duration and latency to mate tested.

There was no interaction between antibiotic treatment and social environment on mating duration ($F_{1,105}$ = 0.234, p = 0.630), and a marginally non-significant effect of antibiotic treatment ($F_{1,105}$ = 3.756, p = 0.055) (Figure 4.11A). There was an effect of social environment, with an increased mating duration in grouped flies ($F_{1,107}$ = 42.876, p < 0.0001, d = 1.268). There was no interaction

or effect of antibiotic treatment or social environment on mating latency (full vs. null model: $\chi^2 = 0.078$, $df = 3$, $p = 0.721$) (Figure 4.11B).

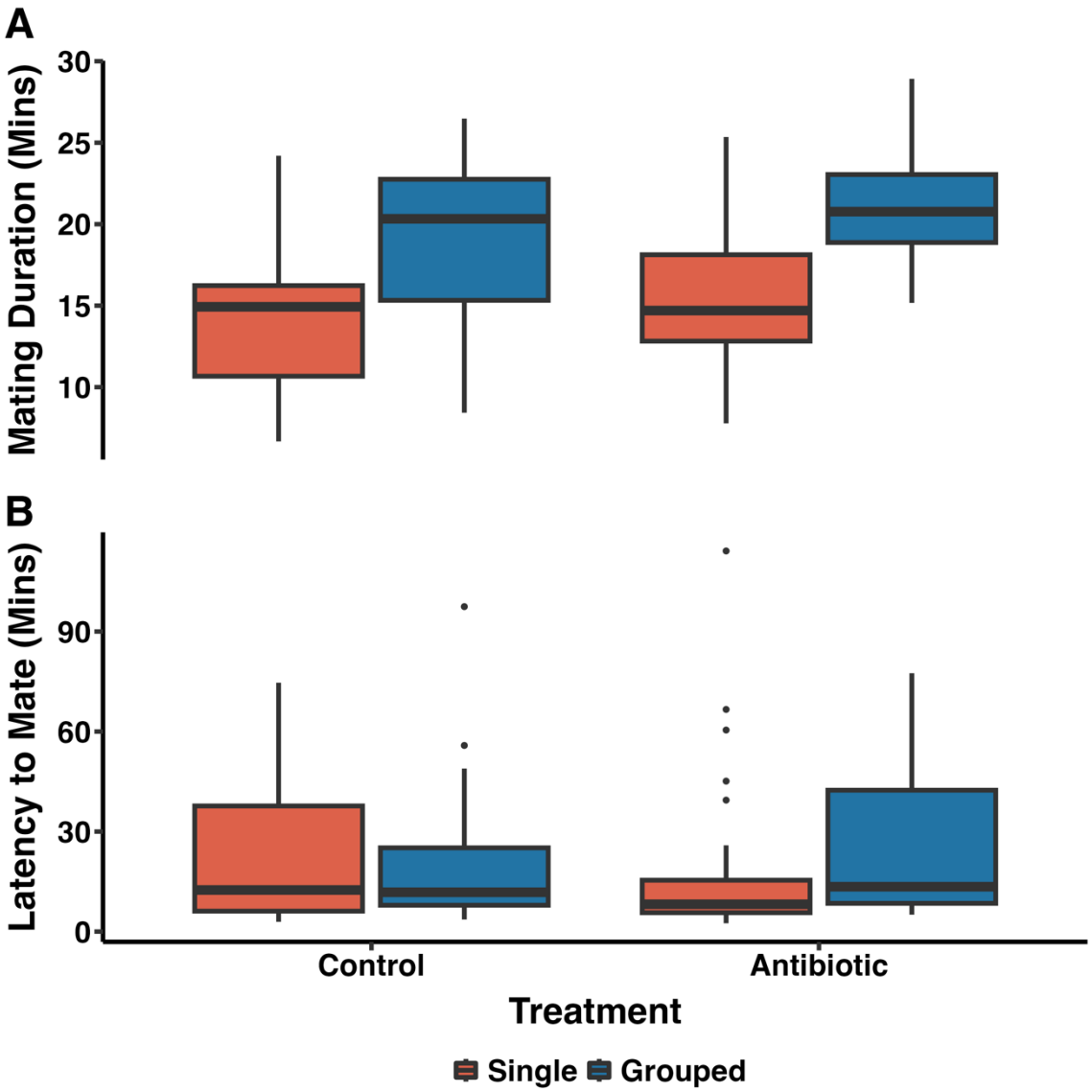

Figure 4.11 Effect of Antibiotics and Social Grouping on Mating Duration and Latency

A) Mating duration and B) latency to mate was measured after males were kept singly on control food (n = 29), in groups of 10 on control food (n = 20), singly on antibiotic food (n = 31), or in groups of 10 on antibiotic food (n = 28) for five days.

4.3.5 Progeny Counts

To test for the effect of antibiotic treatment and social environment on progeny counts, male flies were kept singly or in groups of 10, fed control food or antibiotic food, and mated after five days. There was no interaction or effect of social environment or antibiotic treatment on progeny counts at five days (full vs. null model: $F_{1,189}$ = 0.277, *df* = 3, *p* = 0.842) (Figure 4.12). Because there was no effect of grouping, single flies were kept on control or antibiotic food for 10 days to see if there was an effect of antibiotics after a longer period of time. There was no effect of antibiotic treatment on progeny counts at this age ($F_{1,94}$ = 0.208, *p* = 0.649).

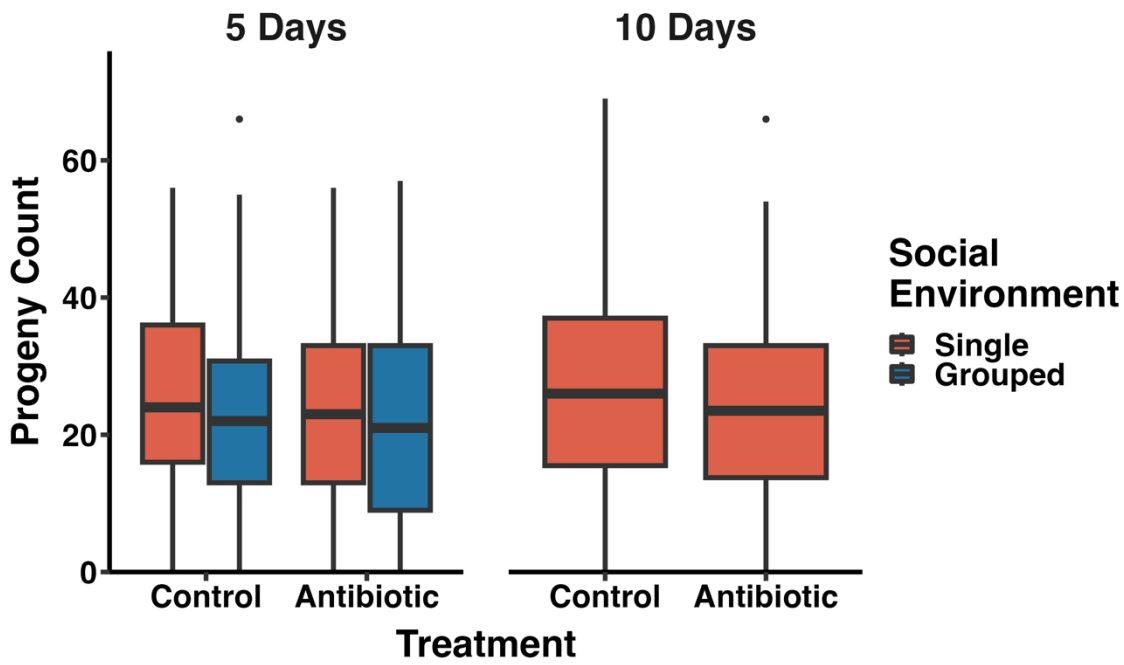

Figure 4.12 Effect of Antibiotics and Grouping on Progeny Counts
For 5 days, males were kept singly on control food (n = 49), groups of 10 on control food (n = 46), singly on antibiotic food (n = 49), or groups of 10 on antibiotic food (n = 49), before mating. For 10 days, males were kept singly on control food (n = 48) or singly on antibiotic food (n = 48), before mating. Mated females were left in vials for 24 hours and progeny counted after two weeks.

4.3.6 Behavioural Analysis

To test for the effect of the social environment and antibiotic treatment on behaviour other than mating, male flies were kept singly, paired with a co-aged consistent rival, paired with a co-aged rival changed weekly, or paired with a young rival changed weekly, and fed control food or food containing antibiotics. Behavioural assays were carried out at an average of 3, 22, 50 and 80 days after eclosion.

4.3.6.1 Activity

For activity, there was no three-way interaction between age, social environment and antibiotic treatment (χ^2 = 7.339 , *df* = 9, *p* = 0.602) (Figure 4.13). There was no interaction between antibiotic treatment and age (χ^2 = 3.192 , *df* = 3, *p* = 0.363), or antibiotic treatment and social environment (χ^2 = 3.882, *df* = 3, *p* = 0.275). There was a significant interaction between social environment and age (χ^2 = 58.249, *df* = 9, *p* < 0.0001), and a significant decrease in activity with antibiotic treatment (χ^2 = 6.191, *df* = 1, *p* = 0.0128).

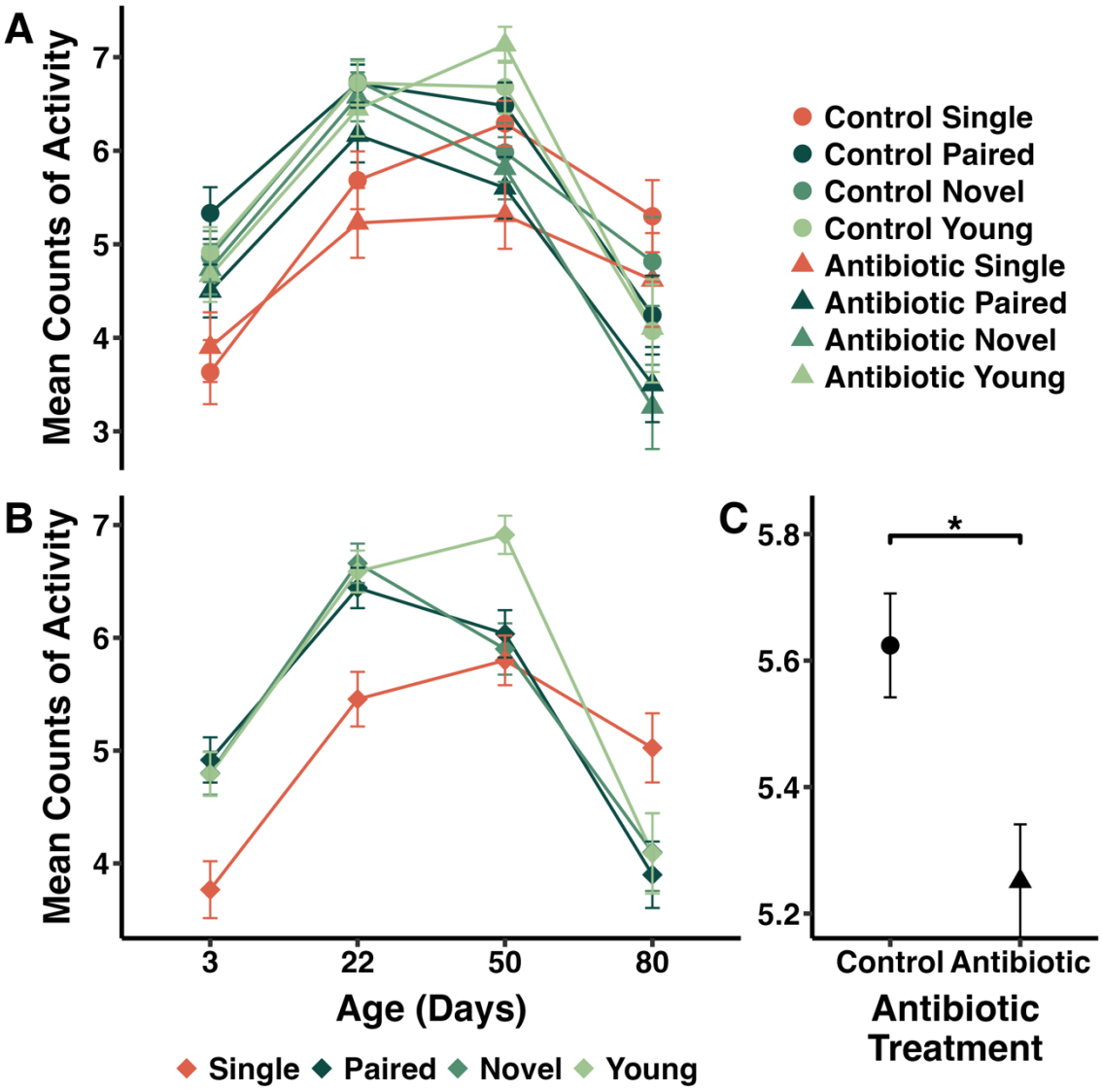

Figure 4.13 Effect of Antibiotics and Pairing on Activity

Counts of activity after being scanned for 10 seconds once every 10 minutes for a total of eight scans. Males were kept singly, paired with a co-aged consistent rival, paired with a co-aged rival changed weekly, or paired with a young rival changed weekly, and fed control food or food containing antibiotics. Behavioural scans were done at 3, 22, 50 or 80 days post eclosion. See Table 4.7 for sample sizes. A) displays all treatments on one graph, but for ease of viewing, B) presents the interaction between social environment and age, and C) shows the effect of antibiotics. Significance levels are indicated (NS. = non-significant, * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$).

The interaction between social treatment and age was driven by an increase in activity from 3 to 22 days in all social treatments, an increase from 22

to 50 days in single flies and flies kept with young rivals, but a decrease from 22 to 50 days in flies paired with a co-aged rivals (Table 4.5). Activity declined in all social treatments from 50 to 80 days, except single flies who's activity stayed the same.

Table 4.5 Pairwise Comparisons of Activity Between Ages

Comparisons between ages for each social environment produced *p* values calculated by emmeans with Tukey adjustment. Asterisks signify significant differences.

Social Environment	Age (Days) Comparisons	*p* value
Single	3 - 22	< 0.0001*
	22 - 50	< 0.0001*
	50 - 80	0.377
Paired	3 - 22	< 0.0001*
	22 - 50	< 0.0001*
	50 - 80	< 0.0001*
Novel	3 - 22	< 0.0001*
	22 - 50	< 0.0001*
	50 - 80	< 0.0001*
Young	3 - 22	< 0.0001*
	22 - 50	< 0.0001*
	50 - 80	0.00013*

At 3 and 22 days, paired flies were more active compared to single flies (Table 4.6). At 50 days, flies paired with young rivals were more active compared to the other social groups, but at 80 days, single flies were more active than paired flies.

Table 4.6 Pairwise Comparisons of Activity Between Social Groups

Comparisons between social groups for each age produced *p* values calculated by emmeans with Tukey adjustment. Asterisks signify significant differences.

Age (Days)	Social Comparisons	*p* value
3	Single - paired	0.002*
	Single - novel	0.0001*
	Single - young	0.0001*
	Paired - novel	0.804
	Paired - young	0.821
	Novel - young	1
22	Single - paired	0.004*
	Single - novel	0.017*
	Single - young	0.009*
	Paired - novel	0.962
	Paired - young	0.992
	Novel - young	0.997
50	Single - paired	0.640
	Single - novel	0.901
	Single - young	0.0001*
	Paired - novel	0.968
	Paired - young	0.009*
	Novel - young	0.003*
80	Single - paired	0.006*
	Single - novel	0.056*
	Single - young	0.044*
	Paired - novel	0.951
	Paired - young	0.955
	Novel - young	1

Table 4.7 Sample Sizes of Behaviour Treatment Groups

Sample sizes of behaviour assay treatment groups at different stages of the experiment. Sample sizes decreased through the experiment due to fly deaths.

Day of the experiment	Treatment	Treatment	Sample size
3	Control	Single	60
		Paired	60
		Novel	60
		Young	60
	Antibiotic	Single	60
		Paired	60
		Novel	60
		Young	60
22	Control	Single	57
		Paired	60
		Novel	59
		Young	58
	Antibiotic	Single	57
		Paired	60
		Novel	59
		Young	56
50	Control	Single	55
		Paired	56
		Novel	53
		Young	50
	Antibiotic	Single	55
		Paired	58
		Novel	48

		Young	53
80	Control	Single	50
		Paired	37
		Novel	27
		Young	27
	Antibiotic	Single	34
		Paired	32
		Novel	23
		Young	29

4.3.6.2 Sitting on Food

For sitting on food, there was a significant three-way interaction between age, social environment and antibiotic treatment ($\chi^2 = 19.868$, $df = 9$, $p = 0.019$) (Figure 4.14).

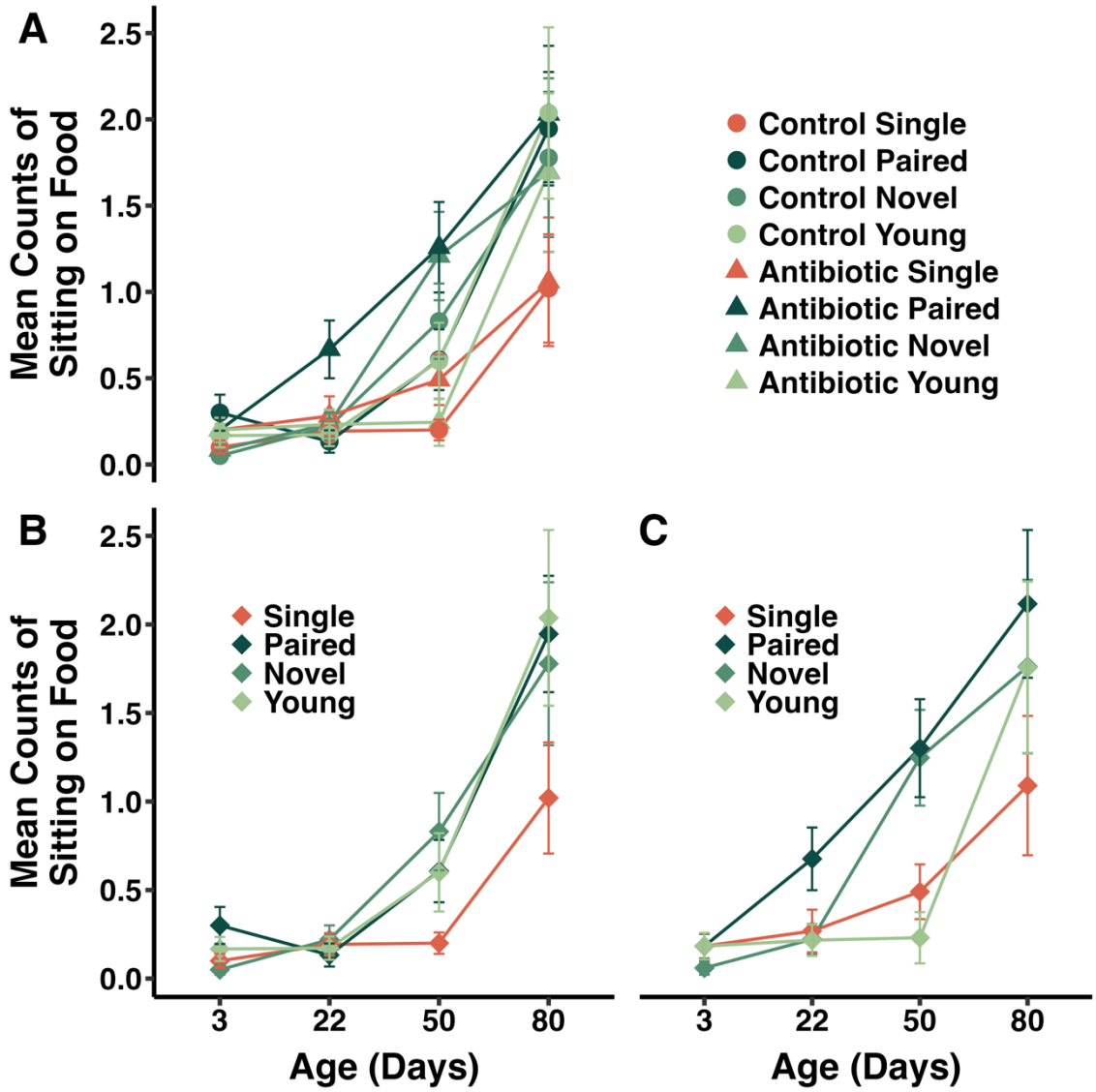

Figure 4.14 Effect of Antibiotics and Pairing on Sitting on Food

Counts of sitting on food after being scanned for 10 seconds once every 10 minutes for a total of eight scans. Males were kept singly, paired with a co-aged consistent rival, paired with a co-aged rival changed weekly, or paired with a young rival changed weekly, and fed control food or food containing antibiotics. Behavioural scans were done at 3, 22, 50 or 80 days post eclosion. See Table 4.7 for sample sizes. A) displays all treatments on one graph, but for ease of viewing, B) presents the effect of the social environment and age on control flies, and C) presents the effect on antibiotic flies.

Across all flies there was a general increase in sitting on food at older ages, but the exact pattern varied depending on social environment and antibiotic treatment. On control food, there was no difference in sitting on food between 3

and 22 days in any social environment (Table 4.8). Sitting on food increased between 22 and 50 days for ‘paired’ pairs or ‘novel’ pairs. Sitting on food then increased between 50 and 80 days for all flies, except ‘novel’ pairs. The only differences between social groups at each age in control flies was between single and ‘novel’ pairs at age 50 days *(p* = 0.030).

On antibiotic food, single flies slowly increased their time sitting on food throughout with age, so that there was no significant differences between adjacent time points but a difference between age 3 and 80 days *(p* = 2 x 10^{-4}). ‘Paired’ flies increased their time sitting on food from 3 to 22 days of age and 22 to 50 days, but this did not increase after 50 days (Table 4.8). ‘Novel’ pairs only increased their time sitting on food from 22 to 50 days of age. ‘Young’ pairs only increased between 50 and 80 days. The only difference between social groups at each age in antibiotic flies was between ‘young’ pairs and ‘paired’ or ‘novel’ pairs at 50 days of age (both $p < 0.0001$).

Antibiotic flies spent more time sitting on food than control flies, at 22 days in ‘paired’ flies *(p* = 0.001) and 50 days in single *(p* = 0.057), ‘paired’ *(p* = 0.018) and ‘young’ flies (p=0.028).

Table 4.8 Pairwise Comparisons of Sitting on Food Between Ages

Comparisons between ages for each treatment produced *p* values calculated by emmeans with Tukey adjustment. Asterisks signify significant differences.

Antibiotic Treatment	Social Environment	Age (Days) Comparisons	*p* value
Control	Single	3 - 22	0.612
		22 - 50	0.973
		50 - 80	< 0.0001*
	Paired	3 - 22	0.317
		22 - 50	2 x 10-4*
		50 - 80	0.001
	Novel	3 - 22	0.073
		22 - 50	0.014*
		50 - 80	0.103
	Young	3 - 22	0.999
		22 - 50	0.060
		50 - 80	< 0.0001*
Antibiotic	Single	3 - 22	0.942
		22 - 50	0.139
		50 - 80	0.121
	Paired	3 - 22	0.010*
		22 - 50	0.001*
		50 - 80	0.898
	Novel	3 - 22	0.310
		22 - 50	< 0.0001*
		50 - 80	0.657
	Young	3 - 22	0.970
		22 - 50	0.824
		50 - 80	< 0.0001*

4.3.6.3 Aggression

The only factor to significantly affect aggression was age (Table 4.9; Figure 4.15). Aggression increased with age from 3 to 22 days *(p* < 0.0001) and then decreased from 50 to 80 days *(p* < 0.0001).

Table 4.9 Interactions and Effect Statistics for Aggression

Interactions and effects	χ^2 value	*df*	*p* value
Antibiotic treatment : Age : Social environment	2.327	6	0.887
Antibiotic treatment : Age	3.722	3	0.293
Antibiotic treatment : Social environment	2.703	2	0.259
Age : Social environment	8.793	6	0.1856
Social environment	0.226	2	0.893
Antibiotic treatment	3.284	1	0.070
Age	357.29	3	<0.0001*

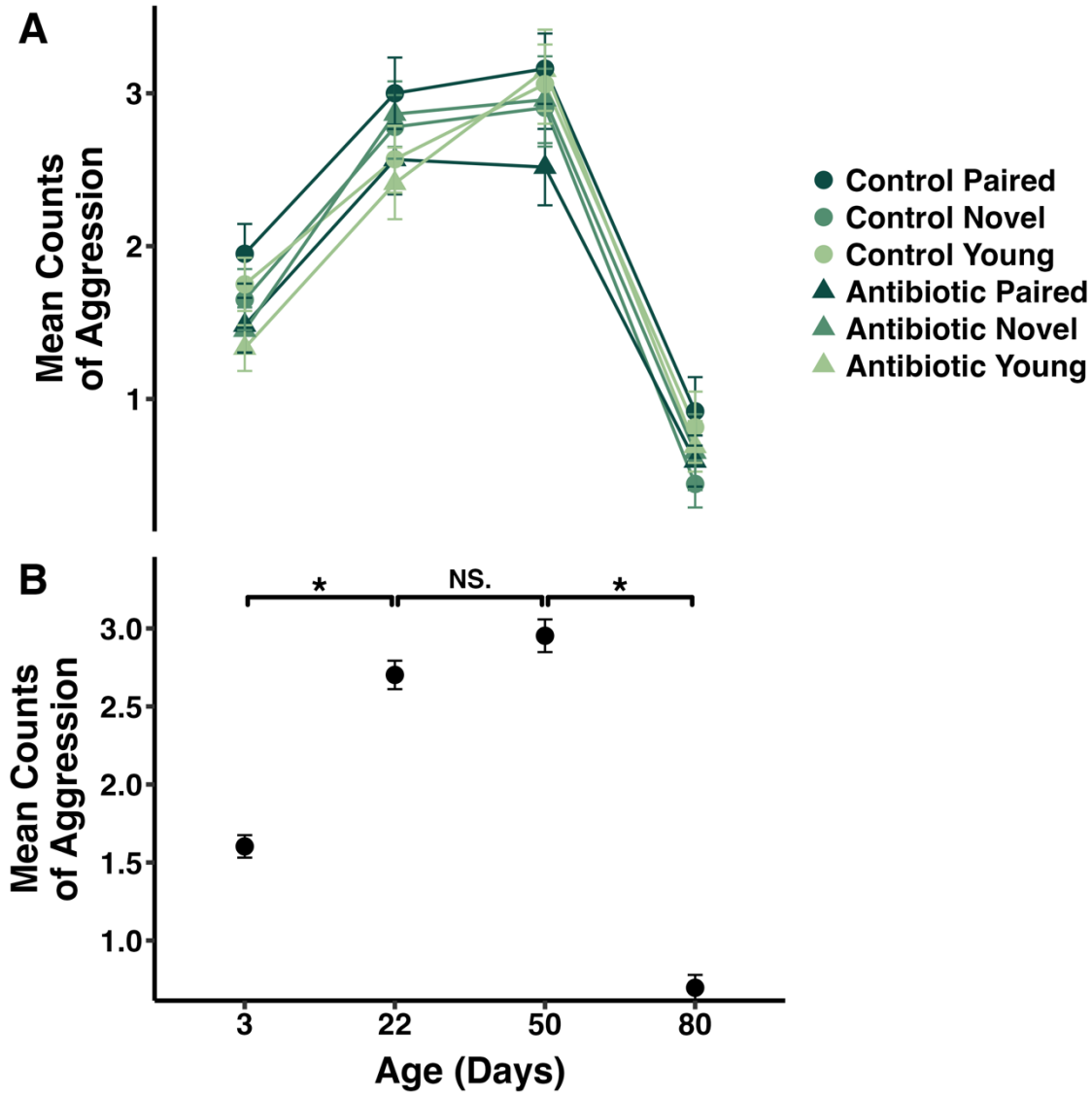

Figure 4.15 Effect of Antibiotics and Pairing on Aggression

Counts of aggression after being scanned for 10 seconds once every 10 minutes for a total of eight scans. Males were paired with a co-aged consistent rival, paired with a co-aged rival changed weekly, or paired with a young rival changed weekly, and fed control food or food containing antibiotics. Behavioural scans were done at 3, 22, 50 or 80 days post eclosion. See Table 4.7 for sample sizes. A) shows all treatments on one graph, but for ease of viewing, B) shows the effect of the only significant factor, age. Significance levels are indicated for pairwise comparisons (NS. = non-significant, * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$).

When comparing aggression and activity, a moderate positive correlation was observed ($R = 0.44$, $p < 0.0001$) (Figure 4.16).

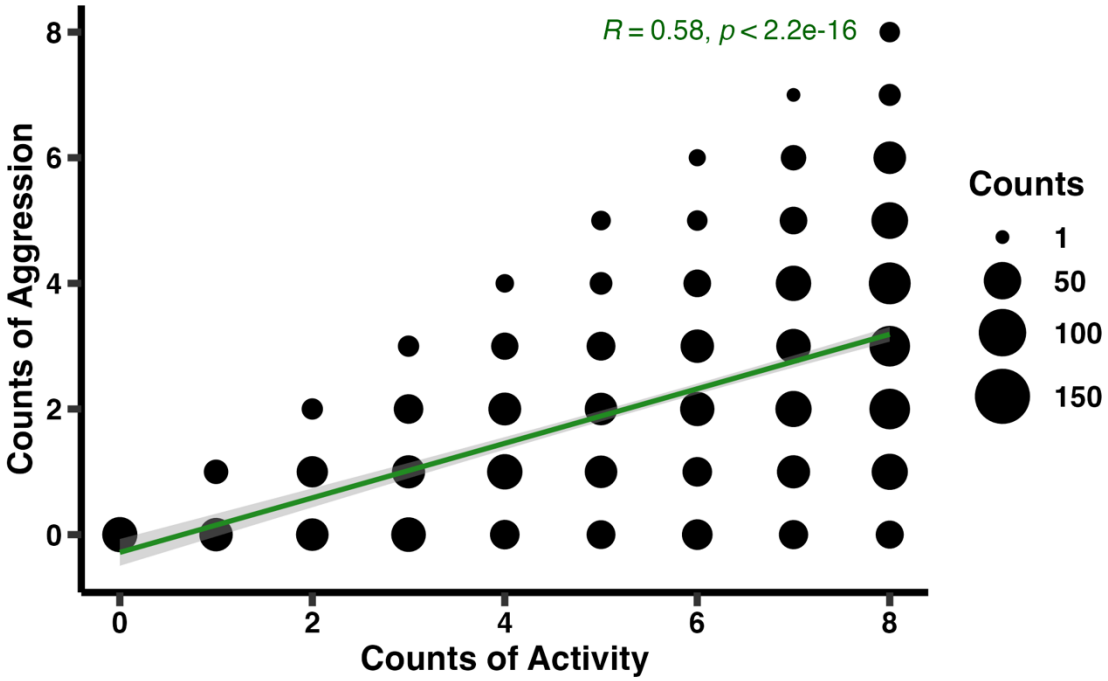

Figure 4.16 Correlation Between Aggression and Activity

The correlation between aggression and activity, when flies were being scanned for 10 seconds once every 10 minutes for a total of eight scans. See Table 4.7 for sample sizes. Point size is proportional to counts of observations. The green line represents the best fit for the correlation between the variables, with shaded regions showing standard error. The spearman's correlation coefficient and *p* value are reported on the graph.

