Drosophila melanogaster and the social environment; ageing, immunity and the microbiome

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The candidate confirms that the work submitted is his/her own, except where work which has formed part of jointly-authored publications has been included. The contribution of the candidate and the other authors to this work has been explicitly indicated below. The candidate confirms that appropriate credit has been given within the thesis where reference has been made to the work of others.

Jointly authored publications

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Abstract

Social environments can influence health and fitness in a variety of organisms. Since both social contact and isolation can be stressful, elucidating the mechanisms underlying the patterns of variation is essential to understanding how social environment contributes to overall phenotypes. Using a *Drosophila melanogaster* fruit fly model, I investigated how social contact influences ageing, immunity and the microbiome, in a sex-specific manner. Throughout, I used same sex pairs or groups to avoid the costs of mating and reproduction. Firstly I examined the effect of social environment on actuarial ageing and functional senescence in unwounded and wounded individuals, as well as stress responses. I found that pairing acted to decrease lifespan and stress resistance for both sexes, but when combined with wounding this effect was more severe for males. Climbing ability decreased with age, but this was more severe for paired females than those kept isolated. I next challenged the immune system directly by using a bacterial injection, as well as measuring gene expression and phagocytosis. In contrast to prior work, I found that older paired flies lived longer post-infection than flies that lived alone. Furthermore, gene expression and flow cytometry data suggested that some immune response pathways are more socially-responsive than others. I next determined the effect of social contact on the bacterial community associated with *D. melanogaster*. I found that the male microbiome is affected disproportionately compared to females, indicating that changes in species richness and evenness are not solely the result of horizontal transfer. I also found that age of cohabitants can have a marked effect on the microbiome composition and found that this can have important physiological implications - paired males are less able to cope with oral infections. Taken together, these findings suggest that the effects of social environments differ both between sexes and between traits.

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Chapter 1 – General Introduction

1.1 The social environment and social living

The social environment is highly variable from organism to organism and can have huge implications for individual health and fitness. Here, I discuss the role that social environment plays in relation to ageing the immune system, and the microbiome, and how these factors interact with each other (**Figure 1**).

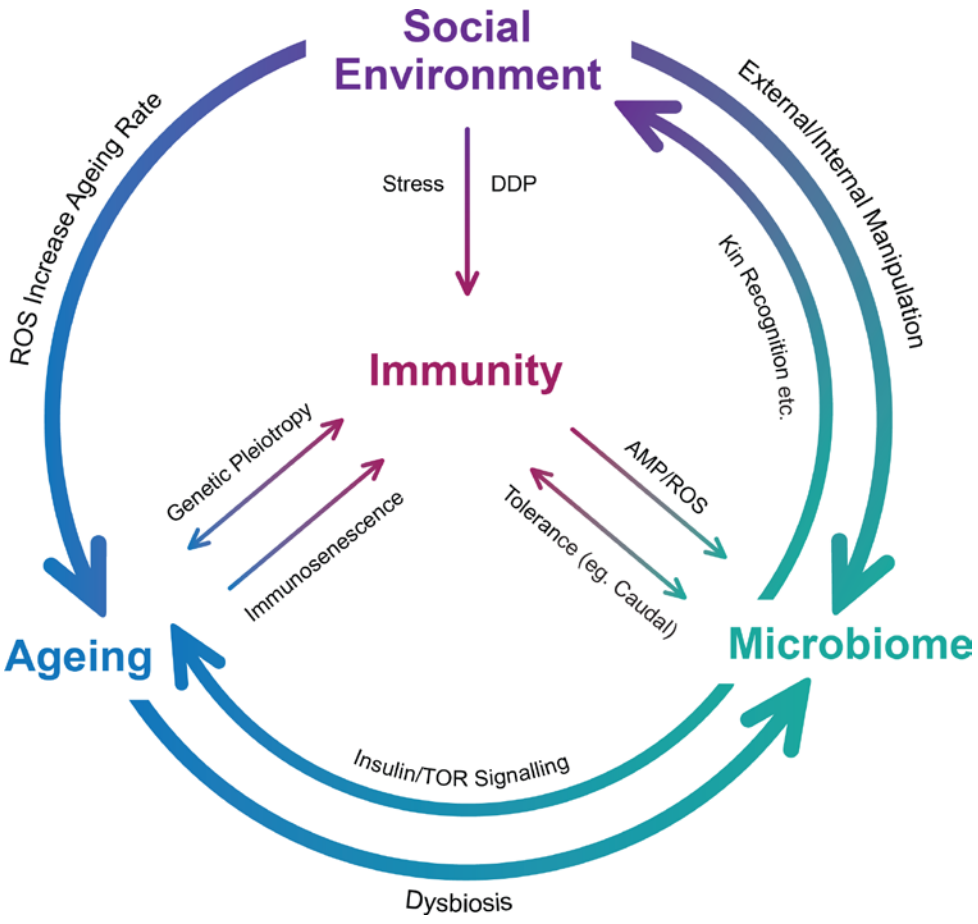

Figure 1 The interconnected relationship between social environment, immunity, ageing and the microbiome, and examples of the mechanisms by which they are able to influence each other. ROS refers to reactive oxygen species, AMP refers to antimicrobial proteins and DDP refers to density dependent prophylaxis.

Groups of conspecific individuals commonly self organise into cooperative units, or societies. These groups, through necessity, elicit a diverse range of social behaviours that can run the gamut from absolute eusociality (Nowak, Tarnita and Wilson 2010), to those that live in small and sometimes transient groups (Molvar and Bowyer 1994). Some researchers have, perhaps controversially, suggested that even microorganisms have diverse and equivalent social lives, including features that were once strictly the preserve of the Animalia, including cooperation, communication and kin recognition (Crespi 2001). Whilst traditional definitions of 'sociality' require 'reciprocal communication of a cooperative nature' (Wilson 1975), a less strict characterisation of the topic may encompass all of the interactions that take place between conspecific individuals and is probably more appropriate for this thesis.

Many animal species, however, still maintain a solitary lifestyle (Karanth and Nichols 1998; Gathmann and Tscharntke 2002) indicating that sociality is only a valuable strategy when the benefits outweigh the costs (Krause and Ruxton 2002). Group size correlates with a large number of costs such as parasite infection, but also a similar number of benefits, like predator avoidance (Brown 1997). Therefore, the level of sociality displayed by a species is a reflection, ultimately, of the optimal strategy for its ecological niche (Jarman 1974) and life history (Silk 2007). Social behaviour and group living can afford the individual many advantages compared to a solitary lifestyle including greater protection from predators; (Cresswell 1994) and greater success in locating resources when foraging or hunting, a hypothesis supported by mathematical models (Clark and Mangel 1986) and bore out by experimental observations (Creel and Creel 1995). In addition, increasing social interactions between conspecifics logically creates more mating opportunities (Lindström and Ranta 1993), but so too does it create more direct competition for mates; a phenomenon responsible in part for the evolution of complex mating systems (Maher and Burger 2011). Similarly, intragroup competition of resources may be equally as important as cooperation, if not more so, as a determinant of group behaviour (Clark and Mangel 1986) - it has been proposed, after all, that

competition for limited resources is the greatest cost of sociality (Elisabeth, Watts and van Schaik 1997).

Of all the detrimental effects that group living can pose, one of the most significant and pervasive is that of infection. Group size is positively correlated with the prevalence and intensity of directly and indirectly transmitted parasites (Patterson and Ruckstuhl 2013) and advances in the fields of network theory and epidemiological modelling have allowed enormous insights into the relationship between social connectedness and risk of communicable disease (Keeling and Eames 2005), revealing that social organization, including the size and composition of social groups, and mating systems can all influence the spread of disease (Altizer *et al.* 2003). Nevertheless, the intuitive link between group size and infection risk is not always as simple as it may ostensibly seem. A meta-analysis comparing parasitism and group size in social animals revealed that parasite number can both increase or decrease with group number (Cote and Poulin 1995) and a more recent meta-analyses found that parasite infection intensity and prevalence can depend strongly on the mode of transmission of the parasite in question (Patterson and Ruckstuhl 2013). Overall, however, the authors found a positive association between group size and parasite number (Rifkin, Nunn and Garamszegi 2012; Patterson and Ruckstuhl 2013). A further consideration in this area of research is that since individuals in a social network do not interact purely at random the effect of larger groups on socially transmitted disease can be mitigated by sub-grouping, a phenomenon known as the social bottleneck hypothesis (Nunn *et al.* 2015). Mathematical modelling also raises the interesting possibility that social complexity could, in fact, have evolved under certain conditions to prevent the spread of pathogens, rather than in spite of it, since evidence suggests that social complexity can lower disease risk without the intervention of immunity or avoidance behaviours in certain circumstances (Hock and Fefferman 2012).

Nonetheless, evidence consistently suggests that social interactions increases the spread of transmissible microorganisms, at least on in individual basis. In zebra finches (*Taeniopygia guttata*) experimentally infected with

Bacillus species social and sexual behaviours aid the transmission of bacteria between birds via an oral-faecal-genital route (Kulkarni and Heeb 2007). Contact during shared visits to flowers can result in the horizontal transmission of parasites between bumblebees (*Bombus* spp.) (Durrer and Schmidhempel 1994) and in similar fashion higher levels of social network connectivity can increase the risk of becoming infected with parasites in gidgee skinks (*Egernia stokesii*). Such is the risk of infection at increased densities that eusocial insects have evolved sophisticated collective anti-parasite defences (Cremer, Armitage and Schmid-Hempel 2007). It should be noted that eusocial species are an extreme form of sociality, where social living in often high density colonies of very related individuals is obligate, and therefore it is hard to extrapolate results to non-eusocial species, but even in mammals and other animals complicated avoidance behaviours help to mitigate the costs of infection (Curtis 2014).

1.2 Sex differences in the effects of social environment on ageing, immunity and the microbiome

Just as the costs and benefits of social contact and isolation vary from species to species, they also vary between sexes within species. Sex differences in ageing are widespread in nature (Austad and Fischer 2016) and are believed to occur via a variety of mechanisms, some of which may interact with the social environment. Males and females acquire fitness in different ways and dissimilarities in lifespan therefore reflect an adaptive response to trade-offs between survival and reproduction (Bonduriansky *et al.* 2008). In short, males can afford to “live fast, die young” for the benefit of enhanced mating success, but for females, whose fitness is limited by the time and resources it takes to make eggs, this is not a viable strategy. In polygynous species, sexual selection results in males performing energetically costly courtship behaviours, multiple mating and male-male competition and aggression, all of which reduce longevity (Partridge and Farquhar 1981). Male lifespan may also be reduced by the cost of sperm and seminal fluid protein production (Bretman *et al.* 2013), although whether or not these costs are any more severe than that of egg

production is not clear. If social interaction between males invokes any of the costs associated with sexual selection, then there is likely to be significant sex differences in the effects of social environment and ageing.

Sex differences in lifespan may also arise by genetic means, but are still important in the context of this thesis since ageing has clear effects on immunity and the microbiome. The effect of sex-linked deleterious alleles on the heterogametic sex is more serious than the homogametic, since the phenotype cannot be rescued by normal alleles on the other X chromosome, a hypothesis known as the unguarded X hypothesis (Trivers 1985). In line with this theory, inbred females suffer greater reductions in lifespan than do males (in XY systems at least) (Carazo *et al.* 2016), confirming that sexual dimorphism in lifespan is, at least in part, down to the 'inbred' nature of sex chromosomes in the heterogametic sex. A further explanation for these differences also relates to the asymmetric inheritance of genetic information – because mitochondrial DNA is solely inherited from the mother, mutations that only effect male offspring (such as defects in sperm) are not negatively selected against and can reach high frequencies in natural populations (Gemmell, Metcalf and Allendorf 2004).

Sex differences in immunity, similarly, may arise as a result of Bateman's principle - the hypothesis that the sexes differ in the reproductive trait limiting their fitness (Arnold 1994), and therefore, as well as influencing sex-specific mating-strategies, this has implications for correlated life-history traits, like immunity (McKean, Nunney and Rowe 2005). These differences often manifest in reduced immune function in males - this is the case in a variety of species and in response to a wide range of infection types (Zuk *et al.* 2004; Klein 2000; Roberts, Walker and Alexander 2001). The immunocompetence handicap posits that the androgenic hormone testosterone, whilst responsible for the production of secondary sexual characteristics, also acts to reduce immunocompetence and thereby provide prospective females with an honest signal of genetic (specifically immune) quality (Folstad and Karter 1992). In short, males are consistently worse at

coping with infection and this is likely a consequence of the combination of their life-history strategy, genetic differences that are attributable to the lack of selection imposed on recessive lethal mutations in the homogametic sex (in similar fashion to sex differences in lifespan) and to differences in the expression of steroid hormones (Fish 2008).

Finally, like the immune system, the microbiome responds to sex hormones in a sex specific manner (Org *et al.* 2016). The growing realisation that sex hormones interact with the microbiome in clinically meaningful ways has initiated a flurry of research into the 'microgenderome' (Flak, Neves and Blumberg 2013), finding links between sex hormones, the gut microbiota and irritable bowel syndrome (Mulak, Tache and Larauche 2014) and autoimmune disease (Markle *et al.* 2013). The composition of the microbiome is also influenced by (and influences) the immune system (Round and Mazmanian 2009), which therefore exerts an additional, sex-specific, layer of control over its structure (Haro *et al.* 2016). Consequently, if the social environment interacts with any of the factors that influence these sex differences it is likely that ageing, immunity and the microbiome will respond to social contact in males and females differently.

1.3 Ageing as the ultimate measure of the effects of social environment

One of the clearest manifestations of the effects of social environment is likely to be on ageing (House, Landis and Umberson 1988). If lifespan (and therefore ageing) is the ultimate phenotype by which we are to measure the effect of social environment, an understanding of the general mechanisms which control it are necessary. Likewise, it is necessary to understand the factors that may act indirectly (or bi-directionally) to alter the rate of ageing, such as the immune system and the microbiome. Ageing is the change in a trait with age (Lopez-Otin *et al.* 2013) and fitness can increase or decrease with age, depending on circumstance. Senescence is defined as a decrease in physiological function with age, manifested in population statistics as an increasing probability of

mortality and decreasing reproductive success – senescence, therefore, always results in a loss of fitness (Ricklefs 2008). Since ageing usually results in the loss of a genetic contribution to future generations selection should act to oppose it (Partridge and Barton 1993), but clearly organisms still die from the accumulating effects of time on physiological functions.

The genetic mechanisms underlying this observation broadly fall into three related categories. The mutation accumulation hypothesis first described by Medawar (1952) posits that selection for survival and fertility weakens with age because extrinsic causes of death (predation or accident for example) may have already killed individuals carrying genes influencing these factors. This would allow the accumulation of deleterious mutations expressed at older ages and consequently reduce the survival and reproductive success of older individuals (Partridge and Barton 1993; Monaghan *et al.* 2008). The related theory of antagonistic pleiotropy (Williams 1957) builds on the mutation accumulation hypothesis by proposing that some genes that infer greater survival or fecundity at young ages can have deleterious effects in old age, but persist due to weaker selective pressures in older individuals (Williams 1957).

The third and perhaps most relevant genetic theory of ageing, in the context of my work on the social environment at least, relates to the accumulation of damage to core cellular functions with age, whilst the costs of controlling this damage become ever more expensive (Ricklefs 2008). Oxidative stress is believed to be the primary mechanism in this accumulation of cellular damage (Kujoth *et al.* 2007) and is caused by the off-target oxidising effects of reactive oxygen species (ROS) that are a natural by-product of mitochondrial aerobic metabolism (Kujoth *et al.* 2007). Increases in oxidative stress are commonly observed under stressful conditions (Schiavone *et al.* 2013) and have been linked to a diverse range of ill-effects including immunosenescence (Cannizzo *et al.* 2011) and inflammatory complications to diseases like diabetes (Evans *et al.* 2002). If one social condition is therefore more stressful than another in *D. melanogaster*, this may result in an increase in oxidative stress that ultimately leads to a shorter lifespan, increased

immunosenescence and which may trade off with other aspects of physiology (i.e. the immune system or control of the microbiome), if a large amount of resources are being used to try and repair this damage.

In the following section I will explore adaptive mechanisms which have evolved to cope with the variation in infection risk that is associated with increased social contact, and the secondary costs which can arise from fluctuations in social environment, specifically those relating to social stress. Social stress in particular has the ability to directly influence ageing rates, via the action of reactive oxygen species (Cook-Wiens and Grotewiel 2002; Epel *et al.* 2004) and so is an important consideration when investigating the effects of social isolation or interaction, one of which is likely to prove more stressful than the other in terms of the resultant physiology observed.

1.4 The adaptive and non-adaptive effects of social environment on immunity

1.4.1 Adaptive immune responses to social environment

Since pathogen exposure generally exhibits a positively density dependent relationship (Patterson and Ruckstuhl 2013) and investment in the immune system is costly, natural selection should favour those able to utilise density-based cues to allocate resources into resistance to infectious disease when appropriate. This would result in animals kept at higher densities exhibiting greater resistance to pathogens compared to those kept at lower densities, a phenomenon which has been termed density dependent prophylaxis (DDP) (Wilson and Reeson 1998). This has proved to be the case in a variety of insect species. Investment in immunity shows density-dependent polyphenism in the mealworm beetle *Tenebrio molitor*, where rearing at increased densities results in increased resistance to entomopathogenic fungi, and which correlates with cuticular melanisation (Barnes and Siva-Jothy 2000). The phase-polyphenic desert locust (*Schistocerca gregaria*) responds to group living by upregulating their immune response, again in response to an entomopathogenic fungal

pathogen (Wilson *et al.* 2002). It was until recently thought that DDP is not present in Eusocial species because of the costs associated with maintaining it constantly (Pie *et al.* 2005), but work in the bumble bee *Bombus terrestris* has demonstrated that DDP may in fact be more phylogenetically widely spread than initially thought (Ruiz-González, Moret and Brown 2009).

1.4.1 Social stress and the immune system

As well as adaptive mechanisms which respond to social contact, non-adaptive, secondary, mechanisms can act to alter immune function (and indeed ageing and the microbiome too), most notably by the action of stress. What constitutes a stressful social environment, however, may be species-specific and context dependent. In humans, social isolation is linked to a diverse range of negative health implications including poor cerebrovascular homeostasis and cognition (Friedler, Crapser and McCullough 2015), depression (Matthews *et al.* 2016) and dysregulation of the immune system (Cruces *et al.* 2015; Cohen *et al.* 1997). Such is its influence over our health that long term longitudinal studies suggest that loneliness and social isolation are risk factors on a par with smoking for the development of coronary heart disease and stroke (Valtorta *et al.* 2016). Interestingly, simply being alone does not elicit these adverse effects, but perceived isolation does (Cacioppo *et al.* 2011), indicating that loneliness and not isolation is the key predictor for ill health in humans. For species that have evolved to live in social aggregations the deleterious effects of isolation are likely an evolutionary response to provoke the reinitiation and maintenance of social connections (Cacioppo *et al.* 2011).

Recent evidence points to social isolation having profound effects on the immune system. A consistent transcriptomic profile is associated with loneliness in humans that is characterised by increased expression of genes controlling pro-inflammatory cytokine signalling and prostaglandin synthesis coupled with the under-expression of other genes, including those involved in innate antiviral resistance (Cole *et al.* 2011; Cole *et al.* 2007b). The authors speculate that this is explained by the relative transmission routes of different pathogens - viruses rarely persist in the environment and are generally

transferred more efficiently at higher densities, whilst in contrast, bacteria and other extracellular pathogens are able to survive for extended periods without a host (Cole *et al.* 2011). Bacteria are also facilitated by wounding and other types of tissue damage associated with both general threat (e.g., predation injury, to which socially isolated individuals are particularly vulnerable) and hostile social interactions with conspecifics (Cole *et al.* 2011). Therefore these transcriptomic changes appear to reflect the relative risks socially isolated or integrated people face (Cole *et al.* 2011).

The interplay between social interaction and the immune system is not limited to humans and is not always as simple as the paradigm described above. In other species socially isolated mice display attenuated T cell function and a prolonged course of viral infection, despite increased macrophage activation (Clausing *et al.* 1994) and prairie voles (*Microtus ochrogaster*) kept alone have significantly lower complement activity and are consequently worse at killing bacteria (Scotti *et al.* 2015). These studies did not measure response to viral infection and so it is difficult to put this work in the context of work on humans described by Cole et al (Cole *et al.* 2007b) above. Insects too are sensitive to social isolation. For the carpenter ant (*Camponotus fellah*) social stress in the form of isolation results in mortality by way of disruptions in energy homeostasis (Koto *et al.* 2015), an observation that is perhaps unsurprising given this is a strictly eusocial animal. Whilst these studies do not implicate the immune system they serve to demonstrate the persistent problem that social isolation represents, across phyla.

Even for species that have evolved to live in social aggregations of some kind, the competitive stress of this environment can have major implications for important aspects of fitness, particularly immunity. Exposure to both chronic and acute social defeat can lead to lower immunoglobulin G concentrations (Jasnow *et al.* 2001) and social subordinacy in general can result in lower numbers of circulating CD8+ T cells, but higher expression levels of key inflammatory genes, suggesting that individuals of a lower social rank suffer immunologically (Tung *et al.* 2012). Intraspecific competition for resources also

leads to increased aggression and compromises individual immunocompetence by reducing antibody responses (Hawley, Lindstrom and Wikelski 2006). Unsurprisingly, aggression between group members can trigger stress responses that are conserved across phyla and are characterised, broadly speaking, once again by dysregulation of the immune system (Koolhaas 2008). So, since both social isolation and interaction can act to influence the immune system in their own right by being differentially stressful, there is a need for a simple laboratory model with which to investigate the fundamental mechanisms mediating these responses. The effects of social stress is also a significant determinant of the composition of the microbiome, which itself can have significant ramifications for immunity and ageing, and which will be discussed below.

1.5 Internal and external manipulation of the microbiome by the social environment

The microbiome encompasses all the microorganisms (bacteria, viruses, fungi, protozoa) that live on or in a host. The advent of next generation sequencing has resulted in an explosion of research into this hidden ‘organ’ and provided insights into the unexpected ways it can modulate a diverse range of negative physiological outcomes including depression (Carabotti *et al.* 2015) and obesity (Ridaura *et al.* 2013), ageing (Vaiserman, Koliada and Marotta 2017) and immune homeostasis (Wu and Wu 2012). The enormous amount of physiological influence that the microbiome can exert therefore makes understanding the factors that shape its composition a valuable subject for research. This topic is made even more pertinent by the fact that there is substantial variation not only between conspecifics (Smith *et al.* 2015), but within individuals over time (Yatsunenko *et al.* 2012) - well studied factors that can shape the microbiome and thereby explain some of this variation include diet (David *et al.* 2014), method of delivery when born (Neu and Rushing 2011) and geographic location (Yatsunenko *et al.* 2012). Less well documented is the effects that social environment can have on the microbiome.

In humans, cohabiting family members and their dogs share more similar microbiomes than those of other families, indicative of the transfer of bacterial species between individuals (Song *et al.* 2013). Transmission of beneficial gut bacteria is an unexpected benefit of sociality in the bumble bee, which gains protection from some parasite infections when exposed to the faeces of nestmates (Koch and Schmid-Hempel 2011). The social transmission of bacteria has been observed in a variety of other species including kittiwakes and baboons, and has been reviewed in some detail by Archie and Tung (2015), but transmission is not the only way the social environment can impact resident microbial communities.

In addition to the physical transmission of bacteria between conspecifics (external effects), physiological changes to, for example, stress or immune responses (internal effects) may also ultimately alter the composition in an intrinsic manner. Social stress has the ability to alter the structure of the intestinal microbiota in male mice, where a reduction in *Lactobacilli* species is observed even after an acute social stress (Galley *et al.* 2014). Here, female mice were not tested because aggression between females is too low to induce a stress response in this particular paradigm, although it is speculated that other stressors specific to females can affect the microbiome in mice (Bangsgaard Bendtsen *et al.* 2012). Similar results have implicated components of the immune system which are socially induced and act directly to reduce species such as *Coprococcus* spp, potentially allowing other, inflammation-inducing species, to bloom (Bailey *et al.* 2011). *Bifidobacterium* spp. are able to confer resistance to social stress, once again confirming the bidirectional nature of the relationship between the microbiota and physiological functions (Yang *et al.* 2017). It remains to be seen if social isolation or stimulation is the more stressful environment, although it is likely to affect the sexes differently due to differences in life history strategy and the different ageing rates of males and females. This, therefore, may lead to sex differences in the manipulation of the microbiota, in relation to social environment.

Finally, the microbiome, like most aspects of physiology, is not invulnerable to the effects of ageing, which represents a further way in which the microbial composition of the host can be internally modulated. In humans, ageing is accompanied by a characteristic shift of the microbial counterpart from that which is dominated by the *Firmicutes* to one with an increase in *Bacteroidetes*, although this a complex and highly country-specific observation (Biagi *et al.* 2012). Such are the physiological implications of the ageing microbiome that it is increasingly being investigated as a target for anti-ageing therapies (Vaiserman, Koliada and Marotta 2017). As discussed, social environment may affect the rate of senescence, and if so this could be a further way in which social stimulation or social isolation is able to alter the microbiome, and in turn the many biological processes that rely on it. Any age or stress related changes in the microbiome are likely to be mediated in part by the gut-brain-microbe axis. This is a multidirectional network of communication which maintains homeostatic balance via extensive cross talk between hormones (e.g. glucocorticoids), immune effectors (e.g. cytokines like IL-6) and neurotransmitters (e.g. dopamine) (El Aidy, Dinan and Cryan 2014). In mammals there is extensive evidence to suggest that the gut-brain-microbe axis is responsible for stress related changes in the microbiome (Foster, Rinaman and Cryan 2017), but how precisely this complex network operates, and how it interacts with the social environment, age and the immune system, understandably, remains somewhat unknown, therefore necessitating a simple lab model with which to understand this complicated topic.