4.3.6.4 Proximity to a Rival

The only significant effect on proximity to rival was age (Table 4.7; Figure 4.17). Time spent in close proximity to a rival decreased from age 3 to 22 days *(p* = 0.001). There was no difference between 22 and 50 days *(p* = 0.086) or 50 and 80 days *(p* = 0.109)

Table 4.10 Interactions and Effect Statistics for Proximity to Rival

Interactions and effects	χ^2 value	*df*	*p* value
Antibiotic treatment : Age : Social environment	7.327	6	0.292
Antibiotic treatment : Social environment	0.787	2	0.675
Antibiotic treatment : Age	2.562	3	0.464
Age : Social environment	8.288	6	0.218
Antibiotic treatment	0.029	1	0.866
Social environment	1.704	2	0.427
Age	36.164	1	<0.0001*

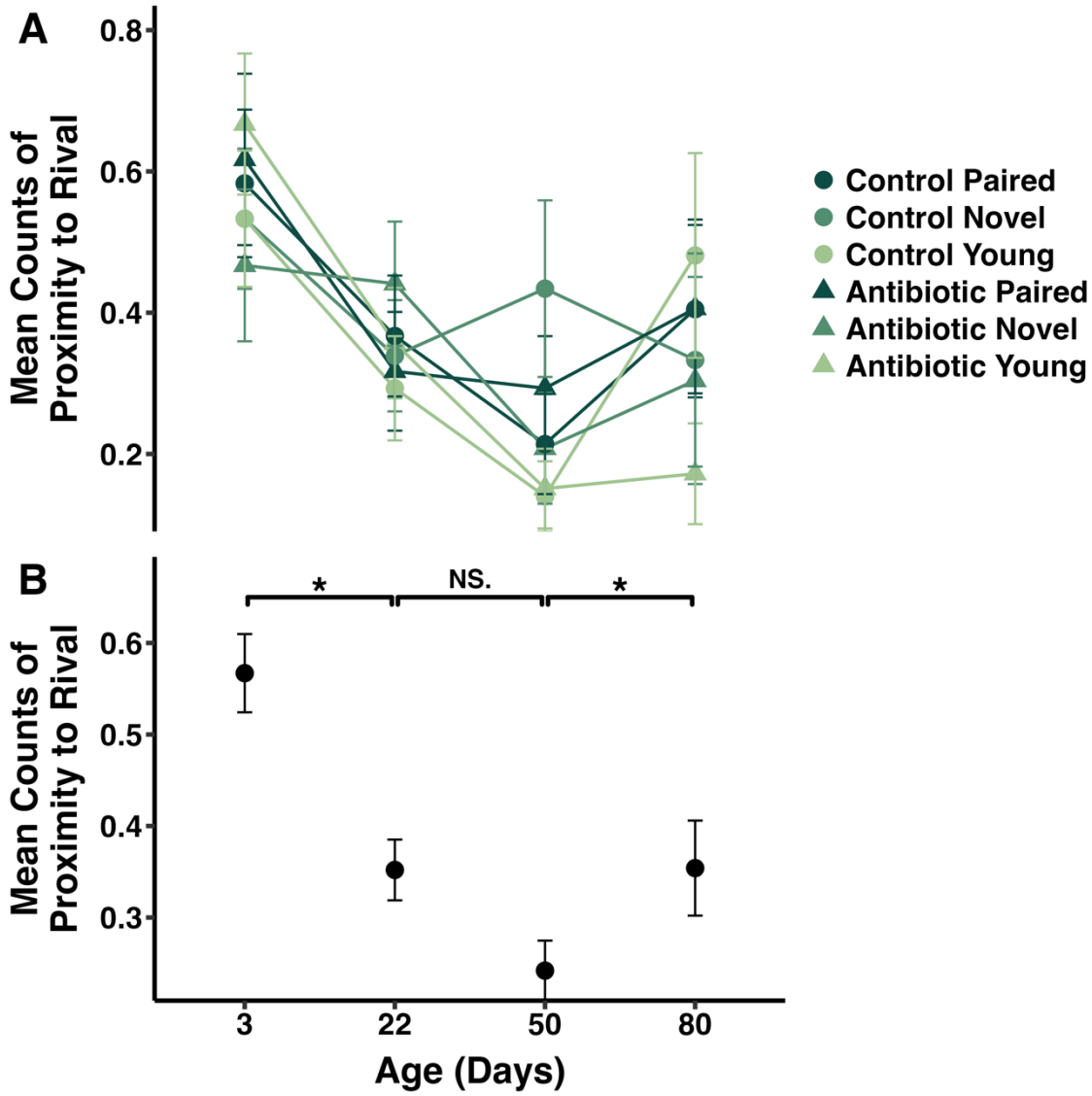

Figure 4.17 Effect of Antibiotics and Pairing on Proximity to Rival

Counts of scans where the fly was in close proximity to a rival after being scanned for 10 seconds once every 10 minutes for a total of eight scans. Males were paired with a co-aged consistent rival, paired with a co-aged rival changed weekly, or paired with a young rival changed weekly, and fed control food or food containing antibiotics. Behavioural scans were done at 3, 22, 50 or 80 days post eclosion. See Table 4.7 for sample sizes. A) displays all treatments on one graph, but for ease of viewing, B) presents the effect of the only significant effect, age. Significance levels are indicated for pairwise comparisons (NS. = non-significant, * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$).

When comparing proximity to a rival and aggression, a weak negative correlation was observed ($R = -0.23$, $p < 0.0001$) (Figure 4.18).

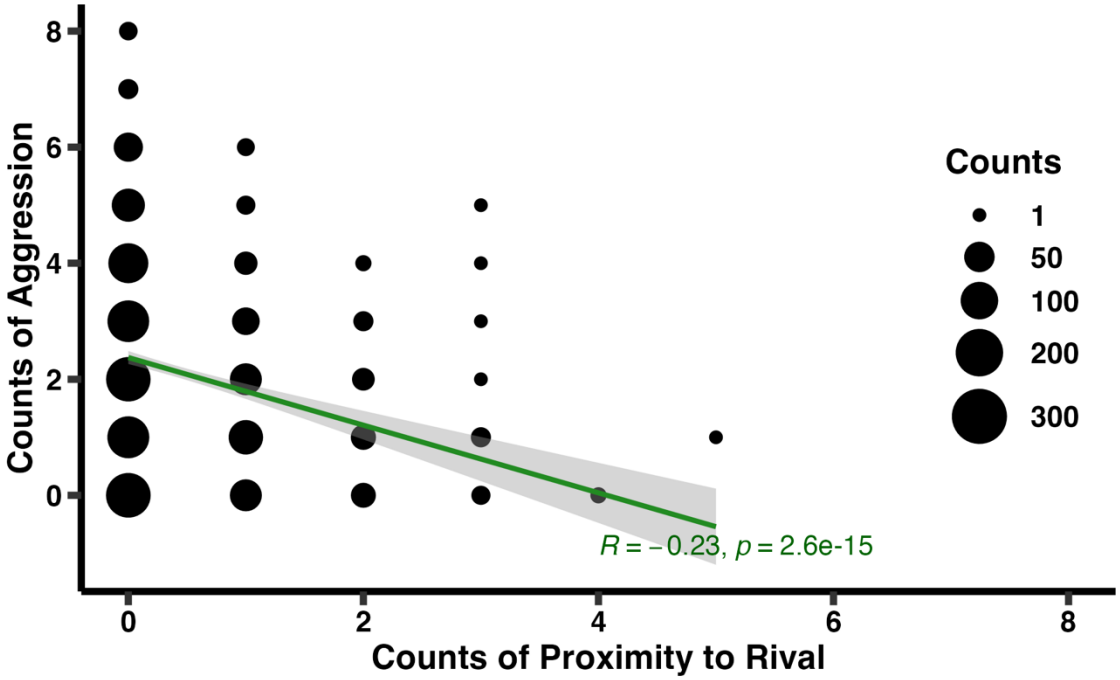

Figure 4.18 Correlation Between Proximity to a Rival and Aggression

The correlation between proximity to a rival and aggression, when flies were being scanned for 10 seconds once every 10 minutes for a total of eight scans. See Table 4.7 for sample sizes. Point size is proportional to counts of observations. The green line represents the best fit for the correlation between the variables, with shaded regions showing standard error. The spearman's correlation coefficient and *p* value are reported on the graph.

4.4 Discussion

The results presented in this chapter convey potential mechanisms underlying the lifespan response to the social environment in *D. melanogaster* (Bretman et al., 2013). To summarise, same-sex social contact was demonstrated to increase cognitive ability, cognitive gene expression and mating duration, with some effects becoming stronger with age. Manipulating the microbiome with antibiotics affected the cognitive response but not the reproductive response. It was also revealed that same-sex social contact decreased activity and increased sitting on food in old aged flies, but this was not dependent on the microbiome.

Previous evidence suggests that both changes in the *Drosophila* microbiome (Brummel et al., 2004; Clark et al., 2015; Fast et al., 2018), and social environment (Bretman et al., 2013; Zajitschek et al., 2013; Leech et al., 2017) can have an impact on lifespan. Additionally, the social environment is known to alter the microbiome (Leech and McDowall et al. 2021), and the data presented in chapter three of this thesis provides evidence to suggest that all three factors are involved in a causal pathway. However, doubt remains regarding whether the reduction in lifespan occurs due to direct effects of the microbiome, or if changes in the microbiome lead to other responses that also affect lifespan.

4.4.1 The Microbiome has a Role in the Cognitive Response to the Social Environment

The results in this chapter revealed that the cognitive response of flies to rival males was impacted by the microbiome. The difference in time spent virgin courting between single and grouped flies was reduced when flies were fed

antibiotics, and this effect was stronger in aged flies. This occurred because single flies increased virgin courting when on antibiotic food, compared to when on control food. Grouped flies, on the other hand, did not.

However, when looking at overall courting, independent of female mating status, single flies increased their overall courting when on antibiotic food compared to control food. To explore whether the increase in virgin courtship was due to this overall increase in courtship, virgin courtship was considered as a proportion out of total courtship attempts. This is a measure of accuracy of courtship attempts, and so directly tests the male's ability to seek out the virgin female. Using this measurement, a similar pattern was apparent, though the effect sizes were reduced. This reduction in effect size suggests that the increased courtship was at least partially responsible for the increase in virgin courtship. However, the antibiotic effect in 49-day old flies remained when inspecting courtship accuracy, as single flies were more able to target the virgin female when on antibiotic food compared to when on control food. This meant that although a difference between single and grouped flies in the antibiotic treatment remained, it was considerably reduced.

The cognitive response's susceptibility to microbiome changes supports the theory that grouping-induced microbiome changes trigger the cognitive response (Figure 4.1b). The increased investment into cognitive assets may explain the socially-driven decrease in lifespan. It may be that certain species of bacteria stimulate pathways that lead to increased cognition in grouped flies. Antibiotics may perturb the microbiome in a similar way to grouping, so that this species can proliferate. On the other hand, microbial species present in single

flies may inhibit cognitive development, perhaps due to increasing investment into other assets such as the immune system. Antibiotic treatment or dysbiosis caused by grouping may decrease the abundance of such species, thus leading to increased cognition. Utilising germ-free flies would help determine which of these explanations is true; as if the latter is true, removing all bacteria would inhibit cognition in the single fly, but if the former is true, this would inhibit cognition in grouped flies. The finding that germ-free flies exhibit reduced memory and learning abilities (DeNieu et al., 2019; Silva et al., 2021), which are able to be rescued by the introduction of *Acetobacter* and *Lactobacillus* (DeNieu et al., 2019), serves as evidence supporting the former mechanism. Furthermore, it has been demonstrated that *L. acidophilus* and *L. rhamnosus* supplementation improved *Drosophila* memory functions (Ho et al., 2024).

Just like the pattern observed in the lifespan response, antibiotics did not fully increase single fly courtship accuracy to that of grouped flies. This would occur if antibiotics do not cause dysbiosis in the exact same way as grouping. Another explanation is that the cognitive response may also be triggered by grouping via other mechanisms, independent of the microbiome. Changes in cognitive investment may then affect the microbiome, and so it is possible that both Figure 4.1a and b are occurring simultaneously. Antibiotics also had a small effect on grouped fly virgin-finding. This may be because antibiotic treatment in combination with social grouping results in a microbiome composition which enhances cognitive development maximally. Antibiotics increasing virgin-finding in both grouped and single flies underscores that the microbiome has a role in triggering cognitive changes.

Although the difference in courtship accuracy between single and grouped flies, in the control groups, were reflected in the mating accuracy, the increase in antibiotic single flies, compared to grouped flies, was not. This is likely due to the increase in single fly accuracy not being large enough to result in a difference in mating in the small sample size of the flies that successfully mated. Additionally, it must be considered that the male is not solely responsible for mating success. Previous research has demonstrated mate choice in females (Byrne & Rice, 2006). If a female is able to detect the previous social environment of a male and senses that he has encountered other males, the female may predict that they have a greater future choice of mates and act more conservative with mating in the present. Male flies can sense female mating status from cuticular hydrocarbons (Friberg, 2006), and so sensing of a male stressed due to their social environment may occur in a similar way. Measuring mating success after manipulation of female sensory perception or male cuticular hydrocarbon synthesis may reveal whether this is occurring. Although not resulting in an increase in mating accuracy in this context, increasing courtship accuracy has other benefits for the fly, as in the wild it would be able to translocate and encounter more females. Greater accuracy in finding the virgin female also means wasting less time identifying, and less time courting mated females that are less likely to mate and contain sperm competition.

Additionally, although an antibiotic-driven increase in single fly overall courtship was observed, the trend for an increase in overall mating success in these flies did not reach statistical significance. Again, lack of statistical power due to the small sample size of flies which mated may have prevented this significance. Nonetheless, this effect on overall courtship reveals a role for the

microbiome in altering mating behaviour, perhaps independent of the cognitive response.

Age-related cognitive decline is a significant concern today due to the growing ageing global population. There is a strong link between social isolation and an increased risk of cognitive decline and dementia, as social engagement also acts as cognitive stimulation (Joyce et al., 2022). The microbiome may play a part in cognitive health, as studies have revealed a link between the microbiome and dementia in humans, with a possible microbial disruption of the neuro-inflammatory system leading to the accumulation of amyloid β in the brain (Minter et al., 2016). Understanding cognitive decline is imperative when searching for potential interventions, and animal studies play a crucial role by providing valuable insights into underlying mechanisms. The *Drosophila* brain encompasses over 300,000 neurons and is compartmentalised for functions such as learning and memory. The processing of sensory information is dependent on neuronal circuits (Grunwald Kadow, 2019), which can be affected by ageing (Matthews & Tye, 2019), and there are already many fruit fly models for age-related cognitive decline and dementia (Reviewed in Giannakou & Crowther, 2011). Findings in this chapter indicate that the social-cognitive link can be modulated by the microbiome in *Drosophila*, laying the groundwork for a hypothesis to be explored in human subjects. This hypothesis could be tested by administrating prebiotics to patients experiencing varying levels of social exposure or isolation. Moreover, conducting additional research in *Drosophila* can facilitate a clearer understanding of the mechanisms underpinning this connection, providing valuable insights before translating these findings to human studies.

4.4.2 Expression of Genes Involved in Cognition is Affected by the Microbiome

The discovered impact of the microbiome on the cognitive response may be explained by differences in cognition-related gene expression. In young flies, the difference in *futsch* expression between single and grouped flies was eliminated in antibiotic flies. However, contrary to the pattern observed in cognitive ability, this appeared to be occurring through a change in both single and grouped fly gene expression when given antibiotics. Regarding *Nrx-1* expression, there was an interaction between social environment and antibiotic treatment in aged but not young flies. Contrary to the pattern observed in cognitive ability, this was driven by a change in grouped fly gene expression with antibiotics.

The finding that antibiotics had an effect on *futsch* gene expression in young single flies but not cognitive behaviour may be because *futsch* gene expression isn't proportionally correlated with cognitive ability, as a host of other genes and pathways are involved in determining this factor. It could also suggest that there may be a delay between cognitive development and the resultant effect to cognitive behaviour, at least when measured using a virgin-finding assay. In aged fly *futsch* expression, no significant interaction between antibiotic treatment and social environment was detected. This could be attributed to a lack of power resulting in an inability to detect differences when effect sizes are small and there is high variation in the data. It must also be noted that gene expression was measured in whole-body flies, potentially obscuring subtle changes in specific tissues such as the gut or brain. Various tissues may have different or even opposing gene expression responses to the microbiome, so measuring whole-body gene expression may lead to misleading interpretations. Thus, future

studies should complement this work with tissue-specific analysis to provide a more comprehensive understanding.

futsch is involved in synaptic development (Roos et al., 2000), whilst *Nrx-1* is involved in synaptic plasticity (Choi et al., 2011). In many species, a reduction in synapses typically correlates with decreased cognition (Spires-Jones & Knafo, 2012), and increased synaptic connectivity improves cognition in mice (Rogers et al., 2011). Therefore, it seems likely that changes in these expression levels are at least partially responsible for any changes in cognition. However, it must be considered that measuring the expression of single genes cannot serve as standalone predictors of cognitive ability, as cognitive development is a multifaceted, complex process influenced by numerous genes and factors.

As previous work has already revealed an association between the microbiome and dementia in humans, the results in this chapter linking changes in cognitive functioning to microbial changes caused by the social environment, could formulate hypotheses for risk factors of cognitive decline in humans. Similarities in the genetic framework between *Drosophila* and humans make this even more valid. Nrx-1, for example, has a homolog in vertebrates, NRXN1, which causes electrophysiological and behavioural changes linked to cognitive impairments if deleted (Etherton et al., 2009). Future research could test the hypothesis that NRXN1 can be modulated by the microbiome in vertebrates, including humans.

4.4.3 More Work is Needed to Establish if Antibiotics Affect Gene Expression if the Microbiome is Restored

To establish whether these gene expression effects were caused by an altered microbiome or through an unwanted effect of the antibiotics, *futsch* expression was tested in flies that had been exposed to antibiotics temporarily and then moved to contaminated food so that the microbiome could recover. The temporary antibiotic treatment had no effect on gene expression, in both single and grouped flies. However, this interpretation requires caution as the previously observed difference in expression between single and grouped flies was not apparent in flies with an intact microbiome. One reason for this may be because to re-establish the microbiome in antibiotic flies, and to keep conditions consistent between treatment groups, all flies had been exposed to contaminated vials containing evidence that other flies were present. Examples of these cues include bacteria or cuticular hydrocarbons shed by flies. These may act as a social signal to single flies that triggers the cognitive response. Therefore, essentially, the single flies are perceiving a grouped environment, explaining why they have the same *futsch* gene expression as a grouped fly.

A future experiment to support the role of the microbiome in this response could involve moving flies from antibiotics to sterile food. If they display the same effect as flies kept on antibiotics for the full 10 days, then this would confirm that the changes are due to experiencing an altered microbiome. Bacterial cultures could also be used to recolonise the microbiome without introducing cues of a social environment. However, it must be considered that certain bacteria may be involved in providing these social cues. Future research could test single fly

futsch gene expression after providing them with potential social cues, aiming to address these knowledge gaps.

4.4.4 There Was No Evidence for a Role of the Microbiome in the Mating Duration Response

The results in this chapter suggest that the microbiome does not have a role in the mating duration response in *Drosophila*. Antibiotics did not alter the difference in mating duration between single and grouped flies. It may be that other mechanisms described could link the microbiome to this response, with either reproductive investment altering the microbiome composition (Figure 4.1a) or the response occurring completely independently of the microbiome (Figure 4.1c). However, it must be considered that mating duration was only measured in young, and not aged flies. For cognitive ability, the interaction between social environment and antibiotic treatment was only apparent at 49 days, and this could be the case for mating duration too. Therefore, in order to make definitive conclusions about the microbiome's role in this response, this experiment would need to be repeated with aged flies.

No evidence for the effect of the microbiome on offspring production was found. Gould et al. (2018) found that although germ-free flies lived for longer, they had a reduced reproductive fitness, suggesting that the effect on lifespan is the result of a trade-off. However, fecundity was measured by counting progeny produced from groups of mixed-sex flies, and so it was not clear whether the microbiome was affecting male or female fecundity, or both. The results in this chapter show that antibiotics did not influence male offspring production after 5 or 10 days of exposure. Further research is needed to see if this continues to be

the case at older ages. Additionally, as there was no difference observed between single and grouped flies, using a measurement that responds to the social environment would be of interest, as it would determine whether antibiotics can affect this response. Previous studies also found no effect of the social environment on offspring production measured using progeny counts, with Hopkins et al. (2019) not observing an effect of pairing or grouping, and Bretman et al. (2012) only finding an effect after the third mating. An alternative method would be to measure the paternity share of the offspring produced by a female that had been mated with other males. Previous studies have found that this increases after exposure to rival males (Bretman et al., 2009; Wigby et al., 2009; Bretman et al., 2013).

4.4.5 The Social Environment Interacts with Age to Affect Behaviour

4.4.5.1 Activity

At a young age, single flies were less active than paired flies. As behaviour was measured within the flies' social treatments, it is unclear whether this effect was due to single flies being disturbed by their rival, causing them to react with aggression or to move away. Leech et al. (2017) found a similar effect, observing slightly more inactivity in single flies aged between 3 and 15 days, but Rouse et al. (2020) found no difference in activity between 10 day single and paired flies.

Nonetheless, in the results presented in this chapter, at age 50 days, activity levels were the same in single and co-aged paired flies, and by 80 days, single flies were more active than paired flies. Rouse et al. (2020) also found this reduction in activity with pairing, but only when measured at midday and in the

afternoon, and not the morning. However, this was only measured in 10-day old flies, and so this effect may have extended into all times of day, with age. Rouse et al. (2020) also observed that this decrease did not occur if the rival was heterospecific, suggesting this was due to the sensing of sexual competition. In the experiments in this chapter, the stress of experiencing this competition either built up with time or begun to have a discernible effect on health during senescence. It appears that paired flies started their decline in activity, a possible marker of senescence, earlier than single flies, as single flies were still increasing their activity between 22 and 50 days of age, an age when co-aged paired fly activity was decreasing. Additionally, flies paired with young rivals differed from co-aged paired flies at 50 days, appearing to act like single flies and delaying their senescence. Despite this, activity sharply declined so that it was indistinguishable from co-aged paired fly activity at 80 days of age. This suggests that being with a young rival may be effective at delaying senescence, but only to a certain point.

Overall, the pattern shown in Figure 4.13 shows that activity peaks later for single flies and flies paired with young rivals, and so also declines later. This premature ageing in the co-aged constant pairs and novel pairs is reflected in the shortened lifespan of these groups (section 3.2.1.4), but the slight delay in young pair ageing is not. Another measurement at 90 or 100 days, where very few paired flies survived to, might have shown that single fly activity declined to the same level as paired fly activity at 80 days.

It may also be that paired flies were less active because they spent more time sleeping. A decrease in sleep in paired flies was also observed by Li et al.

(2021), and aligns with the results from the cognitive experiment as sleep is important in developing synaptic circuitry (Donlea et al., 2014). Additionally, overexpression of *Nrx-1*, which is upregulated in paired flies, increases sleep (Larkin et al., 2015).

Despite previous studies showing germ-free flies had the same (Selkrig et al., 2018; Jia et al., 2021; Grinberg et al., 2022), or higher activity (Schretter et al., 2018; Silva et al., 2021) compared to control flies, the antibiotics used in this study reduced activity. This may be as the previous studies were carried out in four to 10-day old flies. Although the model used for analysis in this chapter did not detect an interaction with antibiotic treatment and age, visual inspection of the graph (Figure 4.13) suggests no effect of treatment at 3 days of age. Grinberg et al. (2022) used the same antibiotic combination and concentrations as what were used in this chapter and yet observed no effect of antibiotics, suggesting that the differing results are either due to a dependence on age, or due to the removal of microbiota that were present in one study but not the other. It may be that there are certain bacterial species increasing activity in control flies in the *Drosophila* stocks used for this research, that are reduced by antibiotics, or species that reduce activity that are only able to proliferate during antibiotic dysbiosis. Schretter et al. (2018) found that mono-association with *L. brevis* was able to reverse the increase in activity in germ-free flies, providing evidence for the latter.

4.4.5.2 Sitting on Food

As flies kept in pairs spent more time sitting on the food than single flies, it can be concluded that the reduction in lifespan in flies kept with rivals was not due to exclusion from the food. It could be conjectured that flies will choose to remain at

the bottom of a vial, on the food, as it is less energetically consumptive than sitting either on the sides of the vial or upside down on the cotton wool. Regardless of mechanism, this appears to act as a more accurate marker of senescence than activity as, unlike activity, it varies somewhat linearly with age, with an overall increase in the time spent sitting on food being noted as flies age. This increase occurred later in single flies and flies paired with a young rival compared to co-aged pairs, similarly to changes in activity.

4.4.5.3 Aggression

Aggression initially increased with age, and then decreased again, similar to activity. An observation made in this study was that many of the observed encounters between flies involved an aggressive interaction. Therefore, it makes sense that the flies that were more active, and more likely to encounter each other, were more likely to have aggressive interactions. It must also be considered that in this assay, aggressive encounters were also classed as activity and so a degree of collinearity may exist. Indeed, a strong correlation between activity and aggression was observed when tested for. Therefore, any effects in aggression also reflected in activity must be considered carefully. The difference in activity between control and antibiotic flies was not reflected in aggression, suggesting antibiotics did not alter aggression, similar to findings in previous studies (Jia et al., 2021; Grinberg et al., 2022). There was also no difference in aggression between social environments, with familiarity and age of the rival not affecting aggression. This suggests that delayed senescence caused by a young rival was not due to decreased aggression, and that the slight decrease in lifespan caused by a novel rival observed in chapter three was not due to increased aggression. It should be noted that this does not mean these rivals

were differentially aggressive innately, as no stimulus, such as a female fly, was used to incite aggression as is often the case in other studies. Carazo et al. (2015) observed that the difference in aggression between familiar and unfamiliar flies was abolished if there was no female present. However, here the intention was to test whether lifespan effects of different social environments could be explained by differences in aggression, and so the conditions used in these assays were appropriate with respect to this context.

4.4.5.4 Proximity to Rival

Chen et al. (2019) found that silencing of the histone demethylase, KDM5, which altered the microbiome, increased fly social space and decreased social contact, both which could be rescued by antibiotics. In this chapter, no such effect of microbiome changes on proximity to rival was observed. Flies reduced their time spent in close proximity to a rival between 3 and 22 days of age.

It must be considered that as this measure aimed to look at non-antagonistic social behaviour, proximity was not counted if the fly was involved in aggressive interactions, and so this increased proximity effect could be due to less aggression at a young age. Indeed, analysis revealed a negative correlation between proximity to rival and aggression, though this was weak. Nonetheless, used in combination with the results exploring aggression, these results suggest that at a younger age, interactions between flies are non-antagonistic, with aggression increasing with age. These results partially align with results from a previous study that discovered that flies moved closer to each other for the first two to three weeks of age and then moved further apart (Brenman-Suttner et al., 2018).

4.4.6 Conclusion

The work presented in this chapter has begun to reveal the microbiome's role in the cognitive response to the social environment. The finding that antibiotics only affect cognitive ability in single flies is of particular interest as this is what occurs during the lifespan response, suggesting that these two responses are somewhat linked. It may be that microbiome composition caused by grouping triggers cognitive investment and this could lead to decreased investment into life-lengthening assets (Figure 4.1b). However, single fly lifespan is not impacted by antibiotics as much as grouping, and so it is likely that the lifespan response occurs due to a mixture of increased cognitive investment and other microbiome effects, as well as other microbiome-independent effects.

No evidence for a microbial role in the mating duration response was uncovered in this chapter. This requires confirmation in later age groups however, as the cognitive effect seems to be dependent on age. If the mating duration response is not altered by antibiotics at any age, then this response may explain the difference between single and grouped flies in lifespan that still occurs, regardless of antibiotic treatment.

Behavioural results suggested that fly activity increased then decreased with age, and was affected by the microbiome. A protective effect of the young age of the rival against the negative effects of being paired, was also revealed. However, the experiments in this chapter did not find any evidence for a role of the microbiome in this protective effect. These results add to existing research demonstrating beneficial effects of pairing with a young rival, including an

increase in lifespan, reduced functional senescence, and reduced stress resistance when compared to their co-aged counterparts (Cho et al., 2021).

Chapter 5
Linking Microbiome Changes to Host Functional Effects

5.1 Introduction

5.1.1 Inferring Functional Effects From Microbial Data

It is accepted that the microbiome is sensitive to perturbation by extrinsic drivers, such as temperature, drug treatment and stress, and there exists extensive research where this change has been measured (Galley et al., 2014; Moghadam et al., 2018; Smith et al., 2019). However, the implications of these changes for the host are often not measured, and it is not always clear whether any recorded changes in host physiology or behaviour are caused by the changing microbiome or by other consequences caused by these stimuli. Understanding the connection between the biological effects caused by an external factor and changes in the microbiome could have large implications for understanding the role of the microbiome in health and disease (Ghaisas et al., 2016). Additionally, measuring the shift in microbiota without considering impacts of this shift may lead to false conclusions as some changes that may seem considerable in terms of community structure, may not significantly alter the biochemical pathways going on in the host and so may have little effect on host health. This is due to the microbiome functional redundancy hypothesis, which states that related and unrelated bacteria may perform the same functions, so one community may be replaced by another with little impact on the overall functional profile of the microbiome, and a similar production of proteins and metabolites (Moya & Ferrer, 2016). Furthermore, this may mean that although different host species' microbiomes differ greatly in taxonomic profile, there may be functional similarities, and these

may change in a similar way in response to stress or changes in the environment. This means a microbiome ‘stress-signature’, a functional response common between species and stressors, could be identified.

To identify the function of microbiota in a host-associated community, predicted functional analysis can be carried out (Yan et al., 2010). Firstly, the Piphillin server takes 16S rRNA sequences and compares them to genomic resources of sequenced bacterial isolates to identify the bacterial composition of the sample (Iwai et al., 2016). Next, the gene function of each bacterium is identified by referring to the Kyoto Encyclopedia of Genes and Genomes (KEGG) database (Kanehisa & Goto, 2000), which is a catalogue linking genomic information to higher-level metabolic functions of the cell, the organism, and the ecosystem. The KEGG database contains a collection of pathway maps for metabolism, genetic information processing, environmental information processing, cellular processes, organismal systems, human diseases and drug development. Compiled from published material, it represents our knowledge of these networks. Functionally annotating sequences involves identifying genes that are known to associate with particular functions or metabolic pathways. Bacteria contain genes that directly impact the host as they produce bioactive compounds, such as neurotransmitters. They also contain genes that have an indirect effect on the host through inducing it to produce bioactive molecules or modifying the bioactivity of certain molecules already present in the host. For example, lipopolysaccharides from different bacterial species are able to impact host immune pathway activation differently, contributing to autoimmunity in humans (Vatanen et al., 2016). In Zebrafish, possible suppression of transcription factor hepatocyte nuclear factor 4A (HNF4α) by the microbiome may perturb the

regulation of host inflammatory pathways (Davison et al., 2017). Some of these gene changes may alter pathways that affect host health and we can compare these between treatment groups. This analysis can be performed on data from any study that looks at microbial changes between groups, without requiring any additional data collection, and yet it is not often carried out. This means published data has been underused and interesting results and conclusions may be left unexplored. Nonetheless, it is important to acknowledge that utilisation of the KEGG database has limitations. Firstly, it doesn't encompass the entirety of biological pathways and functions, and it may not always reflect the latest advancements in scientific understanding. Moreover, functional annotations within KEGG rely heavily on computational predictions rather than direct experimental evidence, warranting cautious interpretation of results.

Many studies have used predicted functional analysis to look at the effects of the microbiome on host functions and conject what this may mean for host health. Xiao et al. (2019) used this method and discovered that when wild bats were moved to captivity, their microbiome diversity increased but their microbial functional diversity decreased. This confirmed that a change in microbiome composition may not always indicate an equivalent change in functional pathways, whilst also exhibiting that the change of diet in captivity may have influenced biological mechanisms within the bats. Bates et al. (2018) revealed that the skin microbial community and predicted functional diversity of the common midwife toad were dependent on the infection state of the fungal pathogen *Batrachochytrium dendrobatidis*, suggesting that skin bacteria may have a role in disease defence. Bharwani et al. (2016) found an overall reduction in functional diversity in the microbiome of mice after social defeat. Moreover,

social defeat caused a decrease in pathways involved in the synthesis and metabolism of SCFAs, potentially explaining the co-occurring negative physiological effects. They also observed less pathways involved in the synthesis and metabolism of tyrosine and tryptophan (Bharwani et al., 2016), precursors for the synthesis of neurotransmitters such as dopamine, serotonin, and melatonin, which could help explain the behavioural changes such as the depression-like symptoms caused by social defeat (Castle et al., 2003). Leech and McDowall et al. (2021) used this method to reveal a microbial functional effect of treatments that reduce lifespan in *Drosophila.* They discovered that microbiome diversity was higher in adult male flies that were kept in groups compared to single flies, and five pathways linked to ageing were predicted to be decreased by the microbiota. Additionally, adult male presence during the larval stage changed the pupal microbiome as well as alternative predicted functional changes (Leech and McDowall et al. 2021).

5.1.2 Measuring Functional Effects From Microbial Data

Predictions from microbial functional analyses can be used to identify which host pathways may be sensitive to microbial changes, and actual measurements of these pathways can help confirm this causal link. Zhou et al. (2021) observed that treating *Drosophila* with sodium butyrate altered the microbiome, and functional prediction suggested that treated flies exhibit a higher abundance of bacteria involved in lipid and carbohydrate metabolism. Subsequent transcriptome and metabolome analysis confirmed that the treatment did indeed upregulate genes involved in this metabolism, leading to an increase in the abundance of lipids (Zhou et al., 2021). Boehme et al. (2021) used a similar approach, when they observed that changes in metabolites, measured in the

hippocampus of mice during faecal microbiota transplantation, correlated with predicted functional changes.

As the microbiome is an important modulator of many host pathways in multiple species, measuring changes in the host transcriptome is one way of measuring the impact of microbial changes. A study in mice focusing on xenobiotic processing genes, found 116 out of 303 to be differentially expressed between control and germ-free mice intestinal tissue (Fu et al., 2017). Broderick et al. (2014) identified 152 genes that differed in expression between control and germ-free *D. melanogaster* guts, including genes associated with immune responses, tissue homeostasis, gut physiology and metabolism. These transcriptome changes can be linked to health, aiding in the identification of a causal relationship between the microbiome and disease. For example, gene expression changes in patients with inflammatory bowel disease (IBD) have been correlated with changes in microbiota, including the increase in expression of antimicrobial gene *DUOX2*, which was associated with an increase in Proteobacteria (Haberman et al., 2014). These changes favour oxidative stress and higher levels of *DUOX2* are associated with more severe mucosal injury, possibly predicting disease progression (Haberman et al., 2014).

5.1.3 Chapter Aims

I sought to identify potential functional effects caused by changes in host microbiomes, by analysing data already collected and published. I focussed my analysis on papers which measured a change in microbiome composition caused by treatments which usually coincide with an impact on host health. I wanted to identify if the microbiome was potentially responsible for health impacts by

inspecting results produced by predicted functional analysis using the Piphillin server and KEGG database.

I included papers in my analyses that used two model species, the fruit fly, *D. melanogaster,* and the house mouse, *Mus musculus*, because they have considerable host genome annotations and extensive genomic resources of genome-sequenced gut microbes, giving greater confidence in the functional analysis. Two papers included in the analyses investigated the effect on microbiota when *D. melanogaster* were developed at lower temperatures (Moghadam et al., 2018; Zare et al., 2018), which causes an increase in longevity (Norry & Loeschcke, 2002). Another paper explored the effect of an oxidant during *Drosophila* development, Tertiary-butyl hydroperoxide (tBH), which resulted in an increase in lifespan (Obata et al., 2018). I hypothesised that if an extension of lifespan by these treatments was caused at least partially through a change in the microbiome, a change in microbial-driven pathways related to longevity would be predicted.