1.6 *Drosophila melanogaster* and the social environment; the interaction between ageing, immunity and the microbiome

1.6.1 The social environment

Whilst *D. melanogaster* is not considered a classically social species, the average fruit fly spends much of its life under dynamic social conditions. In the wild flies likely experience great fluctuations in social interaction; around transient food sources massive aggregations of individuals exist together at high densities (Wertheim *et al.* 2006), before moving between food patches

solitarily. Different genotypes display distinct preferences for specific social conditions, with strains predisposed to clustering in either low or high density groups (Saltz 2011). What is more, within groups, flies form non-random social interaction networks that are mediated by chemosensory cues which could have potentially significant implications for how information spreads throughout the group, but more pertinently, the spread of transmissible infection (Schneider, Dickinson and Levine 2012). Social contact is not confined to the adult life stage; females preferentially lay eggs on food patches occupied by larvae over similar unoccupied patches (Durisko, Anderson and Dukas 2014) and similarly, larvae choose to aggregate early in development, from which they derive an apparent improved ability to dig and burrow into the food substrate (Durisko *et al.* 2014). Male adults are also attracted to food that is occupied by larvae, most likely because the odour that leads them there is a cue that sexually reproductive females are nearby (Durisko, Anderson and Dukas 2014). Females also exhibit the same behaviour but this is more difficult to explain since choosing an oviposition site in the presence of other larvae has both costs and benefits (Durisko, Anderson and Dukas 2014). What is clear, is that *D. melanogaster* have diverse social lives throughout both developmental and adult stages.

1.6.2 Plasticity and male-female interactions

The interactions that take place between male and female *D. melanogaster*, whilst not directly relevant to this thesis (I only investigate same sex interactions) dominate the social environment, and the biology that underlies them has repercussions for all aspects of social interaction in fruit flies. A sexually competitive environment requires the ability of males to assess a variety of sensory cues (Rouse and Bretman 2016; Bretman *et al.* 2011) in order to deduce both female receptivity and level of sperm competition from surrounding males. This is especially relevant to this thesis because investment in this plasticity is likely to invoke trade-offs in other traits, including immunity and ageing. In the presence of strong competition from conspecific males, increased investment in reproduction results in extended mating duration (Bretman, Fricke and Chapman 2009) and the transfer of more seminal fluid

proteins (Wigby *et al.* 2009), which has the ultimate effect of increasing reproductive fitness (Bretman, Fricke and Chapman 2009). This stochasticity in social environment is likely what drives the sexual plasticity observed because without variation, plasticity would not be maintained (Carroll and Corneli 1995) and changes in response to social environment would not occur.

Conspecific interactions understandably, therefore, have ramifications for gene expression, manipulation of which is a principle way of successfully managing dynamic environments. Courting (unmated) males experience rapid (within 5 minutes) transcriptional alterations in differential expression of 43 genes (Carney 2007). These genes have a variety of functions ranging from spermatogenesis to immunity (Carney 2007). In a reciprocal fashion, females exposed to the courtship song of a conspecific male differentially express 412 genes, of which 41 were significantly over or under expressed (Immonen and Ritchie 2012) and which similarly control a diverse range of physiological functions. The frequency of courtship song is used by females of *Drosophila montana* to assess male quality and is a major sexual signal indicating the presence of ‘good genes’ (Immonen and Ritchie 2012), which goes some way to explain the complexity of the genomic response. The integration of multiple cues from an ever changing social environment therefore necessitates rapid and appropriate changes in behaviour and physiology that are mediated by gene expression.

Changes in gene expression are not simply due to the presence of any other organism, but instead reflect who the individual is interacting with. For example, male *D. melanogaster* react differently on a genomic level to conspecific males and females - the expression of 281 genes changes during male–female interactions, while 505 genes are affected by male–male interactions (Ellis and Carney 2011) and there are differences in the male response to conspecific and heterospecific females (Ellis and Carney 2009). In particular, the presence of a mating rival results in the differential expression of more than 100 immune-associated genes, at least in males (Mohorianu *et al.* 2017). As well as gene expression changes social environment can have

tangible effects physiology by, for example, altering brain morphology and increasing the number of neuronal connections in the brain (Donlea and Shaw 2009) indicating that social environment can have profound effects on the fruitfly *D. melanogaster*.

1.6.3 Social environment can alter lifespan and ageing in *D. melanogaster*

As already discussed, the social environment has the ability to influence an enormous amount of physiological functions. Amongst them, ageing is no exception and is also vulnerable to the effects of stimulation by conspecifics, and is perhaps the definitive measure of the impact of social environment. Under certain circumstances, social stimulation can extend lifespan (Ruan and Wu 2008). The gene *Sod* encodes a cytosolic enzyme, Cu/Zn superoxide dismutase, and is an essential component in the antioxidant defence pathway which scavenges ROS generated during aerobic respiration. Flies with mutations in this gene have dramatically shortened lifespan and are especially sensitive to paraquat, a chemical that generates superoxide radicals when ingested (Ruan and Wu 2008). However, these mutants demonstrate a significant lifespan extension and slowing of functional senescence when co-housed with even a single same sex individual, an effect that is more pronounced in males flies, suggesting that they are more sensitive to the social environment than females (Ruan and Wu 2008). Through a series of surgical and genetic manipulations it was discovered that co-habiting flies only provided lifespan extending properties when they were younger than the median age of the focal fly and that physical interaction between the pair was essential in eliciting this response (Ruan and Wu 2008).

Whilst this is an interesting result and provides a mechanistic link between ROS, ageing and the social environment, studies using wild type flies have often found no lifespan extension or even a reduction in lifespan when cohoused (Leech, Sait and Bretman 2017; Iliadi, Iliadi and Boulianne 2009), so drawing general conclusions based on these genetic mutants should be

cautioned. Other studies have looked at the effects of social environment on lifespan in conjunction with dietary restriction (which generally increases lifespan) (Zajitschek *et al.* 2013), and found strong differences in the way the sexes respond. Males appear, once again, to be especially sensitive to social environment – courting males (Cordts and Partridge 1996), those exposed to a rival (Bretman *et al.* 2013) and even those exposed simply to female pheromones all suffer a significant reduction in lifespan (Gendron *et al.* 2014).

1.6.4 Immunosenescence and the link between longevity and immunity

It is well established that the immune system functionally declines with age, resulting in a progressive deterioration in the ability of an individual to combat infection (Aw, Silva and Palmer 2007). This process is known as immunosenescence and is common to all animals. In *D. melanogaster* immunosenescence is characterised by uncontrolled upregulation of immune genes (Pletcher *et al.* 2002; Seroude *et al.* 2002). In particular, these studies report an increase in transcription of peptidoglycan sensing proteins and antimicrobial peptides (AMPs), indicative of a loss of control of NFκB pathways (Seroude *et al.* 2002; Pletcher *et al.* 2002). These studies, however, observe transcript levels in the absence of infection. When subjected to a live infection, older flies are less able to terminate induction of the AMP diptericin (Zerofsky *et al.* 2005). Despite this, the authors suggest the intrinsic capacity to induce antimicrobial peptide genes actually declines with age even though net activity of the immune system following infection with bacteria is elevated in old flies (Zerofsky *et al.* 2005). In contrast, other studies have found that ageing reduces the ability of the fly to survive infection but not via the ability to eliminate bacteria because no correlation between decreased survival and decreased bacterial clearance has been found, suggesting that increased death rates upon infection in older flies is down to a reduced ability to tolerate infections rather than fight them (Seroude *et al.* 2002).

The cellular response to infection also becomes less effective with age. Both sessile and circulating haemocytes are less plentiful in four and five week old flies respectively (this is seen more prominently in females), and are also less able to effectively phagocytose engulfed particles (Horn, Leips and Starz-Gaiano 2014; Mackenzie, Bussiere and Tinsley 2011). There are some discrepancies between these studies but since genetic variation can affect age-specific responses to infection, differences when studies use different fly strains are to be expected (Felix *et al.* 2012). Social environment can affect rate of immunosenescence (Amdam *et al.* 2005; Carroll *et al.* 2013), so this may be a further mechanism by which social environment alters the ability of *D. melanogaster* to fight infection.

Beyond the decline in immune function associated with ageing, longevity and immunity appear to share common functional links. In experimental evolution assays, flies selected for increased longevity also revealed significant enrichment for immunity genes (Remolina *et al.* 2012; Carnes *et al.* 2015). In other studies that extend longevity in *D. melanogaster*, the increase in immune gene expression that is typical of ageing is delayed (Pletcher *et al.* 2002), suggesting that the uncontrolled expression of immunity genes may play a role in ageing and ultimately lifespan. More recent studies indicate that immunity is playing a direct role in lifespan. Tissue-specific overexpression of peptidoglycan recognition protein, PGRP-LE, results in persistent resistance to bacterial infection via robust up-regulation of the IMD cascade, without obvious acute trade-offs, but does eventually reduce lifespan in an NFκB-dependent manner (Libert *et al.* 2006). This is in line with chronic inflammation seen in elderly humans and has been linked to variety of age-related diseases (Franceschi and Campisi 2014). Destabilisation of the same IMD/NF-κB pathway in the brain can affect lifespan in a bidirectional manner. Increases in IMD/NF-κB signalling result in overexpression of AMPs and resultant locomotor defects, neurodegeneration and a shorter lifespan (which are all hallmarks of ageing), while reduction of signalling in healthy flies results in the improved functional and actuarial senescence accompanied by elevated hormonal signalling and increased nutrient availability (Kounatidis *et al.* 2017). Loss of negative regulators of the IMD pathway similarly result in reduced lifespan

(Fernando, Kounatidis and Ligoxygakis 2014), unequivocally indicating that immunity genes can have a causal role in longevity and ageing.

Given the plethora of evidence which link longevity and ageing with the immune system, it is reasonable to assume that if social environment is able to influence the rate of ageing this may be being mediated by changes in the immune system. Likewise, if social interaction or isolation changes the immune system, this may have important repercussions for ageing and lifespan. It is therefore essential to attempt to gain an insight into the true mechanistic effects of the social environment on immunity and ageing.

1.6.5 The immune system of *D. melanogaster*

The fruit fly *Drosophila melanogaster* has long been a laboratory model to study fundamental genetic principles, and has more recently helped to reveal central aspects of innate immunity, most famously enabling the discovery of Toll-like receptors in humans (Medzhitov, PrestonHurlburt and Janeway 1997) after the discovery of the Toll protein in *D. melanogaster* (Lemaitre *et al.* 1996). *D. melanogaster* has evolved multiple mechanisms which respond to bacteria, viruses, fungi and parasites (Lemaitre and Hoffmann 2007; Tzou, De Gregorio and Lemaitre 2002) and these mechanisms often utilise conserved signalling pathways to activate antimicrobial defences, indicating that components of innate immunity in metazoans share evolutionary ancient origins (Lemaitre and Hoffmann 2007), despite the fact that unlike mammals, *D. melanogaster* does not have an adaptive immune system (**Figure 2**).

1.6.5.1 The Imd and Toll pathways and defence against bacteria and fungi

Control over the immune response in *D. melanogaster* is exerted by a number of signalling pathways, the two major ones being the Toll and Imd pathways (De Gregorio *et al.* 2002). The Toll protein in *D. melanogaster* is not a pathogen recognition receptor as in mammals, but instead acts as the major transcriptional regulator responsible for defence against Gram-positive bacteria

and fungi (Medzhitov 2001). Similarly, the Imd pathway has no direct homologue in humans but shares a death domain with an adaptor molecule involved in NF-ƙB activation and apoptosis, RIP (Georgel *et al.* 2001). These pathways are activated upon systemic bacterial infection and control the challenge-induced synthesis of potent antimicrobial peptides which are produced in the fat body and released into the haemolymph to act systemically (Imler and Bulet 2005).

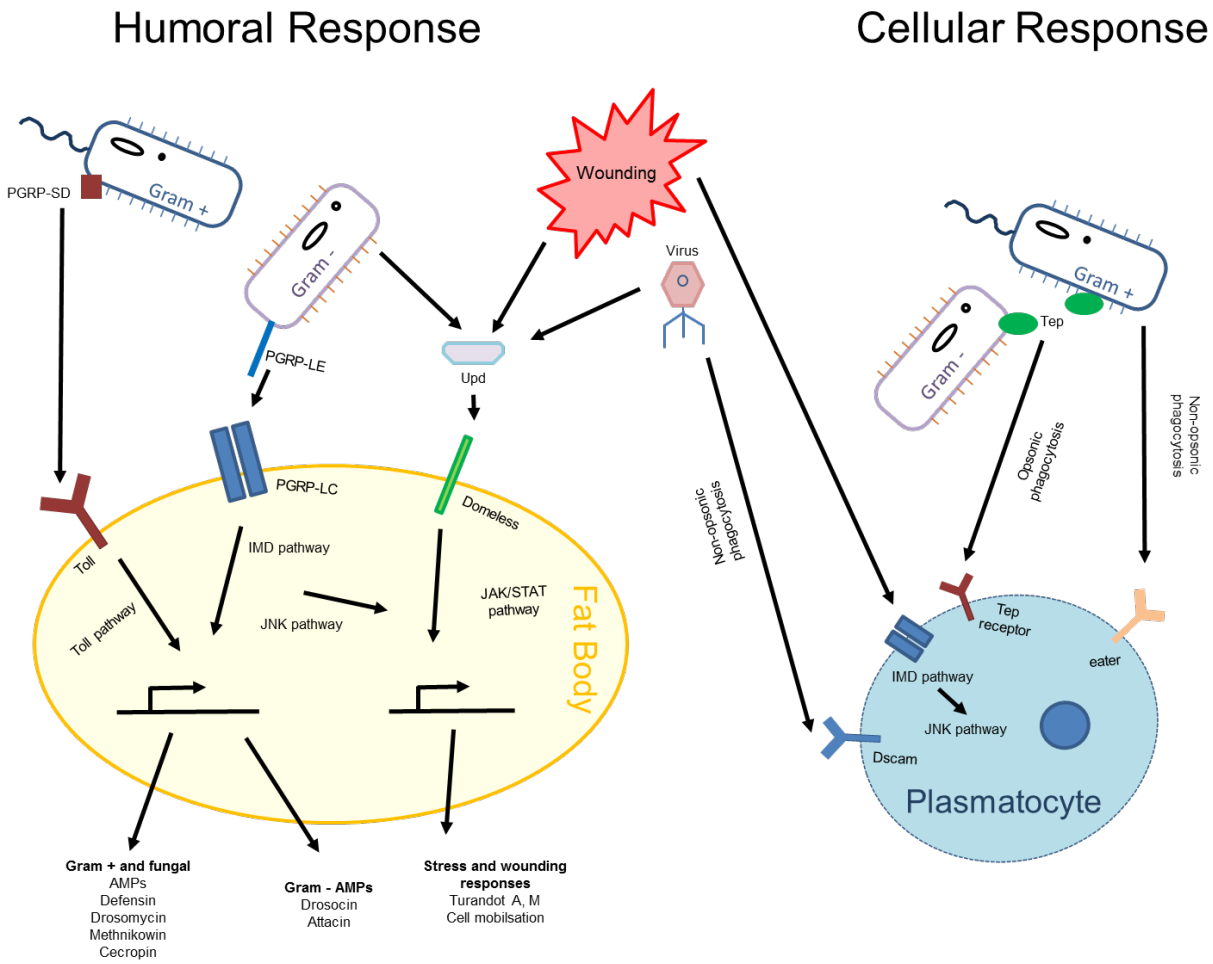

Figure 2 An overview of the *D. melanogaster* immune system. Microbial defence and stress or wounding mechanisms can largely be divided into humoral and cellular responses, with some cross over. Adapted from Lemaitre *et al* (2007).

Antimicrobial activity in *D. melanogaster* is able to discriminate between different types of bacterial pathogens, both at the level of bacterial recognition and at the level of bacterial destruction. Discrimination is initially achieved by an assortment of 13 different peptidoglycan recognition proteins (PGRPs) (Ferrandon *et al.* 2007), which circulate and preferentially attach to either Gram-positive Lys-type peptidoglycan (which contains lysine at the third position in the peptide chain), therefore activating the Toll pathway, or to Gram-negative DAP-type peptidoglycan (which contains *meso*-diaminopimelic acid) and which subsequently activates the Imd pathway (Lemaitre and Hoffmann 2007). A second level of specificity is achieved by the antimicrobial peptides activated by the Toll and Imd pathways themselves. Diptericin, Drosocin and Attacin are most effective against Gram-negative bacteria (Lemaitre, Reichhart and Hoffmann 1997) and are therefore controlled by the Imd pathway. Defensin is preferentially active against Gram-positive microbes, whereas Drosomycin and Metchnikowin are antifungal agents (Lemaitre, Reichhart and Hoffmann 1997), all of which are largely under transcriptional control of the Toll pathway. Cecropin, whilst preferentially attacking Gram-negatives, can act against both bacteria and some fungi (Ekengren and Hultmark 1999). Despite having a strongly compartmentalised defence against bacteria and fungi, there is a degree of overlap between these discrete arms which adds to the complexity of the model (Ferrandon *et al.* 2007).

1.6.5.2 The JAK/STAT pathway, viral infection and wounding response

Drosophila C virus (DCV), when introduced into the body cavity of *D. melanogaster,* invokes an infection response that shares few similarities with that of bacterial or fungal infections (Sabatier *et al.* 2003). Following a series of genetic manipulations it was discovered that infection with DCV results in the activation of the JAK/STAT pathway (Dostert *et al.* 2005), a further highly conserved signal transduction pathway that plays a significant role in human immunity (Stark and Darnell 2012). The JAK/STAT pathway can be activated by 3 ligands, unpaired (upd), upd2 and upd3, each of which is induced under specific conditions (Myllymäki and Rämet 2014). upd3 expression is induced in

adult haemocytes upon bacterial challenge, upd2 and upd3 are both induced in response to viral infection and all three upd molecules are induced locally in response to wounding, indicating that multiple regulatory mechanisms may control their expression (Wright *et al.* 2011; Agaisse *et al.* 2003; Myllymäki and Rämet 2014). In the case of viral infection, JAK/STAT activation results in the transcription of *vir-1*, and although the direct antiviral activity of this gene has never been shown, flies without this gene display higher viral loads (Dostert *et al.* 2005). It was noted at the time that the JAK/STAT pathway is required but not sufficient for the antiviral response, indicating that there are more elements that feed into a complete antiviral response (Dostert *et al.* 2005). More recently, it has been shown that the Imd pathway is involved, but dispensable, in immune defence against Cricket paralysis virus (CPV) and that distinct branches of the Imd pathway may contribute differently to antiviral immunity (Costa *et al.* 2009). Perhaps unsurprisingly given the interconnectedness of these pathways, the Toll pathway has also been linked to defence against viruses, in this case *Drosophila* X virus (DXV) (Zambon *et al.* 2005). The primary means of viral inactivation mediated by the Toll and IMD pathways appears to be via the involvement of cellular responses (Zambon *et al.* 2005; Costa *et al.* 2009).

The cellular response to tissue damage shares many components with that of the immune system. The involvement of the JAK/STAT pathway in response to wounding has already been mentioned (Agaisse *et al.* 2003), but a further pathway, the JNK pathway, is the key regulator of the response to structural perturbations in soft tissues (Ramet *et al.* 2002). Activation results in the initiation of a complex signalling cascade which ultimately prepares cells surrounding the site of injury to enter into a regenerative state (Bosch *et al.* 2005). In an immunological sense, activation of the JNK pathway by TAK1 in response to bacteria does not itself result in the direct transcription of antimicrobial genes such as *attacin* and *cecropin*, but does control expression of other immune inducible genes (Silverman *et al.* 2003). Despite this, more recent work has suggested a role for the JNK pathway in conjunction with NF-kB pathways in antimicrobial gene expression in the fat body (Delaney *et al.* 2006). These JNK-dependent immune genes largely encode proteins involved

in cytoskeleton remodelling, suggestive of a role in haemocyte activation and phagocytosis (Boutros, Agaisse and Perrimon 2002). Response to wounding and infection also share a cellular component, since both result in the recruitment of haemocytes, albeit it for different purposes (Moreira *et al.* 2010; Chambers, Lightfield and Schneider 2012).

1.6.5.3 Phagocytosis

In conjunction to a humoral response to microbial invaders, infection in *D. melanogaster* also results in a potent cellular response, a further similarity with the mammalian immune system. Inhibition of phagocytosis increases susceptibility to a variety of bacterial and viral infections, indicating that phagocytosis is an essential, albeit it non-specific, response to infection (Zambon *et al.* 2005; Nehme *et al.* 2011; Elrod-Erickson, Mishra and Schneider 2000; Hashimoto *et al.* 2009).

Haemolymph fills the body cavity of *D. melanogaster* and contains three classes of blood cell; the plasmatocyte, which is responsible for phagocytosis and constitutes about 95% of circulating larval haemocytes, the crystal cell which mediates the melanisation response and the lamellocyte, which is not commonly seen an adult flies, and is primarily responsible for encapsulation (Lemaitre and Hoffmann 2007; Honti *et al.* 2014). Phagocytosis of pathogens follows a pattern that will be familiar to mammalian immunologists, that of recognition and attachment (both opsonic and non-opsonic), internalisation and destruction, and is conducted by both sessile and circulating plasmatocytes. Recognition of bacterial pathogens is achieved non-opsonically by a variety of cell surface receptors present on plasmatocytes. Of cell surface receptors involved in phagocytosis, *eater* is perhaps the most well characterised (Chung and Kocks 2011; Bretscher *et al.* 2015; Kocks *et al.* 2005). *eater* is a transmembrane epidermal growth factor like (EGF-like) receptor from the Nimrod family expressed exclusively on plasmatocytes (Bretscher *et al.* 2015). *eater* null flies are strongly impaired in the phagocytosis of both Gram-positive and, to a lesser extent, negative bacteria (Kocks *et al.* 2005) and in addition to its role binding bacteria it is required for attachment of plasmatocytes to the

sessile compartment in larvae (Bretscher *et al.* 2015). *eater* works in conjunction with humoral aspects of the immune system, notably ceropin A, which disrupts the bacterial cell surface to reveal previously hidden *eater* ligands, in order to destroy Gram-negative pathogens (Chung and Kocks 2011). Other important cell surface receptors include Dscam (Watson *et al.* 2005) and Draper (Hashimoto *et al.* 2009). Opsonin-directed phagocytosis is thought to occur with the help of the Tep family of proteins (Blandin *et al.* 2004), although Tep deficient flies do not have any obvious susceptibility to bacterial or fungal infections (Bou Aoun *et al.* 2010).

1.6.5.4 Other immune defences

Unrelated to the cellular response, other *Drosophila*-specific humoral peptides are released from the fat body in response to stressful conditions in a similar manner to antimicrobial peptides. The most significant of these stress response proteins is the family of 8 *Turandot* genes which are transcriptionally activated and released under a variety unfavourable conditions including heat shock, UV irradiation, oxidative stress and bacterial infection (Ekengren and Hultmark 2001). Regulation of these genes is done by *MEKK1* (a Mitogen Activated Protein Kinase) and has also been linked to the IMD pathway, both in response to bacterial challenge and other stress responses (Brun *et al.* 2006). Research suggests that some *Turandot* genes are socially sensitive – female flies exposed to courtship song upregulate *TotM* and *TotC* (Immonen and Ritchie 2012) and *TotM* is thought to confer protection from sexually transmitted infections, although interestingly this appears to be via increased tolerance rather than resistance to fungal infections (Zhong *et al.* 2013a).

1.6.6 The microbiome and the immune system of the gut

The host immune system is in constant contact with commensal organisms, and nowhere is this more true than the gut. The gut epithelium, in addition to its role in digestion and metabolism, has to manage the ostensibly opposing tasks of maintaining a healthy mutualistic relationship between host and commensal, as well as detecting and eliminating pathogenic invaders. Early on in the use of

D. melanogaster as a model to study innate immunity, and to an extent even still, research has focussed on the introduction of a systemic infection via injection to the haemocoel. This method clearly has merits, but bypasses entirely aspects of immunity that are in place to combat more natural routes of infection (Vodovar *et al.* 2004). Injection-type infections also potentially circumvent the interactions that take place between the microbiome, which can have protective effects against infection (Blum *et al.* 2013).

Physical (acidity), physiological (peristalsis) and chemical (lysozymes) properties all make the gut a hostile environment for colonisation with would-be pathogens (Hultmark 1996). In addition to these features, immunological components of the gut also help to prevent oral infections. Local AMP production (in contrast to systemic production) is induced upon infection and works in tandem with ROS to combat infections arising from infected food sources. The local, gut epithelium specific, immune response is controlled largely by the IMD pathway and is made up of components familiar from the systemic response, such AMPs and PGRPs (Buchon *et al.* 2009b). Experimentally, this has been observed by infection with *Pseudomonas entomophila* (Vodovar *et al.* 2005) and *Serratia marcessens* (Nehme *et al.* 2007), but unlike systemic infections, AMP production in epithelial cells does not rely on the Toll pathway (Buchon *et al.* 2009b; Ferrandon *et al.* 1998). A role for the JAK-STAT pathway in gut immunity has been outlined however, since it is activated in an Upd3 dependent manner upon oral infection (Buchon *et al.* 2009b). As well as gut immunity exerted by IMD pathway dependent AMPs, the dual oxidase (dDuox) enzyme is also under the control of this crucial pathway (Kim and Lee 2014; Ryu *et al.* 2006) and together they constitute a vital, inducible, partnership in combating orally acquired infections. Should social environment affect the immune system, it is possible that it would act differently for systemic and local infections. It is therefore essential to have an understanding of both types of immunity and the implications to changes in each in order to properly characterise the effect of sociality on the immune system.

Equally, if changes in social environment are able to illicit changes in inducible immunity, they could also alter constitutive defences, eventually resulting in manipulations to the microbiome and the possibility of microbial dysbiosis (Lee and Lee 2014). The homeobox transcription factor Caudal (Cad) prevents chronic activation of the IMD pathway by gut commensals and in doing so stops the emergence of dominant pathobionts (Ryu *et al.* 2008). This, in turn, is significant because the maintenance of a healthy microbiome, as well as being involved in the activation of an appropriate immune response, is responsible for cell proliferation and differentiation, and ultimately gut morphology (Broderick, Buchon and Lemaitre 2014).

1.6.7 The *D. melanogaster* microbiome and the social environment

The microbiome of *D. melanogaster* is an order of magnitude simpler than that of most mammals, making it an ideal model with which to study host-microbe interactions (Kuraishi, Hori and Kurata 2013), in particular in the gut. Host factors are thought to account for the great difference in diversity seen between *D. melanogaster* and mammals, most notably the fact that holometabolous insects, including flies, shed the larval gut during metamorphosis, before production of a new one in adulthood (Buchon *et al.* 2009b). Laboratory reared populations of *D. melanogaster* generally host extremely low diversity bacterial communities dominated by two genera, *Acetobacter* and *Lactobacillus* (Wong, Chaston and Douglas 2013; Wong, Ng and Douglas 2011; Broderick and Lemaitre 2012), but despite this do not appear to have a single, fixed, ‘core’ microbiome. The initial ‘inoculation’ happens early in development, when larvae eat the faeces-contaminated egg chorion (Bakula 1969). In wild caught populations, despite enormous phylogenetic, ecological, and geographical diversity of flies tested, analysis revealed that various *Drosophila* species have a taxonomically restricted bacterial microbiome (Chandler *et al.* 2011). Host diet appears to be the most important factor in shaping the gut microbiome (Chandler *et al.* 2011; Staubach *et al.* 2013), which is partly in line with humans, where diet exerts acute effects on microbial communities in the gut (David *et al.* 2014). The host, however, is able to exercise a level of selectivity as to the composition of the microbiome, since, whilst it is environmentally

acquired and frequent ingestion is required to sustain a healthy microbiome (Blum *et al.* 2013), it does not simply reflect like-for-like that of the external environment (Chandler *et al.* 2011). Chandler et al (2011) report that ultimately composition of the gut microbiome is dictated by diet, host physiology, and chance.

The influence that the microbiota of the fruit fly exerts, like of that of higher order organisms, is multifaceted. The common *Drosophilid* commensal *Lactobacillus plantarum* is able to promote larval growth upon nutrient scarcity via TOR-dependent hormonal modulation (Storelli *et al.* 2011) which suggests the coevolution of both host and commensal. As mentioned previously, TOR is an important regulator of ageing in *D. melanogaster* (Partridge *et al.* 2011), once again highlighting the interconnected nature of these important biological functions. Another frequently encountered commensal, *Acetobacter pomorum*, plays an important role in regulating intestinal stem cell activity and energy metabolism (Shin *et al.* 2011), this time via insulin/insulin-like growth factor signalling, and has, once again, been linked to lifespan (Hwangbo *et al.* 2004). These cases serve to emphasise the importance of the microbiota in the regulation of basic physiological functions.

How the microbiome helps to shape social behaviours in *D. melanogaster* has been revealed in studies that indicate bacterial commensals can play a part in kin recognition (Lize, McKay and Lewis 2014), mate choice (Sharon *et al.* 2010) and aggregation (Venu *et al.* 2014). However, the reciprocal of this question, how social environment shapes the microbiome, has so far remained unaddressed. In *D. melanogaster*, direct evidence for the existence of a gut-brain-microbe axis, as in humans, is yet to be found. It seems likely, however, that the presence of steroid hormones like ecdysone and juvenile hormone, neurotransmitters such as dopamine and many gut-associated immune defences indicate that for *D. melanogaster*, stress may be handled in much the same way as mammals, and it could, therefore, be affected in an intrinsic manner by the social environment.

1.6.8 The microbiome and ageing

Senescence alters the microbiome in *D. melanogaster,* and is tightly linked to the ageing of the immune system. Upon ageing, chronic activation of the transcription factor foxo results in the repression of PGRP-SC2, which is itself an inhibitor of the IMD pathway (Guo *et al.* 2014). Counterintuitively, but in agreement with other literature (Libert *et al.* 2006; Brummel *et al.* 2004), the resultant chronic activation of the IMD pathway leads to commensal dysbiosis represented by an overall increase in bacterial CFU counts, however, the proliferation of specific bacteria was not investigated in this study (Guo *et al.* 2014). Age-associated overexpression of AMP genes in the gut has also been linked to a loss of intestinal homeostasis, (which better predicts onset of death than does chronological age) (Rera, Clark and Walker 2012) as well as stem cell hyperproliferation, and epithelial dysplasia (Guo *et al.* 2014). Establishing the exact cause and effect nature of the relationship between immune gene dysregulation, bacterial dysbiosis and intestinal barrier dysfunction is a challenging task. More recent work has indicated that a distinct shift in microbiota composition follows intestinal barrier dysfunction, leading to systemic immune activation and death (Clark *et al.* 2015). What is clear, however, is that dysbiosis of the microbiome is a principle feature of ageing, is tightly correlated with a loss of immune control and eventually leads to organismal death. Axenic flies have been used to try and clarify the role the microbiome plays in lifespan, with no clear pattern emerging. Germ-free flies have been found to have reduced (Brummel *et al.* 2004), increased (Petkau *et al.* 2014) and no difference (Ren *et al.* 2007) in lifespan.

1.7 Thesis outline

The principal aim of this thesis is to understand how the sex specific effects of social stimulation or isolation alter ageing, the immune system, and the microbiome of *D. melanogaster*. These factors form a complex, interrelated

network of association, all of which have the potential to act upon one another (**Figure 1**). Understanding how these relationships manifest, and more importantly the mechanisms that underlie them is essential to understand the physiological impacts of sociality. I use same-sex groups in socially-stimulated treatments in order to avoid the complications of courtship and mating, and this is consistent throughout the thesis. This research is also pertinent because, aside from providing basic biological insights into a long term laboratory model, social stimulation (or lack of it) is increasingly being recognised as a significant mediator of human health in a variety of capacities.

In Chapter 2 I attempt to gain an overview of the effects of social environment on actuarial senescence, functional senescence and cold tolerance in order to better understand the sex specific effects of social isolation or stimulation on ageing and stress responses. In light of these results, I also explore how changes in behaviour may explain differences in ageing patterns. This chapter serves to establish some basic principles about the effect of social interaction in males and females, in light of which the subsequent chapters can be interpreted.

Chapter 3 uses the same manipulations of social environment to investigate directly the effect that sociality can have on a variety of measures of immunity and how this changes with age. I measure post-infection lifespan in responses to three pathogens, expression of representative immune genes and phagocytosis in order to get a more complete picture of which aspects of immunity are socially-sensitive. There are no studies that directly look at DDP in *D. melanogaster*, and so this work aims to examine which of two competing hypotheses holds true – whether social stress (be it from isolation or interaction) acts to deleteriously affect the immune system, or if DDP results in improved outcomes upon bacterial infection. This chapter builds on work relating to the immune system in Chapter 2 in a more specific way and constitutes the bulk of the work relating to changes in immunity.

Chapter 4 continues the theme of investigating host-microbe interactions in relation to social environment but focusses instead on the non-pathogenic relationship between bacterial commensal and host. The microbiome is both affected by, and affects, the immune system and ageing patterns and therefore gaining an insight into the factors that shape it, and more importantly the functional implications of these changes, is desirable if we are to understand the true impact of sociality on these factors.

The final chapter, Chapter 5, is a general discussion which attempts to form a cohesive picture of the results presented in this thesis and elaborate on the implications of this work.

Chapter 2 – Sex specific effects of social isolation on ageing and immunity in *Drosophila melanogaster*

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2.1 Summary

Social environments can have a major impact on ageing profiles in many animals including humans. However, such patterns in variation in ageing and their underlying mechanisms are not well understood, particularly because both social contact and isolation can be stressful. In order to examine the sex-specific effects of social contact on ageing and the immune system I used *Drosophila melanogaster* fruitflies. I kept flies in isolation versus same-sex pairing throughout life, and measured actuarial (lifespan) and functional senescence (declines in climbing ability), as well as cold stress tolerance. To investigate underlying mechanisms, I determined whether an immune stress (wounding) interacted with effects of social contact, and assessed behaviours that could contribute to differences in ageing rates. Pairing reduced lifespan but this was more severe for males (pairing reduced female lifespan by ~12% whether injured or not, but reduced male lifespan by 20% if uninjured and ~38% if injured), whereas pairing for males, but isolation for females, caused more rapid declines in climbing ability. Wounding reduced lifespan for both sexes, but doubled the negative effect of pairing on male lifespan. Tolerance to cold stress followed a similar pattern to actuarial senescence – pairing reduced the ability of flies to cope with the cold and is more severe for males. I found no evidence that these effects are driven by behavioural interactions. These findings suggest that males and females are differentially sensitive to social contact, that environmental stressors can impact actuarial and functional senescence differently, and that these effects can interact with environmental stressors, such as immune challenges.

2.2 Introduction

It is becoming increasingly clear that social environments can play a significant role in individual ageing rates in animals (Holt-Lunstad, Smith and Layton 2010; Pantell *et al.* 2013; Amdam 2011; Partridge and Prowse 1997), regardless of the related costs of reproduction (Flatt 2011). Often these studies only measure changes in lifespan (actuarial senescence), but functional senescence (decline in physical functions) may also be sensitive to social contact (Behrends *et al.* 2007). Whether social contact is beneficial or costly can depend on both frequency of contact and the identity of the interacting partners (Holt-Lunstad, Smith and Layton 2010). What drives these patterns remains unclear. Social isolation may increase release of stress hormones and off-target inflammatory responses (Hawkley and Cacioppo 2003), whereas social contact could provide beneficial environmental enrichment (Donlea *et al.* 2014), but is likely to increase competition for reproduction, food or territory, and exposure to communicable diseases (Krause and Ruxton 2002). Moreover, social facilitation or ‘group effects’ have been observed whereby insects raised in groups rather than isolation develop faster and invest more in reproduction (Grassé 1946; Schausberger, Gratzer and Strodl 2017; Lihoreau and Rivault 2008), which may have carryover effects on adult lifespan (Lee *et al.* 2016).

Ageing may show sex-specific responses to social environments in humans (Kaplan and Kronick 2006; Moon, Park and Cho 2010; House, Robbins and Metzner 1982) and in other study species numerous studies have shown that females have reduced lifespans in male-biased populations, through male harassment and receipt of toxic seminal proteins (Chapman *et al.* 1995). Males can suffer both from contact with females, through elevated courtship activity (Cordts and Partridge 1996), and also with other males, possibly through direct aggressive interactions though also through increasing investment in reproduction (Bretman *et al.* 2013). Such sex differences can been seen in wild populations, for example, population density affects senescence in male but not female red deer (Mysterud *et al.* 2001). It is therefore likely that what constitutes an adverse social environment, and hence

the consequences for ageing phenotypes, is different for each sex, but the underlying mechanisms driving these differences are poorly understood.

Here I aim to investigate effects of social contact on both actuarial and functional senescence using *Drosophila melanogaster*. Social effects on longevity have previously been reported in *D. melanogaster*, but these studies largely either used mixed-sex groups (hence incorporating the cost of mating) (Iliadi, Iliadi and Boulianne 2009; Zajitschek *et al.* 2013) or measured one sex only (Ruan and Wu 2008; Bretman *et al.* 2013; Gendron *et al.* 2014). There is some evidence that social contact also affects functional senescence in males in terms of locomotor activity (Ruan and Wu 2008) and mating success in later life (Bretman *et al.* 2013). In this latter study, the effect on ageing was attributed partly to responses to sperm competition signalled by the presence of another male (Bretman *et al.* 2013). *D. melanogaster* lifespan in the wild has been estimated as > 50 days (Robson *et al.* 2006), but with animals so hard to observe it is difficult to assess accurately their natural lifespan. It is likely that flies experience stochastic changes in social environment as flies frequently spend time in crowded conditions around ephemeral food sources (Wertheim *et al.* 2006), or are solitary when moving between them, and are hence subject to a great deal of variation in social context. This stochasticity likely underlies the behavioural plasticity that males of many species show in reproductive effort (Wedell, Gage and Parker 2002; Bretman, Gage and Chapman 2011), since without environmental variation, plasticity should not be maintained (Carroll and Corneli 1995). Moreover, *D. melanogaster* show natural genetic variation in propensity to aggregate (Saltz 2011), driving further variation in natural social environments and individual responses to them.

Given that sperm competition is male-specific, and additionally, males are more aggressive towards each other than are females (Nilsen *et al.* 2004), I hypothesised that same-sex social contact would speed ageing in males but not females. To address this I measured the effect of social isolation versus pairing on both actuarial (lifespan) and functional ageing (Grotewiel *et al.* 2005) in both sexes. I chose social isolation versus flies kept in pairs as a single

conspecific is sufficient in males to elicit a sperm competition response, and increases in number or density of rivals does not increase this response (Bretman *et al.* 2010). As the main intention of the study was to assess the effect of social contact *per se*, this design also minimises direct competition for food. I also aimed to gain insights into the underlying mechanisms. Given the intimate link between social environments and immunity (Amdam 2011; Pantell *et al.* 2013), it is possible that if part of these effects are through increased risk of disease or resource allocation to immune function, these patterns would be exacerbated by an immune challenge. Injury is known to interact with ageing and stress resistance (Sepulveda *et al.* 2008) and wounding repair utilises many of the same molecular pathways as infection responses (Felix *et al.* 2012; Ramet *et al.* 2002; Lemaitre and Hoffmann 2007). Wounding was therefore used (amputation of a middle leg) as a general immune challenge, a methodology which has been previously used to investigate stress and ageing in *D. melanogaster* (Sepulveda *et al.* 2008; Carey *et al.* 2007). Since there is strong evidence to suggest a link between stress tolerance/resistance and longevity (and therefore ageing) (Harshman *et al.* 1999) a simple cold stress assay was used to assess the effect of social isolation and stimulation on ability to recover from chill coma, which along with climbing ability, is sensitive to infection status (Linderman *et al.* 2012). How behaviours such as aggression, increased activity or exclusion from the food varied with social environment was also investigated in an attempt to understand the precise effect of social interaction on lifespan.

2.3 Materials and Methods

2.3.1 Fly stocks and maintenance

Drosophila melanogaster (wild-type Dahomey strain) were maintained in mass population cages on standard sugar-yeast agar medium (Bass *et al.* 2007), at a constant 25ºC, 50% humidity with 12:12 light:dark cycle. For experiments, larvae were raised at a density of 100 per vial. Upon eclosion, adult flies (both male and female) were sexed under ice anaesthesia put into groups of 10 and transferred to their experimental treatment the following day. Pairs consisted of

a focal fly (i.e. responses measured only for this individual) and non-focal same-sex partner. Non-focal flies were identified using a small wing clip, carried out the day after eclosion under light CO_2 anaesthesia. Non-focal flies and food was changed weekly, hence non-focals were 2-8 days old throughout.

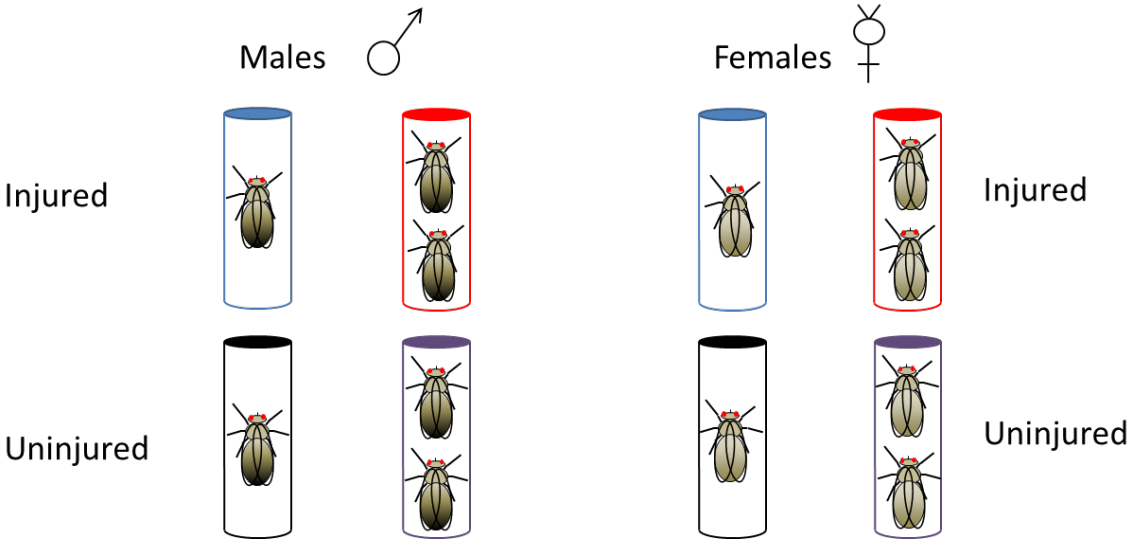

Figure 3 Experimental design. Males and females were kept singly or alone, with or without an immune stimulus. In lieu of a direct immune elicitor, wounding was used, specifically the removal of a middle leg.

2.3.2 Measuring actuarial senescence (lifespan)

Virgin focal flies were maintained in isolation or same-sex pairs and their survival was checked daily until all the flies were dead. In addition, lifespan whilst under immune stress for the focal fly was also tested. Wounding (specifically the amputation of a middle leg) was used as an immune challenge (Carey *et al.* 2009; Carey *et al.* 2007). Amputations were performed under CO_2 anaesthesia on the day after eclosion, with equal numbers having the left or right leg removed. There were eight groups; single or paired, uninjured or injured, male or female, (n = 50 per group; **Figure 3**).

2.3.3 Measuring functional senescence (climbing ability)

Senescence in climbing ability was measured using a standard negative geotaxis assay (Cook-Wiens and Grotewiel 2002). Male and female flies were kept singly or in same sex pairs as before, but all flies were uninjured since climbing ability was being measured. Once a week from 5 to 56 days post eclosion, flies were aspirated into an empty vial (i.e. without food, not used to house flies, and a unique vial for each focal fly) and whether or not focal flies reached a height of 10cm within 120 sec was recorded. Non-focal flies and food were changed as in lifespan assays. Starting n = 60 per treatment, but reduced as flies died, hence the experiment was stopped when only n~20 per treatment remained.

2.3.4 Measuring cold stress tolerance

Flies were raised in the same social conditions as for actuarial and functional senescence assays until the age of 5 days (n = 40 per treatment). Cold tolerance was examined by holding the vials at -20°C for 15 minutes. Following this, flies were kept at 25°C for 60 minutes, and the number of flies lying on their back or side were recorded. This 'fall down' number therefore included both dead individuals and those that, although alive, had not righted after an hour.

2.3.5 Behavioural observations

To evaluate the potential contribution of behavioural variation to the observed ageing patterns, flies were maintained as before; single or paired, uninjured or injured, male or female (n = 20 per treatment). Recorded behaviours were noted as whether the focal fly was inactive, walking, on the food and grooming. Paired flies were also scored for 'social behaviours' - whether they were within a body length of the non-focal fly or involved in an aggressive encounter. Observations were made at 9am, 12pm and 3pm on day 3, 5, 7, 10, 12 and 15 post-eclosion. Vials were placed on a viewing platform to enable easy scans. During each observation period, the behaviour of each focal fly was recorded

each minute for 10 minutes. Each scan of each vial lasted for 10 seconds and the predominant behaviour was recorded.

2.3.6 Statistical Analysis

Data were analysed using R v 3.3.1 and SPSS v 20. As lifespan data violated the assumptions of a Cox regression, this was analysed using a GLM with quasi Poisson errors (to account for over dispersion) with sex, injury and social environment as factors. Our general approach when using GLMs or GLMMs (for repeated measures where fly ID was used a random factor) with appropriate error structures was to simplify from the full model using Analysis of Deviance (AOD), the final model being when no further terms could be removed without significantly reducing the model's descriptive power. I analysed the proportion of flies in each trial successfully reaching 10cm in 120s using a GLM with quasi binomial errors, with sex, age and social environment as fixed factors. To analyse behavioural data, I used GLMMs with Poisson errors, with the number of observations of the behaviour of interest within the 10min scan period as the response, with sex, injury, and social treatment as fixed factors and fly ID, day and time of day as random factors. For behaviours that could only be expressed in pairs (aggression, courting or sitting within a body length of each other) the models were the same but without social treatment as a factor.

2.4 Results

2.4.1 Actuarial senescence (lifespan)

The effect of injury and social environment on lifespan differed between the sexes indicated by the significant 3-way interaction sex, injury and social environment (**Table 1**); female lifespan was longer than that of males and the effect of injury exacerbated that of pairing for males (**Figure 4**). In order to unpick this further I separated the data into males and females in order to remove sex from the model. For males there was a significant interaction between social environment and injury since the reduction in lifespan caused

by pairing was exacerbated by injury (**Figure 4** and **Table 1**). For females, however, there was no significant interaction between social environment and injury, but both injury and social environment significantly reduced lifespan individually (**Figure 4** and **Table 1**). Scaling by sex- and injury treatment-specific median lifespan, pairing reduced female lifespan by ~12% whether injured or not, but reduced male lifespan by 20% if uninjured and ~38% if injured.

Table 1 GLM of lifespan for males and females kept alone or in same sex pairs and either injured or uninjured.

Data	**Explanatory variable**	**Test statistic**	**df**	***p***
Both sexes	Pairing*Injury*Sex	4.052	1, 326	**0.045**
Males	Pairing*Injury	5.431	1, 169	**0.021**
Females	Pairing	17.26	1, 28	**<0.001**
	Injury	9.685	1, 159	**0.002**
	Pairing*Injury	0.113	1, 158	0.738

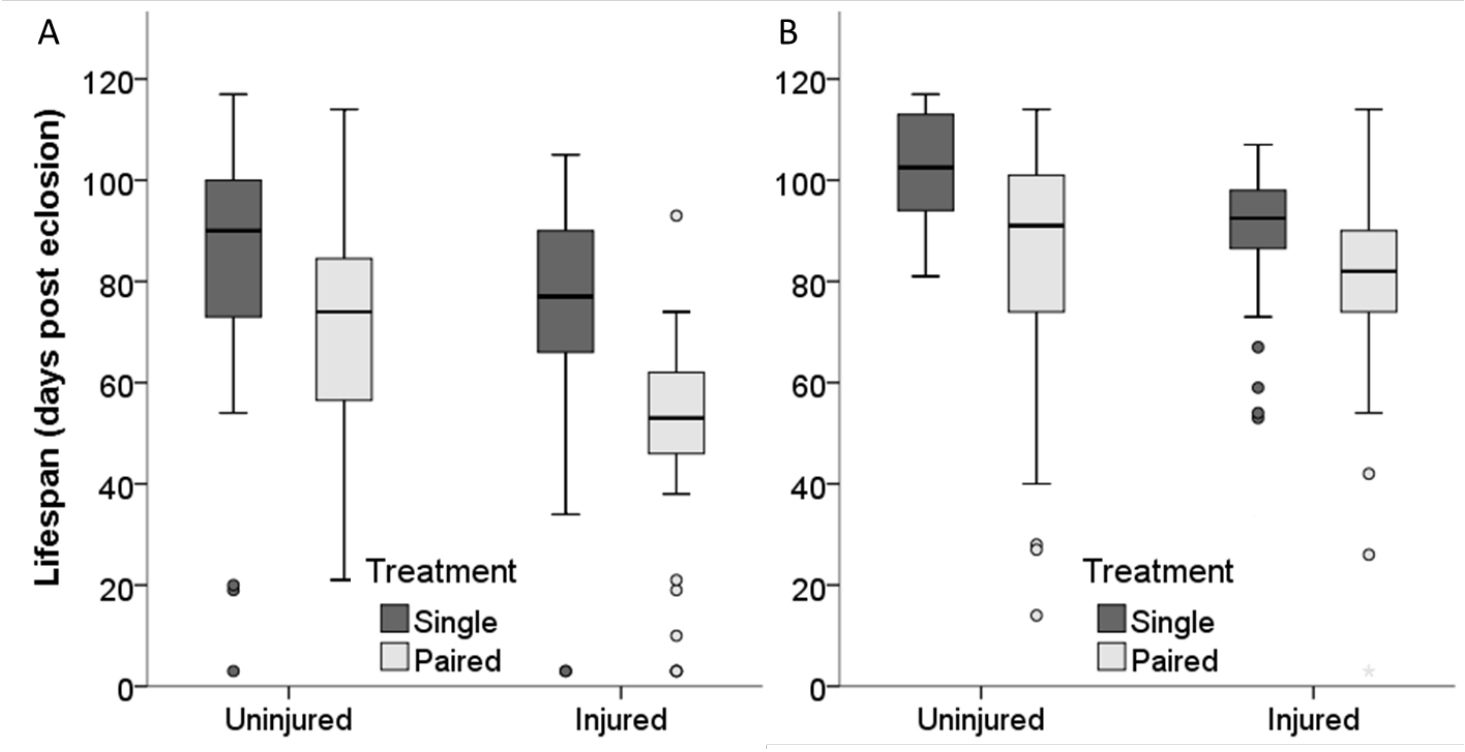

Figure 4 Median lifespan of (A) males and (B) females maintained either alone (dark grey) or with a same sex non-focal partner (Light grey). Injury (removal of a middle leg) was also used to assess if ageing and social environment interact with immune stress. Food and non-focal partners were changed weekly.