The papers identifying microbiome changes in mice focussed on treatments that caused or treated disease. A paper using the antidiabetic drug, acarbose on mice was included as this extended lifespan as well as affecting microbiome composition (Smith et al., 2019). Two other papers included in the analyses looked at the impact of factors that trigger liver disease in mice. These included liver injury, a high fat diet (HFD) (Furuya et al., 2020), and alcohol (ethanol - EtOH) (Yang et al., 2017a; Furuya et al., 2020). The effect on the microbiome of an antifungal agent, amphotericin B, that was able to ameliorate liver disease, was also investigated (Yang et al., 2017a). I aimed to use this

method to implicate the microbiome in disease development. Furthermore, by combining the outcomes across all of the papers in both flies and mice, I aimed to identify a microbiome ‘stress-signature’, looking for any similarities between different stresses.

Results obtained from microbial functional analyses are only predictions, and so should be supported by direct measurements of host pathways. In order to test whether predicted microbial functional effects actually occurred in the host, I measured gene expression in *Drosophila* that were kept in different social environments, that were predicted to produce functional differences in the study by Leech and McDowall et al. (2021). I used antibiotics to see if perturbing the microbiome altered gene expression, hence establishing if, and how much, the microbiota was responsible for differences in gene expression between social environments. Genes involved in apoptosis and the MAPK pathways, both linked to ageing, were chosen because of the association between the *Drosophila* social environment, and senescence and longevity (Bretman et al., 2013). Additionally, these pathways were predicted to be affected considerably by socially-driven microbiome changes in the study by Leech and McDowall et al. (2021).

5.2 Methods

5.2.1 Predicted Functional Meta-Analysis

5.2.1.1 Choice of Data

Studies which reported induced changes in microbiota alongside host health or behaviour were identified (though note a formal systematic review was not performed) (Table 5.1). Papers studying either the fruit fly, *D. melanogaster,* or house mouse, *M. musculus,* were chosen due to the availability of high-quality genomic resources for these species. The number of papers included was restricted by the availability of raw sequencing data and complete metadata (Figure 5.1).

Table 5.1 Studies Used in the Meta-analysis Looking at Treatments That Resulted in Changes to the Microbiome and Host Health

Study	Host Species	Treatment and Comparisons	Reported Effect on Host
Moghadam et al. (2018)	*Drosophila melanogaster*	Developmental temperature. Microbiome sampled from adults (13°C – 23°C, 13°C – 31°C, 23°C – 31°C)	Longer development time and lifespan increase (Norry & Loeschcke, 2002)
Zare et al. (2018)	*Drosophila melanogaster*	Developmental temperature. Microbiome sampled from food used by adults (18°C – 25°C)	Longer development time and lifespan increase (Norry & Loeschcke, 2002)
Obata et al. (2018)	*Drosophila melanogaster*	Developmental oxidant treatment. Microbiome sampled from larvae and adults (Control – tBH)	Acts as an antibiotic and increases lifespan
Furuya et al. (2020)	*Mus musculus*	Diet both in control mice and mice with liver injury (Control – HFD, control – HFD + EtOH, HFD – HFD + EtOH)	HFD developed liver pathology, HFD + EtOH had a larger effect, and liver injury exacerbated these effects
Yang et al. (2017a)	*Mus musculus*	Alcohol and antifungal treatment (Control – EtOH, EtOH – EtOH + antifungal)	Antifungal treatment ameliorated ethanol-induced liver disease
Smith et al. (2019)	*Mus musculus*	Antidiabetic drug (acarbose) (Control – acarbose)	Lifespan increase, delayed intestinal starch breakdown (Laube, 2002)

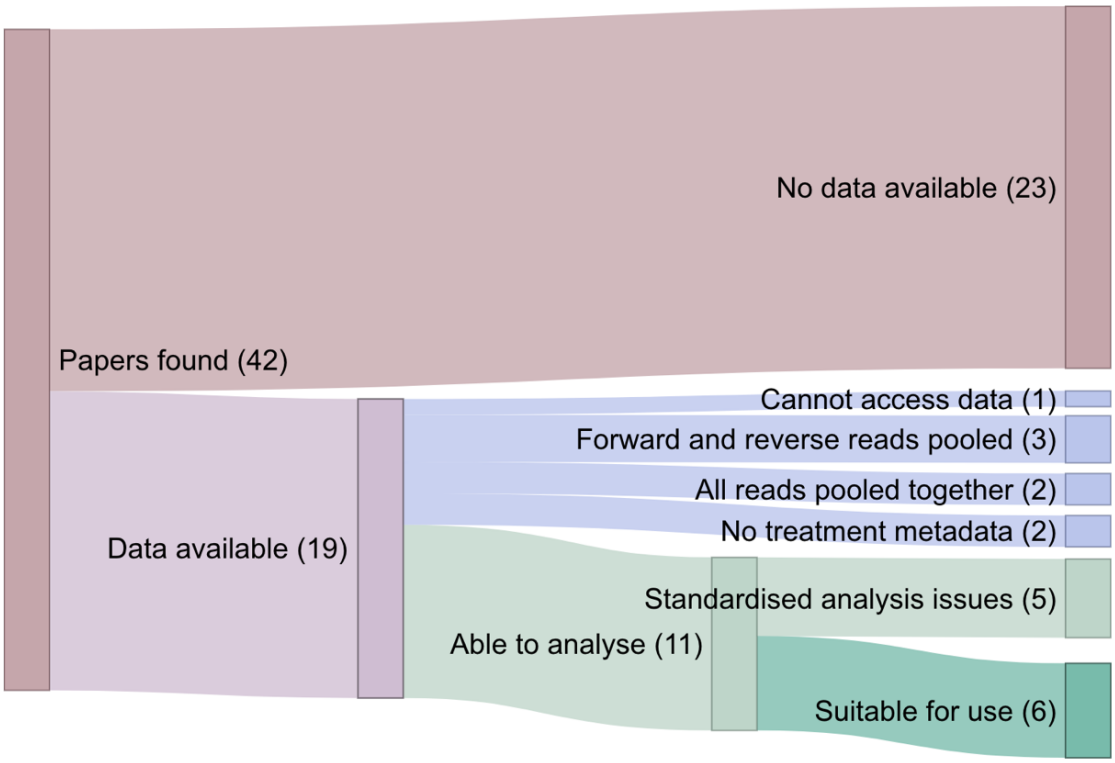

Figure 5.1 Flowchart Presenting Issues Faced When Searching for Suitable 16S Data to Use in the Meta-analysis

The majority of papers first identified did not contain information on where to find data. One had information regarding data availability but the accession number did not exist in the public database. In some cases all sequences for all samples, or forward and reverse sequences of the same sample, were merged together in a single fastq file. In other cases, sequencing data but no metadata was available. If suitable sequencing and metadata was available, data were analysed using a standardised pipeline. Sometimes this produced unusable data, such as if taxonomy could not be assigned.

5.2.1.2 Standardised Bioinformatic Re-analysis of Sequences

To allow for better comparison between studies, all 16S data were analysed using a standardised pipeline. The DADA2 pipeline was used to process raw 16S reads (Callahan et al., 2016). Forward and reverse reads were merged to create amplicon sequence variants (ASVs) and chimeric reads were removed (Table 5.2). Downstream analysis was performed using phyloseq (McMurdie & Holmes,

2013). Data was rarefied to even depths except for the data in the Moghadam et al. (2018) paper, as they specified that they rarefied their data to 5000 reads. As stated in their paper, one sample from the 13°C treatment did not meet this criterion and so was omitted from analysis. In the paper by Smith et al. (2019), sphingolipid species and one outlier sample was removed in their analysis, and so that was also replicated.

Table 5.2 Read and Taxa Information for Datasets After Processing Through the Standardised DADA2 Pipeline

Paper	Total Taxa	Mean Reads Per Sample	Range of Reads Per Sample
Moghadam et al. (2018)	172	37044.67	324 – 200859
Zare et al. (2018)	924	97757.44	38239 – 177858
Obata et al. (2018)	611	135101.4	90244 – 190311
Furuya et al. (2020)	797	2276.711	814 – 7180
Yang et al. (2017a)	370	25851.37	8867 – 38809
Smith et al. (2019)	1302	20592.83	39 – 43609

To ensure that the pipeline used was able to produce results consistent to those presented in the published work, different tests and data visualisation methods were used to analyse the amplicon data and compare results to those reported in the papers. *gplots* was used to plot Venn diagrams to visualize shared and unique ASVs between treatment groups (Warnes et al., 2009). The R package *phyloseq* (McMurdie & Holmes, 2013) was used to calculate alpha

diversity, using the Chao1 diversity index. ANOVA was used to explore the effects of experimental treatment on read count and alpha diversity (Chao1). Beta diversity was analysed using Non-Metric Multidimensional Scaling (NMDS) ordinations on Bray–Curtis distance between bacterial community ASV abundances distances using the R package *vegan* (Oksanen et al., 2007). *Phyloseq* was used to plot heatmaps of the sample community structures and *vegan* was used to carry out a PERMANOVA analysis to test for differences in beta diversity between treatment groups.

5.2.1.3 KEGG Functional Analysis

Raw ASV abundances and sequences were uploaded to the web portal Piphillin (Iwai et al., 2016) using a sequence identity cut-off of 97%. Predicted microbial effects on gene function were identified using the KEGG reference database (Kanehisa & Goto, 2000) (June 2020 release) and DESeq2 identified differentially abundant pathways between treatment groups (Love et al., 2014). The R package *ggplot2* was used to create graphs to visualise log fold differences in pathway abundances between treatment groups (Villanueva & Chen, 2019).

Four pathways of interest involved in ageing and immunity were extracted from the analysis, given that the treatments involved in these studies resulted or would be expected to result in a lifespan effect. This also enabled comparison with predicted functional analysis results from the previous study by Leech and McDowall et al. (2021), which found that social stress during development affected these pathways. Other pathways of interest were identified depending on the context of each study.

To examine differences between treatment groups in the studies by Moghadam et al. (2018) and Yang et al. (2017a), normalised pathway abundances were displayed in a heat map. In the case of Yang et al. (2017a), five pathways selected based on their relation to disease pathology were also displayed in a barchart. The abundances were first logarithmically transformed data with a constant added (log(x + 1)), for visualization purposes and to handle negative values.

5.2.2 Gene Expression

In order to observe the effect of the microbiome on pathways identified in the functional analysis, flies were kept singly or in groups of 10, either on control food or food containing antibiotics, and flash frozen at either 10 (six biological repeats per treatment, each consisting of five pooled whole-body flies) or 49 days from eclosion (six biological repeats per treatment, each consisting of 10 pooled whole-body flies).

Genes coding proteins involved in apoptosis (Table 5.3) were measured at 10 days, and genes coding proteins involved in MAPK pathways were measured at 10 and 49 days (Table 5.4). Genes involved in caspase-dependent apoptosis were chosen; genes for a caspase (*Dcp-1*), and positive (*hid*) and negative (*Diap-1*) downstream regulators known to physically interact with caspases. Hid is also triggered by the c-Jun N-terminal Kinase (JNK) branch of the MAPK signalling pathway. Genes were identified from the two other classes of MAPK signalling present in *Drosophila*; *Vn, Ras85D* and *cnk* from the extracellular signal-regulated kinase (ERK) pathway, and *p38c, Duox* and *Sod2* from the p38c pathway. These genes have all been shown to be expressed in *D.*

melanogaster (Zheng et al., 2005; Ashton-Beaucage et al., 2014; Chakrabarti et al., 2014; Wang et al., 2014; Asbah et al., 2021) (see section 2.3 for methods regarding qPCR analysis).

Table 5.3 Forward and Reverse Oligonucleotides for Apoptosis Genes
Including efficiency and pipetting accuracy (R^2) value.

Gene	Forward	Reverse	Efficiency (%)	R^2
Dcp-1	ATACCCATAC ACGCCGACTT	CCAGGAGCCAT TGTTGATG	94.2	0.988
Hid	GAGAACGACA AAAGGCGAAG	CAAAACGAAAA CGGTCACAAC	98.1	0.996
Diap-1	TCGTCAAATC TCAACGCAAC	CTCTGGCTCCT TTCCTCTGA	110	0.981

Table 5.4 Forward and Reverse Oligonucleotides for MAPK Genes
Including efficiency and pipetting accuracy (R^2) value.

Gene	Forward	Reverse	Efficiency (%)	R^2
cnk	GCCATACGAGC TGGAGAATC	GCAGATTGTCG TTTTTCAGGT	108.9	0.982
Duox	CGTGGCGACAA GTACATACC	CCATCAATCCA AGCAGTCATT	108.9	0.991
p38c	GCATTCTGGCG GAACTTATC	CCTCGTCGGAG TACCCATTA	102.2	0.993
Ras85D	GGACTCTTACC GAAAGCAAGTG	CTCGCCAGTCC GCATATACT	93	0.998
Sod2	TCGGGACTTAG CCTTATTAGCA	AGCTTCGGTAG GGTGTGCTT	99.8	0.999
vn	ATCCAGGCAAC TTCACCATC	CGTGACCTCTG CGTTCTGT	102.8	0.982

5.2.2.1 Statistical Analysis

GLMs with gamma distributions were used to explore gene expression differences between treatments on 10 day *Diap-1,* 10 day *Ras85D* and 49 day *Duox* gene expression. A GLM with an inverse-gaussian distribution was used for 10 day *p38c* gene expression. LMs were used to explore the remaining gene expression differences. Social environment and antibiotic treatment were used as fixed factors.

5.3 Results

5.3.1 Predicted Functional Meta-analysis

16S sequencing data from research presenting an effect of treatments on microbiome composition was analysed using the Piphilin server and KEGG database to infer functional effects caused by microbiota. Predicted functional pathways differing between treatment groups were identified.

5.3.1.1 Pathways Involved in Ageing and Immunity

Analysing the data from the study exploring developmental temperature in *Drosophila* (Moghadam et al., 2018), revealed that flies developed at 13°C had microbiota that were enriched for FoxO signalling, MAPK and apoptosis pathways compared to flies reared at 23°C (Figure 5.2, Table A.1) or 31°C (Figure 5.3; Table A.2), whereas these pathways did not significantly differ between flies reared at 23°C and 31°C (Table A.3). A similar pattern was found when analysing data from another study investigating developmental temperature (Zare et al., 2018). FoxO signalling, longevity regulating and MAPK pathways, but not apoptosis, were predicted to be increased by the microbiota of flies reared at 18°C compared to 25°C (Figure 5.2, Table A.4). When analysing the data from the study exploring the effect of the oxidant, tBH (Obata et al., 2018), FoxO signalling, longevity regulating and MAPK pathways were predicted to be decreased by the microbiota of adult flies when reared with tBH when compared to control flies (Figure 5.2, Table A.5). Interestingly, when the microbiome was sampled in larvae, these pathways were not predicted to be affected by microbiota changes with tBH treatment (Table A.6).

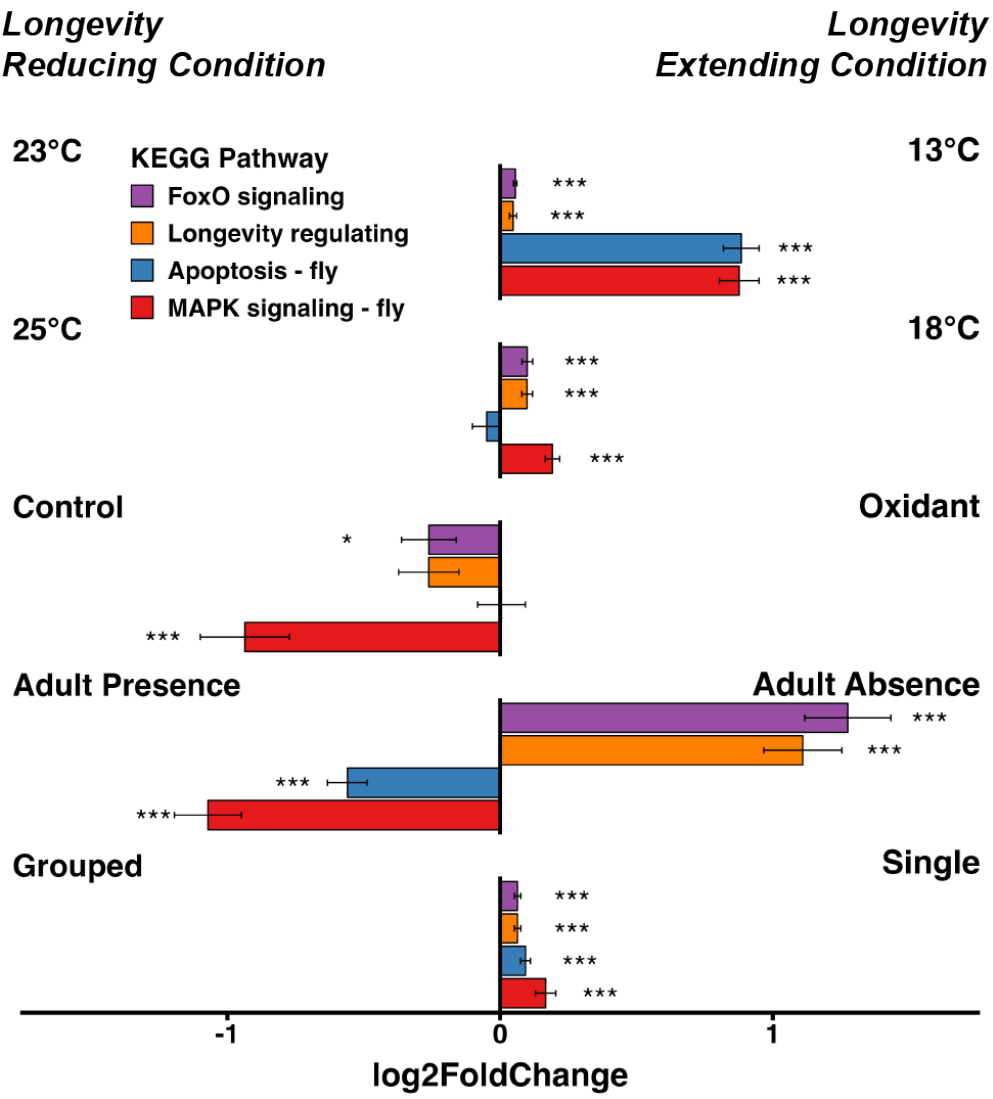

Figure 5.2 The Change in Predicted Microbial Effects on Pathways Between Treatment Groups in *Drosophila* Studies

Piphillin, referring to the KEGG database, was used to predict microbial functional effects from published 16S data. Differentially abundant pathways were determined by DESeq2 analysis. Comparisons were made between *Drosophila* developed at 13°C or 23°C (Moghadam et al., 2018), at 18°C or 25°C (Zare et al., 2018), control flies and flies treated with an oxidant (Obata et al., 2018). These were compared to previous predicted functional analyses, from Leech and McDowall et al. (2021), that compared larvae reared with adults present or absent, as well as adult flies grouped or kept singly. A positive log2 fold change means pathways were represented more in the longevity-extending conditions, whereas a negative change means pathways were represented more in the longevity reducing conditions. Significance levels indicate deviance from 0 when corrected for multiple testing using the Benjamini-Hochberg method (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$).

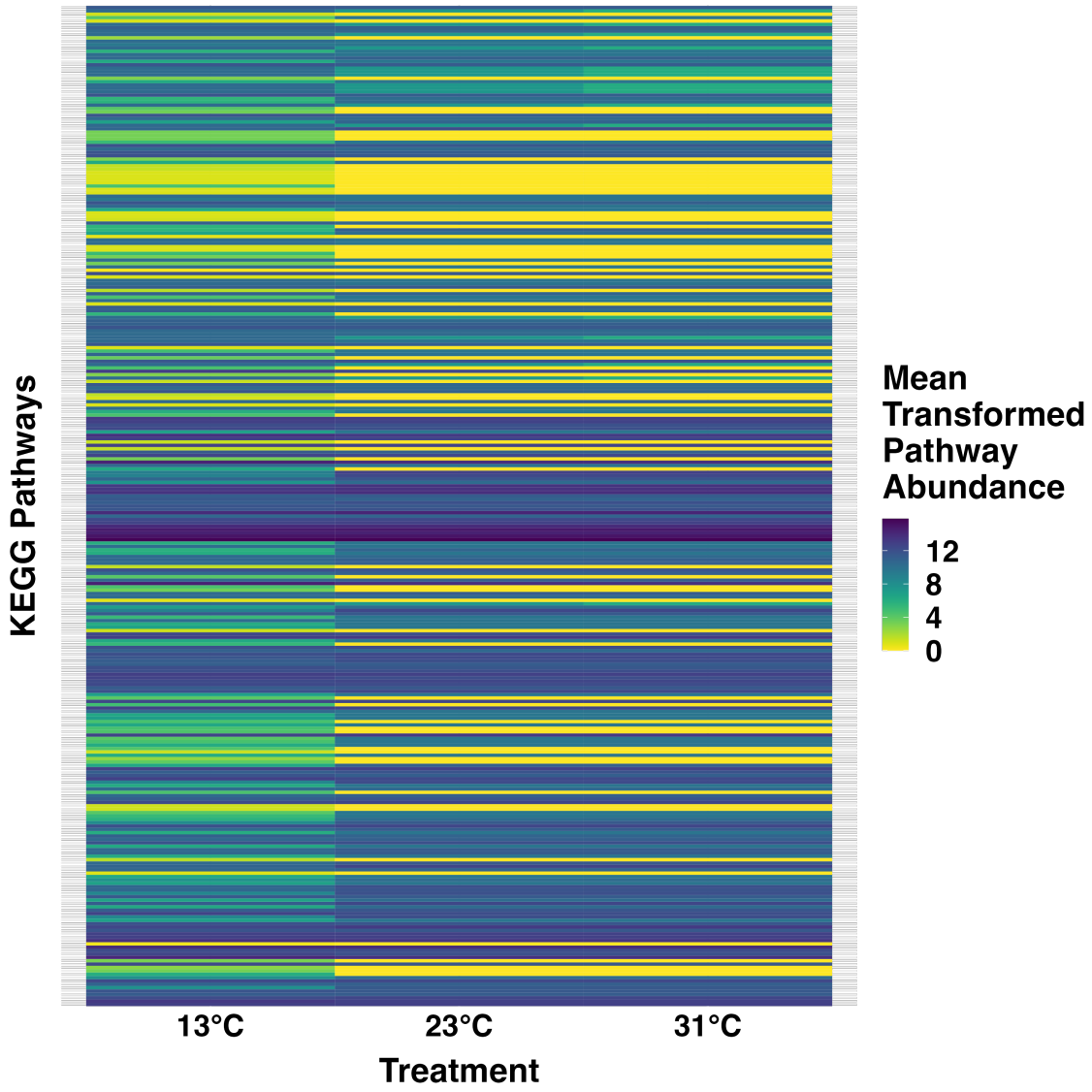

Figure 5.3 All Predicted Microbial Effects on Pathways in *Drosophila* Reared at Different Temperatures

Piphillin, referring to the KEGG database, was used to identify predict microbial functional effects of developmental temperature, using 16S data from the study by Moghadam et al. (2018). Pathway abundances, normalised by DESeq2, were logarithmically transformed with a constant added (log(x + 1)).

When analysing the data from studies which used mice, treatments were not predicted to alter the microbiota in a way that would have a consistent effect on these ageing-related pathways. Using data from the study exploring liver disease in mice (Furuya et al., 2020), it was found that, in mice with induced liver injury, a HFD resulted in microbiota that were enriched for the longevity regulating

pathway (both the multiple species pathway and the general pathway), compared to control mice microbiota (Table A.9). Mice fed a HFD + EtOH had microbiota that were enriched for the longevity regulating pathway, when compared to control mice (Table A.10). In the control mice without induced liver damage, HFD and HFD + EtOH treatments did not result in microbiota that were enriched for any of these ageing-related pathways when compared to control mice (Table A.7, Table A.8). There was no differences in pathways between HFD and HFD + EtOH mice, in both control mice and mice with induced liver injury.

When considering the additional study on liver disease in mice (Yang et al., 2017a), EtOH increased microbiota enriched for apoptosis (Table A.11). When analysing the study where mice were given acarbose to increase longevity (Smith et al., 2019), there was no differences in microbiota enrichment of these longevity-related pathways (Table A.13).

5.3.1.2 Anti-Fungal Treatment in Mice Treated with Alcohol

In the study by Yang et al. (2017a), many predicted pathways that were enriched in mice fed EtOH compared to control mice (Table A.11), were then reduced in mice that were treated with antifungal agents (Figure 5.5, Table A.12). These included alcoholism, dopaminergic synapses, serotonergic synapses and apoptosis, (Figure 5.4), and fatty acid degradation.

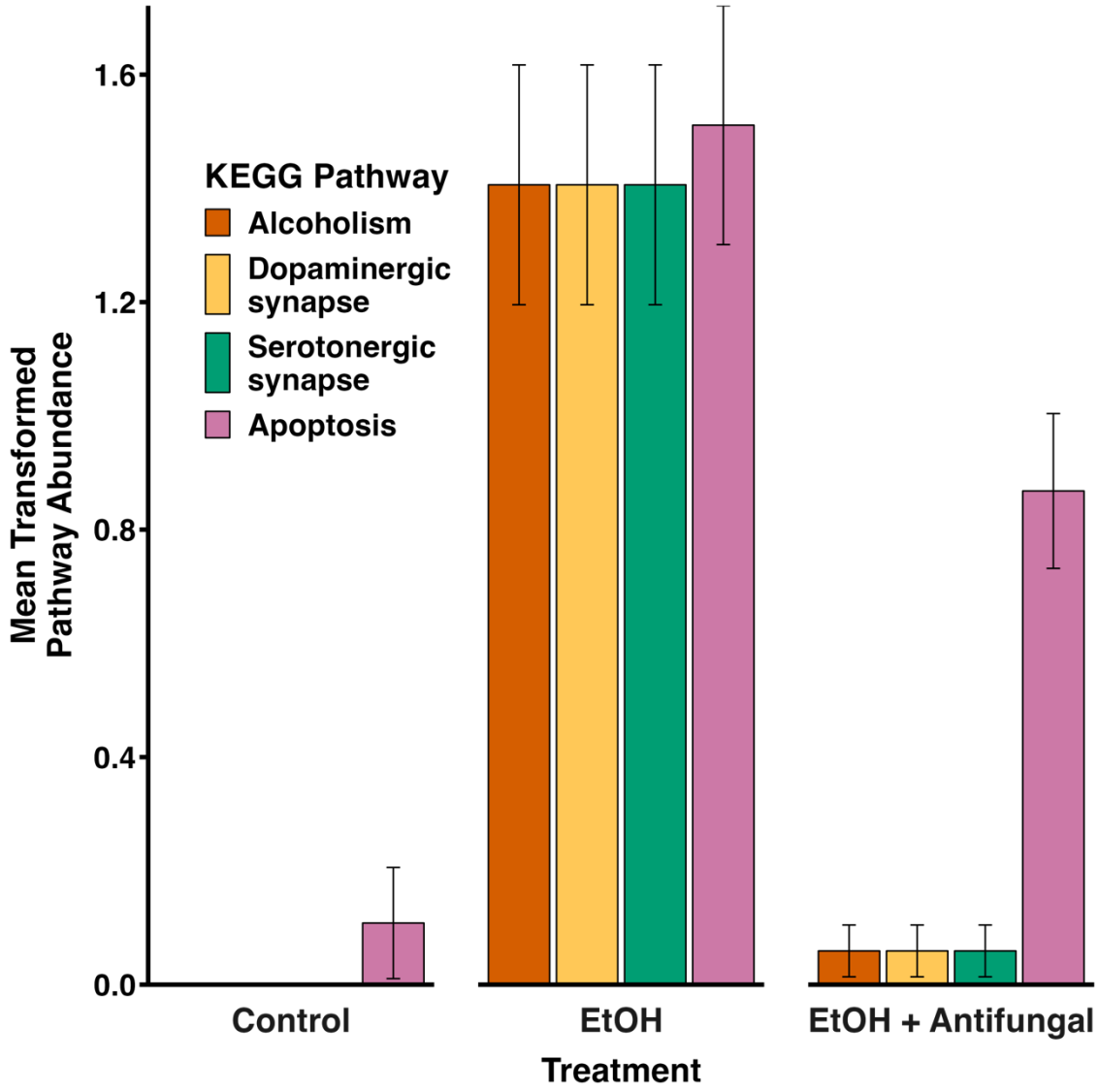

Figure 5.4 Predicted Microbial Effects on Pathways in Mice Treated with Alcohol and an Antifungal Agent

Piphillin, referring to the KEGG database, was used to predict microbial functional effects of alcohol and the antifungal agent, amphotericin B, in mice, using 16S data from the study by Yang et al. (2017a). Pathway abundances, normalised by DESeq2, were logarithmically transformed with a constant added (log(x + 1)).

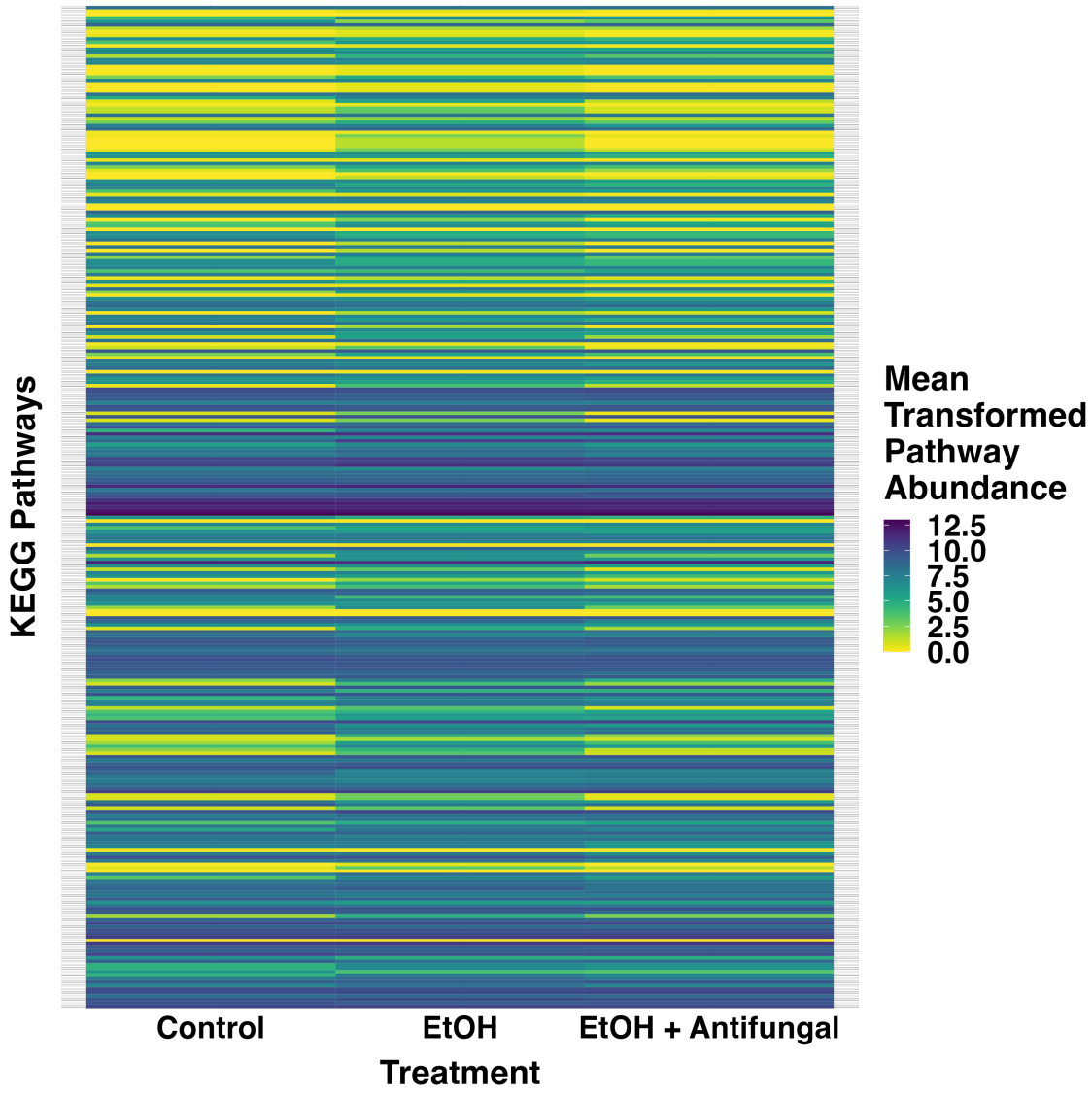

Figure 5.5 All Predicted Microbial Effects on Pathways in Mice Treated with Alcohol and an Antifungal Agent

Piphillin, referring to the KEGG database, was used to predict microbial functional effects of alcohol and the antifungal agent, amphotericin B, in mice, using 16S data from the study by Yang et al. (2017a). Pathway abundances, normalised by DESeq2, were logarithmically transformed with a constant added (log(x + 1)).

5.3.1.3 Other Findings

In both studies exploring liver disease in mice (Yang et al., 2017a; Furuya et al., 2020), fatty acid degradation was predicted to be enriched by the microbiota in mice fed alcohol, compared to the microbiota in control mice (Table A.9, Table

A.10, Table A.11). Smith et al. (2019) observed an effect of acarbose, the drug used to treat type 2 diabetes, on the mice microbiome. Functional analysis predicted that glycosaminoglycan degradation and carbohydrate digestion and absorption would be reduced by the microbiota in mice treated with acarbose, when compared to microbiota in control mice (Table A.13).

5.3.2 Gene Expression

In order to test for the effect of antibiotic treatment on expression of genes of pathways predicted to be increased by the microbiome in single flies, male *D. melanogaster* were kept singly or in groups of 10, either on control food or food containing antibiotics, and gene expression measured at 10 or 49 days of age.

5.3.2.1 Apoptosis Genes

In 10-day old flies, for all of the apoptosis genes, there was no significant interaction or effect of antibiotic treatment or social environment (full vs. null model: *Dcp-1*: $F_{3,21} = 0.526$, $p = 0.670$; *Diap-1*: $\chi^2 = 0.118$, $df = 3$, $p = 0.780$; *hid*: $F_{3,18} = 0.362$, $p = 0.781$) (Figure 4.8).

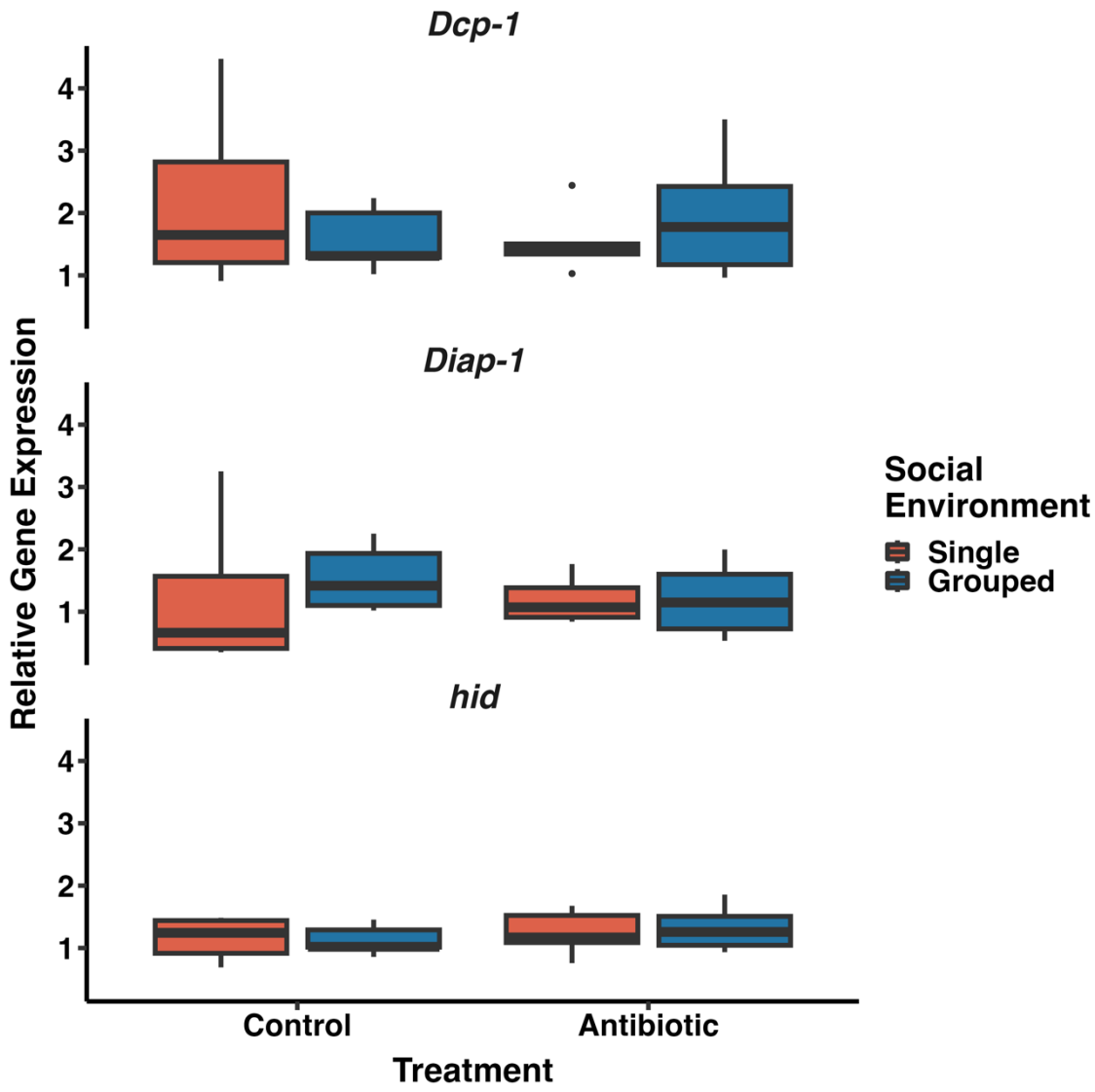

Figure 5.6 Effect of Antibiotics and Social Grouping on Apoptosis Gene Expression

Relative expression of genes involved in apoptosis in males kept singly or in groups of 10, on control food or food containing antibiotics, and snap-frozen at 10 days of age (see Table 2.3 for sample sizes after outlier removal).

5.3.2.2 MAPK Pathway Genes

In 10-day old flies, there was no significant interaction or effect of antibiotic treatment or social environment on the expression of genes involved in the MAPK pathway (full vs. null model: *cnk*: $F_{3,19} = 0.467$, $p = 0.413$; *Duox*: $F_{3,18} = 0.902$, $p = 0.459$; *Ras85D*: $\chi^2 = 0.333$, $df = 3$, $p = 0.776$; *Sod2*: $F_{3,13} = 1.797$, $p = 0.198$;

vn: $F_{3,19}$ = 1.163, p = 0.350) (Figure 5.7). For *p38c*, there was no significant interaction (χ^2 = 0.232, df = 1, p = 0.191), or effect of antibiotic treatment (χ^2 = 0.422, df = 1, p = 0.108), and the effect of social environment was marginally non-significant, with a slight trend for higher expression in grouped flies (χ^2 = 0.997, p = 0.053).

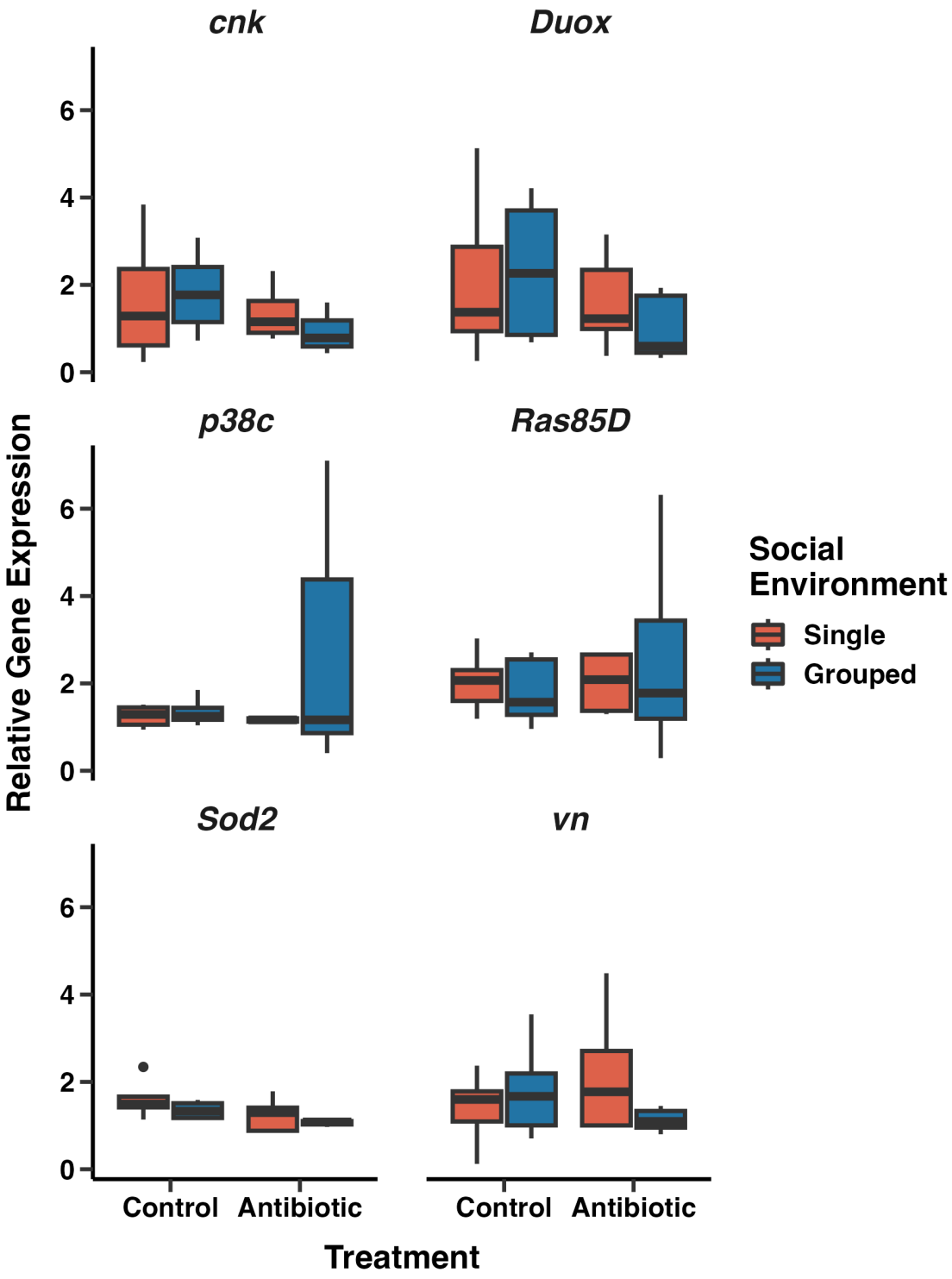

Figure 5.7 Effect of Antibiotics and Social Grouping on 10 Day MAPK Gene Expression

Relative expression of genes involved in MAPK signalling in males kept singly or in groups of 10, on control food or food containing antibiotics, and snap-frozen at 10 days of age (see Table 2.3 for sample sizes after outlier removal).

In 49-day old flies, for genes *p38c, Sod2 and vn*, there was no significant interaction or effect of antibiotic treatment or social environment (full vs. null model: *p38c*: $F_{3,19}$ = 0.4, p = 0.755; *Sod2*: $F_{3,18}$ = 0.925, p = 0.449; *vn*: $F_{3,17}$ = 0.084, p = 0.968) (Figure 5.8).

When measuring *cnk* expression in 49-day old flies, there was a significant interaction between antibiotic treatment and social environment ($F_{1,20}$ = 9.313, p = 0.007) (Figure 5.8). Pairwise comparisons revealed that there was no difference between single and grouped flies in the control group *(*p = 0.732), but grouped flies had a lower expression than single flies in the antibiotic group *(*p = 0.001, d = 2.043). This was driven by a decrease in grouped antibiotic flies compared to control flies *(*$p = 2 \times 10^{-4}$, d = 2.122).

When measuring *Duox* expression in 49-day old flies, there was a significant interaction between antibiotic treatment and social environment (χ^2 = 24.57, $p < 0.0001$) (Figure 5.8). Pairwise comparisons revealed that there was no difference between single and grouped flies in the control group *(*p = 0.776), but grouped flies had a lower expression than single flies in the antibiotic group *(*p = 0.001, d = 3.170). This was driven by a decrease in grouped antibiotic flies compared to control flies *(*p = 0.001, d = 26.694).

When measuring *Ras85D* expression in 49-day old flies, the interaction between antibiotic treatment and social environment was marginally non-significant ($F_{1,19}$ = 3.228, p = 0.089) (Figure 5.8). There was no effect of social environment ($F_{1,20}$ = 0.065, p = 0.802), but control flies had a significantly higher expression than antibiotic flies ($F_{1,21}$ = 5.903, p = 0.025).

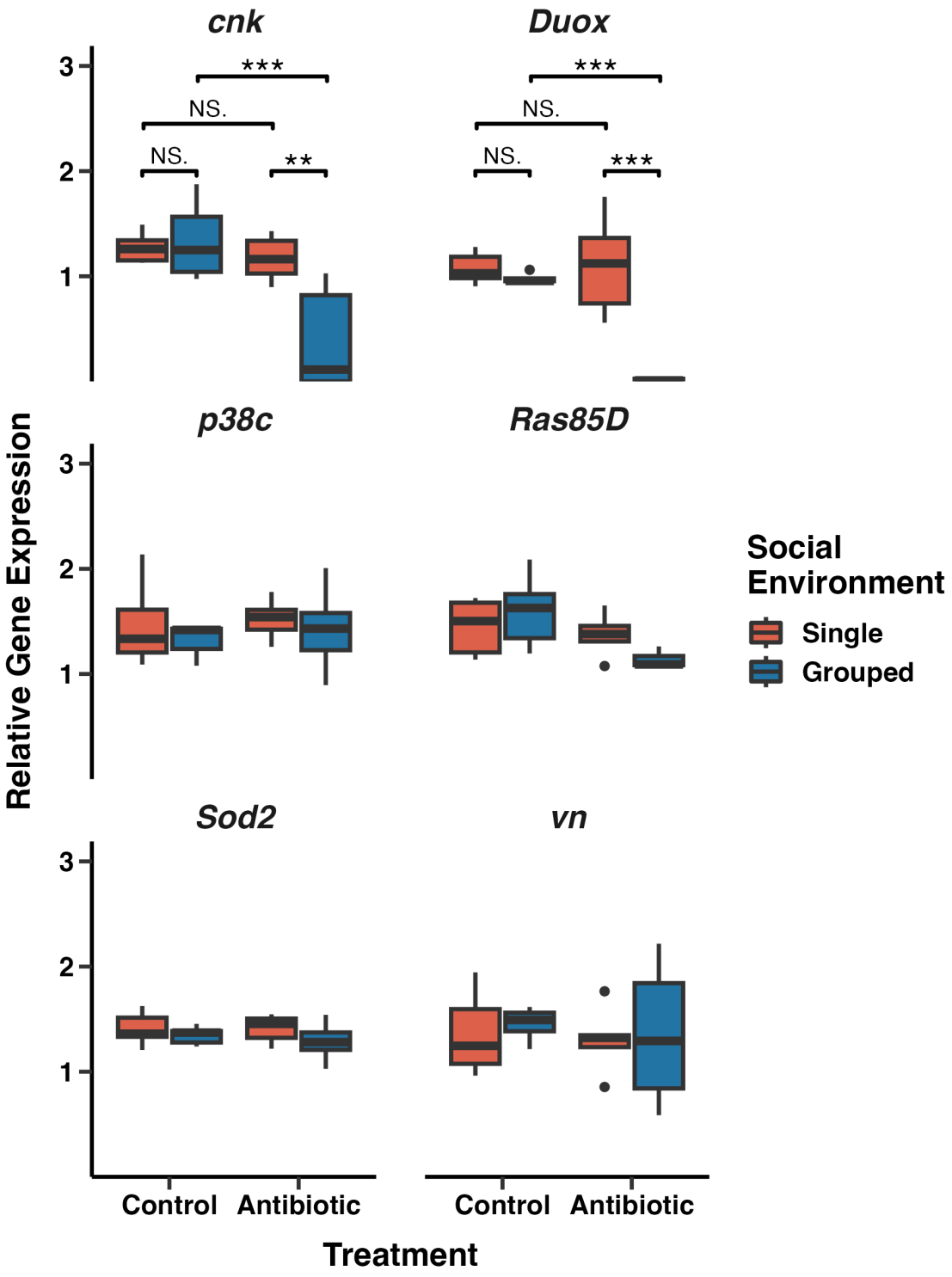

Figure 5.8 Effect of Antibiotics and Social Grouping on 49 Day MAPK Gene Expression

Relative expression of genes involved in MAPK signalling in males kept singly or in groups of 10, on control food or food containing antibiotics, and snap-frozen at 49 days of age (see Table 2.3 for sample sizes after outlier removal). Significance levels are indicated for pairwise comparisons (NS. = non-significant, * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$).

5.4 Discussion

5.4.1 Inferring Functional Effects From Microbial Data

In this chapter, functional effects of an altered microbiota were predicted using previously collected and published microbial data. The first issue encountered when conducting the meta-analysis was a lack of 16S data availability, despite the routine requirement for deposition in a database. Most of the studies first identified did not contain information on where to find this data or stated that data would be provided upon request. Nearly all the corresponding authors did not reply to requests.

Data sharing is important for reproducibility and transparency. It enables researchers to leverage existing data to explore new hypotheses, conduct meta-analyses, and develop a more comprehensive understanding of findings. This data search highlighted a concerning lack of data transparency in many studies measuring 16S changes, discouraging opportunities for scientific progression, and running counter to the FAIR principles for scientific data management (Wilkinson et al., 2016).

5.4.1.1 Pathways Influencing Longevity

Five pathways linked to ageing and immunity were often predicted to be affected by microbiota in treatments that extended lifespan. The longevity regulating pathway involves insulin signalling and target of rapamycin (TOR) signalling, which interact with each other, and both increase lifespan in *Drosophila* when inhibited (Clancy et al., 2001; Kapahi et al., 2004). Studies have established that both are sensitive to the microbiome in *Drosophila*. In previous research, *L.*

plantarum regulated growth via TOR dependent nutrient sensing (Storelli et al., 2011), and the increase in lifespan observed in *Drosophila* after human faecal transplant was linked to TOR downregulation (Ji et al., 2022). In flies, *A. pomorum* was able to quicken development via insulin signalling (Shin et al., 2011), and pro- and prebiotics, which increased lifespan, reduced insulin-like signalling during ageing (Westfall et al., 2018). The FoxO signalling pathway has also been associated with ageing in *Drosophila*; *dFOXO* overexpression in pericerebral fat body lengthened lifespan and fecundity (Giannakou et al., 2004; Hwangbo et al., 2004). Apoptosis, responsible for the removal of damaged or dysfunctional cells, is linked to lifespan in multiple species (Zhang & Herman, 2002), and increases with physiological age in *Drosophila* (Zheng et al., 2005). Lastly, MAPK signalling involves three pathways that use protein phosphorylation to regulate mitosis, apoptosis, and metabolism. Inhibition of RAS or Pnt, involved in ERK signalling, extended lifespan in *Drosophila* (Alic et al., 2014; Slack et al., 2015). Another MAPK pathway, p38 kinase signalling, is important in responding to environmental stimuli, and leads to the production of ROS by the enzyme Duox in response to bacterial activation, important for gut homeostasis (Ha et al., 2009).

A similar pattern of increase in these ageing-related pathways was evident when flies were raised at low temperatures as to when they were raised singly. These pathways were predicted to be enriched by microbiota in single flies compared to grouped flies (Leech and McDowall et al. 2021), and flies reared at 13°C compared to 23°C. Both low temperature (Norry & Loeschcke, 2002), and keeping flies singly (Bretman et al., 2013) increased longevity, and the results presented here indicate that the microbiota could play a part in this increase.

When analysing flies reared at another low, but less extreme temperature, 18°C, only FoxO signalling, longevity regulating and MAPK signalling pathways were enriched by the microbiota, when compared to flies reared at 25°C, and less than when treated with the more extreme temperature, suggesting that effects may be strongest at lower temperatures. Although also permitting an extension in lifespan, rearing larvae without adult flies present showed a slightly different signature, displaying more of an enrichment in the pathways than were enriched modestly in cold-reared flies and adult flies kept singly: FoxO signalling, and longevity regulating pathways (Leech and McDowall et al. 2021). In contrast, they had less of a microbial enrichment of apoptosis and MAPK signalling pathways (Leech and McDowall et al. 2021). Therefore, it seems like longevity may be regulated by the microbiome in different ways depending on the manipulation. Microbiota was not predicted to have as much of a role in modulating longevity via these pathways in the treatments used in mice.

When lifespan was increased with the oxidant, tBH, FoxO and MAPK signalling pathways were predicted to be reduced by the adult fly microbiota when compared to control adult flies. This effect was not present when the larval microbiome was measured, consistent with a smaller difference between treatments in larval microbiome composition than in adults, presented in the paper (Obata et al., 2018). In this study, tBH acted as an antibiotic, reducing *Acetobacter* as well as *Acetobacter*-dependent IMD target gene expression and ISC over-proliferation (Obata et al., 2018). This aligns with the functional analysis prediction that the change in microbiota caused by tBH resulted in less stimulation of pathways linked to immune activation, such as MAPK and FoxO signalling pathways. The MAPK pathway involves the p38 pathway which is involved in the

antimicrobial oxidative response, activated in response to uracil from certain bacteria, such as *G. morbifer* (Ha et al., 2005; Lee et al., 2013). *G. morbifer* has been shown to proliferate in response to IMD overactivation (Roh et al., 2008; Ryu et al., 2008), and so may be present in higher levels in control flies that display a higher IMD activation due to *Acetobacter* stimulation (Obata et al., 2018). Additionally, increased FoxO signalling leads to dysbiosis in aged flies, resulting in an increased activation of the IMD pathway (Guo et al., 2014), and so control fly microbiota enrichment for the FoxO signalling pathway may explain the higher levels of IMD target gene expression observed in control flies (Obata et al., 2018).

5.4.1.2 Pathways Influencing Disease Pathology

Two of the studies included in the meta-analysis explored the development of liver disease, and it's association with microbial changes (Yang et al., 2017a; Furuya et al., 2020). A dysbiotic microbiome, caused by a high fat diet or alcohol consumption, is characteristic of this disease, with alterations in diversity and composition present in human cases (Grander et al., 2018; Mosaferi et al., 2021). This link is to be expected as the liver serves as the initial site for processing nutrients or metabolites generated by the gut. Additionally, dysbiosis can lead to gut barrier dysfunction which leads to translocation of bacterial components to the liver, aiding in disease progression (Fukui, 2015). Faecal microbiota transplantation can transmit liver disease in mice (Llopis et al., 2016), whereas a minor improvement in liver pathology was observed in patients when given microbiota from healthy donors (Philips et al., 2022). However, the interaction between gut and liver is bidirectional, as the liver secretes bile, containing

antimicrobial molecules, into the small intestine. Therefore, it is not always clear when microbiome changes are caused by or causing liver disease.

In the study by Furuya et al. (2020), liver pathology was induced by HFD and augmented by EtOH and induced liver injury. In both control mice and mice with induced liver injury, there was no microbial functional differences predicted between mice on the HFD and mice on the HFD + EtOH, concurrent with the small differences in genus abundances displayed in the paper (Furuya et al., 2020). Alcohol metabolism interferes with the breakdown of fatty acids in the liver, leading to an accumulation of fat within liver cells during alcohol-associated liver disease (Crabb et al., 2004). Indeed, Furuya et al. (2020) observed downregulation of the genes *Acsl1* and *Fabp1*, involved in fatty acid metabolism, in the HFD + EtOH mice. However, in this chapter, fatty acid degradation was predicted to be enriched by microbiota in mice fed HFD or EtOH, independent of liver injury, as well as mice fed EtOH in another study exploring liver disease (Yang et al., 2017a). These results provide evidence that the decrease in fatty acid metabolism usually displayed in mice experiencing liver disease is independent of the microbiome.

Patients with alcohol-associated liver disease have an altered faecal composition of fungi (Lang et al., 2020), that can promote disease progression (Chu et al., 2020). Yang et al. (2017a) found that antifungal treatment impeded the development of ethanol-induced liver disease in mice. They concluded that this was down to a decrease in β-glucan translocation produced by fungi and claimed that the antifungal treatment did not majorly change bacterial composition. However, the results in this chapter reveal that the antifungal

treatment did significantly alter the predicted function of the bacteria in mice treated with EtOH. In fact, antifungal treatment seemed to somewhat reverse the pattern of pathway abundance so that the pathway composition of EtOH + antifungal treated mice appeared more similar to that of control mice, who had not been exposed to alcohol. Pathways reversed by antifungal treatment included alcoholism, serotonergic synapse and dopaminergic synapse, suggesting a possible role for the microbiome in alcohol dependence, reversible by antifungal treatment, warranting further research. The discrepancy between the conclusions made when inspecting microbiome composition differences and when inspecting functional differences highlights the importance of employing functional analysis. Small changes in bacterial community composition that may seem insignificant when examining the microbiome as a whole may be important to the host functionally. As bacteria and fungi occupy similar niches within the gut and so compete with each other, inhibition of fungal growth may have altered the bacterial composition after antifungal treatment, similar to what has been observed in other studies (Wheeler et al., 2016). In the case analysed in this chapter the microbial changes after antifungal treatment resulted in a functional profile more similar to that of control mice

The final paper analysed (Smith et al., 2019) explored the effect of the drug acarbose, used to treat type 2 diabetes mellitus (Laube, 2002). Acarbose inhibits α-glucosidase and α-amylase, which delays the intestinal breakdown of carbohydrates, resulting in reduced increases in blood glucose (Jenkins et al., 1981; Harrison et al., 2019). Predicted microbial pathway analysis presented in this chapter suggested microbial changes may contribute to the drug's antidiabetic effect, as carbohydrate digestion and absorption pathways were

predicted to be reduced by microbiota in mice treated with acarbose. Additionally, the glycosaminoglycan degradation pathway was predicted to be reduced by microbiota in treated mice. Glycosaminoglycans are polysaccharides that decrease during diabetes pathology, leading to leakiness of the kidney membrane (Shimomura & Spiro, 1987). Decrease in this degradation, driven by the microbiota, may maintain glycosaminoglycan levels and so deter the pathology of diabetes.

5.4.2 Measuring Functional Effects of the Microbiota

As microbial functional analysis only predicts pathways that microbiota enrich, these predictions require validation through empirical measurements. One method for such validation involves utilising RT-qPCR to measure gene expression changes. However, no evidence from the gene expression analysis supported the single fly enrichment in MAPK and apoptosis pathways predicted by functional analysis. Yet, it must be considered that the predicted differences in these pathways may not be reflected at the transcriptional level, but could arise from other changes such as post-transcriptional modifications or protein stability, which are not captured by qPCR analysis. Additionally, these pathways are extensive and complicated, and so measurements of more components in the pathway, should be used to measure microbiota dependent enrichment of these pathways overall. This method needs to be complemented by additional methodologies, such as measurements of metabolites, in order to verify the predictions made by the KEGG analysis.

A role of the microbiome in the expression of genes involved in the MAPK pathway was not made clear using 10-day old flies. However, visual inspection

of the results hint at a reversal of the effect of the social environment on *cnk* and *Duox* expression in antibiotic flies. Therefore, gene expression was also measured in 49-day old flies, to determine whether these effects became more prominent with senescence and prolonged exposure to treatment. At this age, antibiotic grouped flies had reduced *cnk* and *Duox* gene expression compared to single flies on antibiotics and flies not receiving antibiotics, with a similar pattern observed in *Ras85D*, though not reaching significance.

In aged flies, there was no difference in *cnk* and *Duox* expression between single and grouped flies in the control treatment, but antibiotics resulted in grouped flies displaying a large reduction in expression compared to single flies. One explanation for this is that the p38 pathway and the ERK pathway, at least when considering *Duox*, *cnk*, and perhaps *Ras85D* gene expression, is activated independently of the microbiome more in single flies than in grouped flies, but microbiota stimulation in grouped flies results in gene expression levels more similar to that of single flies (Figure 5.9a). Subsequently, antibiotics would remove this, leaving only factors independent of the microbiome to increase gene expression. Alternatively, as the antibiotics used do not completely eliminate the microbiome, it must be considered that the antibiotic grouped fly decrease in expression may be triggered by certain microbiota that are capable of inhibiting the pathways measured, rather than a lack of bacteria (Figure 5.9b). It may be that antibiotics work to reduce most bacterial species, and the existing members that are more resistant to the antibiotics are then shaped by dysbiosis caused by grouping. Therefore, only both these treatments result in a reduction in MAPK signalling. As the combination of antibiotics used may not eliminate all species of bacteria, it must be considered that microbiota that are not effected by antibiotics

may also be influencing gene expression in the single fly. Therefore, this method of analyses neither refutes or validates the predictions made by the functional analysis.

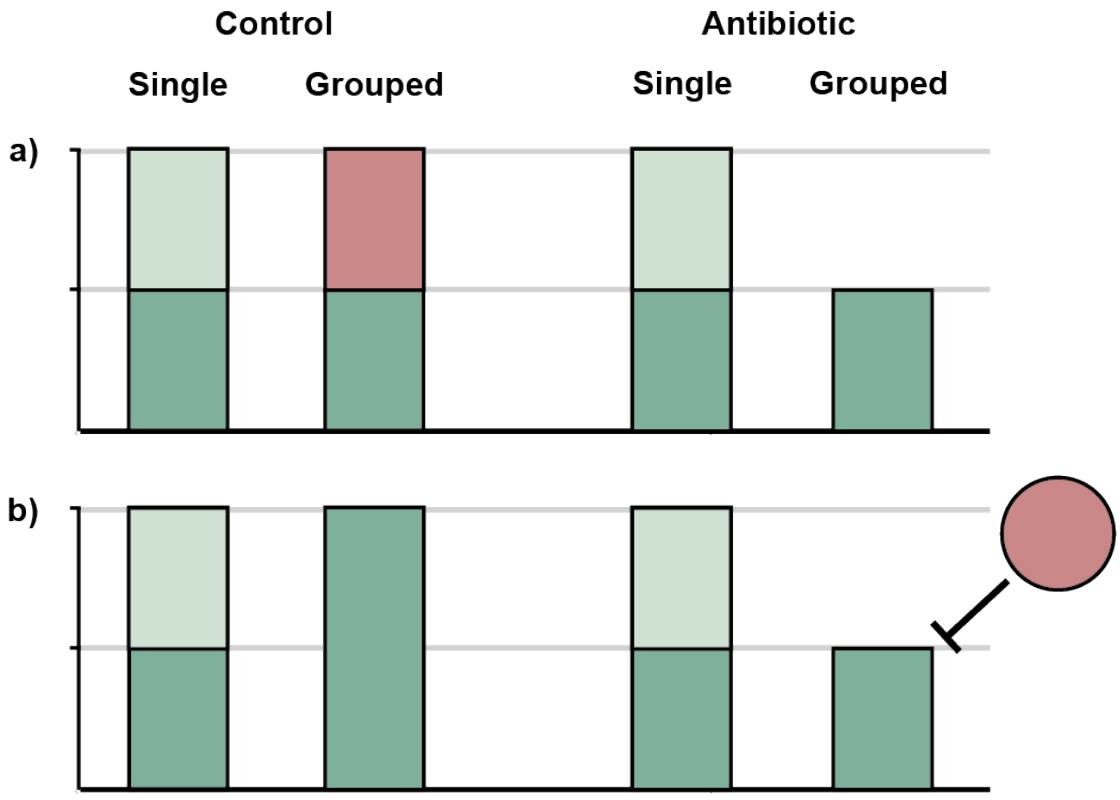

Figure 5.9 Explanations for MAPK Gene Expression Levels in the Different Treatment Groups in Aged Flies

Green bars represent gene expression caused by factors unrelated to the microbiome. The pink area of the bar represents gene expression caused by the microbiome able to be altered by antibiotics and the pink circle represents microbiota that inhibit gene expression. The pale green area of the bar represents gene expression that is possibly caused by microbiota that is not altered by the antibiotics.

If the first explanation is true (Figure 5.9a), grouped fly *Duox* and *Ras85D* expression dependency on the microbiome, particularly during ageing, may occur due to a stimulation of the p38 pathway by members of bacteria present during dysbiosis. The p38 pathway is activated in response to uracil from certain

bacteria, such as *G. morbifer* (Ha et al., 2005; Lee et al., 2013), which has been shown to proliferate in response to IMD overactivation (Roh et al., 2008; Ryu et al., 2008). IMD induction is known to increase with age (Buchon et al., 2009a), and so it is possible this bacteria, or another capable of producing uracil, is present in the grouped flies, activating the p38 response. In this explanation, antibiotics are able to eliminate this species. In the second explanation (Figure 5.9b), bacteria produced by a combination of grouping and antibiotics are able to inhibit MAPK signalling. This could occur via pathogenic bacteria attempting to disrupt the defence systems of the host. A mammalian pathogen, *Yersinia,* is able to prevent host expression of cytokines and anti-apoptotic factors by inhibiting MAPK signalling. Increased pathogenic activity may explain why antibiotic paired flies displayed the lowest activity, a marker of senescence, when aged (Chapter 4), although this does not impact lifespan (Chapter 3).

The decrease in grouped fly gene expression when given antibiotics is also reflected when measuring *fustch* and *Nrx-1* expression (Chapter 4). This is particularly interesting as ERK signalling is involved in the regulation of synaptic plasticity in many species (Reviewed in Sweatt, 2001). This highlights a possible relationship between the microbiome, ERK signalling and neuronal plasticity.

There was little evidence for a role of the microbiome in driving the expression of genes involved in apoptosis. However, this was only measured at 10 days, and results when measuring *Diap-1*, a negative regulator of apoptosis, hinted at a small reversal of the effect of grouping, which may be revealed if measured in aged flies, warranting further study.

5.4.3 Conclusion

The findings outlined in this chapter underscore the significance of examining possible host functional effects driven by alterations in the microbiome. Collecting sequencing data demands considerable resources in terms of both time and cost, making it imperative to maximally benefit on this investment. Inspecting potential effects of microbiome changes not only offers broader context and possible explanations for any observed effects to the host, but it also has the potential to reveal patterns that differ from those identified when focussing solely on sequencing data. Results from functional analysis were able to provide possible explanations for *Drosophila* lifespan changes under different stressors, as well as mice disease pathology, highlighting the remarkable versatility of this method across different biological contexts. However, variations observed among different stressors and organisms did not support the idea of a general stress signature.