2.4.2 Functional senescence (climbing ability)

Once again, a significant 3-way interaction (**Table 2**) determined the proportion of flies completing the climbing task and so the data was again divided by sex in order to further investigate this. For males the interaction between age and social environment is not significant, but climbing ability does decline with age, and pairing reduces the ability of males to complete the climbing task (**Table 2** and **Figure 5** Senescence of climbing ability (proportion of flies that reached 10cm in 120 seconds) was measured for male and female, single or paired flies.. For females there was a significant interaction between social environment and age; at ~35 days after eclosion isolated females become less successful than paired at climbing (**Table 2** and **Figure 5** Senescence of climbing ability (proportion of flies that reached 10cm in 120 seconds) was measured for male and female, single or paired flies..

Table 2 Senescence of climbing ability analysed by GLM for males and females, kept alone or in same-sex pairs and either injured or uninjured.

Data	**Explanatory variable**	**Test statistic**	**df**	***p***
Both sexes	Pairing*Age*Sex	11.685	1, 56	**0.001**
Males	Age	14.456	1, 29	**0.0002**
	Pairing	10.078	1, 29	**0.004**
	Pairing*Age	0.180	1, 28	0.991
Females	Pairing*Age	17.313	1, 28	**<0.0001**

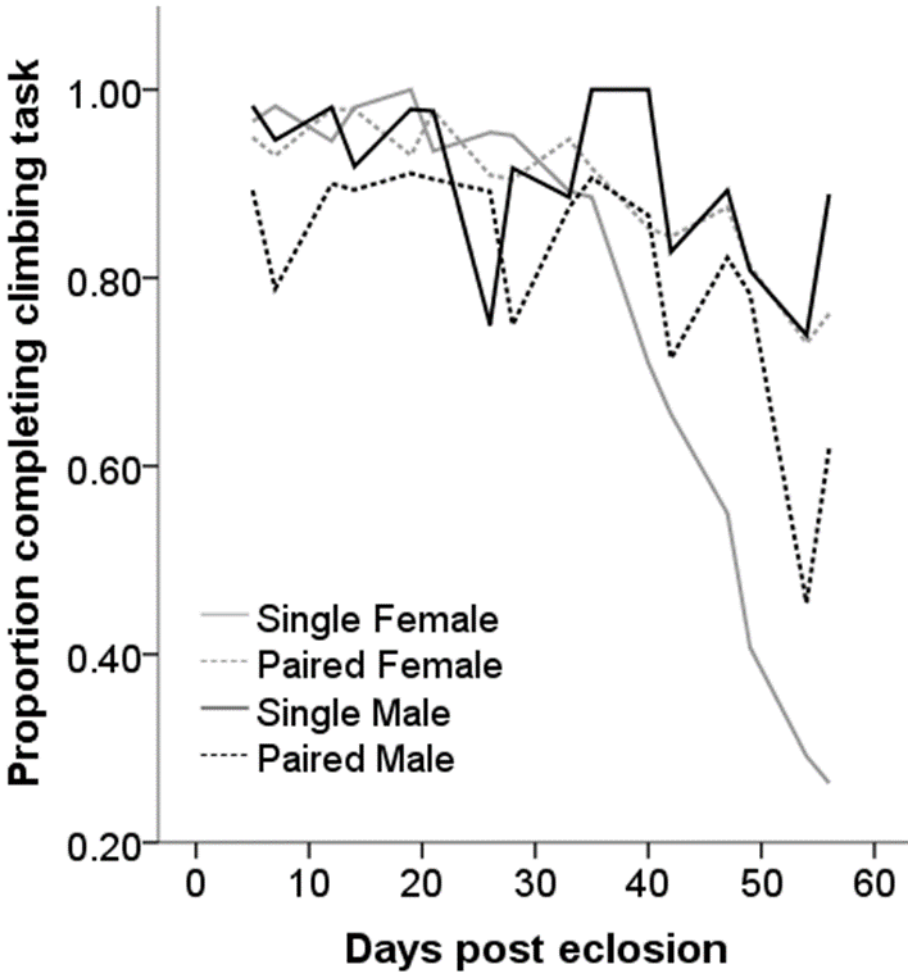

Figure 5 Senescence of climbing ability (proportion of flies that reached 10cm in 120 seconds) was measured for male and female, single or paired flies.

2.4.3 Cold tolerance

Cold stress tolerance was tested only in young flies (5 days old) and always without the additional immune stress of wounding. There was no interaction between social environment and sex, but social stimulation alone reduced the ability of the flies to withstand cold stress (**Figure 6** and **Table 3**).

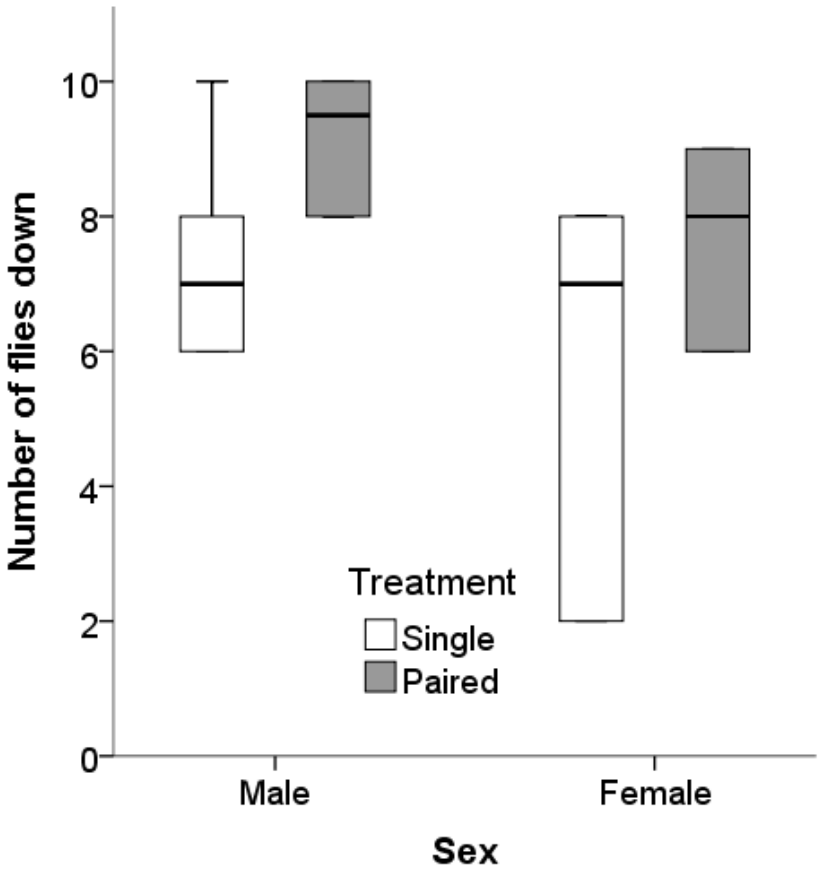

Figure 6 Cold stress tolerance (number of flies that remained knocked down 1 hour post cold exposure) was measured for male and female, single or paired flies.

Table 3 GLM of cold shock resistance for 5 day old flies kept singly or in same sex pairs

Explanatory variable	**Test statistic**	**df**	***p***
Pairing	5.729	1, 22	**0.0261**
Sex	3.906	1, 21	0.061
Pairing*Sex	0.125	1, 21	0.728

2.4.4 Behavioural observations

Overall, we found little evidence that behavioural differences adequately explain the observed differences in actuarial and functional senescence. Males were more inactive than females, and a significant interaction between social environment and injury indicates that injured single flies are more often inactive whereas there is little difference between uninjured flies kept singly or in pairs **Table 4** and **Figure 7**). The time spent on the food differed between the sexes depending on social environment – paired males spent more time on the food (**Table 4** and **Figure 8**). Main effects of injury also revealed that wounded flies spend more time on the food (**Figure 8**). Time spent walking was not affected by sex, but was determined by an interaction between injury and social environment - social environment had little effect in uninjured flies, but for injured flies, isolated flies walked more (**Table 4** and **Figure 9**). There was no effect of social environment or sex but injured flies groomed more (**Table 4** and **Figure 9**). For paired flies interaction behaviours (instances of <1cm between the flies) was affected by an interaction between sex and injury – injury had little influence over the proximity of females to each other but injured males were more often observed close to non-focal partners (**Table 4** and **Figure 10**). Male flies were much more likely to engage in aggressive behaviours, indicated by the highly significant effect of sex, whilst injured flies were less likely to act aggressively towards each other (**Table 4** and **Figure 10**).

Table 4 GLMM for the number of observations of the behaviour of interest within a 10 minute scan period analysed as the response, with sex, injury, and social treatment as fixed factors and fly ID, day and time of day as random factors. Flies were grouped into males and females, either alone or with a same sex partner and either injured or uninjured.

Behaviour	**Explanatory variable**	**AOD χ^2_1**	***p***
Inactivity	Sex	21.246	**< 0.001**
	Pairing*Injury	6.387	**0.012**
On food	Sex	5.435	**0.02**
	Injury	11.337	**< 0.001**
Walking	Pairing*Injury	6.386	**0.011**
Grooming	Injury	5.11	**0.024**
Interaction behaviours	Sex*Injury	11.335	**< 0.001**
Aggression	Sex	116.54	**< 0.0001**
	Injury	7.741	**0.005**

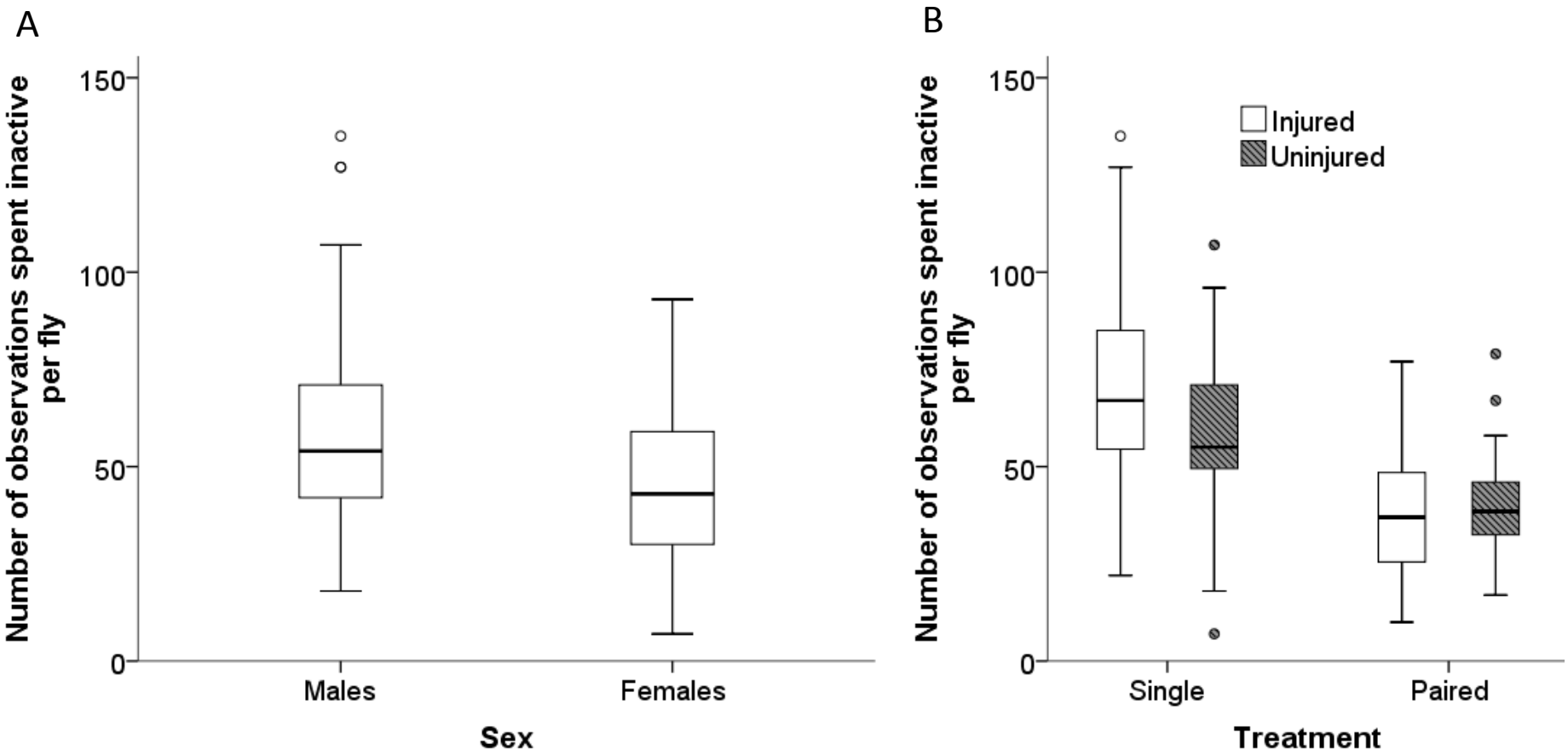

Figure 7 Behavioural observations – inactivity. Males and females, single or in same sex pairs and injured (middle leg amputated) or uninjured were observed 3 times daily for 6 days over two weeks (10 observations per 10 minute scanning period), resulting in 180 individual observations per fly. Plots reflect results of GLMMs, illustrating the respective effect of the three explanatory variables on behaviour. The amount of inactivity was determined by (A) sex and (B) an interaction between social environment and injury.

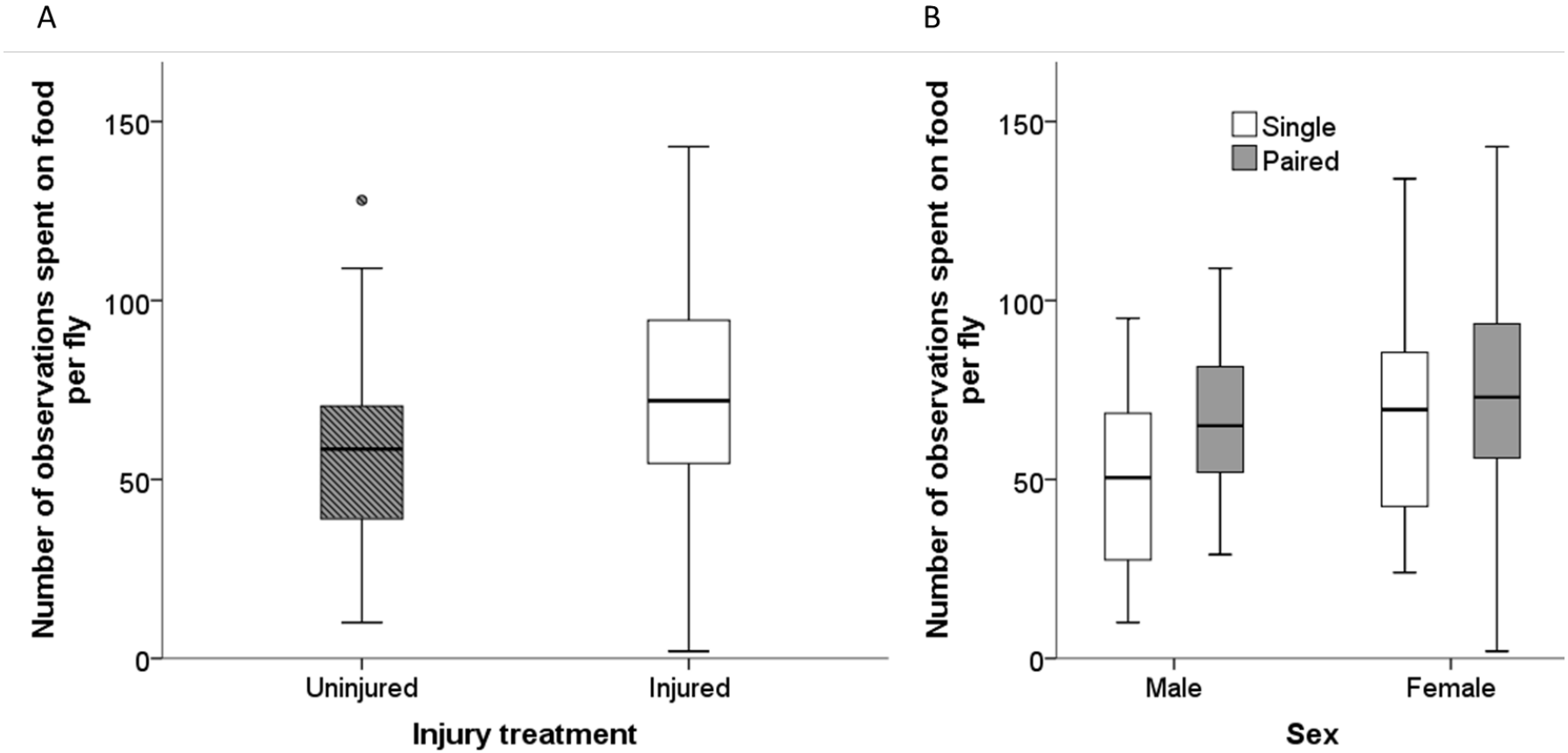

Figure 8 Behavioural observations – time spent on the food. Males and females, single or in same sex pairs and injured (middle leg amputated) or uninjured were observed 3 times daily for 6 days over two weeks (10 observations per vist), resulting in 180 individual observations per fly. Plots reflect results of GLMMs, illustrating the respective effect of the three explanatory variables on behaviour. The amount of inactivity was determined by (A) injury status and (B) an interaction between social environment and sex.

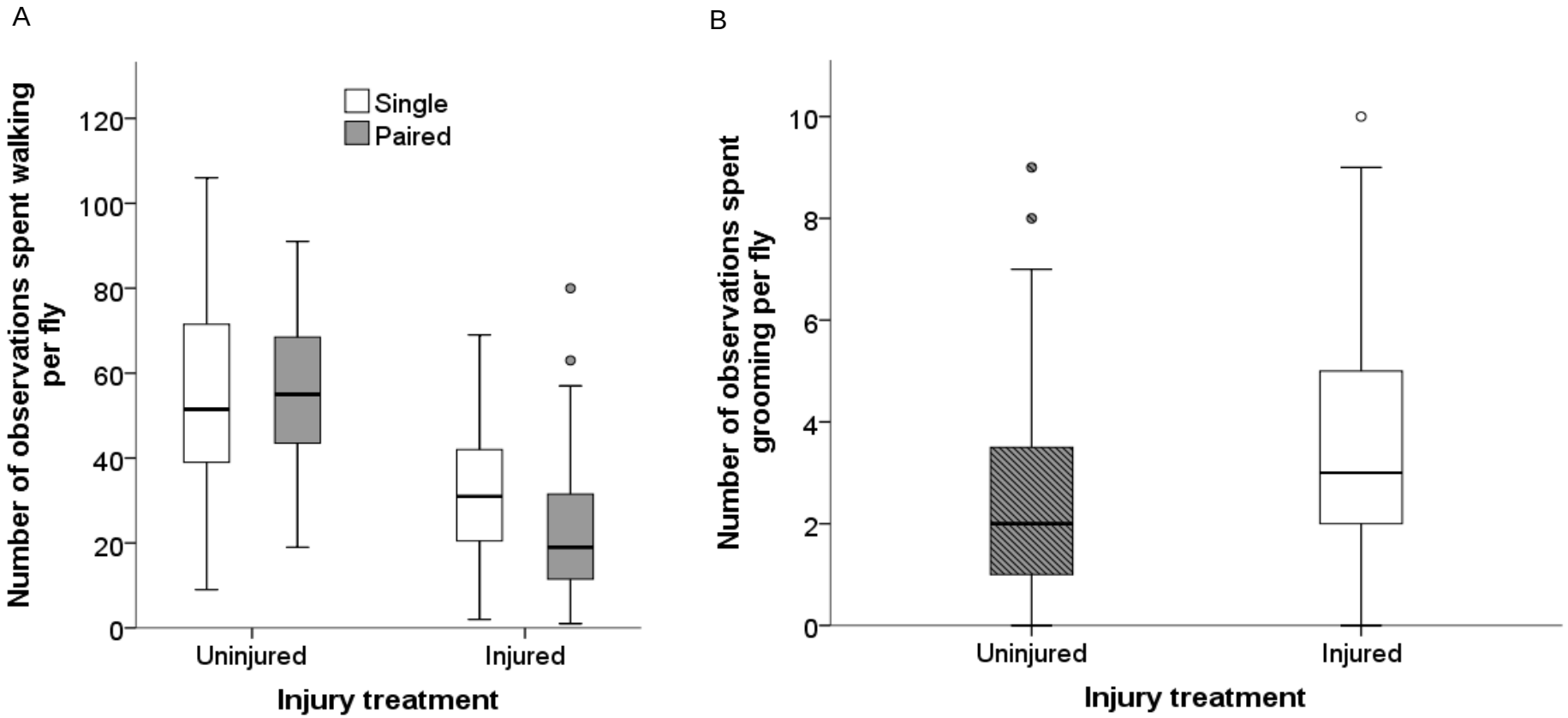

Figure 9 Behavioural observations – walking (panel A) and grooming (panel B). Males and females, single or in same sex pairs and injured (middle leg amputated) or uninjured were observed 3 times daily for 6 days over two weeks (10 observations per visit), resulting in 180 individual observations per fly. Plots reflect results of GLMMs, illustrating the respective effect of the three explanatory variables on behaviour. The amount of walking (A) was determined by an interaction between social treatment and injury status and the amount of grooming (B) was determined by sex.

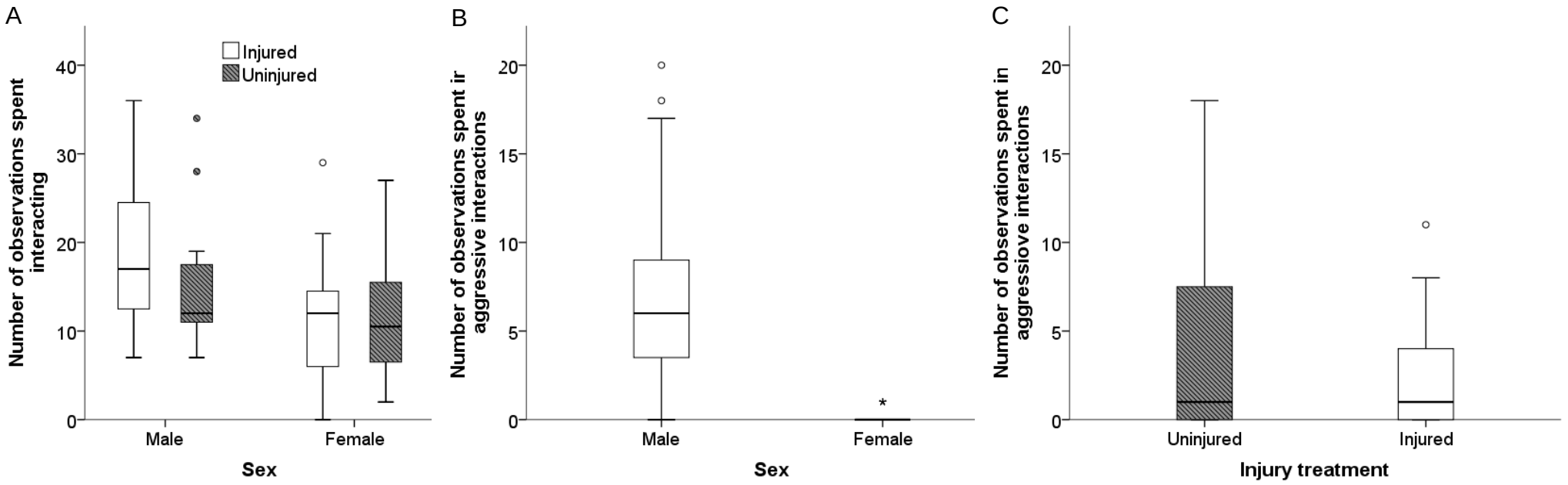

Figure 10 Behavioural observations – Interaction behaviours. Males and females, single or in same sex pairs and injured (middle leg amputated) or uninjured were observed 3 times daily for 6 days over two weeks (10 observations per visit), resulting in 180 individual observations per fly. Plots reflect results of GLMMs, illustrating the respective effect of the three explanatory variables on behaviour. The amount of interaction – the sum of standing, walking or on the food within 1cm of each other, as well as courtship and aggression, (A) was determined by an interaction between sex and injury status. The amount of aggression observed was determined by main effects of sex (B) and injury status (C).

2.5 Discussion

As predicted, social contact had profound effects on actuarial senescence; pairing reduced lifespan, but this was more severe for males (~20% compared to 12% for females). Injury reduced lifespan for both sexes, almost doubling the effect of pairing on male lifespan. A similar pattern was observed for cold stress tolerance where pairing significantly reduces the ability of both sexes to withstand a cold shock. Phenotypic covariance of longevity, stress resistance and immunity is a well-established phenomenon (Amrit, Boehnisch and May 2010), and is mediated at least in part by the overlap of underlying functional pathways (Amrit, Boehnisch and May 2010; Kim *et al.* 2004). It was hypothesised that, due to these partially shared mechanisms, the effect of social stimulation was likely to be the same for stress responses as for lifespan and immunity, which proved to be the case – pairing has a negative effect on stress tolerance. Social contact also affected functional senescence in a sex-specific manner, such that pairing for males, but isolation for females, caused more rapid declines in climbing ability. There was no evidence that these effects are driven directly by behaviour, as neither pairing nor wounding increased activity and flies were not excluded from the food. Whilst there was more aggression between males than between females, this was not more intense for wounded flies, so could not have driven the interactive effect of wounding and social environment on male lifespan. Wounded flies groomed more, but this was unrelated to sex or social environment.

Previous studies on the social effects on ageing in *D. melanogaster* have found reduced lifespan in group-housed flies, but sometimes only in males (Carazo *et al.* 2016) or in both sexes (Iliadi, Iliadi and Boulianne 2009). A further study showed that social environments had an interactive relationship with food resources, as diet affected female lifespan regardless of social environment but dietary restriction reduced male lifespan only in mixed sex groups (Zajitschek *et al.* 2013). Males maintained continuously with other males had longer life spans than those kept with females (Cordts and Partridge 1996), which was attributed to the cost of courtship, specifically mounting

attempts (Partridge and Prowse 1997). Males carrying a mutation in the gene *Sod* (a sulfoxide dismutase involved in responses to oxidative stress) lived longer if housed with “helpers”, but only if those helpers were young wild-type males (Ruan and Wu 2008). Taken together these findings suggest that the effect of social environments on lifespan are complex and dependent on the amount of contact, density and identity of the social partners.

Sex-specific ageing patterns are widely observed amongst animals (Austad and Fischer 2016) and are predicted if one sex suffers from greater extrinsic mortality rates (Williams 1957). In polygynous species this is most often males, likely because of the costs of mating behaviours and secondary sexual traits (Bonduriansky *et al.* 2008). This might contribute to the sex differences I found in ageing *per se* and the response to social contact. Many previous studies show that male *D. melanogaster* respond to the presence of rivals by increasing mating duration (Bretman *et al.* 2012; Bretman *et al.* 2011; Bretman, Fricke and Chapman 2009) and altering ejaculate content (Garbaczewska, Billeter and Levine 2013; Moatt, Dytham and Thom 2014; Wigby *et al.* 2009). These strategies appear to be costly, as starved males are unable to mount this response (Mason, Rostant and Chapman 2016) and paired males die sooner regardless of whether they are actually able to mate (Bretman *et al.* 2013). It is possible, therefore, that anticipating sperm competition elicits a response that is costly even if the ejaculate is not used. Clearly this is a consideration only for males, but whilst females appear less sensitive, they still did respond to social contact, hence I investigated other potential contributing factors.

The ability to withstand stress generally decreases with age (Grotewiel *et al.* 2005). Although I only tested cold tolerance at a single time-point (when flies were 5 days old), this was still enough time for the effect of pairing to become apparent and reduce cold tolerance, indicating that social contact could be increasing the rate of senescence, and doing so quickly. Resistance to oxidative stress declines by 30% in the first 10 days of life (Bonilla, Medina-Leendertz and Diaz 2002) indicating that some traits have the ability to senesce

rapidly under certain conditions. Here, the effect of sex is non-significant, but it is possible that the disproportionate effect of social environment on males (seen as the interaction between sex and social environment in actuarial senescence assays) would become more obvious as the animals age. Given the mechanistic link between longevity, immunity and stress resistance (Eleftherianos and Castillo 2012; Remolina *et al.* 2012; Lin, Seroude and Benzer 1998), these data imply there might be a common underlying cause mediating the effect of social environment on stress resistance and immunity, and suggests the reduction in climbing ability seen in single females may therefore be being conducted via different means. Selection experiments consistently find that resistance to one kind of stress correlates to resistance to others, and likewise the opposite is true (Lin, Seroude and Benzer 1998; Bubliy and Loeschcke 2005). Therefore, if paired flies are more susceptible to cold stress, this may mean that they are also more susceptible to other stresses, most notably oxidative stress. A sensitivity to oxidative stress in paired flies would also help to explain the actuarial senescence results seen here. Cold stress-resistance trades-off against fecundity (Watson and Hoffmann 1996) which may be why paired males are more sensitive to a cold shock, since males increase reproductive investment in the presence of rivals, ultimately increasing fecundity (Bretman, Fricke and Chapman 2009).

One direct consequence of social contact is enhanced competition for resources. The patterns in ageing I observed do not seem to be driven by flies being excluded from food. Injured flies and paired males were more often on the food, though I do not know if they were eating at different rates. Injured flies may simply be on the food more since it is more difficult to rest on the sides of the vial. Likewise, the amount of activity was reduced in shorter-lived paired and injured flies, so does not suggest they were spending more energy in general activity. This contrasts with a recent finding that in the carpenter ant *Camponotus fellah* a decline in lifespan of isolated ants was driven by increased activity and decreased digestion (Koto *et al.* 2015). However, these ants are eusocial and would rarely spend time away from a highly related group, and therefore isolation is likely to be extremely stressful for them. This

highlights the importance of studying the effects of ageing in animals with variation in their social context.

Low levels of aggression were observed in both sexes, but there was clearly more between males, as seen in previous studies (Nilsen *et al.* 2004). Between males, aggression declines quickly with increasing familiarity (Liu *et al.* 2011). Indeed, previous social experience reduces aggression, as previously isolated males (Wang *et al.* 2008) and females (Ueda and Kidokoro 2002) are more aggressive than socially experienced counterparts. Injured males were less often involved in aggressive encounters, though it is difficult to assess whether this is because they were less likely to initiate fights. Wounded males are outcompeted by unimpaired males in gaining matings (Sepulveda *et al.* 2008), so they may be perceived as less of a threat generally. So whilst differences in aggression might contribute to a reduction in male compared to female lifespan, it cannot explain the doubling of the reduction in lifespan for injured paired males found in our study. This is in line with our previous work in males (Bretman *et al.* 2013) and it seems unlikely then that aggression plays a major role in the mechanisms underlying the social effect on ageing.

Injury was used as a simple immune challenge as both wounding and infection responses utilise many shared underlying immunity mechanisms. The effect of injury on lifespan in *D. melanogaster* is not straight forward, as previous studies have found an effect in males only (Sepulveda *et al.* 2008) or in both sexes, but a stronger effect in females (Carey *et al.* 2007). Other invertebrates also show a lifespan cost to wounding (Carey *et al.* 2009) and encapsulation (Armitage *et al.* 2003). The effects of removing a leg could include haemolymph loss and increased risk of infection, plus the cost of wound healing, all of which could be physiologically costly through, for example, increasing metabolic rate (Ardia *et al.* 2012). If the costs of wounding, or even prophylactic increases in immune gene regulation, interact with the cost of being paired in males, this could explain why the effects of injury and pairing in males interact. Wounded flies were less often involved in aggressive encounters and I found a reduction in activity by wounded flies, consistent with

the suggestion that sick animals reduce activity to conserve energy (Sullivan, Fairn and Adamo 2016; Hart 1988), indicating the costs of injury do not seem to arise from behavioural differences. Injured flies groomed more, in line with a previous finding that even decapitated *D. melanogaster* increased grooming if triggered by contact with *Escherichia coli* (Yanagawa, Guigue and Marion-Poll 2014). It is likely that grooming is beneficial to sick invertebrates by removing surface pathogens without increasing heat loss (Sullivan, Fairn and Adamo 2016).

The finding that lifespan, stress resistance and functional senescence show different patterns in response to social contact aligns with the idea that traits do not all necessarily show the same senescence patterns (Grotewiel *et al.* 2005; Nussey *et al.* 2013). The basis of this variation in senescence among traits is not yet understood (Nussey *et al.* 2013). *D. melanogaster* shows senescence in a wide range of traits, with declines becoming apparent at different ages, though comparisons across multiple studies is not straight forward (reviewed by Grotewiel *et al.* 2005). It would therefore be beneficial to explore social effects on senescence in multiple traits, including a variety of stress responses, to fully understand the consequences for later life.

Clearly, only a very simple social environment manipulation was tested. Mixed sex pairs were avoided in order to negate costs of reproduction, but being virgin throughout life is probably unusual, particularly for females (Markow, Beall and Castrezana 2012). In addition, non-focal partners were always less than10 days old as in previous work (Ruan and Wu 2008), but as generations overlap, the age of interacting individuals may alter the effect of social contact on ageing (Souza 2011). The behavioural observations that were conducted were made in relatively young flies, and these interactions could change with age. However, the general pattern is that various behaviours and overall activity declines with age (Grotewiel *et al.* 2005), hence it is likely the observed age is the one that would see the most variation in behaviour. Future work could build on these observations by altering the frequency of social

interactions, the number of flies per group, age of interacting partners and by mating all individuals.

Chapter 3 – Immune function, the social environment and ageing in *Drosophila melanogaster*

Thank you to Sophie Armitage for advice on infection methods, James Rouse and Elizabeth Duncan for help with RT-qPCR and Anna Woolman in setting up injection experiments. Special thanks also to Sophie Evison for help with designing, conducting and analysing phagocytosis assays.

3.1 Summary

Various lines of evidence suggest that an organism's social environment can affect the immune system. Studies in both humans and other animals suggest that social isolation is linked to a host of inflammatory disorders and negative health phenotypes. Conversely, increased social interactions can promote the spread of infectious disease. Here, we use *D. melanogaster*, a common model of the innate immune system, to ask the question, how does social isolation and stimulation affect post-infection lifespan and immunity, and how does this change with age? My previous work has shown that social contact reduces lifespan for both sexes and that injury is twice as detrimental for paired males as paired females. In light of this, I measured post-infection lifespan in single and same-sex pairs. To gain a mechanistic understanding of my results I subsequently analysed gene expression using RT-qPCR and phagocytosis using flow cytometry. In contrast to my prior work, I found that pairing improved post-infection lifespan, or had no negative effects, and there was no interaction between sex and pairing, although males were more susceptible to infection than females. Expression of a subset of immunity genes in paired and single males aged to 52 days is higher, relative to females, which is consistent with the fact that males appear to succumb to immunosenescence quicker. Paired females express less of some stress response genes suggesting that pairing in females is protective in this regard, at least in terms of stress response genes. Flow cytometry experiments revealed that phagocytosis is affected by age and sex, and that social environment reduces phagocytic ability in aged paired females, possibly via the action of haemocyte cell surface receptor, *eater*. These findings suggest that social interaction in *D. melanogaster* affects

immunity in the sexes in different ways, likely interacts with immunosenescence and can act to improve post-infection lifespan under specific conditions.

3.2 Introduction

The social environment has the ability to both improve the immune system via the action of density dependent prophylaxis (Barnes and Siva-Jothy 2000), whilst conversely, “social stress” is associated with disorders of the immune system (Cruces *et al.* 2015; Cohen *et al.* 1997) and immunosenescence (Epel *et al.* 2004) in a variety of organisms. What constitutes social stress is ultimately dependent on the species and situation; isolation can disrupt T cell function (Clausing *et al.* 1994), complement activity (Scotti *et al.* 2015) and reduce lifespan (Koto *et al.* 2015). In humans, the negative clinical manifestations associated with social isolation are mediated in part by a specific transcriptomic profile that is characterised by up regulation of pro-inflammatory cytokine genes and down regulation of genes involved in innate anti-viral resistance (Cole *et al.* 2007a). Conversely, high densities environments can exert significant deleterious effects over physiology, in particular the immune system, due to competitive stress. Accordingly, aggressive encounters common to high density groups negatively influence immunity (Jasnow *et al.* 2001; Hawley, Lindstrom and Wikelski 2006) and glucocorticoids released under stressful conditions reduce immunocompetence (McEwen 2012; Martin 2009).

The relationship, then, between social isolation or stimulation and the immune system is a complex one and is shaped partly by the trade-off that exists between social interaction and the increased transmission of communicable diseases (Godfrey *et al.* 2009). Social and sexual behaviours in zebra finches (*Taeniopygia guttata*) aid the transmission of bacteria via a faecal-oral-genital route (Kulkarni and Heeb 2007) and higher levels of network connectivity in Gidgee skinks (*Egernia stokesii*) increase the risk of parasite infection (Godfrey *et al.* 2009). Since the maintenance of resistance to pathogens is costly it follows that this trait is phenotypically plastic and

investment is increased in line with risk of infection. Accordingly, mealworm beetles (*Tenebrio molitor*) raised at high densities are better able to survive infection with a pathogenic fungus (density-dependent prophylaxis or DDP) (Barnes and Siva-Jothy 2000). Despite an increase in sociality increasing the risk of infection, some complex social network patterns can lower infectious disease risk without the influence of immunity or avoidance behaviours, therefore indicating that social networks can exert a profound influence over the evolutionary course of social behaviour and immunity (Hock and Fefferman 2012).

Age can also influence the immune system, often in a negative manner. Immunosenescence is the gradual deterioration of immune function as an organism ages and is a common trait throughout the animal kingdom (Muller, Fulop and Pawelec 2013). Low social support is associated with shorter leukocyte telomere length in humans later in life, indicating that specific social conditions may contribute to cellular ageing (Carroll *et al.* 2013) and in honey bees (*Apis mellifera)*. Social contact can reverse the effects of immunosenescence (Amdam *et al.* 2005). My own previous work (Chapter 2) confirms a relationship between social environment and actuarial and functional senescence, independent of behaviour, and similarly indicates the involvement of aspects of the immune system (Leech, Sait and Bretman 2017). Together, this work suggests that social environment can interact with age to affect the immune system, but more research is needed in order to decipher precisely how these changes are enacted.

In *D. melanogaster* both sexes have shorter lifespans when kept with same sex partners versus in isolation, but this effect is more severe for males (Leech, Sait and Bretman 2017). When an immune stimulator is applied (wounding), the effect is almost twice as detrimental for paired males compared to paired females, suggesting that immune responses act differentially for males and females in combination with social environment (Leech, Sait and Bretman 2017). Other studies using *D. melanogaster* suggest that the identity of individuals, density and type of contact all act to influence longevity

(Zajitschek *et al.* 2013; Ruan and Wu 2008; Cordts and Partridge 1996). The influence that social environment exerts over the immune system of *D. melanogaster* has, however, mostly been studied in relation to male/female interactions and therefore reproduction, but it is often difficult to disentangle the costs of courtship, mating and reproduction from the underlying effects of simply occupying the same space. Inter-sex interactions are largely mediated by seminal proteins transferred to females during mating and which result in the up-regulation of immune genes responsible for the production of a variety of anti-microbial proteins (Peng, Zipperlen and Kubli 2005). This response is unsurprising given the risk of sexually transmitted disease and injury that comes with reproduction. Curiously, despite the up-regulation of key immunity genes, there is a disparity between potential immune performance and actual survival post infection since increased expression of immune genes does not provide any noticeable benefit to mated females (Short and Lazzaro 2013; Short and Lazzaro 2010; Fedorka *et al.* 2007).

Here, I aimed to test the effect of social interaction on the immune system of *D. melanogaster.* My previous work indicated that social stimulation with a same sex individual is stressful for both sexes, and especially so for males (Leech, Sait and Bretman 2017). There was also an indication that immune-stress exacerbated the effect of pairing on males. This may be because a sexually competitive environment necessitates the ability to accurately integrate a variety of sensory cues into complex behavioural outcomes (Rouse and Bretman 2016; Bretman *et al.* 2010) but at a cost of lifespan and mating success at older ages (Bretman *et al.* 2013). These sex differences may be driven by a trade-off between sexual plasticity and immune plasticity in males, or relatedly, the competitive stress of the shared environment is simply more extreme for males due to, for example, aggression. This could result in males exposed to social contact showing poorer immune responses or faster immunosenescence. Alternatively, in line with previous studies detailing DDP, conspecific density may be used as a cue to increase investment in resistance mechanisms, resulting in the increased ability of paired flies to survive infection. Therefore here I tested these ideas directly by measuring lifespan after infection with different pathogens, differential gene

expression of a sub-set of immunity and stress response-related genes and phagocytosis activity. I hypothesised that post-infection lifespan for paired flies would be reduced for both sexes, but to a greater extent in males, and that differential gene expression and phagocytosis would therefore reflect this by displaying lower gene expression and reduced phagocytic ability.

3.3 Materials and Methods

3.3.1 Fly stocks and maintenance

Drosophila melanogaster wild type (strain Dahomey) were raised on standard sugar-yeast agar (SYA) medium containing 200g brewer's yeast, 100g sugar, 20g agar, 30ml Nipagin (10% w/v solution) and 3ml propionic acid per litre of medium (Bass *et al.* 2007). Flies for all experiments were raised and kept at a constant 25ºC and 50% humidity with 12 hour light – 12 hour dark cycle. Population cages were fed weekly using 3 X 70ml SYA. Eggs were collected on purple grape juice agar plates (275ml water, 12.5g agar, 150ml red grape juice, 10.5ml Nipagin) placed in population cages. Eggs were allowed to develop into larvae for approximately 24 hours and then transferred to 7ml SYA vials supplemented with a loose live yeast paste at a density of 100 larvae per vial.

3.3.2 Experimental design

Upon eclosion adult flies were sexed under ice anaesthesia and transferred to relevant social environment – we had a fully factorial experimental design of eight groups (**Figure 11**): males and females, single or paired, infected or sham infected, young (infection at 10-12 days old) or old (infection at 50-53 days old). Sample sizes ranged from 29-40, for exact numbers per treatment see appendix. In the time prior to infection flies were kept on standard SYA which was changed weekly. In treatments with pairs, the non-focal fly was replaced weekly with a new, 2 day old wing clipped partner to avoid any effect of habituation between the two. For phagocytosis experiments, the same groups were used.

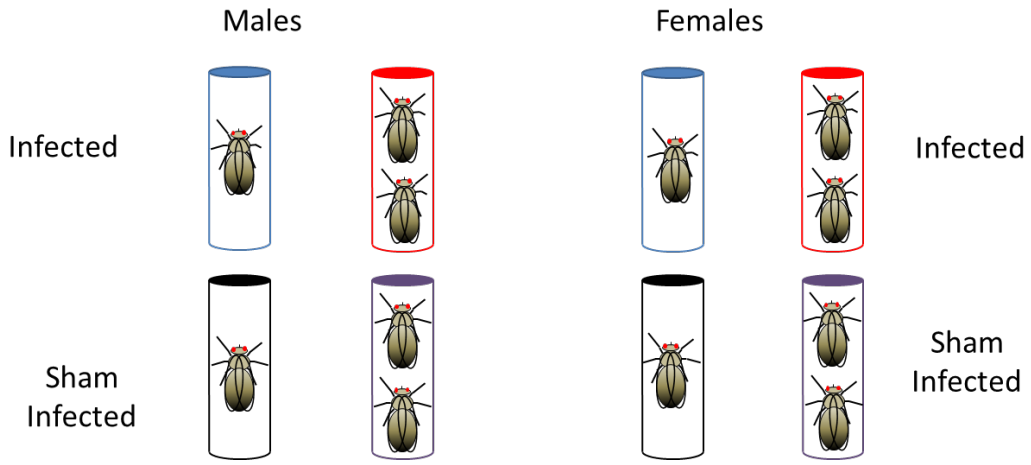

Figure 11 Infection assay experimental design. Males and females maintained either alone or in same-sex pairs for 10 or 52 days, before being injected with one of the three possible bacteria, or a sterile sham injection.

3.3.3 Bacteria and infections

We chose to use three different bacterial species for infections because the innate immune system of *D. melanogaster* operates in principally distinct units that are mostly specific in their ability to combat different types of pathogens, although there is overlap of these largely discrete arms (Lemaitre, Reichhart and Hoffmann 1997; Ferrandon *et al.* 2007). *Pseudomonas aeruginosa* and *Pseudomonas fluorescens* are closely related gram-negative bacteria which have both been used previously to study drosophila immunity (Apidianakis and Rahme 2009; Pimenta, Di Martino and Blight 2006), whilst *Bacillus thuringiensis* is a gram-positive species which commonly infects insects in the wild (Romeis, Meissle and Bigler 2006). *P. aeruginosa* (strain PAO1) and *B. thuringiensis* (DSMZ 2046) were grown for 24 hours at 37ºc with 200rpm shaking. *P. fluorescens* (DSMZ 50090) was grown for 48 hours at 25ºc without shaking.

3.3.3.1 Infections

Flies were injected with 9.2nl of 10^{-2} *P. aeruginosa*, 13.8nl of 10^{-2} *P. fluorescens* and 13.8nl of 10^{-1} *B. thuringiensis*, or the equivalent of between 120-220 CFU's, randomly across groups at the same time of day, using the

Nanoliter 2010 (World Precision Instruments). This dosage was used for *P. aeruginosa* infections as in previous studies (Apidianakis and Rahme 2009), and in light of my own dose response curve studies which showed this dosage to be the lowest dose that killed all the flies. I used similar dosages for the closely related *P. fluorescens* and also *B. thuringiensis*. Uninfected flies were injected first with a sterile sham solution of 10mM $MgSO_4$ in order to control for the wounding itself and the solution the bacteria was diluted in. Sham injected flies were later removed from the analysis as there was no effect of sham injection. The needle was emptied and refilled every 10 injections in order to prevent bacteria pooling in the tip. Flies were then checked hourly for death.

3.3.4 Differential expression of immunity and stress related genes using RT-qPCR

3.3.4.1 Experimental design

Upon eclosion flies were placed into similar groups as above – these groups were, briefly, males and females, single or paired, and maintained as above. In contrast to experiments measuring post-infection lifespan, only one age was tested (52 days old), and flies were not injected with bacteria so gene expression represents basal activity. At 52 days old focal flies from each group were flash frozen individually in liquid nitrogen and stored at -70ºC. Seven biological replicates were completed and 12 flies were pooled per group in order to reduce technical variation between replicates.

3.3.4.2 RNA extraction, cDNA synthesis and RT-qPCR

RNA extractions were performed using the Zymo research Directzol RNA miniprep (Serial no. R2052), including a DNAse treatment. The quantity and purity of resulting RNA was measured using NanoDrop (ND-1000) and the integrity was checked by gel electrophoresis. RNA quantity was standardised across samples and cDNA was generated using the Thermo Scientific first strand synthesis cDNA kit (Serial no. K1612), and the integrity was once again checked by gel electrophoresis. Negative-RT controls were included to check for the presence of contaminating genomic DNA. If there was contaminating

genomic DNA present the reaction was performed again. Primers were designed for *Dro*, *vir-1*, *eater*, *TotA*, *TotM* and *foxo* and housekeeping genes *Actin5c* and *EF1* using primer3plus and where possible, were designed to span intron/exon boundaries in order to detect amplification of contaminating genomic DNA (Appendix A). All genes were chosen to represent a range of immunological defence mechanisms which are under the control of discrete regulatory networks and based on previous research which revealed them to be socially sensitive in males exposed to a rival (Mohorianu *et al.* 2017), with the exception of *foxf* which was not highlighted in this study but has been in others (Giannakou *et al.* 2004). RT-qPCR was completed on the Biorad C1000 touch using the Kicqstart SYBR Green Readymix by Sigma and Biorad plates (HSP9655) and seals (MSB1001).

3.3.5 Investigating phagocytosis using flow cytometry

3.3.5.1 Haemocyte extractions

Haemolymph was extracted from surface-sterilised flies under ice anaesthesia using a flush method. Briefly, following ice-anaesthesia, a small incision was made in the lateral abdomen and a fine gauge (0.3mm) hypodermic needle was used to flush 25µl of chilled Schneider's *Drosophila* media (product number 21720001) (pH adjusted to 6.9) through the fly, from the lateral thorax to the incision made in the abdomen into a 1.5ml eppendorf kept on ice. The resulting haemolymph and media were pooled 3 flies per biological replicate and kept on ice.

3.3.5.2 Sample preparation and flow cytometry

pHrodo Green STP ester (product number P35369) was used to label 50mg of freeze-dried *Pseudomonas aeruginosa* PAO1, following the instructions provided. We chose to use pHrodo Green STP ester because this dye only fluoresces under acidic conditions. This is significant because there is a large reduction in pH inside the phagosome of a haemocyte upon the phagocytosis of bacteria (Luce-Fedrow, Von Ohlen and Chapes 2009). This allowed the non-fluorescing non-phagocytosing haemocytes and those that fluoresced due

to phagocytosis to be distinguished. *P. aeruginosa* was used in line with previous infection assays, where flies from different social environments respond differently to infection with this bacteria.