This analyses should be used in combination with measurements of the proteome, metabolome or transcriptome in order to provide support for any hypotheses formed. Manipulation of the microbiome using germ-free animals or antibiotics allows for identification of which effects are dependent on the microbiome. Using this method, it was revealed that in grouped *Drosophila*, expression of genes involved in p38 and ERK signalling may have been increased by the microbiome. This adds to the evidence that suggests dysbiosis occurs when flies are grouped, with the imbalance in microbiota allowing for pathogenic bacterial species to proliferate and stimulate stress and immune pathways. However, there is still doubt about whether this is occurring. 16S sequencing could be utilised to determine the effect of the combination of

antibiotics used on microbiome composition in *D. melanogaster*. Functional analyses on this data would help identify if the functional signature of antibiotic flies is more similar to that of grouped flies than it is to single flies. This would help establish why grouped flies have a reduced lifespan which can be partially induced in single flies by antibiotic exposure (Chapter 3). Additionally, future research could determine if other pathways, predicted to be enriched in single flies, such as FoxO signalling and longevity regulating pathways are influenced by the microbiome in *Drosophila* kept in different social environments.

As demonstrated in this chapter, predicted functional effects of the microbiota in diseased individuals can help formulate hypotheses on why pathology is occurring, and hence aid in the formulation of treatments. Furthermore, genes differentially regulated in diseased phenotypes could act as biomarkers for disease. Differential gene expression plays a significant part in disease development. For example, numerous studies have connected disease-associated variants in regulatory DNA and diverse human diseases, including cancer (Jiang et al., 2011) and Alzheimer's disease (Gaj et al., 2012). Furthermore, a study used functional analysis to predict that the microbiota increased bacterial invasion of epithelial cells and pathogenic *E. coli* infection in IBD patients (Häsler et al., 2017). Establishing the involvement of the microbiome in gene expression levels that determine health, allows for opportunities for disease prevention and treatments. This is particularly promising given the substantial body of research indicating the ability to alter the microbiome with diet, antibiotics, and probiotics.

Chapter 6
General Discussion

The predominant finding in this thesis was that male responses to the social environment in *D. melanogaster* are modulated at least partially by the microbiome. Male *Drosophila* respond to same-sex social contact by extending their mating duration (Bretman et al., 2009), thus increasing their fitness in the short term (Bretman et al., 2013; Hopkins et al., 2019). This coincides with a reduction in lifespan (Bretman et al., 2013), though it is not clear whether this is a consequence of an increased reproductive response, signifying a trade-off, or alternative impacts of the social environment. As many *Drosophila* traits are sensitive to the microbiome, such as SCFA production (Shin et al., 2011), immune responses (Blum et al., 2013), and metabolism (Wong et al., 2014), and the microbiome can be influenced by the social environment (Leech and McDowall et al. 2021), the microbiome is a promising candidate for a driver of these responses. Work in this thesis revealed that manipulation of the microbiome affects several previously measured social responses, including the reduction in lifespan, improvement in cognitive ability and alteration of gene expression when male *Drosophila* are exposed to rivals.

6.1 Main Findings

A summary of which responses were affected by antibiotic perturbations in this thesis is presented in Table 6.1. Previous work implicated the microbiome in mediating the effect of the social environment on host lifespan (Leech and McDowall et al. 2021), and this thesis presents the first direct test of this hypothesis. The reduction in lifespan displayed by male *Drosophila* when paired

or grouped could be partially replicated in single flies by perturbing the microbiome with antibiotics (Chapter 3). Antibiotics affected single fly, but not grouped or paired fly lifespan, reducing the difference in lifespan between those which had been kept singly and those which had been exposed to rivals. This may be due to dysbiosis, either caused by grouping or antibiotics, reducing lifespan.

Table 6.1 Summary of Findings

The responses tested in this thesis and whether they were observed to be affected by microbial changes in young or aged flies.

Response	Age	Affected by the Microbiome?
Lifespan	_	Yes
Cognitive	Young	No
	Aged	Yes
Mating	Young	No
	Aged	Not investigated
Cognitive gene expression	Young	Sometimes
	Aged	Sometimes
Apoptosis gene expression	Young	No
	Aged	Not investigated
MAPK gene expression	Young	No
	Aged	Yes

Group size influenced lifespan, and hence the role the microbiome played in the social response (Chapter 3). The difference between flies exposed or not exposed to rivals was reduced if flies were kept in pairs, not groups. Furthermore, despite no direct comparison with single flies, antibiotics increased lifespan of flies kept in groups of 20 rather than the usual decrease, and mortality appeared high. The beneficial effect of antibiotics in this case suggests this higher mortality has a microbial reason, which could include increased dysbiosis in groups of 20, or potentially disease.

Findings suggested that accelerated senescence, triggered by the social environment, may depend on the age of the rival. Previous research has exhibited evidence for this, presenting an increase in lifespan if the rival was younger (Cho et al., 2021; Lin et al., 2022). However, in this thesis, any extension in lifespan could not be replicated. Nonetheless, when measuring behaviour, the youth of the rival seemed to have a slight protective effect on socially-driven age-associated changes in behaviour, such as loss of activity and sitting on food (Chapter 4). Work from Leech and McDowall et al. (2021) suggests that this effect could be dependent on the microbiome as when rivals were young the microbiome remained relatively unchanged despite pairing, resulting in flies possessing a microbiome more akin to that of solitary flies. However, work from this thesis uncovered no evidence for a dependency on the microbiome in regards to this effect. This may due to the effect being too subtle to be to be observed consistently. Additionally, using antibiotics meant that the microbiome of single flies may not have been perturbed in the same way as paired flies. Further work could leverage 16S sequencing to explore the effect of the combination of antibiotics used on the microbiome, as well as utilise a germ-free

technique with the experimental design used in this thesis to see if this yields contrasting results.

It was unclear if the socially-driven lifespan response observed occurred due to a direct effect of microbiome alterations, or indirectly, caused by other responses that are triggered by microbiome changes. Results in this thesis provide evidence that the latter may be occurring (Chapter 4). For example, the cognitive response to the social environment was found to be sensitive to microbiome changes. In fact, antibiotics manipulated single fly cognition, reflecting what occurs in the lifespan response. Antibiotics increase single fly cognitive ability to a more similar level to that of grouped flies, suggesting that either certain bacteria present in grouped and antibiotic flies stimulate cognitive development, or bacteria present in single flies on control food inhibit it. Measuring gene expression revealed potential mechanisms underlying this, as antibiotics abolished the difference in the expression of genes involved in cognition between single and grouped flies. This may explain how lifespan is regulated by socially-driven microbial changes, as increased cognitive investment, triggered by microbial changes, may trade-off with lifespan. However, there is insufficient evidence to confirm this at this stage.

Although the mating duration response did not appear to be dependent on the microbiome in young flies (Chapter 4), this was not measured in aged flies and so further validation is needed to see whether the lifespan response is driven by reproductive changes. The outcomes observed when applying antibiotics at different ages implies that the microbiome has a positive effect on lifespan in the young fly, as only antibiotics from eclosion, and not from 30 days, reduced

lifespan (Chapter 3). This is supported by previous work which indicated that a decline in lifespan caused by antibiotics occurred only when applied from eclosion (Brummel et al., 2004).

Performing microbial functional analysis on published 16S data revealed that ageing-related pathways were often predicted to be differentially enriched between treatments that had an influence on lifespan in *Drosophila* (Chapter 5). Enrichment of these pathways when comparing flies reared at low temperatures to those reared at higher temperatures, was also found by Leech and McDowall et al. (2021) when comparing single and grouped flies. Furthermore, microbial functional analysis proved to be promising resource for forming hypotheses relating to the microbiome's role in disease pathology, when exploring liver disease and diabetes treatment in mice. In such research, it is imperative to determine not only how the microbiome is changing, but what this may mean for the host organism. As this analysis requires no further data collection after 16S sequencing, and results from this thesis confirms it yields novel insights beyond those obtained solely by examining microbiome changes, it presents a promising avenue to understand the implications of microbiome alterations. Nonetheless, it is important to note that this approach is most effective in organisms that have very well annotated genomes, and applicability to less extensively studied species is limited. Furthermore, efforts to validify these findings by measuring gene expression were unsuccessful, emphasising the need for caution when interpreting results derived from this method and underscoring the necessity for further verification, involving combining results with other measurements, such as metabolites.

Lastly, it was revealed that the expression of some genes was dependent on the microbiome in grouped *Drosophila* (Chapter 5). When flies were in groups, genes involved in the MAPK signalling pathway were enriched in control flies but not antibiotic flies. This supports the theory that the microbiome of grouped flies stimulates some host pathways more so than in single flies, possibly due to a microbial dysbiosis. Utilising germ-free flies could establish if these pathways are inhibited in grouped flies when the microbiome is removed completely, verifying if pathways were induced by the microbiota.

6.2 How Antibiotics Work to Influence Social Responses in Both Single and Grouped Flies

When interpreting the results presented in this thesis it must be considered that the combination of antibiotics used likely did not completely eliminate bacteria in flies. They also targeted bacteria exclusively, and not other microbial taxa such as fungi, and may exhibit varying effectiveness against different bacterial species. This means antibiotic treatment likely did not completely eliminate differences between single and grouped flies. However, antibiotics were shown to significantly reduce some commensal genera, such as *Lactobacillus*, *Acetobacter*, *Gluconobacter* and *Commensalibacter,* potentially reducing microbial differences between single and grouped flies. Antibiotics were demonstrated to either have an effect on single or grouped flies, depending on the response. This provides evidence that effects measured occurred as a result of antibiotics impacting the microbiome, as opposed to having a non-target effect on the host, as we would expect this to impact both social groups. In single flies, antibiotics may cause an imbalance in the microbiome, removing most of the commensal species and allowing other species to proliferate, both of which may

act to influence social responses. In grouped flies, antibiotics may exacerbate any imbalance already caused by the stress of the social environment, or it may act to reduce some bacterial species that were able to proliferate during the imbalance, both with potential to affect social responses.

It's important to address the potential limitations associated with the use of nipagin and propionic acid which are commonly employed in *Drosophila* research and used in all experiments in this thesis. Nipagin has been demonstrated to influence *Acetobacter* growth (Obadia et al., 2018), and so any effect modulated by *Acetobacter* may not manifest in the experiments conducted in this thesis. Additionally, nipagin is dissolved in ethanol which interacts with variations in the microbiome (Chandler et al., 2022), while acid preservatives, like propionic acid, may affect the fly directly or through effects on the microbiota (Kim et al., 2018). Consequently, caution is warranted when extrapolating the effects observed in this study, as they may not be reflected in wild *Drosophila* populations or laboratories that use different preservatives. For example, Sannino & Dobson (2023) indicated a dependency of microbial effects on triglyceride levels on the preservatives used. These considerations emphasize the need for careful interpretation and contextualization of the findings within the broader scientific landscape.

6.3 The Interaction Between Age and Microbial Impacts

Work in this thesis established that differences between single and grouped flies were often not dependent on the microbiome at a young age, but became dependent at an older age, when other declines in function occur, such as climbing ability (Leech et al., 2017) and mating (Bretman et al., 2013). One explanation for this is that although the antibiotics used seem to reduce bacterial load and remove some commensal species, the composition required for manipulation of the social response may not be established until later in life (Figure 6.1a). It may be that these responses are only influenced by the opportunistic bacterial species exhibiting higher resistance to the antibiotics. These may need time to proliferate, or are only able to do so when immunity is compromised by ageing, or the rest of the microbiome further perturbed by increased IMD signalling with age (Buchon et al., 2009a; Guo et al., 2014). Work from Leech and McDowall et al. (2021) suggests mechanisms independent of the microbiome should be driving the response at a young age as they observed no significant differences in microbial diversity or composition between single and grouped flies at this age. However, the authors did not test for predicted functional differences driven by the microbiome at this age.

Alternatively, bacteria able to manipulate the response may be present, but the host may not be sensitive to the microbiome at a young age, only becoming more sensitive during senescence (Figure 6.1b). This explanation would mean that another mechanism must be driving responses at a young age. Additionally, it permits the explanation that the response is caused by a lack of bacteria instead of a gain. Overall, this work highlights the importance of using sequencing analysis to determine how antibiotics impact the microbial

composition, as well as the importance of measuring microbial impacts at different ages in *Drosophila*.

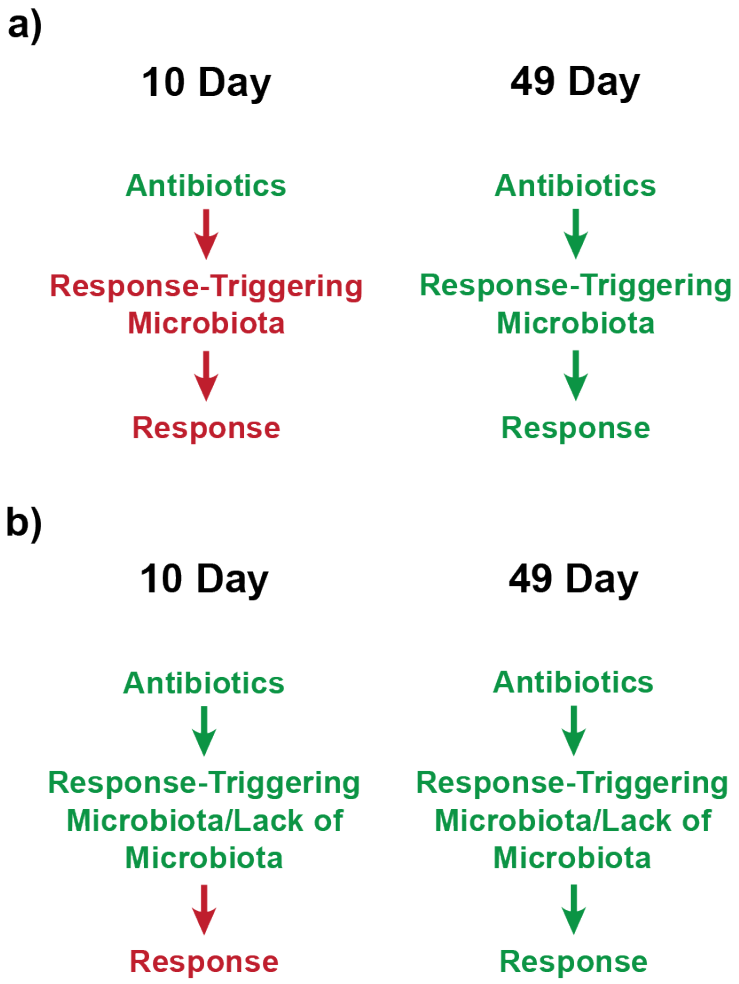

Figure 6.1 Explanations for the Antibiotic Response's Dependency on Age It may be the case that either a) antibiotics at a young age do not change the microbiome sufficiently to cause a change in the response, or that b) antibiotics do cause a change, but the host is not sensitive to the microbiome at this age. Factors altered by the antibiotics are coloured green and those that are not are coloured red.

When *futsch* gene expression was measured, the microbiome did have an effect on the social response in young flies. This suggests this response is sensitive to changes in the microbiome composition that are not able to trigger other responses. However, further measurements of gene expression need to be

considered as the *futsch* gene is only a small part in a complex pathway that controls cognitive ability. Nonetheless, this highlights the importance of testing whether the functional composition of the microbiome is altered during small microbial changes.

The interaction between microbial effects and age highlights the potential application of the *Drosophila* model in investigating the impact of the microbiome on ageing. In humans, certain microbial taxa have been associated with disease, some of which were observed to be elevated solely in aged individuals (Ghosh et al., 2022). Furthermore, some microbiome interventions exhibited greater efficacy in aged individuals (Cancello et al., 2019; Theou et al., 2019; Tran et al., 2019; Del Bo et al., 2021). The microbiome is linked to various theories of ageing through its influence on many age-related factors, including inflammation, immune function, and even telomere shortening. The ageing microbiome can lead to intestinal barrier dysfunction and increased pro-inflammatory cytokines (Thevaranjan et al., 2017), which can result in inflamm-ageing, chronic inflammation that accompanies ageing and is associated with many age-related diseases (Franceschi et al., 2000). Moreover, studies suggest a link between the microbiome and telomere length, further linking the microbiome to ageing (Reviewed in Assis et al., 2022). Metagenomic analysis revealed that age-related microbial alterations in *Drosophila* shared similarities to those observed in the aged human gastrointestinal tract and those with inflammatory disorders (Rera et al., 2012). Additionally, results in this thesis support the antagonistic pleiotropy theory of ageing (Williams, 1957), indicating that the *Drosophila* microbiome transitions from a beneficial trait at an early age to detrimental later in life. The findings in this thesis contribute to the body of research that supports *Drosophila*

as an invaluable model for understanding the role of the microbiome in immunity and inflammation during ageing (Brummel et al., 2004; Libert et al., 2006; Ren et al., 2007; Guo et al., 2014).

6.4 Areas for Future Research

The work presented in this thesis ultimately raises new questions that need to be addressed in future work. The microbiome's involvement in socially-driven responses has been revealed, but the specific mechanisms by which this occurs are yet to be determined. It was often unclear whether it was the loss or gain of bacteria that influenced social responses, a limitation occurring due to the use of antibiotics, which did not completely eliminate the microbiome. Utilising germ-free flies would result in the uniformization of the microbiome between single and grouped flies, providing a clearer understanding of the specific role played by microbiota. Combining results from axenic flies in this way, with studies using antibiotics and 16S sequencing could lead to the identification of certain bacteria that are able to influence social responses.

Work in this thesis began to explore how bacteria interact with host pathways to confer effects, yet further research is needed to examine additional mechanisms. No evidence for dopamine's role in the lifespan effect was uncovered in this thesis, but some evidence was found that supported an involvement of the expression of genes that are altered by the social environment, or even genes involved in the MAPK pathway. The microbiome has been shown to regulate *Drosophila* behaviour through octopamine signalling (Schretter et al., 2018; Jia et al., 2021), so further work should explore the involvement of octopamine in socially-driven responses. Future research should also aim to address the involvement of dopamine, and other neurotransmitters, in the cognitive response, as if the lifespan and cognitive responses occur independently, they may be modulated by different mechanisms. Dopamine, serotonin, octopamine and GABA all have roles in *Drosophila* learning

(Schwaerzel et al., 2003; Schroll et al., 2006; Kim et al., 2007; Liu & Davis, 2009; Monier et al., 2018; Pavlowsky et al., 2018), making them promising candidates.

The extent of lifespan reduction was dependent on group size, with groups of 20 displaying the highest mortality, which was reduced by antibiotics. Group size has previously been shown to influence some social responses, like offspring production (Hopkins et al., 2019), but not others, such as mating duration (Bretman et al., 2009; Bretman et al., 2010). It is yet to be determined whether group size influences the cognitive response. If an increased group size leads to a greater cognitive enhancement, this would provide support that the cognitive response drives lifespan effects. If this was the case, we would expect antibiotics to reduce cognition in flies in groups of 20, mirroring the pattern observed in lifespan. In chapter 3, it was conjectured that disease may be a reason for the high mortality in these groups of 20 (Figure 3.11). Measuring other responses such as the cognitive response in these groups would further test this theory, as increased disease would not be expected to increase the cognitive response. Additionally, the effect of these increased group densities on the microbiome could be explored by 16S sequencing.

As work in this thesis discovered that decreased cognition in single flies was dependent on the microbiome, it was theorised that increased cognitive investment, triggered by microbial changes, resulted in a trade-off with lifespan (Figure 6.2). To confirm whether this is occurring, cognitive development could be inhibited to explore whether this prevents lifespan reduction in grouped flies and single flies fed antibiotics. The finding that genes associated with neural development were altered with social environment and the microbiome implies

that these have a role in enhancing cognition. Therefore, it would be interesting to explore if reducing *futsch* or *Nrx-1* gene expression results in a change in cognitive performance and lifespan. Furthermore, it is yet to be ascertained whether the mating duration response could be the reason behind the microbiome-driven lifespan reduction (Figure 6.2).

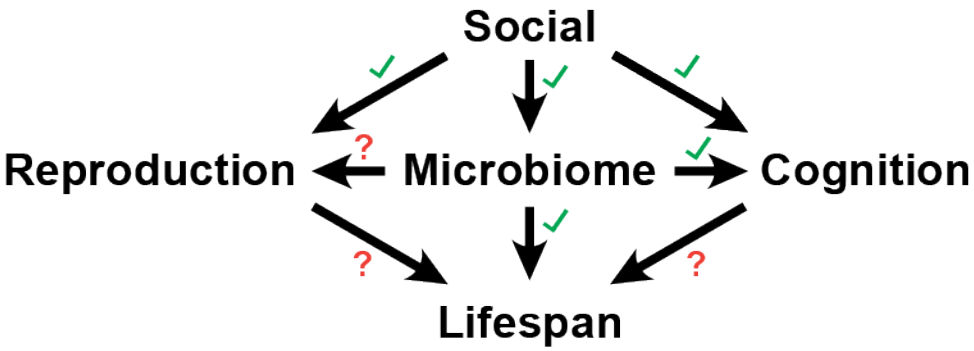

Figure 6.2 Established Interactions Between the Social Environment and Lifespan in *Drosophila* and Those That Require Further Study

Previous research has established that the social environment influences both reproductive and cognitive responses, as well as the microbiome. Work in this thesis has demonstrated that changes in the microbiome alter cognition and lifespan. Further work needs to establish whether the microbiome also alters reproduction, and whether the reduction in lifespan is driven by the cognitive or reproductive response.

6.5 Concluding Remarks

To conclude, research in this thesis provides evidence for a role of the microbiome in socially-driven responses in male *Drosophila,* influencing changes in lifespan, cognition and gene expression, often in an age-dependent manner. This highlights the significant involvement of the *Drosophila* microbiome in linking external stimuli to health outcomes. This *Drosophila* model underscores the relevance of harnessing the microbiome during targeted interventions to protect health and fight disease in other taxa, particularly in the context of ageing-associated conditions.

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Appendix

Table A.1 Effect of 13°C Rearing Temperature, Compared to 23°C, on Predicted Microbial Functional Pathway Abundance in *Drosophila*

Piphillin, referring to the KEGG database, was used to predict microbial functional effects of 13°C rearing temperature in *Drosophila*, using 16S data from the study by (Moghadam et al., 2018). Differentially abundant pathways were determined by DESeq2 analysis. A positive log2 fold change means pathways were represented more in flies reared at 13°C whereas a negative change means more pathways represented in flies reared at 23°C.

KEGG Number	Log2 Fold Change	LFC SE	Adj. *p* Value	Pathway
ko00270	-0.907	0.019	<0.0001	Cysteine and methionine metabolism
ko00330	-1.832	0.045	<0.0001	Arginine and proline metabolism
ko00401	-1.414	0.026	<0.0001	Novobiocin biosynthesis
ko00460	-0.872	0.019	<0.0001	Cyanoamino acid metabolism
ko00473	-1.191	0.028	<0.0001	D-Alanine metabolism
ko00480	-0.651	0.015	<0.0001	Glutathione metabolism
ko00523	-0.864	0.023	<0.0001	Polyketide sugar unit biosynthesis
ko00960	-1.178	0.022	<0.0001	Tropane, piperidine and pyridine alkaloid biosynthesis
ko00999	-0.876	0.018	<0.0001	Biosynthesis of various plant secondary metabolites

ko04146	0.580	0.014	<0.0001	Peroxisome
ko04922	0.432	0.009	<0.0001	Glucagon signaling pathway
ko00030	-0.492	0.013	<0.0001	Pentose phosphate pathway
ko00780	-0.393	0.011	<0.0001	Biotin metabolism
ko00627	0.965	0.027	<0.0001	Aminobenzoate degradation
ko00010	0.109	0.003	<0.0001	Glycolysis / Gluconeogenesis
ko03008	0.941	0.026	<0.0001	Ribosome biogenesis in eukaryotes
ko04151	0.937	0.028	<0.0001	PI3K-Akt signaling pathway
ko04016	-0.859	0.026	<0.0001	MAPK signaling pathway - plant
ko05200	0.485	0.015	<0.0001	Pathways in cancer
ko04931	0.970	0.033	<0.0001	Insulin resistance
ko04217	0.928	0.032	<0.0001	Necroptosis
ko00051	0.265	0.009	<0.0001	Fructose and mannose metabolism
ko00860	-0.337	0.012	<0.0001	Porphyrin metabolism
ko01210	-0.215	0.008	<0.0001	2-Oxocarboxylic acid metabolism
ko01200	0.054	0.002	<0.0001	Carbon metabolism
ko00562	-0.920	0.044	<0.0001	Inositol phosphate metabolism
ko00521	-0.661	0.032	<0.0001	Streptomycin biosynthesis
ko00750	-0.247	0.012	<0.0001	Vitamin B6 metabolism
ko01230	-0.451	0.024	<0.0001	Biosynthesis of amino acids
ko04626	-0.518	0.031	<0.0001	Plant-pathogen interaction

ko04070	1.368	0.081	<0.0001	Phosphatidylinositol signaling system
ko00983	0.491	0.029	<0.0001	Drug metabolism - other enzymes
ko00770	-0.998	0.061	<0.0001	Pantothenate and CoA biosynthesis
ko00130	1.073	0.067	<0.0001	Ubiquinone and other terpenoid-quinone biosynthesis
ko00511	-5.716	0.378	<0.0001	Other glycan degradation
ko04122	-0.453	0.030	<0.0001	Sulfur relay system
ko04141	1.359	0.090	<0.0001	Protein processing in endoplasmic reticulum
ko00471	0.406	0.027	<0.0001	D-Glutamine and D-glutamate metabolism
ko05418	0.053	0.004	<0.0001	Fluid shear stress and atherosclerosis
ko00430	-1.076	0.073	<0.0001	Taurine and hypotaurine metabolism
ko00450	-0.259	0.018	<0.0001	Selenocompound metabolism
ko04142	-5.824	0.408	<0.0001	Lysosome
ko00561	0.886	0.064	<0.0001	Glycerolipid metabolism
ko04214	0.884	0.065	<0.0001	Apoptosis - fly
ko05152	0.882	0.066	<0.0001	Tuberculosis
ko01523	0.881	0.067	<0.0001	Antifolate resistance
ko00710	0.766	0.060	<0.0001	Carbon fixation in photosynthetic organisms
ko00760	-1.156	0.093	<0.0001	Nicotinate and nicotinamide metabolism
ko00510	-5.717	0.459	<0.0001	N-Glycan biosynthesis
ko02060	11.865	0.977	<0.0001	Phosphotransferase system (PTS)
ko04013	0.877	0.072	<0.0001	MAPK signaling pathway - fly

ko04714	0.872	0.075	<0.0001	Thermogenesis
ko05134	0.873	0.076	<0.0001	Legionellosis
ko00909	-5.544	0.484	<0.0001	Sesquiterpenoid and triterpenoid biosynthesis
ko04940	0.874	0.077	<0.0001	Type I diabetes mellitus
ko00350	-1.558	0.140	<0.0001	Tyrosine metabolism
ko00720	0.123	0.011	<0.0001	Carbon fixation pathways in prokaryotes
ko00310	-0.612	0.056	<0.0001	Lysine degradation
ko00333	0.269	0.025	<0.0001	Prodigiosin biosynthesis
ko04727	0.871	0.084	<0.0001	GABAergic synapse
ko04724	0.871	0.084	<0.0001	Glutamatergic synapse
ko00195	0.863	0.084	<0.0001	Photosynthesis
ko00970	1.076	0.108	<0.0001	Aminoacyl-tRNA biosynthesis
ko02026	-1.366	0.140	<0.0001	Biofilm formation - Escherichia coli
ko00900	0.600	0.062	<0.0001	Terpenoid backbone biosynthesis
ko05014	0.055	0.006	<0.0001	Amyotrophic lateral sclerosis
ko01501	-0.429	0.045	<0.0001	beta-Lactam resistance
ko00360	-1.739	0.181	<0.0001	Phenylalanine metabolism
ko01055	0.860	0.091	<0.0001	Biosynthesis of vancomycin group antibiotics
ko04066	1.187	0.127	<0.0001	HIF-1 signaling pathway
ko00020	0.644	0.071	<0.0001	Citrate cycle (TCA cycle)
ko00908	0.856	0.095	<0.0001	Zeatin biosynthesis

ko05205	0.856	0.095	<0.0001	Proteoglycans in cancer
ko05133	0.052	0.006	<0.0001	Pertussis
ko04152	3.909	0.437	<0.0001	AMPK signaling pathway
ko04115	3.797	0.427	<0.0001	p53 signaling pathway
ko04215	3.797	0.427	<0.0001	Apoptosis - multiple species
ko04621	0.385	0.043	<0.0001	NOD-like receptor signaling pathway
ko05145	3.797	0.427	<0.0001	Toxoplasmosis
ko05161	3.797	0.427	<0.0001	Hepatitis B
ko05163	3.797	0.427	<0.0001	Human cytomegalovirus infection
ko05164	3.797	0.427	<0.0001	Influenza A
ko05167	3.797	0.427	<0.0001	Kaposi sarcoma-associated herpesvirus infection
ko05168	3.797	0.427	<0.0001	Herpes simplex virus 1 infection
ko05170	3.797	0.427	<0.0001	Human immunodeficiency virus 1 infection
ko05210	3.797	0.427	<0.0001	Colorectal cancer
ko05222	3.797	0.427	<0.0001	Small cell lung cancer
ko05416	3.797	0.427	<0.0001	Viral myocarditis
ko00940	3.793	0.427	<0.0001	Phenylpropanoid biosynthesis
ko05120	3.802	0.430	<0.0001	Epithelial cell signaling in Helicobacter pylori infection
ko00620	-0.712	0.081	<0.0001	Pyruvate metabolism
ko05010	0.854	0.097	<0.0001	Alzheimer disease
ko00053	-0.369	0.042	<0.0001	Ascorbate and aldarate metabolism

ko04072	3.781	0.435	<0.0001	Phospholipase D signaling pathway
ko05231	3.781	0.435	<0.0001	Choline metabolism in cancer
ko04068	0.056	0.007	<0.0001	FoxO signaling pathway
ko05225	-0.424	0.050	<0.0001	Hepatocellular carcinoma
ko05111	-1.945	0.233	<0.0001	Biofilm formation - Vibrio cholerae
ko05206	-4.450	0.540	<0.0001	MicroRNAs in cancer
ko05204	-0.740	0.090	<0.0001	Chemical carcinogenesis - DNA adducts
ko01503	-0.981	0.120	<0.0001	Cationic antimicrobial peptide (CAMP) resistance
ko04112	0.806	0.099	<0.0001	Cell cycle - Caulobacter
ko00550	0.755	0.093	<0.0001	Peptidoglycan biosynthesis
ko03410	0.590	0.073	<0.0001	Base excision repair
ko04932	0.847	0.105	<0.0001	Non-alcoholic fatty liver disease
ko05012	0.847	0.105	<0.0001	Parkinson disease
ko00300	0.793	0.099	<0.0001	Lysine biosynthesis
ko02024	-0.617	0.078	<0.0001	Quorum sensing
ko04216	10.953	1.382	<0.0001	Ferroptosis
ko00190	0.663	0.084	<0.0001	Oxidative phosphorylation
ko04920	11.351	1.470	<0.0001	Adipocytokine signaling pathway
ko00400	-2.235	0.290	<0.0001	Phenylalanine, tyrosine and tryptophan biosynthesis
ko01040	-0.726	0.094	<0.0001	Biosynthesis of unsaturated fatty acids
ko00410	-0.954	0.128	<0.0001	beta-Alanine metabolism

ko00785	0.841	0.115	<0.0001	Lipoic acid metabolism
ko04612	0.839	0.117	<0.0001	Antigen processing and presentation
ko04657	0.839	0.117	<0.0001	IL-17 signaling pathway
ko04659	0.839	0.117	<0.0001	Th17 cell differentiation
ko04914	0.839	0.117	<0.0001	Progesterone-mediated oocyte maturation
ko04915	0.839	0.117	<0.0001	Estrogen signaling pathway
ko05215	0.839	0.117	<0.0001	Prostate cancer
ko00140	25.424	3.607	<0.0001	Steroid hormone biosynthesis
ko00072	11.891	1.698	<0.0001	Synthesis and degradation of ketone bodies
ko04113	11.430	1.650	<0.0001	Meiosis - yeast
ko01120	0.038	0.006	<0.0001	Microbial metabolism in diverse environments
ko00966	9.629	1.420	<0.0001	Glucosinolate biosynthesis
ko04910	11.035	1.634	<0.0001	Insulin signaling pathway
ko03010	0.854	0.127	<0.0001	Ribosome
ko00524	9.591	1.426	<0.0001	Neomycin, kanamycin and gentamicin biosynthesis
ko03450	12.380	1.845	<0.0001	Non-homologous end-joining
ko01130	-0.124	0.019	<0.0001	Biosynthesis of antibiotics
ko00571	11.123	1.698	<0.0001	Lipoarabinomannan (LAM) biosynthesis
ko00740	0.832	0.128	<0.0001	Riboflavin metabolism
ko00791	10.988	1.723	<0.0001	Atrazine degradation
ko04138	11.044	1.764	<0.0001	Autophagy - yeast

ko00981	11.819	1.902	<0.0001	Insect hormone biosynthesis
ko00625	-3.011	0.485	<0.0001	Chloroalkane and chloroalkene degradation
ko00361	-3.139	0.513	<0.0001	Chlorocyclohexane and chlorobenzene degradation
ko00643	-3.132	0.514	<0.0001	Styrene degradation
ko00622	12.715	2.096	<0.0001	Xylene degradation
ko00362	-2.801	0.463	<0.0001	Benzoate degradation
ko05142	8.748	1.445	<0.0001	Chagas disease
ko00980	-0.687	0.115	<0.0001	Metabolism of xenobiotics by cytochrome P450
ko00071	-2.824	0.475	<0.0001	Fatty acid degradation
ko00903	-3.151	0.535	<0.0001	Limonene degradation
ko00621	12.483	2.119	<0.0001	Dioxin degradation
ko00281	-3.088	0.525	<0.0001	Metabolism of terpenoids and polyketides
ko00624	9.956	1.702	<0.0001	Polycyclic aromatic hydrocarbon degradation
ko05016	0.616	0.105	<0.0001	Huntington disease
ko00680	-0.328	0.056	<0.0001	Methane metabolism
ko00364	-3.292	0.568	<0.0001	Fluorobenzoate degradation
ko00440	-3.318	0.574	<0.0001	Phosphonate and phosphinate metabolism
ko02025	-2.978	0.515	<0.0001	Biofilm formation - Pseudomonas aeruginosa
ko03320	-3.019	0.522	<0.0001	PPAR signaling pathway
ko00623	-3.241	0.564	<0.0001	Toluene degradation
ko00630	0.257	0.045	<0.0001	Glyoxylate and dicarboxylate metabolism