A preliminary dose response curve was conducted in order to determine a suitable concentration and a simple *in vitro*-versus-*in vivo* assay was done to see which system provided the best results. Based on these results we chose to make up pHrodo-labelled bacterial suspensions of 0.33mg per 50µl and proceed with the *in vitro* assay as the results were more reliable.

To prepare the samples 0.33mg of dyed bacteria was sonicated in 50µl of Hanks Balanced Salt Solution (Thermo Fisher product number 14170112) for 45 minutes, before 5µl pHrodo-labelled bacteria/buffer mixture was added to the extracted haemolymph and allowed to incubate for 1 hour at 25˚C in the dark. 150µl of 0.5% paraformaldehyde solution was added next in order to fix the cells and finally 40µl NucBlue (Invitrogen™ product number R37605) was added to differentiate whole intact haemocytes from other debris, then incubated for a further 15 minutes, again without light. Fixed and stained samples were kept on ice until they were analysed using a BD LSRII flow cytometer (BD Biosciences) using the slowest flow rate for 3 minutes.

Instrument thresholds and software analysis gates were first established based on relevant unlabelled haemocyte (unstained), NucBlue-labelled haemocyte (UV), and pHrodo-bacteria conjugate (green only) control samples. Firstly, in order to distinguish between irrelevant autofluorescent debris and NucBlue labelled haemocytes, unstained and UV only controls were compared. Next, in order to differentiate between haemocytes and non-phagocytosed bacterial particles, UV only and green only control samples were compared. To calculate phagocytic activity the number of UV+ green- (non-phagocytosing haemocytes) was compared to UV+ green+ (phagocytosing haemocytes).

UV labelled haemocytes (NucBlue) were detected in the UV gated (780/60 band-pass) and green labelled phagocytes in the Blue gated (530/30 band-pass) instrument parameters. Data analysis was limited to haemocytes by selection based on the UV fluorescent signal and then displaying the phagocytes associated in a dualparameter logarithmic dot plot of green signal vs. side scatter-area. Phagocytic activity was calculated for each sample by counting non-phagocytic (UV+ green-) and phagocytic (UV+ green+) haemocytes to determine their relative number.

3.3.6 Statistical analysis

3.3.6.1 Infection assays

All statistics were conducted using R (i386 3.2.0) and SPSSv21. Post-infection lifespan was analysed using a GLM (as the assumptions of the Cox Proportional Hazards model were violated) with quasi Poisson errors (to account for over dispersion) with sex and social environment as factors. Models were simplified from the full model using Analysis of Deviance (AOD), the final model being when no further terms could be removed without significantly reducing the model's descriptive power. Sham infected flies were removed from the analysis as only one died over the course of the experiment.

3.3.6.2 Gene expression

The average normalised expression (for formula see Appendix) was calculated and then $\log_2$ transformed in order to make the data distribution symmetrical (Hellemans and Vandesompele 2011). The data was subsequently separated by gene and analysed using a GLM with a Gaussian error structure, the full model being reduced using AOD as above. The Bonferroni-Holm method (Holm 1979) was used to correct for multiple testing.

3.3.6.3 Flow cytometry

Total haemocyte counts for each of the eight treatments was first assessed using a generalised linear model fitted with a quasipoisson error distribution. An

interaction was fitted between the fixed factors of sex, age and treatment before subtracting the terms. Next, the proportion of phagocytic haemocytes out of total haemocyte counts (phagocytic index, or PI) was analysed using a generalised linear model fitted with a quasibinomial error distribution. Once again, we fitted a three-way interaction between sex, age and treatment before models were simplified using Analysis of Deviance (AOD) before splitting the data by sex and age.

3.4 Results

3.4.1 Post infection survival

Using the full models with age, sex and treatment as fixed factors, revealed age effects for all bacteria (older flies were less able to survive infection) (**Table 5**, **Table 6** and **Table 7**), hence to probe the patterns further, data from young and old flies were analysed separately. *P. aeruginosa* was the only bacterial infection where lifespan was significantly affected by social environment, but in young flies there was no effect of sex or pairing (**Table 5**) – there was no difference in lifespan when flies were young, but once they were aged to 52 days, pairing acted to improve post-infection lifespan for both sexes (**Table 5** and **Figure 13**). Additionally, in old flies there was a main effect of sex (**Table 5**), whereby females survived for longer after infection (**Figure 13**).

Table 5 Post *P. aeruginosa* infection lifespan analysed using GLM and AOD. Young and old groups were initially analysed together using treatment, age and sex as fixed factors, before being separated and analysed independently.

Age	**Response variable**	**Explanatory variable**	**df**	***F***	***p***
All data	Lifespan	Pairing*Age	1, 292	4.807	**0.029**
		Sex*Age	1, 293	4.495	**0.035**
		Sex*Age*Pairing	1, 289	0.196	0.658
Young	Lifespan	Pairing	1, 147	0.013	0.911
		Sex	1, 149	0.169	0.681
		Pairing*Sex	1, 147	0.340	0.561
Old	Lifespan	Pairing	1, 145	4.916	**0.028**
		Sex	1, 147	4.553	**0.035**
		Pairing*Sex	1, 143	0.093	0.761

For flies infected with *P. fluorescens,* once again there were strong age effects (old flies die quicker - **Table 6** and **Figure 13**) and so young and old groups were analysed separately. Treatment had no effect on ability to survive infection with *P. fluorescens*, but as with *P. aeruginosa*, female survival is superior to males in old flies, and in contrast to *P. aeruginosa*, in young flies too (**Table 6** and **Figure 13**).

Table 6 Post *P. fluorescens* infection lifespan analysed using GLM and AOD. Young and old groups were initially analysed together before being separated. Age, sex and treatment were entered as fixed factors.

Age	**Response variable**	**Explanatory variable**	**df**	***F***	***p***
All data	Lifespan	Age	1, 275	25.538	**<0.0001**
		Pairing	1, 273	0.767	0.38
		Sex	1, 277	8.641	**0.004**
		Sex*Age*Pairing	1, 269	2.368	0.125
Young	Lifespan	Pairing	1, 145	0.343	0.559
		Sex	1, 147	10.316	**<0.001**
		Pairing*Sex	1, 145	0.137	0.712
Old	Lifespan	Pairing	1, 125	0.631	0.429
		Sex	1, 127	5.949	**0.016**
		Pairing*Sex	1, 125	1.795	0.183

A similar pattern also emerges with flies injected with *B. thuringiensis*. Again, the significant effect of sex as seen in older flies (**Table 7**) is explained by the females living longer after infection than males. The overall effects of age (**Table 7**) once again indicate that older flies are less able to survive infection with this bacteria, with more flies dying earlier than their young counterparts (**Figure 13**).

Table 7 Post *B. thuringiensis* infection lifespan analysed using GLM and AOD. Young and old groups were once again initially analysed together before being divided by age. Age, sex and treatment were entered as fixed factors.

Age	**Response variable**	**Explanatory variable**	**df**	***F***	***p***
All data	Lifespan	Age	1, 301	23.066	**<0.0001**
		Pairing	1, 300	0.100	0.752
		Sex	1, 301	7.606	**<0.01**
		Sex*Age*Pairing	1, 296	1.298	0.257
Young	Lifespan	Pairing	1, 152	0.046	0.828
		Sex	1, 150	2.314	0.130
		Pairing*Sex	1, 150	0.143	0.706
Old	Lifespan	Pairing	1, 147	0.044	0.835
		Sex	1, 149	6.562	**0.011**
		Pairing*Sex	1, 147	1.709	0.193

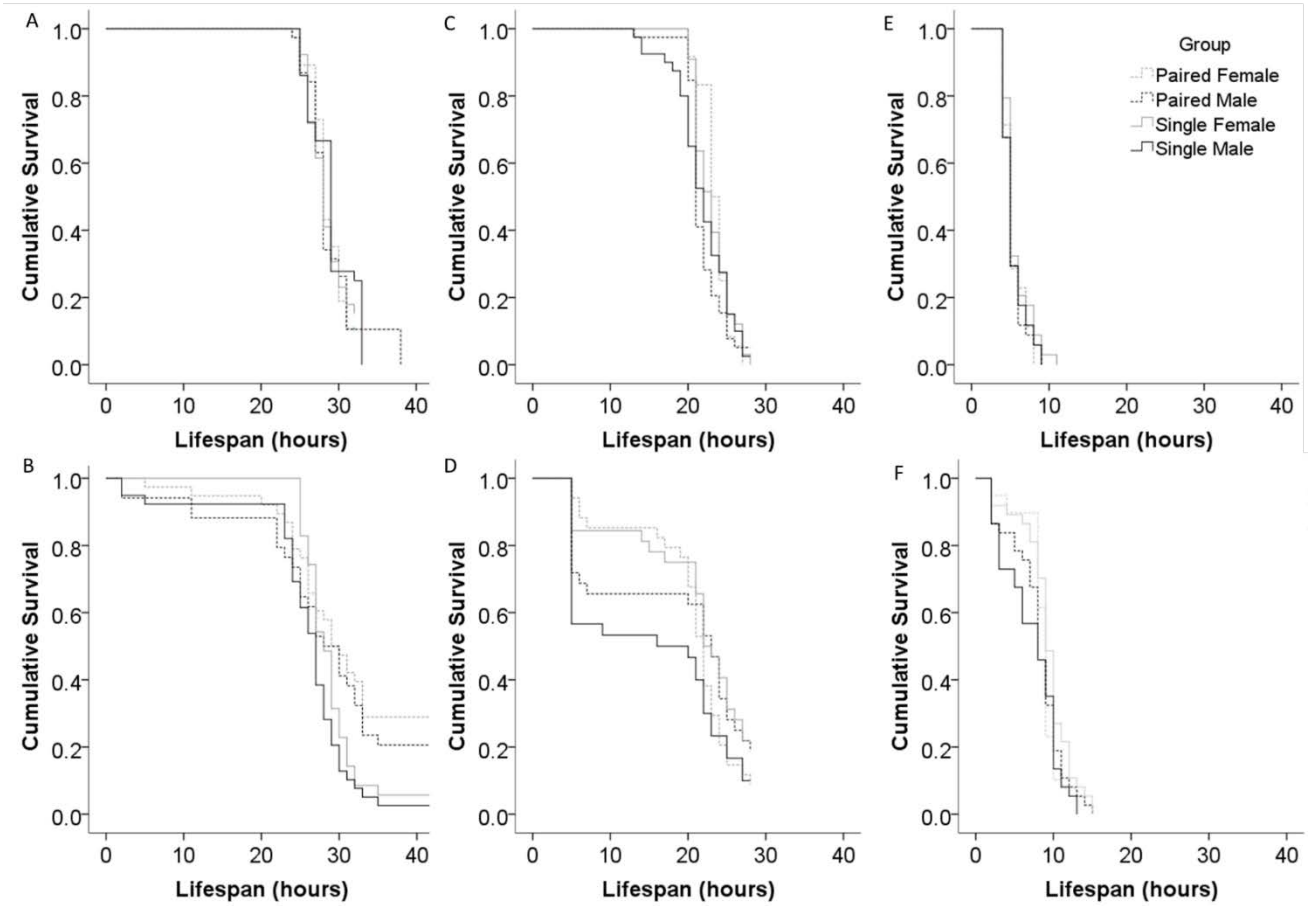

Figure 12 Kaplan Meier curves showing cumulative survival post infection. Males are shown in black and females in grey, whilst dotted lines denote paired groups and solid lines are single flies. The top row (panels A, C and E) show young flies and the bottom row (panels B, D and F) show old flies. Panels in the same column were infected with the same bacteria – A and B were infected with *P. aeruginosa*, C and D were infected with *P. fluorescens* and E and F were infected with *B. thuringiensis.*

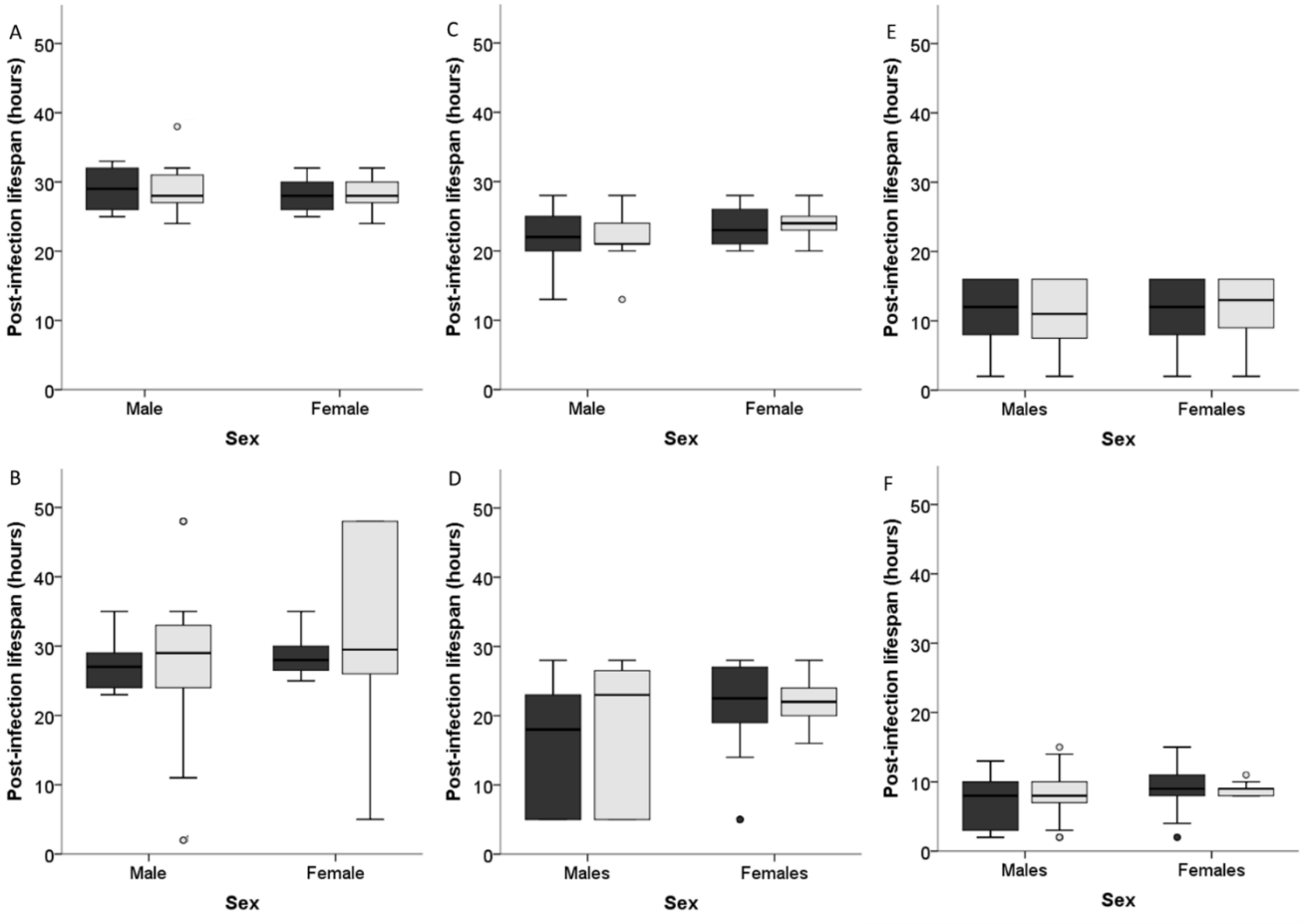

Figure 13 Median survival post infection. Singles are shown in black and pairs in grey. The top row (panels A, C and E) show young flies and the bottom row (panels B, D and F) show old flies. Panels in the same column were infected with the same bacteria – A and B were infected with *P. aeruginosa*, C and D were infected with *P. fluorescens* and E and F were infected with *B. thuringiensis.* Whiskers represent maximum and minimum non-outlier values. Circles indicate outliers (Q1/Q3±1.5 x IQ range).

3.4.2 Differential gene expression

A panel of immune-associated, stress response and ageing genes were chosen based on existing literature and RNASeq data from previous work. Only older flies were used in light of infection assays which indicated that the effects of social environment only become apparent with age. The antimicrobial peptide, *Dro*, was significantly lower expressed in females compared to males, regardless of being kept singly or in pairs (**Table 8** and **Figure 14**). A similar pattern was observed for the antiviral gene *vir-1* (**Table 8** and **Figure 14**). For *eater*, the transmembrane phagocytosis receptor, sex is again the only significant factor (**Table 8** and **Figure 14**). The stress induced humoral peptides *TotA* and *TotM* belong to the same family of 8 closely related proteins which are expressed under a variety of stressful conditions. Once again, *TotA* is expressed to different extents by the sexes, with normalised expression in females significantly lower than in males (**Figure 14**). There is also a significant main effect of pairing which appears to be acting in different ways for the sexes, although there is no significant interaction. For males the median normalised expression value is similar for both single and paired groups. For females, pairing has resulted in significantly lower normalised expression of this gene (**Table 8** and **Figure 14**). For *TotM*, there is a significant interaction between sex and treatment – there is a strong effect of pairing for females reducing expression, but not males (**Table 8** and **Figure 14**). *foxo* is a transcription factor involved in regulation of the insulin signalling pathway and is implicated in the ageing process. Expression of this gene, in agreement with all of my other gene expression data, was relatively lower for females compared to male counterparts (**Table 8** and **Figure 14**).

Table 8 Log2 normalised expression was divided by gene and analysed using a GLM, with sex and treatment as fixed factors, significant results are shown in bold. Results were corrected for multiple testing using the Holm-Bonferroni method.

Gene	**Explanatory Variable**	**df**	***F***	***P***
Dro	Sex	1, 25	90.233	**<0.0001**
	Pairing	1, 23	0.134	0.365
	Sex*Pairing	1, 23	1.896	0.717
vir-1	Sex	1, 26	98.25	**<0.0001**
	Pairing	1, 24	0.824	0.442
	Sex*Pairing	1, 24	1.583	0.442
eater	Sex	1, 25	56.184	**<0.0001**
	Pairing	1, 23	2.647	0.235
	Sex*Pairing	1, 23	2.474	0.13
TotA	Sex	1, 24	34.243	**<0.0001**
	Pairing	1, 23	9.336	**0.011**
	Sex*Pairing	1, 23	3.058	0.094
TotM	Sex	1, 24	15.758	**0.002**
	Pairing	1, 22	6.908	**0.002**
	Sex*Pairing	1, 22	16.535	**0.015**
foxo	Sex	1, 27	14.013	**0.001**
	Pairing	1, 25	0.4539	0.507
	Sex*Pairing	1, 25	0.0419	0.840

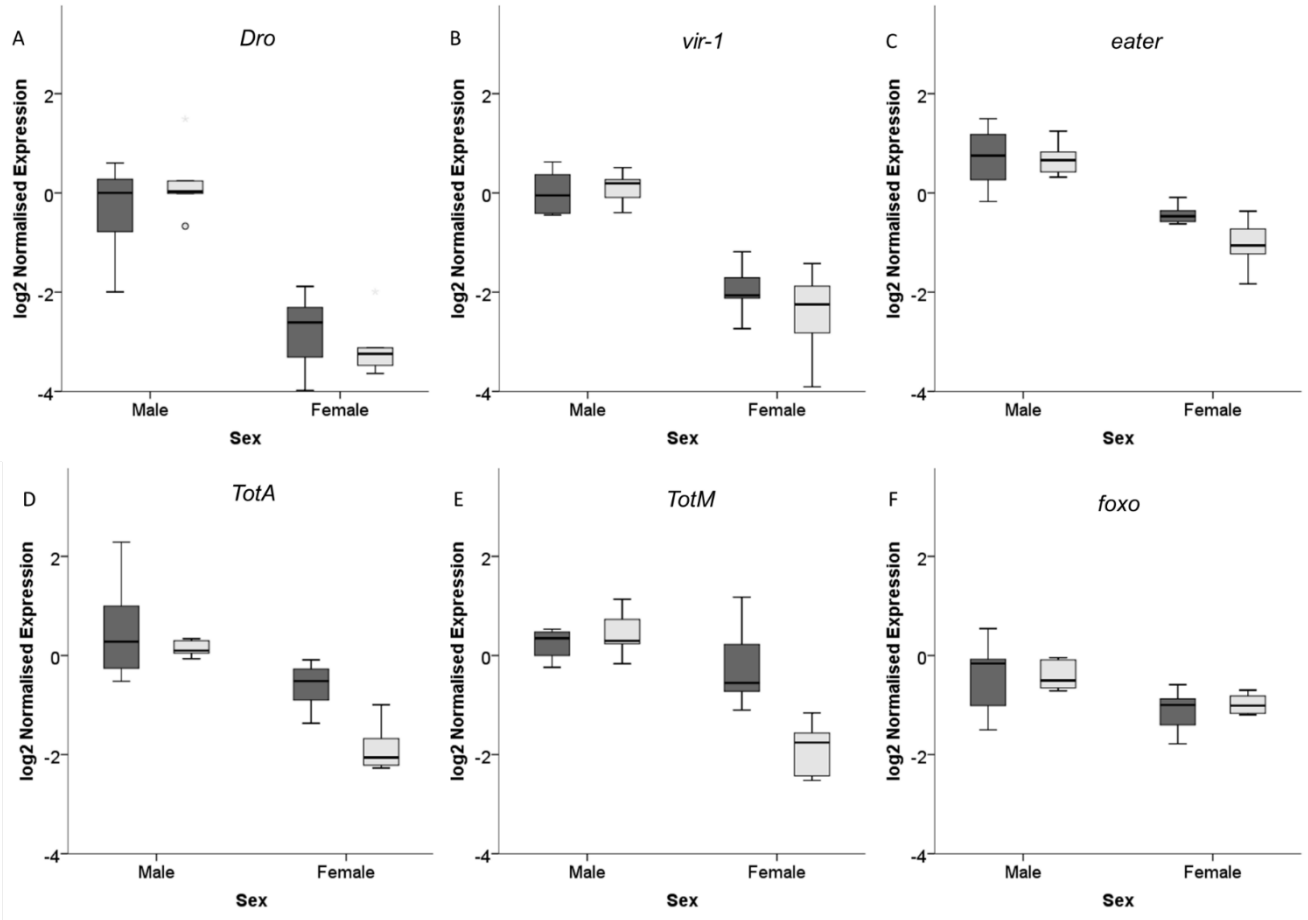

Figure 14 Differential gene expression for 52 day old flies kept singly or in pairs, divided by gene. Darker bars indicate single flies, whilst lighter bars represent paired. Six genes were analysed in total: (A) *Dro*, the antimicrobial peptide, (B) the anti-viral gene *vir-1*, (C) the phagocytosis receptor *eater*, (D) the stress induced humoral peptide *TotA*, (E) the stress induced humoral peptide *TotM* and (F) *foxo*, the transcriptional activator. Whiskers represent maximum and minimum non-outlier values. Circles indicate outliers (Q1/Q3±1.5 x IQ range).

3.4.3 Phagocytosis activity in relation to age, sex and pairing

Analysis of total haemocytes (NucBlue positive cells) revealed that there was no effect of age, sex or treatment (**Table 9** and **Figure 15**), although there is a non-significant trend for young males, regardless of social environment to have more haemocytes. The proportion of phagocytosing cells (proportion of phagocytic haemocytes out of total haemocyte counts, also known as phagocytic index) was analysed next – here there were main effects of sex (males appear to have a slightly higher PI), but nothing else (**Table 9** and **Figure 16**).

The data were subsequently divided by sex. Pairing and age had no effect on males, but for females, pairing reduces the proportion of phagocytosing cells (**Table 9** and **Figure 16**).The data was next divided by age and once again the proportion of successful phagocytic events, first for young flies and then for old flies was analysed. Sex had a significant effect on the number of phagocytosing cells in young flies, with males having more successful phagocytic events than females (**Table 9** and **Figure 16**), but this effect was abolished with age.

Table 9 Phagocytosis results analysed by GLM. Males and females were kept alone or in same sex pairs and either aged to 10 or 52 days. Haemolymph was extracted and phagocytosis was analysed *in vitro* using flow cytometry. 2 separate response variables were examined – total number of haemocytes and proportion of phagocytosing cells (proportion of phagocytic haemocytes out of total haemocyte counts or phagocytic index). The full model with sex, age and treatment was initially analysed before being divided by sex and age to better interpret the results.