ko00982	-0.678	0.119	<0.0001	Drug metabolism - cytochrome P450
ko04964	10.109	1.777	<0.0001	Proximal tubule bicarbonate reclamation
ko03060	0.551	0.098	<0.0001	Protein export
ko03020	0.493	0.089	<0.0001	RNA polymerase
ko00120	11.255	2.048	<0.0001	Primary bile acid biosynthesis
ko01220	-0.366	0.067	<0.0001	Degradation of aromatic compounds
ko00626	-3.265	0.596	<0.0001	Naphthalene degradation
ko00592	9.645	1.763	<0.0001	alpha-Linolenic acid metabolism
ko00830	-3.280	0.609	<0.0001	Retinol metabolism
ko00906	-3.319	0.620	<0.0001	Carotenoid biosynthesis
ko03440	0.444	0.083	<0.0001	Homologous recombination
ko00405	-3.275	0.619	<0.0001	Phenazine biosynthesis
ko00930	-3.195	0.605	<0.0001	Caprolactam degradation
ko00500	-3.085	0.585	<0.0001	Starch and sucrose metabolism
ko00531	-3.280	0.622	<0.0001	Glycosaminoglycan degradation
ko04930	-3.246	0.617	<0.0001	Type II diabetes mellitus
ko05165	-3.246	0.617	<0.0001	Human papillomavirus infection
ko05203	-3.246	0.617	<0.0001	Viral carcinogenesis
ko05143	9.256	1.768	<0.0001	African trypanosomiasis
ko00472	-3.338	0.639	<0.0001	D-arginine and D-ornithine metabolism
ko01053	-3.326	0.637	<0.0001	Biosynthesis of siderophore group nonribosomal peptides

ko00590	-3.293	0.633	<0.0001	Arachidonic acid metabolism
ko00052	-3.159	0.612	<0.0001	Galactose metabolism
ko01062	-3.344	0.651	<0.0001	Biosynthesis of terpenoids and steroids
ko00121	11.187	2.206	<0.0001	Secondary bile acid biosynthesis
ko00600	-3.212	0.635	<0.0001	Sphingolipid metabolism
ko04011	-3.336	0.660	<0.0001	MAPK signaling pathway - yeast
ko02030	-3.123	0.620	<0.0001	Bacterial chemotaxis
ko04918	-3.267	0.654	<0.0001	Thyroid hormone synthesis
ko00332	-3.266	0.654	<0.0001	Carbapenem biosynthesis
ko00920	-3.134	0.630	<0.0001	Sulfur metabolism
ko00290	-3.141	0.633	<0.0001	Valine, leucine and isoleucine biosynthesis
ko00340	-3.135	0.632	<0.0001	Histidine metabolism
ko04726	11.004	2.222	<0.0001	Serotonergic synapse
ko04728	11.004	2.222	<0.0001	Dopaminergic synapse
ko05030	11.004	2.222	<0.0001	Cocaine addiction
ko05031	11.004	2.222	<0.0001	Amphetamine addiction
ko05034	11.004	2.222	<0.0001	Alcoholism
ko03070	0.735	0.149	<0.0001	Bacterial secretion system
ko00280	0.338	0.069	<0.0001	Valine, leucine and isoleucine degradation
ko00061	-0.043	0.009	<0.0001	Fatty acid biosynthesis
ko02040	-3.148	0.647	<0.0001	Flagellar assembly

ko00540	-3.154	0.649	<0.0001	Lipopolysaccharide biosynthesis
ko05132	-3.283	0.678	<0.0001	Salmonella infection
ko04978	-3.331	0.692	<0.0001	Mineral absorption
ko00633	11.642	2.430	<0.0001	Nitrotoluene degradation
ko00261	0.354	0.074	<0.0001	Monobactam biosynthesis
ko00311	-3.331	0.698	<0.0001	Penicillin and cephalosporin biosynthesis
ko04080	10.936	2.313	<0.0001	Neuroactive ligand-receptor interaction
ko04979	10.936	2.313	<0.0001	Cholesterol metabolism
ko05166	10.936	2.313	<0.0001	Human T-cell leukemia virus 1 infection
ko04212	0.353	0.075	<0.0001	Longevity regulating pathway - worm
ko00240	0.403	0.089	<0.0001	Pyrimidine metabolism
ko05020	-3.338	0.740	<0.0001	Prion disease
ko05219	7.677	1.704	<0.0001	Bladder cancer
ko00565	-3.342	0.743	<0.0001	Ether lipid metabolism
ko00603	-3.354	0.747	<0.0001	Glycosphingolipid biosynthesis - globo and isoglobo series
ko05146	-3.368	0.753	<0.0001	Amoebiasis
ko03018	0.235	0.053	<0.0001	RNA degradation
ko04614	-3.361	0.761	<0.0001	Renin-angiotensin system
ko00220	0.047	0.011	<0.0001	Arginine biosynthesis
ko00642	9.898	2.328	<0.0001	Ethylbenzene degradation
ko05150	-3.374	0.795	<0.0001	Staphylococcus aureus infection

ko01054	-3.378	0.799	<0.0001	Nonribosomal peptide structures
ko03030	0.374	0.089	<0.0001	DNA replication
ko05340	-3.365	0.800	<0.0001	Primary immunodeficiency
ko00572	8.648	2.074	<0.0001	Arabinogalactan biosynthesis - Mycobacterium
ko04919	-3.394	0.818	<0.0001	Thyroid hormone signaling pathway
ko03420	0.345	0.083	<0.0001	Nucleotide excision repair
ko00513	-3.377	0.816	<0.0001	Various types of N-glycan biosynthesis
ko00604	-3.377	0.816	<0.0001	Glycosphingolipid biosynthesis - ganglio series
ko00730	-0.100	0.025	<0.0001	Thiamine metabolism
ko00790	-0.314	0.081	1.27E-04	Folate biosynthesis
ko00520	-0.238	0.061	1.37E-04	Amino sugar and nucleotide sugar metabolism
ko00650	-0.539	0.140	1.47E-04	Butanoate metabolism
ko00965	8.965	2.406	2.48E-04	Betalain biosynthesis
ko01524	-0.177	0.048	3.36E-04	Platinum drug resistance
ko01212	-0.091	0.025	3.41E-04	Fatty acid metabolism
ko04211	0.048	0.013	3.73E-04	Longevity regulating pathway
ko03013	8.753	2.434	4.06E-04	Nucleocytoplasmic transport
ko04260	0.464	0.131	4.92E-04	Cardiac muscle contraction
ko04210	0.048	0.014	9.70E-04	Apoptosis
ko01502	0.333	0.100	0.001	Vancomycin resistance
ko00670	0.193	0.059	0.001	One carbon pool by folate

ko03430	0.220	0.068	0.002	Mismatch repair
ko02010	-0.289	0.092	0.002	ABC transporters
ko00640	-0.190	0.062	0.002	Propanoate metabolism
ko02020	-0.380	0.132	0.005	Two-component system
ko01100	-0.056	0.020	0.005	Metabolic pathways
ko01051	0.045	0.017	0.008	Biosynthesis of ansamycins
ko05230	0.044	0.016	0.008	Central carbon metabolism in cancer
ko00525	0.044	0.017	0.013	Acarbose and validamycin biosynthesis
ko00660	-0.294	0.117	0.014	C5-Branched dibasic acid metabolism
ko04622	7.113	2.964	0.019	RIG-I-like receptor signaling pathway
ko00404	8.302	3.609	0.025	Staurosporine biosynthesis
ko03022	6.822	3.101	0.033	Basal transcription factors
ko04974	6.820	3.166	0.037	Protein digestion and absorption
ko04024	7.619	3.611	0.041	cAMP signaling pathway
ko00591	6.667	3.185	0.042	Linoleic acid metabolism

Table A.2 Effect of 13°C Rearing Temperature, Compared to 31°C, on Predicted Microbial Functional Pathway Abundance in *Drosophila*

Piphillin, referring to the KEGG database, was used to predict microbial functional effects of 13°C rearing temperature in *Drosophila*, using 16S data from the study by (Moghadam et al., 2018). Differentially abundant pathways were determined by DESeq2 analysis. A positive log2 fold change means pathways were represented more in flies reared at 13°C whereas a negative change means more pathways represented in flies reared at 31°C.

KEGG Number	Log2 Fold Change	LFC SE	Adj. *p* Value	Pathway
ko00627	1.034	0.027	<0.0001	Aminobenzoate degradation
ko03008	1.010	0.026	<0.0001	Ribosome biogenesis in eukaryotes
ko04146	0.615	0.014	<0.0001	Peroxisome
ko04922	0.456	0.009	<0.0001	Glucagon signaling pathway
ko00780	-0.414	0.011	<0.0001	Biotin metabolism
ko00030	-0.517	0.013	<0.0001	Pentose phosphate pathway
ko00480	-0.682	0.015	<0.0001	Glutathione metabolism
ko00523	-0.902	0.023	<0.0001	Polyketide sugar unit biosynthesis
ko00460	-0.909	0.019	<0.0001	Cyanoamino acid metabolism
ko00999	-0.914	0.018	<0.0001	Biosynthesis of various secondary metabolites - part 1
ko00270	-0.946	0.019	<0.0001	Cysteine and methionine metabolism
ko00960	-1.223	0.022	<0.0001	Tropane, piperidine and pyridine alkaloid biosynthesis
ko00473	-1.236	0.028	<0.0001	D-Alanine metabolism

ko00401	-1.464	0.026	<0.0001	Novobiocin biosynthesis
ko00330	-1.890	0.045	<0.0001	Arginine and proline metabolism
ko00010	0.112	0.003	<0.0001	Glycolysis / Gluconeogenesis
ko04151	1.005	0.028	<0.0001	PI3K-Akt signaling pathway
ko04016	-0.897	0.026	<0.0001	MAPK signaling pathway - plant
ko05200	0.513	0.015	<0.0001	Pathways in cancer
ko04931	1.039	0.033	<0.0001	Insulin resistance
ko04217	0.996	0.032	<0.0001	Necroptosis
ko00051	0.276	0.009	<0.0001	Fructose and mannose metabolism
ko00860	-0.356	0.012	<0.0001	Porphyrin and chlorophyll metabolism
ko01210	-0.229	0.008	<0.0001	2-Oxocarboxylic acid metabolism
ko01200	0.054	0.002	<0.0001	Carbon metabolism
ko00562	-0.957	0.044	<0.0001	Inositol phosphate metabolism
ko00521	-0.693	0.032	<0.0001	Streptomycin biosynthesis
ko00750	-0.263	0.012	<0.0001	Vitamin B6 metabolism
ko01230	-0.475	0.024	<0.0001	Biosynthesis of amino acids
ko04070	1.501	0.081	<0.0001	Phosphatidylinositol signaling system
ko00983	0.521	0.029	<0.0001	Drug metabolism - other enzymes
ko04626	-0.543	0.031	<0.0001	Plant-pathogen interaction
ko00130	1.164	0.067	<0.0001	Ubiquinone and other terpenoid-quinone biosynthesis
ko00770	-1.041	0.061	<0.0001	Pantothenate and CoA biosynthesis

ko04141	1.492	0.090	<0.0001	Protein processing in endoplasmic reticulum
ko04122	-0.478	0.030	<0.0001	Sulfur relay system
ko00471	0.429	0.027	<0.0001	D-Glutamine and D-glutamate metabolism
ko00430	-1.121	0.073	<0.0001	Taurine and hypotaurine metabolism
ko00511	-5.792	0.378	<0.0001	Other glycan degradation
ko00450	-0.274	0.018	<0.0001	Selenocompound metabolism
ko05418	0.052	0.003	<0.0001	Fluid shear stress and atherosclerosis
ko00561	0.955	0.064	<0.0001	Glycerolipid metabolism
ko04214	0.953	0.065	<0.0001	Apoptosis - fly
ko04142	-5.900	0.408	<0.0001	Lysosome
ko05152	0.951	0.066	<0.0001	Tuberculosis
ko01523	0.950	0.067	<0.0001	Antifolate resistance
ko00710	0.823	0.060	<0.0001	Carbon fixation in photosynthetic organisms
ko04152	5.991	0.437	<0.0001	AMPK signaling pathway
ko04115	5.836	0.427	<0.0001	p53 signaling pathway
ko04215	5.836	0.427	<0.0001	Apoptosis - multiple species
ko05145	5.836	0.427	<0.0001	Toxoplasmosis
ko05161	5.836	0.427	<0.0001	Hepatitis B
ko05163	5.836	0.427	<0.0001	Human cytomegalovirus infection
ko05164	5.836	0.427	<0.0001	Influenza A
ko05167	5.836	0.427	<0.0001	Kaposi sarcoma-associated herpesvirus infection

ko05168	5.836	0.427	<0.0001	Herpes simplex virus 1 infection
ko05170	5.836	0.427	<0.0001	Human immunodeficiency virus 1 infection
ko05210	5.836	0.427	<0.0001	Colorectal cancer
ko05222	5.836	0.427	<0.0001	Small cell lung cancer
ko05416	5.836	0.427	<0.0001	Viral myocarditis
ko05120	5.873	0.430	<0.0001	Epithelial cell signaling in Helicobacter pylori infection
ko00940	5.819	0.427	<0.0001	Phenylpropanoid biosynthesis
ko04072	5.760	0.436	<0.0001	Phospholipase D signaling pathway
ko05231	5.760	0.436	<0.0001	Choline metabolism in cancer
ko02060	12.781	0.971	<0.0001	Phosphotransferase system (PTS)
ko04013	0.946	0.072	<0.0001	MAPK signaling pathway - fly
ko00760	-1.204	0.093	<0.0001	Nicotinate and nicotinamide metabolism
ko00510	-5.793	0.459	<0.0001	N-Glycan biosynthesis
ko04714	0.941	0.075	<0.0001	Thermogenesis
ko05134	0.942	0.076	<0.0001	Legionellosis
ko04940	0.943	0.077	<0.0001	Type I diabetes mellitus
ko00909	-5.621	0.484	<0.0001	Sesquiterpenoid and triterpenoid biosynthesis
ko00350	-1.615	0.140	<0.0001	Tyrosine metabolism
ko00310	-0.645	0.056	<0.0001	Lysine degradation
ko00333	0.283	0.025	<0.0001	Prodigiosin biosynthesis
ko00720	0.126	0.011	<0.0001	Carbon fixation pathways in prokaryotes

ko04727	0.940	0.084	<0.0001	GABAergic synapse
ko04724	0.940	0.084	<0.0001	Glutamatergic synapse
ko00195	0.932	0.084	<0.0001	Photosynthesis
ko00970	1.171	0.108	<0.0001	Aminoacyl-tRNA biosynthesis
ko00900	0.642	0.062	<0.0001	Terpenoid backbone biosynthesis
ko01055	0.929	0.090	<0.0001	Biosynthesis of vancomycin group antibiotics
ko04066	1.299	0.127	<0.0001	HIF-1 signaling pathway
ko02026	-1.420	0.140	<0.0001	Biofilm formation - Escherichia coli
ko01501	-0.454	0.045	<0.0001	beta-Lactam resistance
ko00360	-1.799	0.181	<0.0001	Phenylalanine metabolism
ko05014	0.055	0.006	<0.0001	Amyotrophic lateral sclerosis (ALS)
ko00020	0.690	0.071	<0.0001	Citrate cycle (TCA cycle)
ko00908	0.925	0.095	<0.0001	Zeatin biosynthesis
ko05205	0.925	0.095	<0.0001	Proteoglycans in cancer
ko05010	0.922	0.097	<0.0001	Alzheimer disease
ko04621	0.408	0.043	<0.0001	NOD-like receptor signaling pathway
ko00620	-0.749	0.081	<0.0001	Pyruvate metabolism
ko00053	-0.388	0.042	<0.0001	Ascorbate and aldarate metabolism
ko05133	0.052	0.006	<0.0001	Pertussis
ko05225	-0.449	0.050	<0.0001	Hepatocellular carcinoma
ko04112	0.870	0.099	<0.0001	Cell cycle - Caulobacter

ko00550	0.814	0.093	<0.0001	Peptidoglycan biosynthesis
ko04932	0.916	0.105	<0.0001	Non-alcoholic fatty liver disease (NAFLD)
ko05012	0.916	0.105	<0.0001	Parkinson disease
ko05204	-0.777	0.090	<0.0001	Chemical carcinogenesis
ko05111	-2.009	0.233	<0.0001	Biofilm formation - Vibrio cholerae
ko00300	0.856	0.099	<0.0001	Lysine biosynthesis
ko04216	11.858	1.378	<0.0001	Ferroptosis
ko03410	0.632	0.073	<0.0001	Base excision repair
ko01503	-1.026	0.120	<0.0001	Cationic antimicrobial peptide (CAMP) resistance
ko04068	0.055	0.006	<0.0001	FoxO signaling pathway
ko00190	0.712	0.084	<0.0001	Oxidative phosphorylation
ko05206	-4.526	0.540	<0.0001	MicroRNAs in cancer
ko02024	-0.651	0.078	<0.0001	Quorum sensing
ko04920	12.254	1.466	<0.0001	Adipocytokine signaling pathway
ko01040	-0.764	0.094	<0.0001	Biosynthesis of unsaturated fatty acids
ko00400	-2.303	0.290	<0.0001	Phenylalanine, tyrosine and tryptophan biosynthesis
ko00785	0.910	0.115	<0.0001	Lipoic acid metabolism
ko00410	-0.999	0.128	<0.0001	beta-Alanine metabolism
ko04612	0.908	0.117	<0.0001	Antigen processing and presentation
ko04657	0.908	0.117	<0.0001	IL-17 signaling pathway
ko04659	0.908	0.117	<0.0001	Th17 cell differentiation

ko04914	0.908	0.117	<0.0001	Progesterone-mediated oocyte maturation
ko04915	0.908	0.117	<0.0001	Estrogen signaling pathway
ko05215	0.908	0.117	<0.0001	Prostate cancer
ko00072	12.790	1.695	<0.0001	Synthesis and degradation of ketone bodies
ko04113	12.331	1.647	<0.0001	Meiosis - yeast
ko00966	10.533	1.416	<0.0001	Glucosinolate biosynthesis
ko00524	10.495	1.422	<0.0001	Neomycin, kanamycin and gentamicin biosynthesis
ko00140	26.582	3.607	<0.0001	Steroid hormone biosynthesis
ko04910	11.936	1.630	<0.0001	Insulin signaling pathway
ko03010	0.925	0.127	<0.0001	Ribosome
ko03450	13.278	1.842	<0.0001	Non-homologous end-joining
ko01130	-0.132	0.019	<0.0001	NA
ko00571	12.022	1.695	<0.0001	Lipoarabinomannan (LAM) biosynthesis
ko00740	0.900	0.128	<0.0001	Riboflavin metabolism
ko00791	11.887	1.719	<0.0001	Atrazine degradation
ko04138	11.943	1.761	<0.0001	Autophagy - yeast
ko00981	12.716	1.899	<0.0001	Insect hormone biosynthesis
ko05142	9.651	1.441	<0.0001	Chagas disease (American trypanosomiasis)
ko00622	13.611	2.093	<0.0001	Xylene degradation
ko01120	0.036	0.006	<0.0001	Microbial metabolism in diverse environments
ko00624	10.856	1.698	<0.0001	Polycyclic aromatic hydrocarbon degradation

ko00625	-3.088	0.485	<0.0001	Chloroalkane and chloroalkene degradation
ko00621	13.379	2.117	<0.0001	Dioxin degradation
ko05016	0.663	0.105	<0.0001	Huntington disease
ko00980	-0.725	0.115	<0.0001	Metabolism of xenobiotics by cytochrome P450
ko00361	-3.215	0.513	<0.0001	Chlorocyclohexane and chlorobenzene degradation
ko00643	-3.208	0.514	<0.0001	Styrene degradation
ko00680	-0.350	0.056	<0.0001	Methane metabolism
ko00362	-2.877	0.462	<0.0001	Benzoate degradation
ko04964	11.008	1.774	<0.0001	Proximal tubule bicarbonate reclamation
ko00071	-2.900	0.475	<0.0001	Fatty acid degradation
ko00903	-3.228	0.535	<0.0001	Limonene and pinene degradation
ko00281	-3.164	0.525	<0.0001	Geraniol degradation
ko03060	0.592	0.098	<0.0001	Protein export
ko00982	-0.716	0.119	<0.0001	Drug metabolism - cytochrome P450
ko00592	10.544	1.759	<0.0001	alpha-Linolenic acid metabolism
ko00120	12.152	2.045	<0.0001	Primary bile acid biosynthesis
ko02025	-3.054	0.515	<0.0001	Biofilm formation - Pseudomonas aeruginosa
ko03320	-3.095	0.522	<0.0001	PPAR signaling pathway
ko00364	-3.369	0.568	<0.0001	Fluorobenzoate degradation
ko00440	-3.394	0.574	<0.0001	Phosphonate and phosphinate metabolism
ko03020	0.528	0.089	<0.0001	RNA polymerase

ko00630	0.265	0.045	<0.0001	Glyoxylate and dicarboxylate metabolism
ko00623	-3.317	0.564	<0.0001	Toluene degradation
ko01220	-0.391	0.067	<0.0001	Degradation of aromatic compounds
ko05143	10.155	1.765	<0.0001	African trypanosomiasis
ko03440	0.475	0.083	<0.0001	Homologous recombination
ko00626	-3.341	0.596	<0.0001	Naphthalene degradation
ko00830	-3.357	0.609	<0.0001	Retinol metabolism
ko00061	-0.048	0.009	<0.0001	Fatty acid biosynthesis
ko00121	12.083	2.204	<0.0001	Secondary bile acid biosynthesis
ko00906	-3.395	0.620	<0.0001	Carotenoid biosynthesis
ko00405	-3.351	0.619	<0.0001	Phenazine biosynthesis
ko00930	-3.271	0.605	<0.0001	Caprolactam degradation
ko00500	-3.161	0.585	<0.0001	Starch and sucrose metabolism
ko00531	-3.356	0.622	<0.0001	Glycosaminoglycan degradation
ko04930	-3.322	0.617	<0.0001	Type II diabetes mellitus
ko05165	-3.322	0.617	<0.0001	Human papillomavirus infection
ko05203	-3.322	0.617	<0.0001	Viral carcinogenesis
ko04726	11.900	2.219	<0.0001	Serotonergic synapse
ko04728	11.900	2.219	<0.0001	Dopaminergic synapse
ko05030	11.900	2.219	<0.0001	Cocaine addiction
ko05031	11.900	2.219	<0.0001	Amphetamine addiction

ko05034	11.900	2.219	<0.0001	Alcoholism
ko00472	-3.414	0.639	<0.0001	D-Arginine and D-ornithine metabolism
ko01053	-3.402	0.637	<0.0001	Biosynthesis of siderophore group nonribosomal peptides
ko03070	0.795	0.149	<0.0001	Bacterial secretion system
ko00590	-3.369	0.633	<0.0001	Arachidonic acid metabolism
ko00052	-3.236	0.612	<0.0001	Galactose metabolism
ko01062	-3.420	0.651	<0.0001	Biosynthesis of terpenoids and steroids
ko00600	-3.288	0.635	<0.0001	Sphingolipid metabolism
ko04011	-3.412	0.660	<0.0001	MAPK signaling pathway - yeast
ko00633	12.536	2.428	<0.0001	Nitrotoluene degradation
ko02030	-3.199	0.620	<0.0001	Bacterial chemotaxis
ko04080	11.830	2.310	<0.0001	Neuroactive ligand-receptor interaction
ko04979	11.830	2.310	<0.0001	Cholesterol metabolism
ko05166	11.830	2.310	<0.0001	Human T-cell leukemia virus 1 infection
ko04918	-3.343	0.654	<0.0001	Thyroid hormone synthesis
ko00332	-3.343	0.654	<0.0001	Carbapenem biosynthesis
ko00261	0.378	0.074	<0.0001	Monobactam biosynthesis
ko00920	-3.210	0.630	<0.0001	Sulfur metabolism
ko00290	-3.217	0.633	<0.0001	Valine, leucine and isoleucine biosynthesis
ko00340	-3.212	0.632	<0.0001	Histidine metabolism
ko05219	8.577	1.701	<0.0001	Bladder cancer

ko00280	0.348	0.069	<0.0001	Valine, leucine and isoleucine degradation
ko04212	0.376	0.075	<0.0001	Longevity regulating pathway - worm
ko02040	-3.224	0.647	<0.0001	Flagellar assembly
ko00540	-3.230	0.649	<0.0001	Lipopolysaccharide biosynthesis
ko05132	-3.359	0.678	<0.0001	Salmonella infection
ko04978	-3.407	0.692	<0.0001	Mineral absorption
ko00311	-3.407	0.698	<0.0001	Penicillin and cephalosporin biosynthesis
ko00240	0.431	0.089	<0.0001	Pyrimidine metabolism
ko03018	0.249	0.053	<0.0001	RNA degradation
ko00642	10.793	2.325	<0.0001	Ethylbenzene degradation
ko05020	-3.415	0.740	<0.0001	Prion diseases
ko00572	9.544	2.072	<0.0001	Arabinogalactan biosynthesis - Mycobacterium
ko00565	-3.418	0.743	<0.0001	Ether lipid metabolism
ko00603	-3.431	0.747	<0.0001	Glycosphingolipid biosynthesis - globo and isoglobo series
ko05146	-3.444	0.753	<0.0001	Amoebiasis
ko03030	0.400	0.089	<0.0001	DNA replication
ko04614	-3.437	0.761	<0.0001	Renin-angiotensin system
ko03420	0.368	0.083	<0.0001	Nucleotide excision repair
ko00220	0.046	0.011	<0.0001	Arginine biosynthesis
ko05150	-3.451	0.795	<0.0001	Staphylococcus aureus infection
ko01054	-3.454	0.799	<0.0001	Nonribosomal peptide structures

ko05340	-3.441	0.800	<0.0001	Primary immunodeficiency
ko00730	-0.107	0.025	<0.0001	Thiamine metabolism
ko04919	-3.470	0.818	<0.0001	Thyroid hormone signaling pathway
ko00513	-3.453	0.816	<0.0001	Various types of N-glycan biosynthesis
ko00604	-3.453	0.816	<0.0001	Glycosphingolipid biosynthesis - ganglio series
ko00520	-0.257	0.061	<0.0001	Amino sugar and nucleotide sugar metabolism
ko00790	-0.337	0.081	<0.0001	Folate biosynthesis
ko00650	-0.574	0.140	<0.0001	Butanoate metabolism
ko00965	9.859	2.403	<0.0001	Betalain biosynthesis
ko01212	-0.100	0.025	<0.0001	Fatty acid metabolism
ko03013	9.647	2.432	<0.0001	RNA transport
ko01524	-0.192	0.048	<0.0001	Platinum drug resistance
ko04260	0.498	0.131	1.74E-04	Cardiac muscle contraction
ko04211	0.047	0.013	4.13E-04	Longevity regulating pathway
ko01502	0.356	0.100	4.48E-04	Vancomycin resistance
ko00670	0.204	0.059	6.64E-04	One carbon pool by folate
ko03430	0.234	0.068	7.89E-04	Mismatch repair
ko02010	-0.312	0.092	8.70E-04	ABC transporters
ko00640	-0.207	0.062	9.65E-04	Propanoate metabolism
ko04210	0.048	0.014	0.001	Apoptosis
ko01100	-0.061	0.020	0.002	Metabolic pathways

ko02020	-0.409	0.132	0.002	Two-component system
ko00660	-0.319	0.117	0.008	C5-Branched dibasic acid metabolism
ko04622	8.005	2.962	0.008	RIG-I-like receptor signaling pathway
ko01051	0.044	0.016	0.008	Biosynthesis of ansamycins
ko05230	0.043	0.016	0.009	Central carbon metabolism in cancer
ko00404	9.193	3.607	0.013	Staurosporine biosynthesis
ko00525	0.043	0.017	0.014	Acarbose and validamycin biosynthesis
ko03022	7.714	3.100	0.015	Basal transcription factors
ko04974	7.712	3.164	0.017	Protein digestion and absorption
ko00591	7.559	3.183	0.021	Linoleic acid metabolism
ko04024	8.511	3.610	0.021	cAMP signaling pathway
ko03050	8.164	3.611	0.028	Proteasome
ko00984	7.984	3.612	0.031	Steroid degradation
ko04976	7.895	3.613	0.033	Bile secretion
ko04071	7.732	3.614	0.037	Sphingolipid signaling pathway
ko00365	7.467	3.616	0.044	Furfural degradation
ko00514	7.418	3.616	0.046	Other types of O-glycan biosynthesis
ko00515	7.418	3.616	0.046	Mannose type O-glycan biosynthesis
ko04014	7.251	3.618	0.048	Ras signaling pathway
ko04022	7.251	3.618	0.048	cGMP-PKG signaling pathway
ko04144	7.251	3.618	0.048	Endocytosis

ko04261	7.251	3.618	0.048	Adrenergic signaling in cardiomyocytes
ko04666	7.251	3.618	0.048	Fc gamma R-mediated phagocytosis
ko04911	7.251	3.618	0.048	Insulin secretion
ko04912	7.251	3.618	0.048	GnRH signaling pathway
ko04925	7.251	3.618	0.048	Aldosterone synthesis and secretion
ko04928	7.251	3.618	0.048	Parathyroid hormone synthesis, secretion and action
ko04960	7.251	3.618	0.048	Aldosterone-regulated sodium reabsorption
ko04961	7.251	3.618	0.048	Endocrine and other factor-regulated calcium reabsorption
ko04970	7.251	3.618	0.048	Salivary secretion
ko04971	7.251	3.618	0.048	Gastric acid secretion
ko04972	7.251	3.618	0.048	Pancreatic secretion
ko04973	7.251	3.618	0.048	Carbohydrate digestion and absorption
ko00901	7.211	3.618	0.049	Indole alkaloid biosynthesis
ko04924	7.207	3.618	0.049	Renin secretion
ko05410	7.207	3.618	0.049	Hypertrophic cardiomyopathy (HCM)

Table A.3 Effect of 31°C Rearing Temperature, Compared to 23°C, on Predicted Microbial Functional Pathway Abundance in *Drosophila*

Piphillin, referring to the KEGG database, was used to predict microbial functional effects of 31°C rearing temperature in *Drosophila*, using 16S data from the study by (Moghadam et al., 2018). Differentially abundant pathways were determined by DESeq2 analysis. A positive log2 fold change means pathways were represented more in flies reared at 31°C whereas a negative change means more pathways represented in flies reared at 23°C.

KEGG Number	Log2 Fold Change	LFC SE	Adj. *p* Value	Pathway
ko04115	2.040	0.427	2.96E-05	p53 signaling pathway
ko04152	2.082	0.437	2.96E-05	AMPK signaling pathway
ko04215	2.040	0.427	2.96E-05	Apoptosis - multiple species
ko05120	2.070	0.430	2.96E-05	Epithelial cell signaling in Helicobacter pylori infection
ko05145	2.040	0.427	2.96E-05	Toxoplasmosis
ko05161	2.040	0.427	2.96E-05	Hepatitis B
ko05163	2.040	0.427	2.96E-05	Human cytomegalovirus infection
ko05164	2.040	0.427	2.96E-05	Influenza A
ko05167	2.040	0.427	2.96E-05	Kaposi sarcoma-associated herpesvirus infection
ko05168	2.040	0.427	2.96E-05	Herpes simplex virus 1 infection
ko05170	2.040	0.427	2.96E-05	Human immunodeficiency virus 1 infection
ko05210	2.040	0.427	2.96E-05	Colorectal cancer
ko05222	2.040	0.427	2.96E-05	Small cell lung cancer

ko05416	2.040	0.427	2.96E-05	Viral myocarditis
ko00940	2.026	0.426	2.99E-05	Phenylpropanoid biosynthesis
ko04072	1.979	0.435	7.17E-05	Phospholipase D signaling pathway
ko05231	1.979	0.435	7.17E-05	Choline metabolism in cancer

Table A.4 Effect of 18°C Rearing Temperature, Compared to 25°C, on Predicted Microbial Functional Pathway Abundance in *Drosophila*

Piphillin, referring to the KEGG database, was used to predict microbial functional effects of 18°C rearing temperature in *Drosophila*, using 16S data from the study by (Zare et al., 2018). Differentially abundant pathways were determined by DESeq2 analysis. A positive log2 fold change means pathways were represented more in flies reared at 18°C whereas a negative change means more pathways represented in flies reared at 25°C.

KEGG Number	Log2 Fold Change	LFC SE	Adj. *p* Value	Pathway
ko00640	-0.059	0.005	<0.0001	Propanoate metabolism
ko00010	-0.152	0.016	<0.0001	Glycolysis / Gluconeogenesis
ko00521	-0.077	0.008	<0.0001	Streptomycin biosynthesis
ko00300	-0.145	0.017	<0.0001	Lysine biosynthesis
ko01230	-0.035	0.004	<0.0001	Biosynthesis of amino acids
ko00500	-0.839	0.102	<0.0001	Starch and sucrose metabolism

ko00520	-0.264	0.034	<0.0001	Amino sugar and nucleotide sugar metabolism
ko00472	0.202	0.027	<0.0001	D-Arginine and D-ornithine metabolism
ko00281	0.195	0.026	<0.0001	Geraniol degradation
ko00903	0.194	0.026	<0.0001	Limonene and pinene degradation
ko04922	-0.341	0.046	<0.0001	Glucagon signaling pathway
ko00051	-0.575	0.078	<0.0001	Fructose and mannose metabolism
ko00513	0.193	0.026	<0.0001	Various types of N-glycan biosynthesis
ko00604	0.193	0.026	<0.0001	Glycosphingolipid biosynthesis - ganglio series
ko00550	-0.089	0.012	<0.0001	Peptidoglycan biosynthesis
ko04013	0.192	0.027	<0.0001	MAPK signaling pathway - fly
ko00531	0.207	0.029	<0.0001	Glycosaminoglycan degradation
ko00970	-0.127	0.018	<0.0001	Aminoacyl-tRNA biosynthesis
ko02030	0.156	0.022	<0.0001	Bacterial chemotaxis
ko00930	0.188	0.027	<0.0001	Caprolactam degradation
ko02040	0.165	0.024	<0.0001	Flagellar assembly
ko00290	0.119	0.017	<0.0001	Valine, leucine and isoleucine biosynthesis
ko00950	0.187	0.027	<0.0001	Isoquinoline alkaloid biosynthesis
ko00310	0.162	0.024	<0.0001	Lysine degradation
ko00510	0.186	0.027	<0.0001	N-Glycan biosynthesis
ko00540	0.151	0.022	<0.0001	Lipopolysaccharide biosynthesis

ko00440	-0.270	0.040	<0.0001	Phosphonate and phosphinate metabolism
ko04151	0.185	0.028	<0.0001	PI3K-Akt signaling pathway
ko00364	0.185	0.028	<0.0001	Fluorobenzoate degradation
ko00565	0.183	0.028	<0.0001	Ether lipid metabolism
ko04919	0.183	0.028	<0.0001	Thyroid hormone signaling pathway
ko04612	0.184	0.028	<0.0001	Antigen processing and presentation
ko04657	0.184	0.028	<0.0001	IL-17 signaling pathway
ko04659	0.184	0.028	<0.0001	Th17 cell differentiation
ko04914	0.184	0.028	<0.0001	Progesterone-mediated oocyte maturation
ko04915	0.184	0.028	<0.0001	Estrogen signaling pathway
ko05215	0.184	0.028	<0.0001	Prostate cancer
ko04210	0.183	0.028	<0.0001	Apoptosis
ko04614	0.183	0.028	<0.0001	Renin-angiotensin system
ko00052	-0.543	0.083	<0.0001	Galactose metabolism
ko00909	0.183	0.028	<0.0001	Sesquiterpenoid and triterpenoid biosynthesis
ko03070	0.116	0.018	<0.0001	Bacterial secretion system
ko00190	0.113	0.018	<0.0001	Oxidative phosphorylation
ko02060	-2.520	0.404	<0.0001	Phosphotransferase system (PTS)
ko00020	0.081	0.013	<0.0001	Citrate cycle (TCA cycle)
ko04918	-0.215	0.036	<0.0001	Thyroid hormone synthesis

ko04141	-0.206	0.034	<0.0001	Protein processing in endoplasmic reticulum
ko04964	4.390	0.732	<0.0001	Proximal tubule bicarbonate reclamation
ko00240	-0.038	0.007	<0.0001	Pyrimidine metabolism
ko01110	-0.023	0.004	<0.0001	Biosynthesis of secondary metabolites
ko00561	-0.431	0.077	<0.0001	Glycerolipid metabolism
ko00710	-0.025	0.004	<0.0001	Carbon fixation in photosynthetic organisms
ko00860	0.283	0.053	<0.0001	Porphyrin and chlorophyll metabolism
ko04068	0.100	0.019	<0.0001	FoxO signaling pathway
ko00620	-0.062	0.012	<0.0001	Pyruvate metabolism
ko04714	0.121	0.024	<0.0001	Thermogenesis
ko04211	0.099	0.019	<0.0001	Longevity regulating pathway
ko00280	0.090	0.018	<0.0001	Valine, leucine and isoleucine degradation
ko04066	-0.153	0.031	<0.0001	HIF-1 signaling pathway
ko00603	-0.135	0.027	<0.0001	Glycosphingolipid biosynthesis - globo and isoglobo series
ko05016	0.117	0.024	<0.0001	Huntington disease
ko04940	-0.141	0.030	<0.0001	Type I diabetes mellitus
ko01523	-0.149	0.031	<0.0001	Antifolate resistance
ko04931	-0.127	0.027	<0.0001	Insulin resistance
ko04727	-0.139	0.029	<0.0001	GABAergic synapse
ko05014	0.095	0.020	<0.0001	Amyotrophic lateral sclerosis (ALS)

ko00906	-0.139	0.031	<0.0001	Carotenoid biosynthesis
ko04152	-2.831	0.624	<0.0001	AMPK signaling pathway
ko04910	-2.799	0.628	<0.0001	Insulin signaling pathway
ko02010	-0.190	0.044	<0.0001	ABC transporters
ko00600	-0.414	0.096	<0.0001	Sphingolipid metabolism
ko04138	-2.770	0.652	<0.0001	Autophagy - yeast
ko04726	2.828	0.673	1.01E-04	Serotonergic synapse
ko04728	2.828	0.673	1.01E-04	Dopaminergic synapse
ko05030	2.828	0.673	1.01E-04	Cocaine addiction
ko05031	2.828	0.673	1.01E-04	Amphetamine addiction
ko05034	2.828	0.673	1.01E-04	Alcoholism
ko03022	2.875	0.687	1.06E-04	Basal transcription factors
ko01053	1.910	0.460	1.23E-04	Biosynthesis of siderophore group nonribosomal peptides
ko05219	2.839	0.685	1.25E-04	Bladder cancer
ko05230	-0.099	0.024	1.29E-04	Central carbon metabolism in cancer
ko00514	2.853	0.693	1.35E-04	Other types of O-glycan biosynthesis
ko00515	2.853	0.693	1.35E-04	Mannose type O-glycan biosynthesis
ko05133	0.536	0.131	1.51E-04	Pertussis
ko00360	0.310	0.076	1.51E-04	Phenylalanine metabolism
ko00720	-0.017	0.004	1.57E-04	Carbon fixation pathways in prokaryotes

ko00572	-2.570	0.632	1.60E-04	Arabinogalactan biosynthesis - Mycobacterium
ko00633	3.290	0.809	1.60E-04	Nitrotoluene degradation
ko04978	0.534	0.132	1.62E-04	Mineral absorption
ko05146	1.878	0.466	1.79E-04	Amoebiasis
ko00040	-0.108	0.027	1.79E-04	Pentose and glucuronate interconversions
ko05020	1.878	0.468	1.94E-04	Prion diseases
ko01220	-0.373	0.093	2.07E-04	Degradation of aromatic compounds
ko03410	-0.104	0.026	2.34E-04	Base excision repair
ko04920	2.961	0.751	2.45E-04	Adipocytokine signaling pathway
ko04216	2.954	0.749	2.45E-04	Ferroptosis
ko00780	0.072	0.019	3.82E-04	Biotin metabolism
ko00960	0.385	0.101	4.14E-04	Tropane, piperidine and pyridine alkaloid biosynthesis
ko03050	2.828	0.754	5.21E-04	Proteasome
ko00380	0.304	0.082	5.86E-04	Tryptophan metabolism
ko00564	0.041	0.011	6.30E-04	Glycerophospholipid metabolism
ko00480	0.035	0.009	6.89E-04	Glutathione metabolism
ko03450	5.335	1.460	7.31E-04	Non-homologous end-joining
ko02024	-0.034	0.009	8.45E-04	Quorum sensing
ko00401	0.302	0.084	9.53E-04	Novobiocin biosynthesis
ko00571	2.807	0.794	0.001	Lipoarabinomannan (LAM) biosynthesis

ko04626	0.062	0.018	0.001	Plant-pathogen interaction
ko04917	-3.107	0.882	0.001	Prolactin signaling pathway
ko02020	-0.072	0.021	0.001	Two-component system
ko04071	5.016	1.460	0.002	Sphingolipid signaling pathway
ko00523	-0.070	0.020	0.002	Polyketide sugar unit biosynthesis
ko00562	0.138	0.040	0.002	Inositol phosphate metabolism
ko01100	-0.034	0.010	0.002	Metabolic pathways
ko00592	4.231	1.241	0.002	alpha-Linolenic acid metabolism
ko00624	3.472	1.027	0.002	Polycyclic aromatic hydrocarbon degradation
ko00340	-0.021	0.006	0.002	Histidine metabolism
ko03013	-3.090	0.935	0.002	RNA transport
ko00621	-3.019	0.919	0.003	Dioxin degradation
ko00072	-2.887	0.882	0.003	Synthesis and degradation of ketone bodies
ko00622	-3.048	0.931	0.003	Xylene degradation
ko00120	-3.298	1.007	0.003	Primary bile acid biosynthesis
ko00121	-3.300	1.009	0.003	Secondary bile acid biosynthesis
ko04080	-3.199	0.983	0.003	Neuroactive ligand-receptor interaction
ko04979	-3.199	0.983	0.003	Cholesterol metabolism
ko05166	-3.199	0.983	0.003	Human T-cell leukemia virus 1 infection
ko01120	-0.102	0.032	0.003	Microbial metabolism in diverse environments

ko04213	0.210	0.065	0.003	Longevity regulating pathway - multiple species
ko00405	-0.304	0.094	0.003	Phenazine biosynthesis
ko04212	0.057	0.018	0.003	Longevity regulating pathway - worm
ko00511	-0.316	0.100	0.003	Other glycan degradation
ko00730	-0.014	0.004	0.003	Thiamine metabolism
ko01054	1.298	0.413	0.004	Nonribosomal peptide structures
ko04973	4.076	1.320	0.004	Carbohydrate digestion and absorption
ko00642	3.596	1.170	0.005	Ethylbenzene degradation
ko03018	-0.015	0.005	0.005	RNA degradation
ko01130	-0.011	0.004	0.005	NA
ko01210	0.053	0.018	0.006	2-Oxocarboxylic acid metabolism
ko00965	3.110	1.036	0.006	Betalain biosynthesis
ko04016	0.054	0.018	0.006	MAPK signaling pathway - plant
ko03060	0.080	0.027	0.006	Protein export
ko00030	-0.110	0.037	0.006	Pentose phosphate pathway
ko00999	0.375	0.129	0.008	Biosynthesis of various secondary metabolites - part 1
ko05204	0.087	0.031	0.009	Chemical carcinogenesis
ko04260	0.087	0.031	0.010	Cardiac muscle contraction
ko04024	5.779	2.076	0.011	cAMP signaling pathway
ko00750	0.050	0.018	0.012	Vitamin B6 metabolism

ko05231	3.542	1.317	0.014	Choline metabolism in cancer
ko04072	3.413	1.282	0.015	Phospholipase D signaling pathway
ko00981	3.726	1.414	0.016	Insect hormone biosynthesis
ko00910	-0.084	0.032	0.016	Nitrogen metabolism
ko03320	-0.252	0.097	0.018	PPAR signaling pathway
ko04146	0.165	0.064	0.018	Peroxisome
ko00627	-0.379	0.154	0.027	Aminobenzoate degradation
ko03440	0.149	0.062	0.030	Homologous recombination
ko00650	0.069	0.029	0.030	Butanoate metabolism
ko00350	-0.119	0.049	0.030	Tyrosine metabolism
ko01200	-0.038	0.016	0.033	Carbon metabolism
ko00626	-0.445	0.192	0.037	Naphthalene degradation
ko04113	3.025	1.309	0.038	Meiosis - yeast
ko00790	-0.124	0.054	0.038	Folate biosynthesis
ko00311	0.910	0.394	0.038	Penicillin and cephalosporin biosynthesis
ko00071	-0.292	0.129	0.042	Fatty acid degradation
ko00625	-0.485	0.218	0.047	Chloroalkane and chloroalkene degradation

Table A.5 Effect of an Oxidant on Predicted Microbial Functional Pathway Abundance in Adult *Drosophila*

Piphillin, referring to the KEGG database, was used to predict microbial functional effects of the oxidant, tBH in *Drosophila*, using 16S data from the study by (Obata et al., 2018). Differentially abundant pathways were determined by DESeq2 analysis. A positive log2 fold change means pathways were represented more in the oxidant-treated flies whereas a negative change means more pathways represented in control flies.

KEGG Number	Log2 Fold Change	LFC SE	Adj. *p* Value	Pathway
ko00970	0.223	0.023	<0.0001	Aminoacyl-tRNA biosynthesis
ko04714	-0.981	0.112	<0.0001	Thermogenesis
ko02040	-1.337	0.166	<0.0001	Flagellar assembly
ko05016	-1.187	0.152	<0.0001	Huntington disease
ko02030	-1.349	0.177	<0.0001	Bacterial chemotaxis
ko05204	-0.775	0.102	<0.0001	Chemical carcinogenesis
ko04932	-1.215	0.164	<0.0001	Non-alcoholic fatty liver disease
ko05012	-1.215	0.164	<0.0001	Parkinson disease
ko00550	0.196	0.028	<0.0001	Peptidoglycan biosynthesis
ko00510	-4.795	0.688	<0.0001	N-Glycan biosynthesis
ko00860	-1.104	0.164	<0.0001	Porphyrin and chlorophyll metabolism
ko04260	-1.647	0.246	<0.0001	Cardiac muscle contraction
ko04919	-2.492	0.373	<0.0001	Thyroid hormone signaling pathway

ko00909	-5.293	0.798	<0.0001	Sesquiterpenoid and triterpenoid biosynthesis
ko05133	-0.995	0.150	<0.0001	Pertussis
ko00565	-2.008	0.318	<0.0001	Ether lipid metabolism
ko05200	-0.422	0.068	<0.0001	Pathways in cancer
ko01524	-0.424	0.069	<0.0001	Platinum drug resistance
ko05132	-2.047	0.338	<0.0001	Salmonella infection
ko04210	-1.280	0.216	<0.0001	Apoptosis
ko05225	-0.665	0.114	<0.0001	Hepatocellular carcinoma
ko04978	-2.185	0.375	<0.0001	Mineral absorption
ko04151	-1.010	0.174	<0.0001	PI3K-Akt signaling pathway
ko00950	-1.317	0.228	<0.0001	Isoquinoline alkaloid biosynthesis
ko04013	-0.936	0.163	<0.0001	MAPK signaling pathway - fly
ko05146	-1.924	0.341	<0.0001	Amoebiasis
ko05230	0.197	0.035	<0.0001	Central carbon metabolism in cancer
ko00540	-0.989	0.177	<0.0001	Lipopolysaccharide biosynthesis
ko00040	0.286	0.052	<0.0001	Pentose and glucuronate interconversions
ko00930	-0.717	0.133	<0.0001	Caprolactam degradation
ko04612	-1.418	0.268	<0.0001	Antigen processing and presentation
ko04657	-1.418	0.268	<0.0001	IL-17 signaling pathway
ko04659	-1.418	0.268	<0.0001	Th17 cell differentiation

ko04914	-1.418	0.268	<0.0001	Progesterone-mediated oocyte maturation
ko04915	-1.418	0.268	<0.0001	Estrogen signaling pathway
ko05215	-1.417	0.268	<0.0001	Prostate cancer
ko05150	0.276	0.052	<0.0001	Staphylococcus aureus infection
ko00513	-4.196	0.831	<0.0001	Various types of N-glycan biosynthesis
ko00604	-4.196	0.831	<0.0001	Glycosphingolipid biosynthesis - ganglio series
ko04614	-1.930	0.394	<0.0001	Renin-angiotensin system
ko00360	-0.353	0.073	<0.0001	Phenylalanine metabolism
ko05020	-1.520	0.314	<0.0001	Prion disease
ko00600	0.317	0.067	<0.0001	Sphingolipid metabolism
ko03013	0.349	0.076	<0.0001	RNA transport
ko00472	-1.108	0.242	<0.0001	D-Arginine and D-ornithine metabolism
ko03070	-0.277	0.063	<0.0001	Bacterial secretion system
ko03010	0.161	0.037	<0.0001	Ribosome
ko00561	0.236	0.055	<0.0001	Glycerolipid metabolism
ko00310	-0.369	0.086	<0.0001	Lysine degradation
ko00520	0.192	0.045	1.28E-04	Amino sugar and nucleotide sugar metabolism
ko00030	0.146	0.035	1.31E-04	Pentose phosphate pathway
ko00052	0.219	0.052	1.32E-04	Galactose metabolism
ko05010	-0.445	0.107	1.66E-04	Alzheimer disease

ko04918	0.202	0.050	2.65E-04	Thyroid hormone synthesis
ko00960	-0.548	0.137	3.55E-04	Tropane, piperidine and pyridine alkaloid biosynthesis
ko05206	-0.997	0.257	5.35E-04	MicroRNAs in cancer
ko04917	0.464	0.120	5.99E-04	Prolactin signaling pathway
ko00524	0.346	0.091	6.84E-04	Neomycin, kanamycin and gentamicin biosynthesis
ko00140	2.481	0.651	6.84E-04	Steroid hormone biosynthesis
ko00330	-0.248	0.066	8.86E-04	Arginine and proline metabolism
ko00944	2.506	0.676	9.99E-04	Flavone and flavonol biosynthesis
ko00511	0.325	0.090	0.002	Other glycan degradation
ko04922	0.202	0.057	0.002	Glucagon signaling pathway
ko00190	-0.247	0.070	0.002	Oxidative phosphorylation
ko03020	0.168	0.048	0.002	RNA polymerase
ko00410	-0.273	0.081	0.004	beta-Alanine metabolism
ko00020	-0.197	0.060	0.004	Citrate cycle (TCA cycle)
ko05111	-0.167	0.051	0.004	Biofilm formation - Vibrio cholerae
ko00500	0.202	0.061	0.004	Starch and sucrose metabolism
ko00364	-0.576	0.175	0.004	Fluorobenzoate degradation
ko04066	0.169	0.052	0.005	HIF-1 signaling pathway
ko00130	0.131	0.040	0.005	Ubiquinone and other terpenoid-quinone biosynthesis
ko00940	0.401	0.125	0.005	Phenylpropanoid biosynthesis

ko05340	0.291	0.092	0.006	Primary immunodeficiency
ko00240	0.122	0.039	0.007	Pyrimidine metabolism
ko00626	0.205	0.067	0.008	Naphthalene degradation
ko01502	0.153	0.050	0.009	Vancomycin resistance
ko01523	0.166	0.055	0.010	Antifolate resistance
ko00401	-0.350	0.117	0.011	Novobiocin biosynthesis
ko00061	0.095	0.032	0.011	Fatty acid biosynthesis
ko00730	0.145	0.049	0.011	Thiamine metabolism
ko02010	0.106	0.036	0.011	ABC transporters
ko01501	0.150	0.051	0.012	beta-Lactam resistance
ko00622	0.274	0.094	0.012	Xylene degradation
ko00903	-0.503	0.177	0.016	Limonene and pinene degradation
ko00983	0.109	0.039	0.018	Drug metabolism - other enzymes
ko00630	-0.123	0.045	0.021	Glyoxylate and dicarboxylate metabolism
ko05014	-0.258	0.095	0.021	Amyotrophic lateral sclerosis
ko00625	0.191	0.072	0.026	Chloroalkane and chloroalkene degradation
ko04068	-0.261	0.100	0.029	FoxO signaling pathway
ko00300	0.124	0.048	0.030	Lysine biosynthesis
ko02060	0.200	0.077	0.030	Phosphotransferase system (PTS)
ko04940	0.259	0.100	0.030	Type I diabetes mellitus

ko00900	0.109	0.042	0.031	Terpenoid backbone biosynthesis
ko04727	0.239	0.097	0.041	GABAergic synapse
ko04213	-0.218	0.089	0.043	Longevity regulating pathway - multiple species

Table A.6 Effect of an Oxidant on Predicted Microbial Functional Pathway Abundance in *Drosophila* Larvae

Piphillin, referring to the KEGG database, was used to predict microbial functional effects of the oxidant, tBH in *Drosophila* larvae, using 16S data from the study by (Obata et al., 2018). Differentially abundant pathways were determined by DESeq2 analysis. A positive log2 fold change means pathways were represented more in the oxidant-treated larvae whereas a negative change means more pathways represented in control larvae.

KEGG Number	Log2 Fold Change	LFC SE	Adj. *p* Value	Pathway
ko04142	-2.501	0.220	<0.0001	Lysosome
ko00513	-4.080	0.448	<0.0001	Various types of N-glycan biosynthesis
ko00604	-4.080	0.448	<0.0001	Glycosphingolipid biosynthesis - ganglio series
ko00510	-4.868	0.556	<0.0001	N-Glycan biosynthesis
ko04919	-1.872	0.251	<0.0001	Thyroid hormone signaling pathway
ko04978	-1.601	0.274	<0.0001	Mineral absorption
ko00565	-1.424	0.247	<0.0001	Ether lipid metabolism
ko00909	-5.916	1.045	<0.0001	Sesquiterpenoid and triterpenoid biosynthesis
ko05132	-1.490	0.262	<0.0001	Salmonella infection

ko00860	-0.823	0.151	<0.0001	Porphyrin and chlorophyll metabolism
ko05146	-1.388	0.267	<0.0001	Amoebiasis
ko04260	-1.265	0.251	<0.0001	Cardiac muscle contraction
ko04614	-1.413	0.301	<0.0001	Renin-angiotensin system
ko00531	-0.929	0.213	2.81E-04	Glycosaminoglycan degradation
ko00540	-0.752	0.176	4.01E-04	Lipopolysaccharide biosynthesis
ko00190	-0.128	0.034	0.003	Oxidative phosphorylation
ko02040	-0.821	0.220	0.003	Flagellar assembly
ko00625	0.195	0.052	0.003	Chloroalkane and chloroalkene degradation
ko00944	-1.499	0.405	0.003	Flavone and flavonol biosynthesis
ko02030	-0.827	0.225	0.004	Bacterial chemotaxis
ko05215	-1.029	0.299	0.006	Prostate cancer
ko04612	-1.029	0.299	0.006	Antigen processing and presentation
ko04657	-1.029	0.299	0.006	IL-17 signaling pathway
ko04659	-1.029	0.299	0.006	Th17 cell differentiation
ko04914	-1.029	0.299	0.006	Progesterone-mediated oocyte maturation
ko04915	-1.029	0.299	0.006	Estrogen signaling pathway
ko00290	-0.565	0.162	0.006	Valine, leucine and isoleucine biosynthesis
ko00960	-0.285	0.081	0.006	Tropane, piperidine and pyridine alkaloid biosynthesis
ko01220	0.176	0.051	0.006	Degradation of aromatic compounds
ko00622	0.204	0.059	0.006	Xylene degradation

ko00950	-0.911	0.266	0.006	Isoquinoline alkaloid biosynthesis
ko05020	-0.995	0.298	0.007	Prion disease
ko00051	0.269	0.080	0.007	Fructose and mannose metabolism
ko00440	0.198	0.060	0.008	Phosphonate and phosphinate metabolism
ko02060	0.298	0.091	0.008	Phosphotransferase system (PTS)
ko05016	-0.799	0.247	0.010	Huntington disease
ko00120	0.362	0.112	0.010	Primary bile acid biosynthesis
ko04932	-0.838	0.262	0.010	Non-alcoholic fatty liver disease
ko05012	-0.838	0.262	0.010	Parkinson disease
ko00121	0.364	0.114	0.010	Secondary bile acid biosynthesis
ko00401	-0.170	0.054	0.012	Novobiocin biosynthesis
ko04210	-0.868	0.281	0.014	Apoptosis
ko05010	-0.286	0.093	0.014	Alzheimer disease
ko02010	0.085	0.028	0.014	ABC transporters
ko00660	-0.432	0.142	0.015	C5-Branched dibasic acid metabolism
ko01210	-0.223	0.074	0.016	2-Oxocarboxylic acid metabolism
ko00626	0.173	0.057	0.016	Naphthalene degradation
ko00472	-0.762	0.257	0.019	D-Arginine and D-ornithine metabolism
ko00500	0.227	0.077	0.020	Starch and sucrose metabolism
ko03070	-0.161	0.056	0.023	Bacterial secretion system
ko02020	0.091	0.032	0.023	Two-component system

ko00640	0.112	0.039	0.023	Propanoate metabolism
ko04622	0.379	0.132	0.023	RIG-I-like receptor signaling pathway
ko01053	-1.686	0.602	0.028	Biosynthesis of siderophore group nonribosomal peptides
ko04138	0.336	0.121	0.028	Autophagy - yeast
ko04141	0.212	0.077	0.030	Protein processing in endoplasmic reticulum
ko04910	0.303	0.110	0.031	Insulin signaling pathway
ko04080	0.341	0.126	0.033	Neuroactive ligand-receptor interaction
ko04979	0.341	0.126	0.033	Cholesterol metabolism
ko05166	0.341	0.126	0.033	Human T-cell leukemia virus 1 infection
ko01120	0.068	0.025	0.036	Microbial metabolism in diverse environments
ko00830	0.210	0.079	0.036	Retinol metabolism
ko00572	0.342	0.131	0.042	Arabinogalactan biosynthesis - Mycobacterium
ko02026	0.172	0.066	0.043	Biofilm formation - Escherichia coli
ko00350	0.083	0.033	0.047	Tyrosine metabolism
ko04152	0.289	0.114	0.049	AMPK signaling pathway
ko04714	-0.621	0.245	0.050	Thermogenesis
ko05206	-0.468	0.185	0.050	MicroRNAs in cancer

Table A.7 Effect of a High Fat Diet on Predicted Microbial Functional Pathway Abundance in Mice

Piphillin, referring to the KEGG database, was used to predict microbial functional effects of alcohol in mice, using 16S data from mice without induced liver damage from the study by (Furuya et al., 2020). Differentially abundant pathways were determined by DESeq2 analysis. A positive log2 fold change means pathways were represented more in the HFD mice whereas a negative change means more pathways represented in control mice.

KEGG Number	Log2 Fold Change	LFC SE	Adj. *p* Value	Pathway
ko00510	-6.696	1.392	<0.0001	N-Glycan biosynthesis
ko03015	-6.696	1.392	<0.0002	mRNA surveillance pathway
ko04080	-6.684	1.384	<0.0003	Neuroactive ligand-receptor interaction
ko04260	-6.696	1.392	<0.0004	Cardiac muscle contraction
ko04932	-6.696	1.392	<0.0005	Non-alcoholic fatty liver disease
ko04979	-6.684	1.384	<0.0006	Cholesterol metabolism
ko05012	-6.696	1.392	<0.0007	Parkinson disease
ko05100	-6.696	1.392	<0.0008	Bacterial invasion of epithelial cells
ko05110	-6.696	1.392	<0.0009	Vibrio cholerae infection
ko05166	-6.684	1.384	<0.0010	Human T-cell leukemia virus 1 infection
ko00565	-6.077	1.353	1.04E-04	Ether lipid metabolism
ko00965	-6.077	1.353	1.04E-04	Betalain biosynthesis
ko04072	-6.077	1.353	1.04E-04	Phospholipase D signaling pathway
ko04622	-6.077	1.353	1.04E-04	RIG-I-like receptor signaling pathway

ko05142	-6.077	1.353	1.04E-04	Chagas disease
ko05143	-6.077	1.353	1.04E-04	African trypanosomiasis
ko05219	-6.077	1.353	1.04E-04	Bladder cancer
ko05150	-6.008	1.356	1.29E-04	Staphylococcus aureus infection
ko01054	-5.637	1.333	2.91E-04	Nonribosomal peptide structures
ko04978	-5.643	1.331	2.91E-04	Mineral absorption
ko04714	-5.573	1.341	3.86E-04	Thermogenesis
ko00591	-5.497	1.339	4.59E-04	Linoleic acid metabolism
ko00791	-5.270	1.344	9.53E-04	Atrazine degradation
ko00930	-4.962	1.294	0.001	Caprolactam degradation
ko00981	-4.844	1.269	0.001	Insect hormone biosynthesis
ko00944	-5.241	1.378	0.001	Flavone and flavonol biosynthesis
ko00572	-4.640	1.245	0.002	Arabinogalactan biosynthesis - Mycobacterium
ko04917	-3.983	1.070	0.002	Prolactin signaling pathway
ko00364	-4.309	1.166	0.002	Fluorobenzoate degradation
ko00623	-4.309	1.166	0.002	Toluene degradation
ko03450	-4.871	1.321	0.002	Non-homologous end-joining
ko04138	-3.918	1.072	0.002	Autophagy - yeast
ko00514	-4.243	1.194	0.003	Other types of O-glycan biosynthesis
ko00515	-4.243	1.194	0.003	Mannose type O-glycan biosynthesis
ko00130	2.002	0.566	0.003	Ubiquinone and other terpenoid-quinone biosynthesis

ko00642	-4.535	1.293	0.003	Ethylbenzene degradation
ko00970	2.300	0.657	0.003	Aminoacyl-tRNA biosynthesis
ko00540	1.851	0.536	0.003	Lipopolysaccharide biosynthesis
ko04934	-4.320	1.251	0.003	Cushing syndrome
ko05020	-4.522	1.301	0.003	Prion diseases
ko05204	-4.699	1.358	0.003	Chemical carcinogenesis
ko05211	-4.320	1.251	0.003	Renal cell carcinoma
ko05225	-4.699	1.358	0.003	Hepatocellular carcinoma
ko00120	-3.675	1.088	0.004	Primary bile acid biosynthesis
ko00121	-3.675	1.088	0.004	Secondary bile acid biosynthesis
ko00592	-4.370	1.292	0.004	alpha-Linolenic acid metabolism
ko05146	-3.667	1.083	0.004	Amoebiasis
ko00361	-3.660	1.090	0.004	Chlorocyclohexane and chlorobenzene degradation
ko00531	2.438	0.746	0.006	Glycosaminoglycan degradation
ko00903	-4.066	1.249	0.006	Limonene and pinene degradation
ko04142	1.896	0.590	0.006	Lysosome
ko03013	-3.365	1.060	0.007	RNA transport
ko04216	-3.655	1.164	0.008	Ferroptosis
ko00621	-3.598	1.154	0.008	Dioxin degradation
ko00622	-3.598	1.153	0.008	Xylene degradation
ko00472	-3.684	1.186	0.008	D-Arginine and D-ornithine metabolism

ko05340	-3.894	1.254	0.008	Primary immunodeficiency
ko00643	-3.952	1.289	0.009	Styrene degradation
ko05133	-4.225	1.378	0.009	Pertussis
ko04918	-3.999	1.355	0.013	Thyroid hormone synthesis
ko00590	-4.008	1.363	0.013	Arachidonic acid metabolism
ko00511	1.606	0.551	0.014	Other glycan degradation
ko00281	-3.741	1.298	0.016	Geraniol degradation
ko05132	-3.207	1.116	0.016	Salmonella infection
ko05206	-1.736	0.607	0.016	MicroRNAs in cancer
ko00513	2.179	0.765	0.016	Various types of N-glycan biosynthesis
ko00604	2.179	0.765	0.016	Glycosphingolipid biosynthesis - ganglio series
ko00790	1.439	0.509	0.017	Folate biosynthesis
ko00860	1.394	0.496	0.018	Porphyrin and chlorophyll metabolism
ko00940	-2.894	1.033	0.018	Phenylpropanoid biosynthesis
ko00740	1.546	0.553	0.018	Riboflavin metabolism
ko00603	1.525	0.547	0.018	Glycosphingolipid biosynthesis - globo and isoglobo series
ko00190	1.529	0.573	0.026	Oxidative phosphorylation
ko00460	-2.547	0.957	0.026	Cyanoamino acid metabolism
ko00280	1.280	0.499	0.035	Valine, leucine and isoleucine degradation
ko00400	1.379	0.540	0.035	Phenylalanine, tyrosine and tryptophan biosynthesis

Table A.8 Effect of a High Fat Diet and Ethanol on Predicted Microbial Functional Pathway Abundance in Mice

Piphillin, referring to the KEGG database, was used to predict microbial functional effects of alcohol in mice, using 16S data from mice without induced liver damage from the study by (Furuya et al., 2020). Differentially abundant pathways were determined by DESeq2 analysis. A positive log2 fold change means pathways were represented more in the HFD + EtOH mice whereas a negative change means more pathways represented in control mice.

KEGG Number	Log2 Fold Change	LFC SE	Adj. *p* Value	Pathway
ko02030	-3.066	0.764	0.005	Bacterial chemotaxis
ko02040	-6.375	1.499	0.005	Flagellar assembly
ko02060	4.040	0.998	0.005	Phosphotransferase system (PTS)
ko00940	-4.294	1.243	0.034	Phenylpropanoid biosynthesis

Table A.9 Effect of a High Fat Diet on Predicted Microbial Functional Pathway Abundance in Mice With Induced Liver Injury

Piphillin, referring to the KEGG database, was used to predict microbial functional effects of alcohol in mice, using 16S data from mice with induced liver damage from the study by (Furuya et al., 2020). Differentially abundant pathways were determined by DESeq2 analysis. A positive log2 fold change means pathways were represented more in the HFD mice whereas a negative change means more pathways represented in control mice.