Data separated by	**Response Variable**	**Explanatory Variable**	**df**	**F**	***p***
Full model	PI	Sex	1, 127	5.484	**0.021**
		Age	1, 125	1.037	0.311
		Pairing	1, 125	2.849	0.094
		Pairing*Sex	1, 123	3.733	0.056
		Sex*Age*Pairing	1, 121	0.270	0.604
	Total haemocytes	Sex	1, 125	0.656	0.420
		Age	1, 125	0.368	0.545
		Pairing	1, 127	0.746	0.389
		Sex*Age*Pairing	1, 121	0.438	0.509
Sex - males	PI	Age	1, 63	0.469	0.496
		Pairing	1, 61	0.021	0.884
		Pairing*Age	1, 61	<0.0001	0.992
	Total haemocytes	Age	1, 62	0.650	0.423
		Pairing	1, 62	1.200	0.278
		Pairing*Age	1, 60	0.443	0.508
Sex - females	PI	Age	1, 63	0.965	0.330
		Pairing	1, 61	7.331	**0.009**
		Pairing*Age	1, 61	0.533	0.468
	Total haemocytes	Age	1, 62	2.117	0.151

		Pairing	1, 62	0.053	0.819
		Pairing*Age	1, 60	1.732	0.193
Age - young	PI	Sex	1, 63	8.629	**0.005**
		Pairing	1, 61	0.887	0.350
		Pairing*Sex	1, 60	2.156	0.147
	Total haemocytes	Sex	1, 62	2.643	0.109
		Pairing	1, 62	0.111	0.740
		Pairing*Sex	1, 61	0.701	0.406
Age - old	PI	Sex	1, 63	1.695	0.198
		Pairing	1, 61	1.605	0.210
		Pairing*Sex	1, 60	1.903	0.173
	Total haemocytes	Sex	1, 63	0.382	0.539
		Pairing	1, 61	3.122	0.082
		Pairing*Sex	1, 61	<0.0001	1.000

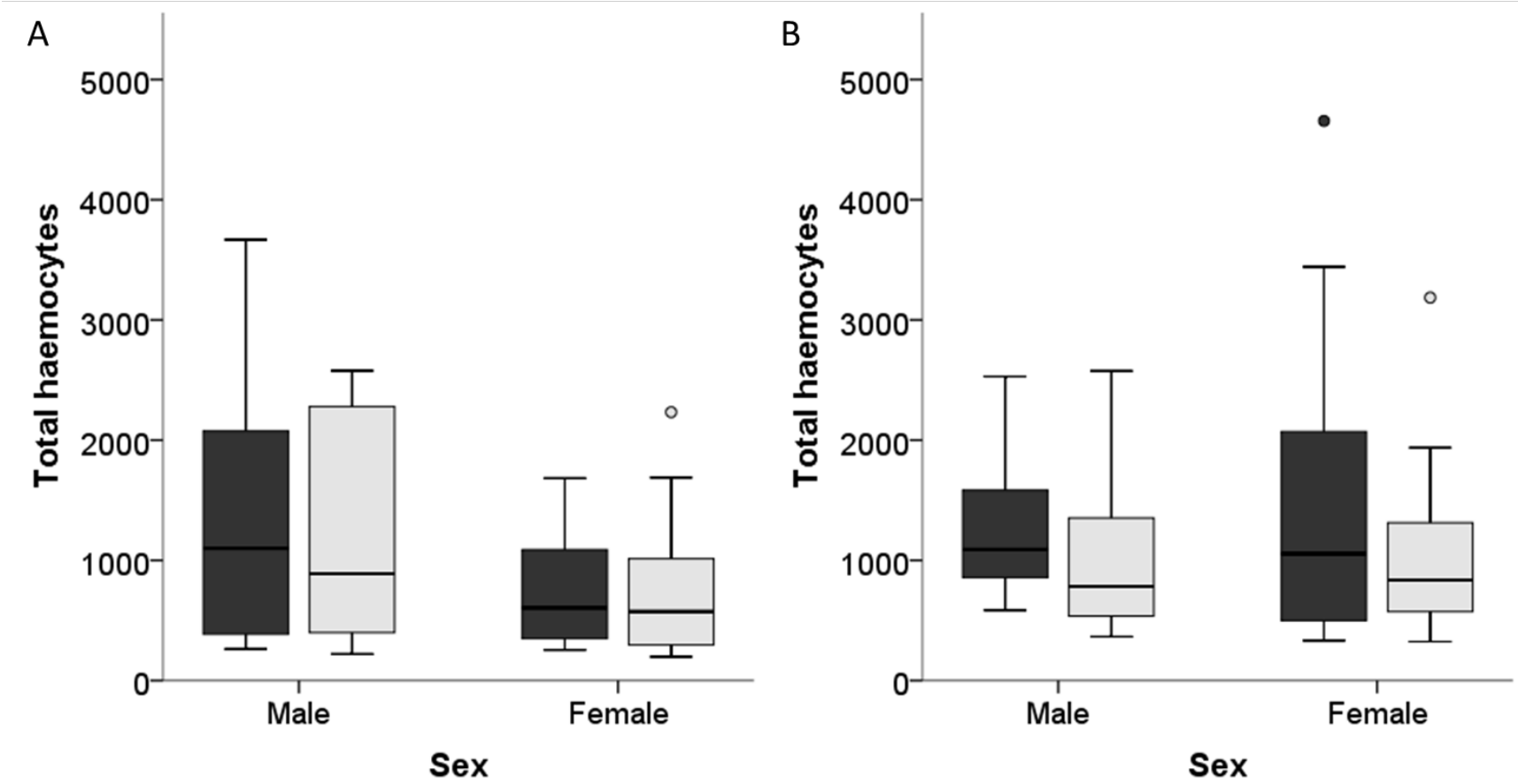

Figure 15 Number of haemocytes (NucBlue positive cells) for single flies (darker) and paired flies (grey), divided by sex for (A) young flies and old flies (B). Whiskers represent maximum and minimum non-outlier values. Circles indicate outliers (Q1/Q3±1.5 x IQ range).

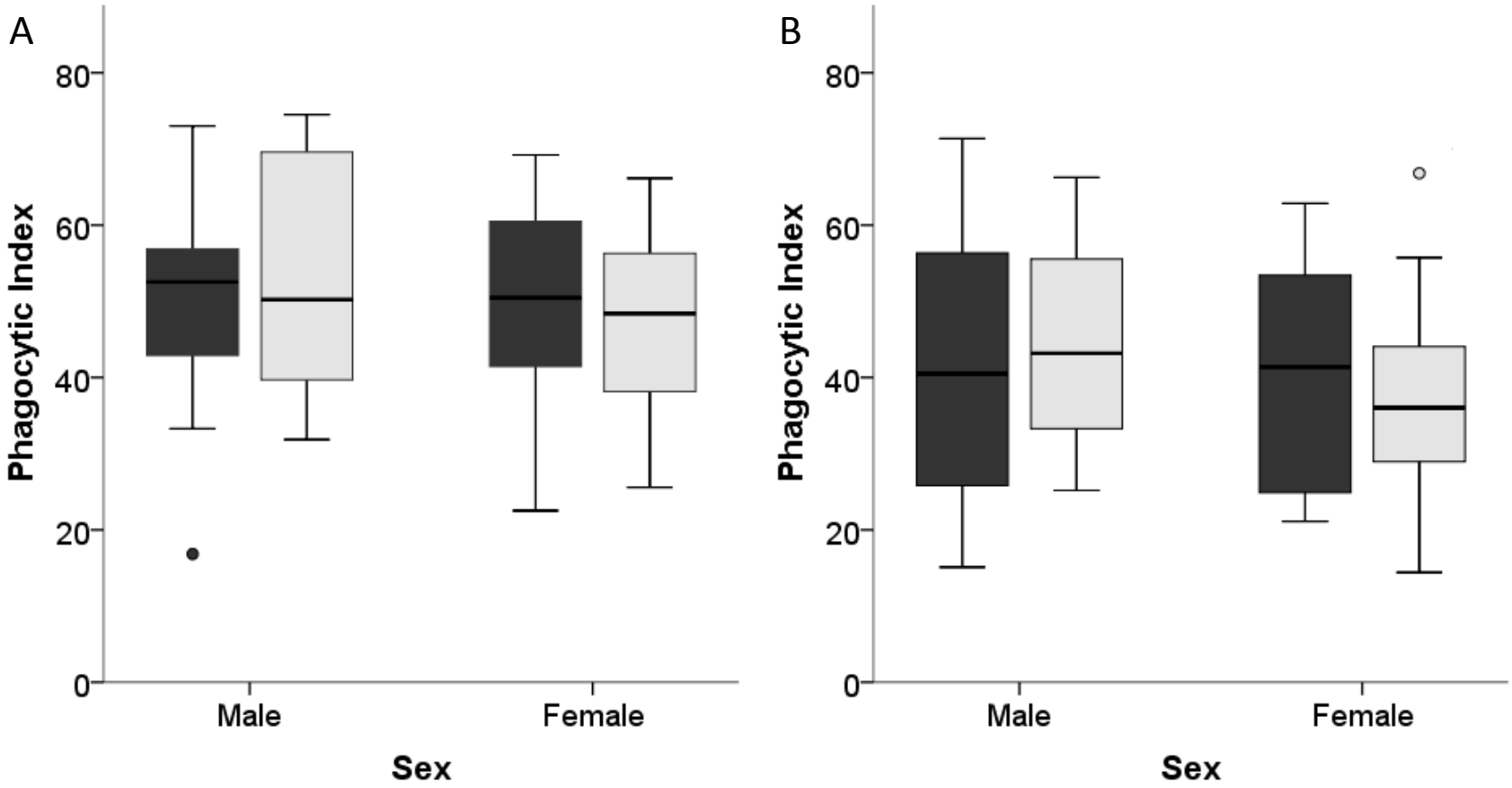

Figure 16 Phagocytic Index (Phrodo positive cells/NucBlue positive cells*100), for single flies (darker) and paired flies (grey), divided by sex. Figure shows young flies (A) and old flies (B). Whiskers represent maximum and minimum non-outlier values. Circles indicate outliers (Q1/Q3±1.5 x IQ range).

3.5 Discussion

3.5.1 Social contact can improve post-infection lifespan

These results indicate a complicated relationship between social environment, immunity and ageing. It was hypothesised that, based on previous work (Bretman *et al.* 2013; Leech, Sait and Bretman 2017), social stimulation would act as a stressor, especially in males (specifically due to either the competitive or reproductive stress of the environment), and consequently, infected flies would be less-able to mount an effective immune response to bacterial challenges. This did not prove to be the case, and in fact, paired flies either performed the same or better than single flies in terms of post-infection lifespan. Indeed, where they did better this was at older ages, suggesting the possibility that DDP only provides fitness benefits in older flies. In females, gene expression analysis revealed that paired females display reduced expression and more sensitivity to social environment for some genes, however, overall we found no evidence that the underlying mechanism for DDP was socially-stimulated differential expression of immune-related genes. A functional phagocytosis assay also revealed that phagocytic ability is reduced in paired females.

Whilst we found evidence for effects on post-infection lifespan, this was not consistent across bacteria. It is not clear whether the time course of infection (i.e. how long the infection takes to kill the fly) is responsible for the significant effect of social environment on lifespan for flies infected with *P. aeruginosa,* or if the unique pathogenesis caused by this particular infection is the cause, since this effect is absent for the other two infections. I used three different bacterial infections in order to test the theory that different parts of the immune system are differentially sensitive to the social environment. Since the two Gram negative *Pseudomonas* species activate broadly similar immune pathways in the fly, but they display different patterns in terms of social sensitivity, this indicates that the individual pathogenesis of the bacteria is more likely to be responsible.

All three infections resulted in differing time course to death patterns with *P. aeruginosa* infected flies starting to die approximately 22 hours post-infection, *P. fluorescens* approximately 15 hours post-infection and *B. thuringiensis* infected flies almost immediately after infection. The improvement in post-infection lifespan for paired flies could be due to their increasing investment in immune defences in order to guard against the perceived risk of infection that social contact brings, or density dependent prophylaxis (DDP) (Wilson *et al.* 2001). This is a common strategy and has been shown previously in mealworm beetles (*Tenebrio molitor*) and desert locusts (*Schistocerca gregaria*) (Wilson *et al.* 2002), as well as other insects and echinoderms. The DDP hypothesis assumes that all traits associated with disease resistance should be up-regulated in response to an increased threat of disease at high density (Mills 2012). However, trade-offs within the immune system may result in certain arms being prioritised over others (Cotter, Kruuk and Wilson 2004), and the senescence of individual immune traits does not always occur at the same rate (Reavey *et al.* 2015) which could explain why lifespan, gene expression data and phagocytosis do not show a consistent pattern.

One possible explanation for the observed results is that as virulence of the infecting bacteria increases, so too does the ability of the bacteria to overwhelm the host immune system (Schmid-Hempel and Frank 2007; Frank and Schmid-Hempel 2008).This would mean that the benefit provided by being in pairs is only beneficial in real terms with less virulent infections. This would explain why flies infected with the least pathogenic of my three challenges, *P. aeruginosa*, perform better when in pairs (an effect seen for both sexes), and why there seems to be a trend towards a similar pattern for the slightly more virulent *P. fluorescens*, but no sign of the effect for *B. thuringiensis* infected flies, which starts to kill the flies almost immediately. This may indicate that there is a trade-off between the sustained investment in immunity (constitutive defence) associated with increased conspecific density and lifespan, should the investment prove to be unnecessary. In the event that the fly does become infected, providing it is not so virulent as to swamp the innate immune defences, an improvement in lifespan is the result.

3.5.2 All genes are expressed at higher levels in males

Differential gene expression in response to social contact is to be expected as an organism's fitness largely depends on its ability to integrate a variety of environmental and social cues into plastic behavioural and physiological responses. Previous work has shown that exposure to a mating rival in males results in the differential expression of over a thousand genes, of which approximately one hundred are immune-associated (Mohorianu *et al.* 2017). Multiple studies have also described differential expression of immunity genes for male-female interactions (Ellis and Carney 2011; Carney 2007; Short and Lazzaro 2013). Pre-emptive immune activation is predicted to be more efficient than a purely reactionary stimulation, thereby conferring an advantage to those able to successfully predict when an immune challenge is likely to occur (Zhong *et al.* 2013b). I therefore decided to investigate the expression of six immune, stress and age related genes in order to see if their expression profiles could help to explain the patterns in post-infection lifespan.

Briefly, *Dro* encodes the antimicrobial peptide Drosocin, which is under regulatory control of the Imd pathway (Lemaitre and Hoffmann 2007). *vir-1* is largely under the control of the JAK-STAT pathway but is generally specific in nature, unlike the other genes we have looked at, i.e. it is not induced by bacteria, fungi or other environmental stressors but almost exclusively by viral infection (Dostert *et al.* 2005). *eater* is a transmembrane EGF-like receptor expressed on haemocytes and pro-haemocytes which binds to bacteria either directly (gram-positive bacteria) or after disruption of the outer membrane (gram-negative) and was initially thought to be required for efficient phagocytosis of all bacteria (Kocks *et al.* 2005). It also plays an important role in the adhesion of sessile haemocytes to the sessile compartment, which often occurs post-infection (Bretscher *et al.* 2015). Both *TotM* and *TotA* genes (and indeed all the genes in the Turandot family) encode humoral factors that are induced under a number of stressful conditions including heat shock, UV irradiation, oxidative stress and bacterial infection (Ekengren and Hultmark 2001). *foxo* is an extremely well-characterised transcription factor involved in

regulation of the insulin signalling pathway. It has been implicated in an enormous variety of relevant physiological functions including regulation of longevity and ageing (Hwangbo *et al.* 2004), gut homeostasis and immunity (Guo *et al.* 2014), as well as organ-specific phenotypic plasticity (Tang *et al.* 2011).

In *D. melanogaster*, transcription of immune-related genes increases with age (Landis *et al.* 2004; Zerofsky *et al.* 2005), which should be interpreted as a gradual deterioration in control of the immune response (immunosenescence), given this coincides with poorer immune performance. In virgin females challenged with a bacterial infection, older flies express more of the antimicrobial peptide *diptericin A* than younger flies, and are less able to terminate this induction (Zerofsky *et al.* 2005), an effect that was found when flies were kept in cages of 200. My own results indicate that there is no effect of social environment on the expression profile of antimicrobial peptide *Dro* for either sex. If ageing is characterised by a continuing decline in regulatory control of antimicrobial peptide genes (Landis *et al.* 2004; Zerofsky *et al.* 2005) the relative difference in expression of *Dro* in females as compared to males may explain why females in general have an improved post-infection lifespan. The lack of social-effects suggests that any post-infection lifespan improvement observed in paired flies is not because of differences in antimicrobial peptide gene expression (assuming transcription of these genes equates to an equivalent increase in peptide). As *Dro* is an antimicrobial peptide that acts directly on pathogens rather than a negative regulator within a gene regulatory network, the lack of an effect of social environment seems to indicate that pairing in flies primes the immune system against a pathogenic insult independently of this AMP.

The gene expression pattern we observed for *vir*-1 is almost identical to *Dro.* This may be to be expected given that the Imd pathway, which regulates *Dro,* is also partly involved in antiviral responses (Costa *et al.* 2009), despite *vir-1* primarily being under the control of the JAK-STAT pathway (Dostert *et al.* 2005). In contrast to the other genes examined, *vir-1* is highly specific in nature

– it is not induced by bacteria or fungi, but almost exclusively by viral infection (Dostert *et al.* 2005). Expression of *vir-1* is not modified by a number of stressful conditions including heat shock, cold shock, mechanical pressure, dehydration or ultraviolet irradiation (Dostert *et al.* 2005), which, interestingly, is a result confirmed by my study, if we assume one of my social conditions to be more stressful than the other. However, *vir-1* has been shown to be sensitive to stimuli beyond that of viral infection - it is upregulated (along with some*Turandot* genes) in female virgins held under diapause conditions (11ºC with a reduced photoperiod) (Kucerova *et al.* 2016), confirming the role of *vir-1* is not purely a response to viral pathogens.

Upregulation of *foxo* in the brain and fat body has been linked to increased ageing rates (Hwangbo *et al.* 2004) and in the gut chronic upregulation with age leads eventually to death via inhibition of PGRP-SC2 (a negative regulator of the IMD innate immune pathway) (Guo *et al.* 2014). Since previous work has shown that social environment affects ageing to differing extents in males and females, it was necessary to investigate if this is being mediated in some part by *foxo.* Whilst clear sex effects in expression of *foxo* were found (males always expressed relatively more than females), there was no evidence that social environment is affecting ageing in a *foxo* dependent manner, suggesting that other important regulators of immunosenescence and ageing (e.g. Tor) may be regulating the differences observed. To the best of my knowledge, this is the first time that a relative constitutive increase in expression of *foxo* in males relative to females has been shown in aged individuals. Previous work has, however, implicated *foxo* in sex-specific ageing patterns – in males, *foxo* interacts with tumour repressor p53 to reduce lifespan (Shen and Tower 2010) and overexpression of *foxo* in the fat body increases lifespan in females but has no noticeable effect in males (Giannakou *et al.* 2004).

eater also displayed similar patterns of expression to *Dro*, *vir-1* and *foxo* – relatively higher in males, regardless of social environment, compared to females. There was no statistically significant effect of social environment for

expression of *eater*, but phagocytosis experiments revealed PI values which indicate a reduction phagocytosis in paired females. Given RNAi knockdown of *eater* results in a profound decrease in phagocytosis of heat-killed bacteria (Chung and Kocks 2012), this result is consistent with eater being a major player in *D. melanogaster* phagocytosis. Counterintuitively, if dysregulation with age results in higher expression levels of immune genes , the lower relative expression we see in paired females translates into less phagocytosis without trade-offs in post-infection lifespan, this could also strengthen the argument that pairing delays immunosenescence in females.

The sex differences in post-infection lifespan data (males always perform worse than females) only became apparent once the flies were tested at the older time-point of 52 days (with the exception of 10 day old flies infected with *P. fluorescens*). This suggests that the immune system of *D. melanogaster* deteriorates to differing extents for male and female flies, which is in line with previous studies that report the severity of infection is generally worse for males than females (Ramsden, Cheung and Seroude 2008) and that phagocytosis senesces in different ways for males and females (Mackenzie, Bussiere and Tinsley 2011) in *D. melanogaster*.

3.5.3 Expression of *Turandot* genes is sensitive to social environment in females

In contrast to the four strictly immunological genes, social environment has a clear effect on stress response genes - a pattern of lower expression in paired females is seen in two of the six genes I tested - *TotA* and *TotM*. For females, it appears that being in pairs is less stressful than social isolation, a reflection supported by the *TotA* and *TotM* gene expression profiles. These peptides are secreted from the fat body into the haemolymph and act systemically under stressful conditions, although their exact method of action is still unknown. They are controlled via a complex set of regulatory cascades including the Imd, JAK/STAT and the MAPK pathways (Lemaitre and Hoffmann 2007). Since these genes are upregulated in single females relative to those paired it may

indicate being alone is stressful for females, a result that is mirrored in the functional senescence assay from Chapter 2. *TotM*, and another member of the Turandot family that was not measured in this study, *TotC*, are upregulated in females exposed to courtship song, confirming that *TotM* is a "socially sensitive" gene. The Tot M protein product does not appear to be directly antimicrobial in the same way as Dro but instead may help *D. melanogaster* to tolerate fungal infections, rather than resist them (Zhong *et al.* 2013b). In previous studies the effect of *TotM* has been examined in the context of reproduction, but since we have only used same sex pairs rather than mating pairs, and differential gene expression responses are known to vary in accordance with the sex of the interacting partner (Ellis and Carney 2011), there would be no need to upregulate a gene that seems to primarily promote immunity against sexually transmitted infections (Zhong *et al.* 2013b) in my experiment. Additionally, since up regulation of immune genes with age is a hallmark of immunosenescence (Zerofsky *et al.* 2005), paired females expressing less of these stress genes may have slowed immunosenescence compared to those kept singly.

3.5.4 Phagocytosis differs by sex, and is sensitive to social stimulation in females

Previous literature has suggested that for virgin female *D. melanogaster* both circulating (Mackenzie, Bussiere and Tinsley 2011) and sessile (Horn, Leips and Starz-Gaiano 2014) haemocyte density is reduced at four and five weeks old respectively. However, we found no significant reductions in haemocyte numbers with age in either sex. Interestingly, Mackenzie et al (2011) reported that for males the number of circulating haemocytes remained the same at four weeks old, but in females there was a 20% decline in haemocyte density (Mackenzie, Bussiere and Tinsley 2011). The disparity between my own results and these are likely due to the fly strains used – genotype can have marked effects on the senescence of phagocytosis (Mackenzie, Bussiere and Tinsley 2011) and this study used Samarkand and Oregon R strains, whilst my own work used Dahomey. In terms of the ability of haemocytes to phagocytose foreign objects, my work tentatively echoes previous studies - age may act to

reduce the ability of cells to actively phagocytose in both sexes, but this is not a statistically significant effect (Mackenzie, Bussiere and Tinsley 2011; Horn, Leips and Starz-Gaiano 2014).

In young flies, males have a significantly higher proportion of phagocytosing haemocytes than females, but this did not translate into clear fitness benefits when infected with *P. aeruginosa*, *P. fluorescens* or *B. thuringiensis*. Since an effective immune response requires the orchestration of many discrete facets of the immune system (Lemaitre and Hoffmann 2007) simply having more actively phagocytosing cells may not be enough to impact positively on fitness, especially as parts of the immune system trade-off against each other (Cotter, Kruuk and Wilson 2004). Social stimulation results in a significantly lower proportion of phagocytosing cells for females, an effect that is true at both ages, but more obviously in older flies and which may be explained by the fact that females don't need to invest as much in standing immunity to still be better than males. This result is perhaps best discussed in tandem with gene expression data, where we looked at phagocytosis receptor *eater*. It is difficult to draw direct conclusions between these studies and my own in respect to social environment and sex as either a single sex was used and no information provided regarding rearing densities (Horn, Leips and Starz-Gaiano 2014) or flies were taken directly from population cages (Mackenzie, Bussiere and Tinsley 2011).

3.5.5 Conclusions

One of the primary observations that this work has elicited is the higher transcript levels of immune genes in males compared to females. If, as mentioned previously, immunosenescence results in a loss of control in these usually strictly regulated genes (Landis *et al.* 2004; Zerofsky *et al.* 2005), this may help to explain the overall pattern of reduced post-infection lifespan in males, compared to females. If, using this paradigm, lower gene expression levels represent greater control over the immune system and lower levels of immunosenescence, the effect of pairing that we observe for genes *TotA* and *TotM* indicates that pairing for females may act to reduce immunosenescence

and improve lifespan. This does not adequately explain why pairing also acts to improve post-infection lifespan in males, however. This may be because we have looked at a very small number of the total immune genes that are likely to be affected by ageing and we know that ageing affects the sexes differentially (Leech, Sait and Bretman 2017). Males may be suffering in other ways, for example in terms of reduced fecundity, or stress responses where, females do not. Relatedly, it is also a possibility they are simply having to work harder to ultimately achieve the same result. In the context of immunosenescence, the relative under-expression of these genes may mean that certain traits are ageing more slowly in paired females. The relative fitness strategy of the sexes may also be playing a part - males aim to mate as successfully as possible as many times as possible and therefore valuable resources are employed in the behavioural and neuronal plasticity required in integrate social cues into changes in reproductive investment, resulting in trade-offs with immunological functions. For females, the principle fitness strategy is to mate and then survive, meaning that investments into plastic immune responses are favoured. The highly significant sex differences that my post-infection lifespan, gene expression assays and phagocytosis experiments (with males always performing worse, regardless of social environment) indicate that post-infection lifespan and gene expression and phagocytosis are tightly linked. The reduced lifespan of male flies after infection may also be a function of their already reduced lifespan under normal conditions (Leech, Sait and Bretman 2017), which could be due to, in part, the accumulation of recessive deleterious alleles in the heterogametic sex (the unguarded X hypothesis) (Carazo *et al.* 2016).