KEGG Number	Log2 Fold Change	LFC SE	Adj. *p* Value	Pathway
ko02060	4.458	0.645	<0.0001	Phosphotransferase system (PTS)
ko00940	-5.926	0.898	<0.0001	Phenylpropanoid biosynthesis
ko00364	-6.304	0.985	<0.0001	Fluorobenzoate degradation
ko00623	-6.304	0.985	<0.0001	Toluene degradation
ko00280	1.387	0.231	<0.0001	Valine, leucine and isoleucine degradation
ko00514	-5.077	0.857	<0.0001	Other types of O-glycan biosynthesis
ko00515	-5.077	0.857	<0.0001	Mannose type O-glycan biosynthesis
ko05206	-2.442	0.442	<0.0001	MicroRNAs in cancer
ko00944	-6.406	1.181	<0.0001	Flavone and flavonol biosynthesis
ko00531	3.552	0.661	<0.0001	Glycosaminoglycan degradation
ko00740	1.840	0.347	<0.0001	Riboflavin metabolism
ko00130	2.253	0.432	<0.0001	Ubiquinone and other terpenoid-quinone biosynthesis
ko00460	-3.104	0.611	<0.0001	Cyanoamino acid metabolism
ko00970	1.845	0.363	<0.0001	Aminoacyl-tRNA biosynthesis

ko00510	-5.681	1.143	<0.0001	N-Glycan biosynthesis
ko00540	1.655	0.336	<0.0001	Lipopolysaccharide biosynthesis
ko00380	3.235	0.665	<0.0001	Tryptophan metabolism
ko03015	-5.476	1.123	<0.0001	mRNA surveillance pathway
ko03320	-1.618	0.332	<0.0001	PPAR signaling pathway
ko05100	-5.476	1.123	<0.0001	Bacterial invasion of epithelial cells
ko00513	3.287	0.680	<0.0001	Various types of N-glycan biosynthesis
ko00604	3.287	0.680	<0.0001	Glycosphingolipid biosynthesis - ganglio series
ko04142	2.041	0.432	<0.0001	Lysosome
ko00860	1.264	0.271	<0.0001	Porphyrin metabolism
ko00310	1.557	0.338	<0.0001	Lysine degradation
ko04213	3.062	0.677	<0.0001	Longevity regulating pathway - multiple species
ko04626	-1.669	0.371	<0.0001	Plant-pathogen interaction
ko04260	-5.115	1.146	<0.0001	Cardiac muscle contraction
ko04932	-5.115	1.146	<0.0001	Non-alcoholic fatty liver disease
ko05012	-5.115	1.146	<0.0001	Parkinson disease
ko00621	-3.423	0.778	<0.0001	Dioxin degradation
ko04138	-3.950	0.900	<0.0001	Autophagy - yeast
ko04917	-4.123	0.940	<0.0001	Prolactin signaling pathway
ko00361	-3.064	0.704	<0.0001	Chlorocyclohexane and chlorobenzene degradation
ko02030	-2.352	0.540	<0.0001	Bacterial chemotaxis

ko01051	-1.701	0.397	1.24E-04	Biosynthesis of ansamycins
ko05110	-4.914	1.161	1.55E-04	Vibrio cholerae infection
ko00965	-5.044	1.240	2.82E-04	Betalain biosynthesis
ko04622	-5.044	1.240	2.82E-04	RIG-I-like receptor signaling pathway
ko05142	-5.044	1.240	2.82E-04	Chagas disease
ko05143	-5.044	1.240	2.82E-04	African trypanosomiasis
ko05219	-5.044	1.240	2.82E-04	Bladder cancer
ko04016	2.911	0.717	2.83E-04	MAPK signaling pathway - plant
ko00785	2.837	0.721	4.68E-04	Lipoic acid metabolism
ko00120	-3.840	0.999	6.58E-04	Primary bile acid biosynthesis
ko00121	-3.840	0.999	6.58E-04	Secondary bile acid biosynthesis
ko00591	-5.015	1.318	7.46E-04	Linoleic acid metabolism
ko00622	-3.080	0.819	8.75E-04	Xylene degradation
ko00603	1.368	0.366	9.47E-04	Glycosphingolipid biosynthesis - globo and isoglobo series
ko00633	2.714	0.732	0.001	Nitrotoluene degradation
ko05146	-2.977	0.825	0.002	Amoebiasis
ko04146	1.186	0.336	0.002	Peroxisome
ko00565	-4.375	1.245	0.002	Ether lipid metabolism
ko00999	-1.056	0.303	0.002	Biosynthesis of various plant secondary metabolites
ko04072	-4.003	1.148	0.002	Phospholipase D signaling pathway
ko00790	1.068	0.312	0.003	Folate biosynthesis

ko00471	-0.949	0.280	0.003	D-Glutamine and D-glutamate metabolism
ko04211	2.498	0.752	0.004	Longevity regulating pathway
ko00405	2.425	0.740	0.004	Phenazine biosynthesis
ko05132	-3.070	0.945	0.005	Salmonella infection
ko04216	-2.420	0.755	0.005	Ferroptosis
ko04714	-4.021	1.263	0.006	Thermogenesis
ko01220	-1.152	0.363	0.006	Degradation of aromatic compounds
ko04931	-0.943	0.308	0.008	Insulin resistance
ko00791	-3.997	1.318	0.009	Atrazine degradation
ko02040	-2.979	0.994	0.010	Flagellar assembly
ko00190	0.979	0.332	0.012	Oxidative phosphorylation
ko00562	-0.862	0.293	0.012	Inositol phosphate metabolism
ko03013	-2.226	0.759	0.012	Nucleocytoplasmic transport
ko00511	1.166	0.416	0.018	Other glycan degradation
ko04066	0.867	0.309	0.018	HIF-1 signaling pathway
ko00410	1.036	0.371	0.018	beta-Alanine metabolism
ko00909	2.193	0.793	0.019	Sesquiterpenoid and triterpenoid biosynthesis
ko05020	-2.981	1.105	0.024	Prion disease
ko00053	1.420	0.534	0.026	Ascorbate and aldarate metabolism
ko00400	0.856	0.325	0.027	Phenylalanine, tyrosine and tryptophan biosynthesis
ko00450	0.660	0.250	0.027	Selenocompound metabolism

ko00472	-2.025	0.772	0.028	d-arginine and d-ornithine metabolism
ko04621	-0.822	0.314	0.028	NOD-like receptor signaling pathway
ko05225	-3.411	1.306	0.028	Hepatocellular carcinoma
ko00071	1.032	0.399	0.030	Fatty acid degradation
ko04080	-2.825	1.135	0.038	Neuroactive ligand-receptor interaction
ko04979	-2.825	1.135	0.038	Cholesterol metabolism
ko05166	-2.825	1.135	0.038	Human T-cell leukemia virus 1 infection
ko00362	-1.007	0.409	0.041	Benzoate degradation
ko00950	1.873	0.762	0.041	Isoquinoline alkaloid biosynthesis
ko00051	0.936	0.390	0.046	Fructose and mannose metabolism
ko00930	-2.731	1.138	0.046	Caprolactam degradation

Table A.10 Effect of a High Fat Diet and Ethanol on Predicted Microbial Functional Pathway Abundance in Mice With Induced Liver Injury

Piphillin, referring to the KEGG database, was used to predict microbial functional effects of alcohol in mice, using 16S data from mice with induced liver damage from the study by (Furuya et al., 2020). Differentially abundant pathways were determined by DESeq2 analysis. A positive log2 fold change means pathways were represented more in the HFD + EtOH mice whereas a negative change means more pathways represented in control mice.

KEGG Number	Log2 Fold Change	LFC SE	Adj. *p* Value	Pathway
ko02060	4.132	0.645	<0.0001	Phosphotransferase system (PTS)
ko00280	1.283	0.232	<0.0001	Valine, leucine and isoleucine degradation
ko00514	-4.679	0.864	<0.0001	Other types of O-glycan biosynthesis
ko00515	-4.679	0.864	<0.0001	Mannose type O-glycan biosynthesis
ko05206	-2.430	0.444	<0.0001	MicroRNAs in cancer
ko00130	2.247	0.432	<0.0001	Ubiquinone and other terpenoid-quinone biosynthesis
ko00510	-5.859	1.176	<0.0001	N-Glycan biosynthesis
ko00531	3.309	0.661	<0.0001	Glycosaminoglycan degradation
ko00970	1.789	0.363	<0.0001	Aminoacyl-tRNA biosynthesis
ko04260	-5.921	1.206	<0.0001	Cardiac muscle contraction
ko04932	-5.921	1.206	<0.0001	Non-alcoholic fatty liver disease
ko05012	-5.921	1.206	<0.0001	Parkinson disease
ko00380	3.239	0.665	<0.0001	Tryptophan metabolism

ko05110	-5.963	1.228	<0.0001	Vibrio cholerae infection
ko00740	1.657	0.348	<0.0001	Riboflavin metabolism
ko00540	1.573	0.337	<0.0001	Lipopolysaccharide biosynthesis
ko00513	3.058	0.680	<0.0001	Various types of N-glycan biosynthesis
ko00604	3.058	0.680	<0.0001	Glycosphingolipid biosynthesis - ganglio series
ko00944	-4.916	1.134	1.85E-04	Flavone and flavonol biosynthesis
ko04213	2.933	0.677	1.85E-04	Longevity regulating pathway - multiple species
ko04142	1.815	0.432	3.19E-04	Lysosome
ko00621	-3.251	0.782	3.63E-04	Dioxin degradation
ko00860	1.120	0.272	4.08E-04	Porphyrin metabolism
ko00364	-3.849	0.958	5.80E-04	Fluorobenzoate degradation
ko00623	-3.849	0.958	5.80E-04	Toluene degradation
ko03320	-1.322	0.333	6.91E-04	PPAR signaling pathway
ko00310	1.328	0.339	8.32E-04	Lysine degradation
ko03015	-4.146	1.101	0.001	mRNA surveillance pathway
ko05100	-4.146	1.101	0.001	Bacterial invasion of epithelial cells
ko04016	2.678	0.717	0.002	MAPK signaling pathway - plant
ko00472	-2.884	0.779	0.002	d-arginine and d-ornithine metabolism
ko00785	2.642	0.721	0.002	Lipoic acid metabolism
ko00622	-2.848	0.821	0.004	Xylene degradation
ko00999	-1.056	0.305	0.004	Biosynthesis of various plant secondary metabolites

ko00633	2.494	0.733	0.005	Nitrotoluene degradation
ko01051	-1.354	0.397	0.005	Biosynthesis of ansamycins
ko00790	1.054	0.313	0.005	Folate biosynthesis
ko00460	-2.015	0.611	0.006	Cyanoamino acid metabolism
ko00405	2.436	0.741	0.006	Phenazine biosynthesis
ko00603	1.203	0.367	0.006	Glycosphingolipid biosynthesis - globo and isoglobo series
ko00361	-2.300	0.704	0.007	Chlorocyclohexane and chlorobenzene degradation
ko04146	1.093	0.337	0.007	Peroxisome
ko00071	1.289	0.400	0.007	Fatty acid degradation
ko00410	1.148	0.372	0.011	beta-Alanine metabolism
ko00940	-2.726	0.887	0.011	Phenylpropanoid biosynthesis
ko04626	-1.144	0.371	0.011	Plant-pathogen interaction
ko04978	-3.201	1.043	0.011	Mineral absorption
ko04138	-2.736	0.898	0.012	Autophagy - yeast
ko02030	-1.630	0.540	0.013	Bacterial chemotaxis
ko04211	2.276	0.753	0.013	Longevity regulating pathway
ko05146	-2.467	0.827	0.014	Amoebiasis
ko00471	-0.803	0.281	0.020	D-Glutamine and D-glutamate metabolism
ko01054	-2.973	1.061	0.023	Nonribosomal peptide structures
ko04216	-2.123	0.757	0.023	Ferroptosis
ko04931	-0.863	0.309	0.023	Insulin resistance

ko00190	0.906	0.332	0.028	Oxidative phosphorylation
ko00400	0.842	0.325	0.042	Phenylalanine, tyrosine and tryptophan biosynthesis
ko01220	-0.928	0.364	0.045	Degradation of aromatic compounds
ko04080	-2.915	1.146	0.045	Neuroactive ligand-receptor interaction
ko04979	-2.915	1.146	0.045	Cholesterol metabolism
ko05166	-2.915	1.146	0.045	Human T-cell leukemia virus 1 infection

Table A.11 Effect of Ethanol on Predicted Microbial Functional Pathway Abundance in Mice

Piphillin, referring to the KEGG database, was used to predict microbial functional effects of alcohol in mice, using 16S data from the study by Yang et al. (2017a). Differentially abundant pathways were determined by DESeq2 analysis. A positive log2 fold change means pathways were represented more in the ethanol-treated mice whereas a negative change means more pathways represented in control mice.

KEGG Number	Log2 Fold Change	LFC SE	Adj. *p* Value	Pathway
ko00643	6.677	0.523	<0.0001	Styrene degradation
ko00981	7.114	0.629	<0.0001	Insect hormone biosynthesis
ko00903	6.259	0.657	<0.0001	Limonene and pinene degradation
ko04138	7.759	0.873	<0.0001	Autophagy - yeast
ko05146	7.987	0.939	<0.0001	Amoebiasis
ko00362	1.938	0.263	<0.0001	Benzoate degradation
ko00590	6.053	0.887	<0.0001	Arachidonic acid metabolism
ko00625	1.336	0.199	<0.0001	Chloroalkane and chloroalkene degradation
ko00791	7.088	1.072	<0.0001	Atrazine degradation
ko00053	1.875	0.287	<0.0001	Ascorbate and aldarate metabolism
ko04216	5.737	0.907	<0.0001	Ferroptosis
ko00910	0.859	0.141	<0.0001	Nitrogen metabolism
ko00071	1.373	0.236	<0.0001	Fatty acid degradation
ko03450	7.787	1.421	<0.0001	Non-homologous end-joining

ko00010	0.299	0.055	<0.0001	Glycolysis / Gluconeogenesis
ko04714	5.344	0.998	<0.0001	Thermogenesis
ko04964	-3.009	0.583	<0.0001	Proximal tubule bicarbonate reclamation
ko03070	-0.379	0.073	<0.0001	Bacterial secretion system
ko00450	-0.321	0.062	<0.0001	Selenocompound metabolism
ko03320	0.752	0.146	<0.0001	PPAR signaling pathway
ko00592	5.579	1.083	<0.0001	alpha-Linolenic acid metabolism
ko02010	0.986	0.193	<0.0001	ABC transporters
ko02024	0.527	0.106	<0.0001	Quorum sensing
ko00944	9.468	1.909	<0.0001	Flavone and flavonol biosynthesis
ko00650	0.492	0.103	<0.0001	Butanoate metabolism
ko00909	-4.082	0.856	<0.0001	Sesquiterpenoid and triterpenoid biosynthesis
ko00500	1.008	0.214	<0.0001	Starch and sucrose metabolism
ko00642	5.932	1.262	<0.0001	Ethylbenzene degradation
ko00100	-3.706	0.803	<0.0001	Steroid biosynthesis
ko00513	-2.847	0.621	<0.0001	Various types of N-glycan biosynthesis
ko00604	-2.847	0.621	<0.0001	Glycosphingolipid biosynthesis - ganglio series
ko00531	-2.235	0.485	<0.0001	Glycosaminoglycan degradation
ko00524	-0.706	0.153	<0.0001	Neomycin, kanamycin and gentamicin biosynthesis
ko01100	-0.090	0.020	<0.0001	Metabolic pathways
ko00510	4.825	1.043	<0.0001	N-Glycan biosynthesis

ko04978	5.826	1.260	<0.0001	Mineral absorption
ko04919	8.243	1.793	<0.0001	Thyroid hormone signaling pathway
ko00281	5.146	1.141	<0.0001	Geraniol degradation
ko04152	-0.789	0.176	<0.0001	AMPK signaling pathway
ko00565	5.316	1.191	<0.0001	Ether lipid metabolism
ko00190	-0.626	0.143	<0.0001	Oxidative phosphorylation
ko00361	3.084	0.710	<0.0001	Chlorocyclohexane and chlorobenzene degradation
ko00360	0.930	0.216	1.01E-04	Phenylalanine metabolism
ko05100	7.598	1.764	1.01E-04	Bacterial invasion of epithelial cells
ko05020	6.511	1.523	1.15E-04	Prion diseases
ko04113	5.484	1.287	1.21E-04	Meiosis - yeast
ko04144	8.383	2.000	1.60E-04	Endocytosis
ko03018	-0.323	0.078	1.77E-04	RNA degradation
ko00350	0.468	0.113	1.77E-04	Tyrosine metabolism
ko02060	1.898	0.456	1.77E-04	Phosphotransferase system (PTS)
ko05134	-0.298	0.073	2.08E-04	Legionellosis
ko00440	-1.095	0.274	3.29E-04	Phosphonate and phosphinate metabolism
ko00030	0.747	0.188	3.50E-04	Pentose phosphate pathway
ko01502	-0.341	0.086	3.93E-04	Vancomycin resistance
ko03420	-0.307	0.079	4.48E-04	Nucleotide excision repair
ko00364	5.646	1.443	4.48E-04	Fluorobenzoate degradation

ko04080	3.582	0.924	4.82E-04	Neuroactive ligand-receptor interaction
ko04979	3.582	0.924	4.82E-04	Cholesterol metabolism
ko05166	3.582	0.924	4.82E-04	Human T-cell leukemia virus 1 infection
ko05204	3.936	1.016	4.82E-04	Chemical carcinogenesis
ko04940	-0.592	0.154	5.17E-04	Type I diabetes mellitus
ko00930	4.732	1.240	5.94E-04	Caprolactam degradation
ko00514	4.845	1.279	6.42E-04	Other types of O-glycan biosynthesis
ko00515	4.845	1.279	6.42E-04	Mannose type O-glycan biosynthesis
ko00020	-0.731	0.193	6.60E-04	Citrate cycle (TCA cycle)
ko00920	-0.514	0.137	6.98E-04	Sulfur metabolism
ko00965	4.480	1.206	8.27E-04	Betalain biosynthesis
ko00600	-1.660	0.448	8.34E-04	Sphingolipid metabolism
ko00624	4.617	1.245	8.34E-04	Polycyclic aromatic hydrocarbon degradation
ko01120	0.175	0.049	0.002	Microbial metabolism in diverse environments
ko00550	0.331	0.096	0.002	Peptidoglycan biosynthesis
ko00633	-3.168	0.923	0.002	Nitrotoluene degradation
ko00983	0.478	0.140	0.002	Drug metabolism - other enzymes
ko00511	-2.047	0.607	0.003	Other glycan degradation
ko00730	-0.391	0.116	0.003	Thiamine metabolism
ko02026	0.927	0.275	0.003	Biofilm formation - Escherichia coli
ko03050	4.581	1.378	0.003	Proteasome

ko04112	-0.165	0.050	0.003	Cell cycle - Caulobacter
ko00571	4.319	1.311	0.003	Lipoarabinomannan (LAM) biosynthesis
ko03022	4.573	1.390	0.003	Basal transcription factors
ko05143	4.207	1.286	0.004	African trypanosomiasis
ko05142	4.216	1.288	0.004	Chagas disease
ko00680	0.352	0.109	0.004	Methane metabolism
ko05231	-4.362	1.354	0.004	Choline metabolism in cancer
ko00627	2.334	0.735	0.005	Aminobenzoate degradation
ko04727	-0.864	0.273	0.005	GABAergic synapse
ko00261	-0.275	0.088	0.005	Monobactam biosynthesis
ko04910	-0.665	0.213	0.006	Insulin signaling pathway
ko00770	-0.258	0.084	0.007	Pantothenate and CoA biosynthesis
ko04210	7.149	2.335	0.007	Apoptosis
ko04974	7.922	2.631	0.008	Protein digestion and absorption
ko00603	-1.613	0.540	0.008	Glycosphingolipid biosynthesis - globo and isoglobo series
ko00540	-1.956	0.658	0.009	Lipopolysaccharide biosynthesis
ko04142	-2.054	0.698	0.009	Lysosome
ko05132	3.167	1.085	0.010	Salmonella infection
ko00330	-0.389	0.134	0.010	Arginine and proline metabolism
ko04918	2.791	0.980	0.012	Thyroid hormone synthesis
ko05340	1.972	0.700	0.013	Primary immunodeficiency

ko01501	-0.289	0.105	0.016	beta-Lactam resistance
ko00720	-0.244	0.090	0.018	Carbon fixation pathways in prokaryotes
ko02025	0.580	0.216	0.018	Biofilm formation - Pseudomonas aeruginosa
ko04626	0.768	0.286	0.018	Plant-pathogen interaction
ko04726	7.509	2.795	0.018	Serotonergic synapse
ko04728	7.509	2.795	0.018	Dopaminergic synapse
ko05030	7.509	2.795	0.018	Cocaine addiction
ko05031	7.509	2.795	0.018	Amphetamine addiction
ko05034	7.509	2.795	0.018	Alcoholism
ko00940	2.627	0.990	0.020	Phenylpropanoid biosynthesis
ko00626	0.617	0.237	0.023	Naphthalene degradation
ko00572	1.977	0.760	0.023	Arabinogalactan biosynthesis - Mycobacterium
ko04724	-0.668	0.259	0.024	Glutamatergic synapse
ko01062	-1.982	0.774	0.025	Biosynthesis of terpenoids and steroids
ko00072	1.942	0.776	0.029	Synthesis and degradation of ketone bodies
ko01051	0.915	0.367	0.030	Biosynthesis of ansamycins
ko05010	0.379	0.153	0.032	Alzheimer disease
ko02030	1.407	0.574	0.033	Bacterial chemotaxis
ko00460	1.633	0.679	0.037	Cyanoamino acid metabolism
ko01220	0.869	0.371	0.044	Degradation of aromatic compounds
ko00970	-0.381	0.163	0.044	Aminoacyl-tRNA biosynthesis

ko03410	-0.092	0.040	0.046	Base excision repair
ko00473	-0.543	0.234	0.046	D-Alanine metabolism
ko00332	-0.261	0.113	0.047	Carbapenem biosynthesis
ko01200	0.084	0.036	0.047	Carbon metabolism
ko05111	0.338	0.147	0.047	Biofilm formation - Vibrio cholerae
ko00140	-1.875	0.825	0.050	Steroid hormone biosynthesis

Table A.12 Effect of an Antifungal Agent on Predicted Microbial Functional Pathway Abundance in Mice fed Ethanol

Piphillin, referring to the KEGG database, was used to predict microbial functional effects of the antifungal agent, amphotericin B, in mice that had been fed alcohol, using 16S data from the study by Yang et al. (2017a). Differentially abundant pathways were determined by DESeq2 analysis. A positive log2 fold change means pathways were represented more in the EtOH + antifungal treated mice whereas a negative change means more pathways represented in EtOH mice.

KEGG Number	Log2 Fold Change	LFC SE	Adj. *p* Value	Pathway
ko00903	-4.539	0.485	<0.0001	Limonene and pinene degradation
ko00981	-4.573	0.499	<0.0001	Insect hormone biosynthesis
ko04138	-5.349	0.594	<0.0001	Autophagy - yeast
ko00910	-1.060	0.121	<0.0001	Nitrogen metabolism
ko05146	-5.946	0.688	<0.0001	Amoebiasis
ko00053	-1.724	0.202	<0.0001	Ascorbate and aldarate metabolism

ko00071	-1.222	0.153	<0.0001	Fatty acid degradation
ko03420	0.530	0.068	<0.0001	Nucleotide excision repair
ko04113	-7.065	0.945	<0.0001	Meiosis - yeast
ko00965	-6.131	0.825	<0.0001	Betalain biosynthesis
ko00510	-4.642	0.705	<0.0001	N-Glycan biosynthesis
ko04714	-4.050	0.621	<0.0001	Thermogenesis
ko00624	-5.768	0.893	<0.0001	Polycyclic aromatic hydrocarbon degradation
ko04112	0.312	0.049	<0.0001	Cell cycle - Caulobacter
ko02024	-0.366	0.058	<0.0001	Quorum sensing
ko05143	-5.269	0.851	<0.0001	African trypanosomiasis
ko00791	-4.424	0.717	<0.0001	Atrazine degradation
ko05142	-5.197	0.854	<0.0001	Chagas disease
ko00970	0.666	0.110	<0.0001	Aminoacyl-tRNA biosynthesis
ko03450	-5.052	0.868	<0.0001	Non-homologous end-joining
ko00565	-4.649	0.806	<0.0001	Ether lipid metabolism
ko03050	-5.801	1.030	<0.0001	Proteasome
ko00592	-3.579	0.639	<0.0001	alpha-Linolenic acid metabolism
ko03022	-5.794	1.038	<0.0001	Basal transcription factors
ko04216	-3.093	0.558	<0.0001	Ferroptosis
ko00473	0.735	0.139	<0.0001	D-Alanine metabolism
ko00571	-5.570	1.062	<0.0001	Lipoarabinomannan (LAM) biosynthesis

ko00281	-3.780	0.723	<0.0001	Geraniol degradation
ko01502	0.310	0.060	<0.0001	Vancomycin resistance
ko00590	-2.935	0.568	<0.0001	Arachidonic acid metabolism
ko00350	-0.347	0.067	<0.0001	Tyrosine metabolism
ko00930	-4.258	0.839	<0.0001	Caprolactam degradation
ko00362	-1.084	0.223	<0.0001	Benzoate degradation
ko04144	-6.522	1.356	<0.0001	Endocytosis
ko00514	-4.859	1.014	<0.0001	Other types of O-glycan biosynthesis
ko00515	-4.859	1.014	<0.0001	Mannose type O-glycan biosynthesis
ko04919	-6.108	1.284	<0.0001	Thyroid hormone signaling pathway
ko05205	0.320	0.069	<0.0001	Proteoglycans in cancer
ko03070	0.309	0.067	<0.0001	Bacterial secretion system
ko00642	-3.563	0.775	<0.0001	Ethylbenzene degradation
ko00625	-0.721	0.161	<0.0001	Chloroalkane and chloroalkene degradation
ko03010	0.226	0.051	<0.0001	Ribosome
ko05100	-5.350	1.214	<0.0001	Bacterial invasion of epithelial cells
ko00760	0.195	0.045	<0.0001	Nicotinate and nicotinamide metabolism
ko00908	0.259	0.060	<0.0001	Zeatin biosynthesis
ko02025	-0.835	0.199	1.68E-04	Biofilm formation - Pseudomonas aeruginosa
ko03018	0.281	0.068	2.23E-04	RNA degradation
ko05111	-0.368	0.089	2.28E-04	Biofilm formation - Vibrio cholerae

ko03410	0.208	0.051	2.51E-04	Base excision repair
ko04152	0.558	0.137	2.79E-04	AMPK signaling pathway
ko03008	0.259	0.065	3.41E-04	Ribosome biogenesis in eukaryotes
ko00730	0.363	0.091	3.65E-04	Thiamine metabolism
ko05020	-3.722	0.936	3.72E-04	Prion diseases
ko00525	0.389	0.098	3.72E-04	Acarbose and validamycin biosynthesis
ko03030	0.291	0.074	4.44E-04	DNA replication
ko00364	-3.551	0.906	4.51E-04	Fluorobenzoate degradation
ko04978	-2.884	0.744	5.28E-04	Mineral absorption
ko01055	0.405	0.105	5.68E-04	Biosynthesis of vancomycin group antibiotics
ko00521	0.260	0.068	6.10E-04	Streptomycin biosynthesis
ko05230	0.417	0.110	7.39E-04	Central carbon metabolism in cancer
ko00944	-3.942	1.049	8.00E-04	Flavone and flavonol biosynthesis
ko05134	0.239	0.065	9.74E-04	Legionellosis
ko00643	-1.977	0.536	0.001	Styrene degradation
ko04066	0.420	0.114	0.001	HIF-1 signaling pathway
ko00720	0.244	0.066	0.001	Carbon fixation pathways in prokaryotes
ko00333	0.708	0.199	0.002	Prodigiosin biosynthesis
ko04080	-2.106	0.609	0.002	Neuroactive ligand-receptor interaction
ko04979	-2.106	0.609	0.002	Cholesterol metabolism
ko05166	-2.106	0.609	0.002	Human T-cell leukemia virus 1 infection

ko00250	0.158	0.046	0.002	Alanine, aspartate and glutamate metabolism
ko00360	-0.794	0.232	0.003	Phenylalanine metabolism
ko04070	0.370	0.109	0.003	Phosphatidylinositol signaling system
ko00195	0.253	0.075	0.003	Photosynthesis
ko00780	0.632	0.191	0.004	Biotin metabolism
ko01051	-0.719	0.218	0.004	Biosynthesis of ansamycins
ko03430	0.275	0.084	0.004	Mismatch repair
ko04726	-5.507	1.790	0.007	Serotonergic synapse
ko04728	-5.507	1.790	0.007	Dopaminergic synapse
ko05030	-5.507	1.790	0.007	Cocaine addiction
ko05031	-5.507	1.790	0.007	Amphetamine addiction
ko05034	-5.507	1.790	0.007	Alcoholism
ko00010	-0.123	0.040	0.007	Glycolysis / Gluconeogenesis
ko04940	0.379	0.124	0.008	Type I diabetes mellitus
ko01100	0.091	0.030	0.008	Metabolic pathways
ko00564	0.229	0.076	0.009	Glycerophospholipid metabolism
ko01040	0.368	0.124	0.010	Biosynthesis of unsaturated fatty acids
ko00340	-1.190	0.400	0.010	Histidine metabolism
ko01230	-0.265	0.090	0.011	Biosynthesis of amino acids
ko01503	0.365	0.125	0.011	Cationic antimicrobial peptide (CAMP) resistance
ko04614	-3.556	1.227	0.012	Renin-angiotensin system

ko00450	0.259	0.090	0.013	Selenocompound metabolism
ko00380	-1.045	0.365	0.013	Tryptophan metabolism
ko02010	-0.426	0.149	0.013	ABC transporters
ko05152	0.143	0.050	0.013	Tuberculosis
ko00591	-3.326	1.185	0.015	Linoleic acid metabolism
ko04212	0.262	0.094	0.016	Longevity regulating pathway - worm
ko00650	-0.205	0.076	0.020	Butanoate metabolism
ko05133	-3.308	1.225	0.020	Pertussis
ko04210	-3.370	1.267	0.023	Apoptosis
ko00600	1.039	0.395	0.024	Sphingolipid metabolism
ko05132	-2.010	0.770	0.026	Salmonella infection
ko00190	0.341	0.134	0.030	Oxidative phosphorylation
ko04974	-3.622	1.423	0.030	Protein digestion and absorption
ko00230	0.135	0.053	0.032	Purine metabolism
ko03020	0.204	0.081	0.032	RNA polymerase
ko00520	0.197	0.079	0.034	Amino sugar and nucleotide sugar metabolism
ko01524	0.695	0.280	0.035	Platinum drug resistance
ko02030	-0.947	0.383	0.035	Bacterial chemotaxis
ko00330	0.271	0.110	0.036	Arginine and proline metabolism
ko04973	-1.063	0.434	0.037	Carbohydrate digestion and absorption
ko03060	0.150	0.062	0.040	Protein export

ko00280	-0.318	0.132	0.041	Valine, leucine and isoleucine degradation
ko00626	-0.330	0.139	0.044	Naphthalene degradation
ko00670	0.114	0.048	0.046	One carbon pool by folate
ko03320	-0.350	0.149	0.047	PPAR signaling pathway

Table A.13 Effect of Acarbose on Predicted Microbial Functional Pathway Abundance in Mice

Piphillin, referring to the KEGG database, was used to predict microbial functional effects of alcohol in mice, using 16S data from the study by Smith et al. (2019). Differentially abundant pathways were determined by DESeq2 analysis. A positive log2 fold change means pathways were represented more in the acarbose-treated mice whereas a negative change means more pathways represented in control mice.

KEGG Number	Log2 Fold Change	LFC SE	Adj. *p* Value	Pathway
ko00710	0.065	0.012	<0.0001	Carbon fixation in photosynthetic organisms
ko02010	0.219	0.040	<0.0001	ABC transporters
ko05100	2.722	0.537	<0.0001	Bacterial invasion of epithelial cells
ko00600	-0.405	0.085	1.23E-04	Sphingolipid metabolism
ko00030	0.105	0.024	4.57E-04	Pentose phosphate pathway
ko00531	-1.355	0.309	5.29E-04	Glycosaminoglycan degradation
ko00603	-0.551	0.126	5.29E-04	Glycosphingolipid biosynthesis - globo and isoglobo series
ko02024	0.107	0.027	0.003	Quorum sensing
ko00670	-0.085	0.022	0.004	One carbon pool by folate
ko00625	0.185	0.050	0.006	Chloroalkane and chloroalkene degradation

ko00750	-0.246	0.066	0.006	Vitamin B6 metabolism
ko04917	0.303	0.085	0.009	Prolactin signaling pathway
ko01501	0.163	0.048	0.015	beta-Lactam resistance
ko03070	0.092	0.028	0.015	Bacterial secretion system
ko04146	-0.244	0.073	0.015	Peroxisome
ko00410	-0.590	0.177	0.016	beta-Alanine metabolism
ko05225	2.338	0.714	0.018	Hepatocellular carcinoma
ko00562	0.256	0.079	0.020	Inositol phosphate metabolism
ko00140	-2.588	0.860	0.037	Steroid hormone biosynthesis
ko00190	-0.079	0.027	0.037	Oxidative phosphorylation
ko00361	0.817	0.276	0.037	Chlorocyclohexane and chlorobenzene degradation
ko00550	0.067	0.023	0.037	Peptidoglycan biosynthesis
ko04080	-1.087	0.373	0.037	Neuroactive ligand-receptor interaction
ko04919	1.474	0.499	0.037	Thyroid hormone signaling pathway
ko04979	-1.087	0.374	0.037	Cholesterol metabolism
ko05166	-1.087	0.374	0.037	Human T-cell leukemia virus 1 infection
ko05204	2.149	0.713	0.037	Chemical carcinogenesis
ko05219	2.019	0.695	0.037	Bladder cancer
ko01523	-0.102	0.035	0.040	Antifolate resistance
ko00511	-0.408	0.143	0.042	Other glycan degradation
ko04973	-0.863	0.310	0.049	Carbohydrate digestion and absorption