One further possible explanation for the lack of a plastic response at the genomic level in males is that senescence has reduced their ability to respond to environmental cues appropriately. In humans, epigenetic drift is known to affect cellular plasticity in line with ageing (Li and Tollefsbol 2016) and models predict that as age increases phenotypic adjustments are disfavoured since any beneficial effects would have less time to take effect before death (Fischer *et al.* 2014). Even though male and female flies in my experiment were the same actuarial age, differential ageing rates could mean that the males were “biologically” older than females and may therefore have lost this ability. This

hypothesis could be tested by repeating the experiment with younger males and older females (where one would expect to see males display the same differential gene expression patterns as the females tested here, since they hadn't yet lost his ability, and females would lose the genetic plasticity we observed as they too reach an age where plasticity is no longer able to be maintained).

Future work will attempt to further elucidate the link between the social environment, immunity and ageing. It would be useful to look at gene expression levels of paired and single flies post-infection. This would help us to understand if any of the physiological benefits or disadvantages to social stimulation are constitutive, or if they are latent until an immunological stressor necessitates their activation. Other molecular assays to explore the mechanisms underpinning ageing, and in particular immunosenescence, would help to precisely explain how females differ from males in terms of immunological decline. Manipulating the age and density of cohabitants may also be a useful means of addressing the remaining questions we have regarding how stressful the different sexes find the respective social environments.

In conclusion, social stimulation acts to improve lifespan post-infection or does not affect it at all. Pairing is not deleterious to lifespan post bacterial infection in this instance, which is in contrast to other types of immune elicitor, such as injury, suggesting that DDP may be an important immunologically plastic fitness strategy under certain conditions in *D. melanogaster*. It is also possible that pairing in these circumstances slows immunosenescence of some traits in females, which is in contrast to males, which are generally more sensitive to social stress. Gene expression analysis of single and paired flies suggests a complicated situation whereby certain arms of the immune response are more socially-responsive than others (notably stress response genes in females) and males are succumbing to immune dysregulation quicker than females, regardless of social environment.

Chapter 4 – The *Drosophila melanogaster* microbiome; ageing and the social environment

Thanks to Xav Harrison for help with bioinformatics and data analysis, Kevin Hopkins for sample prep and sequencing and Laurin McDowall for sample collection and Zahra Nikakhtari for helping with infection feeding assays.

4.1 Summary

The microbiome is a complex assemblage of microorganisms that live symbiotically with their host. Its composition is determined by a network of regulatory pathways which integrates the host's immune, endocrine and neural systems. The social environment is known to influence a diverse range of host functions including immune, ageing and stress related aspects of physiology, often in sex specific ways. Given how these factors often influence each other, social environments could alter microbiomes both extrinsically through transmission and in an intrinsic manner (compositional alterations arising from more than just the physical transmission of bacteria). I set out to investigate how social isolation and stimulation altered the composition of the microbiome of *Drosophila melanogaster*. I hypothesised that, social stimulation would act to alter the composition of the whole-body microbiome (sampled using 16S rRNA sequencing), although these changes may only become apparent in older flies because previous work indicates that *D. melanogaster* become more sensitive to social environment with age. In addition, I predicted that the male microbiome would be more sensitive to changes in social environment, since social contact appears to disproportionately affect ageing and lifespan in cohoused males. My results indicated that this was the case, and the male microbiome is especially sensitive to changes in the social environment. I also investigated how the age of co-habitants affects the microbiome by housing ageing flies with constantly young co-habitants. Housing with young non-focal flies resulted in microbial profiles more closely related to that of young flies than those of the same biological age. In order to investigate the functional effect of changes in bacterial composition on the immune system, I orally infected old flies raised with and without social stimulation with the pathogen *Pseudomonas fluorescens.* I found that paired males were significantly worse at dealing with

infection than those kept singly, indicating that the shifts I saw in the microbiome of these flies may be having a serious deleterious effect on the immune system and therefore infection outcome.

4.2 Introduction

All metazoans play host to a microbial assemblage known as the microbiome (Adair and Douglas 2016). The collection of bacteria, fungi, viruses and archaea that form the microbiome can impact a diverse range of host traits including ageing (Heintz and Mair 2014), obesity (Ridaura *et al.* 2013), development (Hsiao *et al.* 2013), mental health (Carabotti *et al.* 2015) and mate choice (Venu *et al.* 2014). In light of the importance of the microbiome, an understanding of the factors that drive its composition and alteration is necessary. Factors known to influence the composition of the microbiome include diet (David *et al.* 2014), genetics (Goodrich *et al.* 2014) and microbe-microbe interactions (Buffie *et al.* 2015).The social environment (interactions that take place between conspecific individuals) is one such factor that has been shown to affect the structure of the microbiome, but so far evidence largely comes from group living mammals. In humans, cohabitation results in shared microbial profiles (Song *et al.* 2013), in ring-tailed lemurs (*Lemur catta*) taxonomic composition of the gut microbiota is influenced by social group (Bennett *et al.* 2016) and in wild baboons (*Simia hamadryas*) social group is the strongest single predictor of gut microbiome composition (Tung *et al.* 2015). The structural similarities in microbiome composition between organisms that occupy shared environs (Lax *et al.* 2014) are to be expected given that the horizontal transmission of bacteria is aided by social interaction (Kulkarni and Heeb 2007) and (often shared) diet reproducibly alters the microbiome (David *et al.* 2014).

Apart from the extrinsic horizontal transfer of bacteria, however, an additional intrinsic means of control is consistently being exerted by the host to shape the taxonomic composition of the microbiome via the immune system and other homeostatic pathways. This is a reciprocal relationship in which the

microorganisms in the intestine play a fundamental role in the training and induction of the immune system (Lemaitre and Hoffmann 2007; Belkaid and Hand 2014), and in turn, the immune system prevents the unchecked growth of symbionts and pathobionts to ensure a healthy host (Chow, Tang and Mazmanian 2011; Thaiss *et al.* 2016). In addition to the immune system, microbes are manipulated via neuroendocrine mechanisms, often in response to stress and via the action of hormones and neurotransmitters (Freestone *et al.* 2008). These factors therefore constitute both an external and internal means by which the social environment can alter the microbiome, but at present little research has addressed this issue. Given that the immune system plays a vital role in shaping the microbiome, any external stimuli that can exert an influence over immune function have the potential to affect the microbiota. My previous work addressed in Chapter 2 (Leech, Sait and Bretman 2017) has implicated the immune system in the differential ageing profiles of flies exposed to different social stresses and a host of other studies have expounded on the link between social stress and the immune system (Koto *et al.* 2015; Scotti *et al.* 2015; Cole 2014; Ellis and Carney 2011). The proposed connection between social stress, the immune system and the microbiome is supported by recent evidence which demonstrates that social stress in mice alters relative proportions of key bacterial genera and their metabolites, as well as altering gut immune gene expression (Foster, Rinaman and Cryan 2017; Galley *et al.* 2014).

An additional layer of complexity is added by the ageing process and its interaction with social stress and the immune system. As mentioned previously, our prior work has demonstrated that both actuarial and functional senescence are affected by social environment and that social interaction affects the sexes differently; male lifespan is reduced disproportionately by the presence of same-sex cohabitants when given an immune challenge (Leech, Sait and Bretman 2017). Dysbiosis of the microbiome is a common hallmark of ageing (Clark *et al.* 2015), so too various disease states. In *D. melanogaster* this is characterised by the expansion of the Gammaproteobacteria, which precedes the onset of intestinal barrier dysfunction (Clark *et al.* 2015) but likely comes after the chronic activation of f*oxo*, which disrupts immune homeostasis and

therefore compromises efficacy of the immune response in gut epithelial cells (Guo *et al.* 2014). Thus, the significant impacts we observe on health and lifespan in relation to differential social stimulation may be explained in part by the changes in microbiome composition and its interaction with ageing.

In this study I investigated the effect of social environment on microbiome composition and how this altered with age by comparing socially isolated flies to flies kept in same sex groups of 10, in both young and old animals. Here, I predicted that social contact would alter the microbiome, that this effect was likely to become stronger over time and that males were likely to be affected more so than females. I predicted that these changes would be mediated in an intrinsic manner caused by the differential levels of social stress across ages and sexes – in short, extrinsic effects would be explained by an effect of social environment that is the same across sexes and ages, whereas intrinsic manipulation of the microbiome would present as interactions between social environment, age and sex. I also investigated the effect of the age of the cohabiting fly by housing consistently young flies with an ageing focal, for which I hypothesised that cohabitation with young flies would result in younger bacterial assemblages associated with the focals. Finally, I predicted that these shifts were likely to have deleterious consequences on the ability of flies to survive an oral infection and so the implications of microbiome changes were examined in the context of immunity by providing socially stimulated and isolated flies with an oral infection.

4.3 Materials and Methods

4.3.1 Fly stocks and maintenance

Drosophila melanogaster wild type (strain Dahomey) were raised on standard sugar-yeast agar (SYA) medium containing 100g brewer's yeast, 100g sugar, 20g agar, 30ml Nipagin (10% w/v solution) and 3ml propionic acid per litre of medium (Bass *et al.* 2007). Flies for all experiments were raised and kept at a constant 25ºC and 50% humidity with 12 hour light – 12 hour dark cycle.

Population cages were fed weekly using 3 X 70ml SYA. Eggs were collected on purple grape juice agar plates (275ml water, 12.5g agar, 150ml red grape juice, 10.5ml Nipagin) placed in population cages. Eggs were allowed to develop into larvae for approximately 24 hours and then transferred to 7ml SYA vials supplemented with a loose live yeast paste at a density of 100 larvae per vial. Upon eclosion adult flies were sexed under ice anaesthesia and transferred to the relevant social environment.

4.3.2 Experimental design

4.3.2.1 Singles vs groups in co-aged groups

I employed a fully factorial experimental design consisting of eight groups: males or females, kept alone or in groups of 10 and either 11 days old or 49 days old when frozen (**Figure 17**A and B). These ages were chosen in line with previous work conducted in Chapters 2 and 3, which showed that the effects of age become apparent around this time. In contrast to the work in Chapters 2 and 3 which used the addition of a single non-focal in socially stimulated groups, here I used groups of 10 (9 non-focals) to exacerbate the effect of social environment. Food was changed weekly and focal flies in groups of 10 were identified at the start of the experiment with a small wing clip. Flies kept alone were also wing clipped in order to control for any effect wing-clipping may have on the microbiome.

4.3.2.2 Effect of mixed aged groups

An additional social environment was used in order to identify the effect that the age of the cohabiting flies has on a focal fly's microbiome. To do this males and females were housed in groups of 10 and a focal fly was identified at the start of the experiment with a small wing clip, as for the previously described social environments (**Figure 17**C). However, in contrast to the other groups, focal flies were transferred to a new vial containing nine 1 day old non-focal flies of the same sex weekly. Consequently, these focal flies were constantly in the presence of young cohabitants always between the age of 1 and 7 days old. For clarity, these flies are named 'babysitters', since an older fly was kept in the

presence of younger ones. Focal flies were aged to 49 days, as in the prior experiment.

4.3.3 DNA extraction and 16s rRNA sequencing

For sequencing, each biological replicate was a pool of 10 flies, with eight replicates per social environment. DNA was extracted using the Mobio PowerSoil® DNA Isolation Kit according to the instructions provided and quality checked using NanoDrop (ND-1000). The V4 region of the 16S gene was then PCR amplified in triplicate using 5x HOT FIREPol Blend Master Mix and indexed primer sets to give unique combination of barcodes to each sample. Reactions were set up in the following manner: 2µl Master Mix, 4µl PCR-grade water, 3µl of the 2µM primers added to each well, and 1µl of gDNA. PCR cycling conditions were as follows: 95°C for 15 mins, 95°C for 20s, 50°C for 1 min, 72°C for 1 min x 28 cycles, 72°C for 10 mins and held at 4°C. The PCR product was checked on 2% agarose gel with Gel Red and replicates were pooled prior to sample clean-up using Ampure XP beads (replicates that failed to amplify were removed). Resulting DNA was then further quality checked using Qubit (to determine the concentration of each sample) and TapeStation (to assess primer dimers) before being sequenced using paired end 250bp v2 chemistry on an Illumina MiSeq.

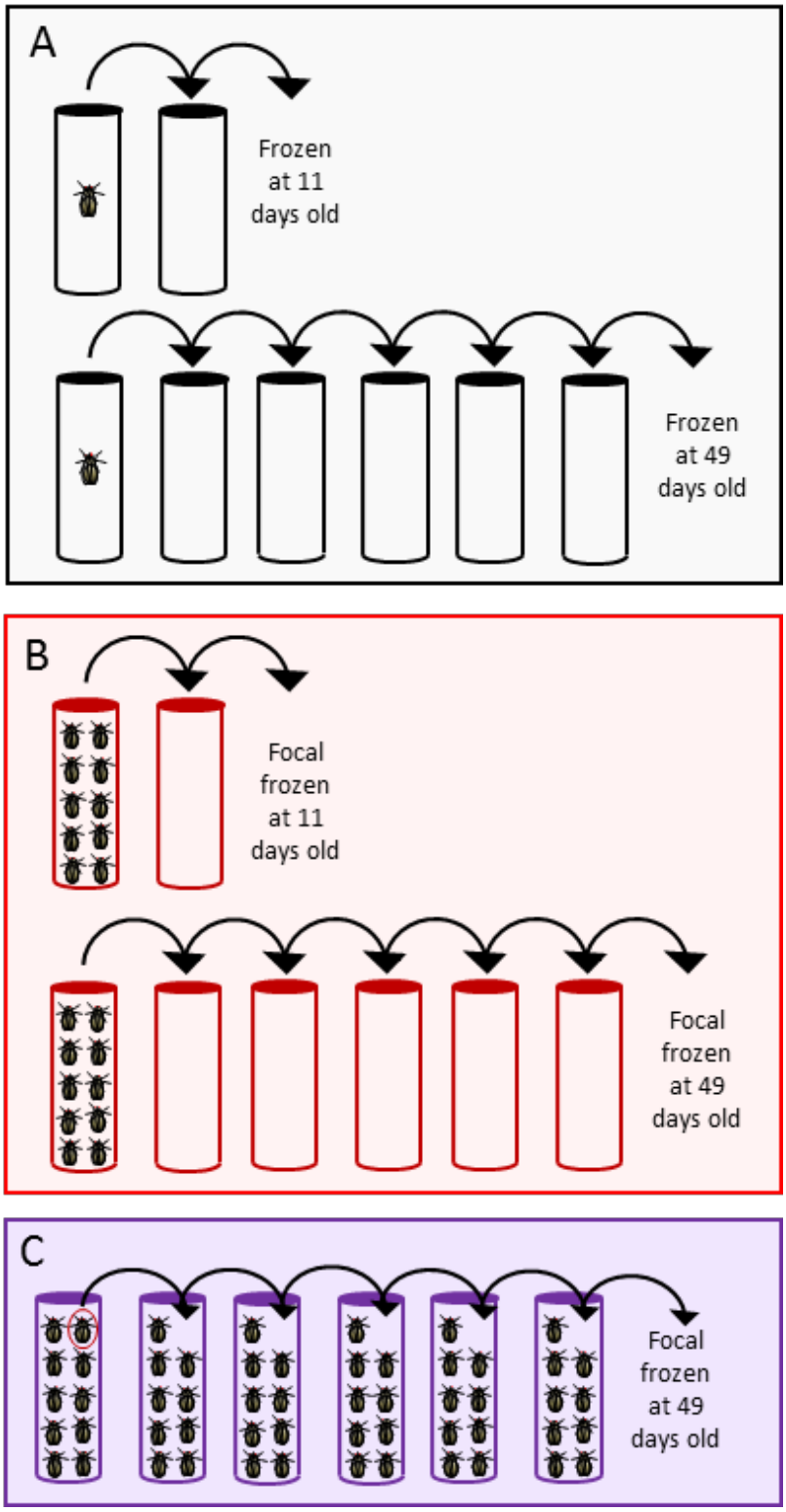

Figure 17 Males and females were kept singly and sampled at 11 days old or 49 days old (A) with food changed weekly throughout, or in groups of 10 (B). Babysitter flies were housed with non-focal flies replaced each week with a fresh batch of young flies (1-6 days old) (C) and frozen at 49 days old.

4.3.4 Bioinformatics

Post-sequencing bioinformatics were conducted using mothur (version 38.2) (Schloss *et al.* 2009), largely following the protocol detailed by Schloss *et al* (2013). Briefly, contigs were assembled from paired end reads, aligned to the SILVA SEED reference database, filtered for potential chimeras and clustered into Operational Taxonomic Units (OTUs). Sequences from Archaea, chloroplasts, and mitochondria were identified and removed. The SILVA database was also used for taxonomic assignment of OTUs. The average library size was ~40k reads per sample after passing quality control. The resulting shared file was finally exported as a .biom file for statistical analysis.

4.3.5 Oral infection assay

Pseudomonas fluorescens (DSMZ 50090) was grown for 48 hours at 25ºc without shaking, centrifuged, and the bacterial pellet was re-suspended in a solution of 5% sucrose 5% yeast. To administer the bacteria, 30µl was pipetted onto 7ml agar gel ("no-food" vial). Males and females were raised either singly (males n = 34, females n = 39) or with a same sex partner (males n = 31, females n = 30) to the age of 50 days (food and non-focal flies were changed weekly) before being starved for 3 hours prior to infection and placed into the no-food vial to feed on the bacteria solution. Pairs were used in this experiment, rather than groups of 10, since previous work had shown a single partner is enough to elicit both a sperm competition response (Bretman *et al.* 2010) and changes in ageing patterns (Leech, Sait and Bretman 2017). 30µl of fresh bacterial-sucrose solution was added for 3 days, prior to being transferred onto standard SYA and observed every 24 hours until death for 1 week. Sham controls were included and subsequently removed from the analysis since no flies died.

Social interaction does not alter appetite in *D. melanogaster* (Ja *et al.* 2007), but this study did investigate groups consisting of different ages and using food infected with bacteria. Therefore, in order to confirm that the observed patterns

were not due to differences in the amount of infected food eaten between treatments, the CAFE assay was used to quantify how much food was eaten by flies kept in the same groups (Ja *et al.* 2007). Briefly, food was made and infected as above, before the liquid medium was filled into glass capillary tubes. Flies were kept singly or in same-sex pairs and aged to 50 days (as per all previous work) before being transferred into no-food vials containing agar gel to be starved for 3 hours prior to infection. After 3 hours, capillary tubes containing bacterial solutions and topped with mineral oil to avoid evaporation were made accessible to the flies. To ensure that only focal flies from social treatment groups could eat the food, non-focals were kept in transparent Eppendorf tubes with a net lid, inside no-food vials. This ensured they were unable to feed, but that focal flies were still able to detect visual, auditory and olfactory cues from the cohabitant. Flies were allowed to feed for 24 hours before the amount of food eaten was measured and normalised using evaporation controls that contained no flies.

4.3.6 Statistical Analysis

All statistical analysis was conducted using R (version 3.3.2) using the phyloseq (McMurdie and Holmes 2013), vegan (Dixon 2003), ggplot2 (Wickham 2009), ape (Paradis, Claude and Strimmer 2004), DESeq2 (Love, Huber and Anders 2014) and lme4 (Bates *et al.* 2015) cran packages. Prior to analysis contaminating OTUs present in the negative controls were removed and sequences were rarefied in order to normalise library sizes.

4.3.6.1 Alpha Diversity

Alpha diversity was first estimated using the Chao1 species richness indicator (Chao 1984) and the resulting Chao1 values were analysed using GLM with social environment (single vs grouped), sex and age as fixed factors. Models were simplified from the full model using Analysis of Deviance (AOD), the final model being when no further terms could be removed without significantly reducing the model’s descriptive power.

4.3.6.2 Beta Diversity

Non-metric multidimensional scaling (NMDS) was used to partition distance matrices among sources of variation and PERMANOVA was used examine the effect of social environment, sex and age on species richness and abundance between groups.

4.3.6.3 Relative abundance of bacterial species

To identify specific bacterial species that alter significantly in their relative abundance between groups we used DESeq2 (Love, Huber and Anders 2014) to first identify OTUs of interest that change significantly between groups (of which there were 2) and then blasted these against the GreenGenes database to identify to species level (DeSantis *et al.* 2006).

4.3.6.4 Oral infection assay

In order to test for differences in the amount of food eaten between groups, firstly, one was added to all values in order to make all values positive. The amount of food eaten was subsequently analysed using a GLM with quasi-Poisson errors to account for over dispersion, the full model was subtracted using the AOD, as with previous data. Individual groups of interest were finally compared to each other using Mann-Whitney U test, since the data were not normally distributed. The results were corrected for multiple testing using the Bonferroni method. For post-infection lifespan, due to the data being highly censored (a high number of individuals were still alive at the end of the one week testing period) a chi squared test was used to determine if the number of flies that were dead at the end of one week differed by sex and social environment.

4.4 Results

4.4.1 Singles vs groups alpha diversity

Alpha diversity was measured using the Chao1 species richness indicator which was then entered into a GLM with social environment, sex and age as fixed factors. There was a significant interaction between social environment and age (**Table 10**) - grouping had the effect of increasing alpha diversity, but only for older flies. There was no effect of sex.

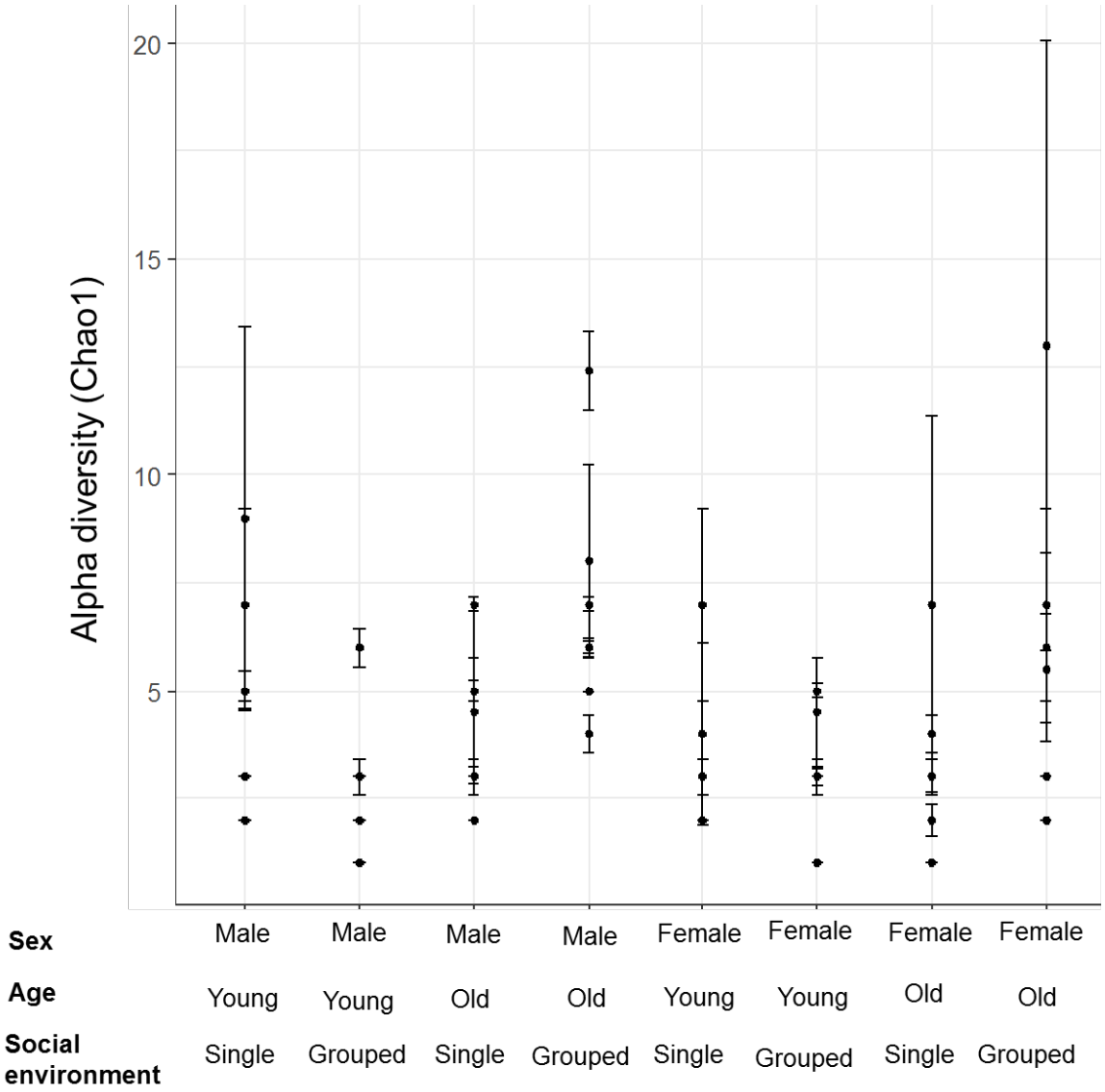

Figure 18 Chao1 Alpha diversity index scores for male and female, grouped or single, young and old flies.

Table 10 GLM AOD results of Chao1 values to assess alpha diversity. Social environment, age and sex were entered as fixed factors.

Explanatory Variable	**Test Statistic**	**df**	***P***
Sex	2.578	1, 60	0.114
Social environment*Age	5.956	3, 60	**0.001**

4.4.2 Singles vs groups beta diversity

In terms of beta diversity, there is a highly significant 3 way interaction between social environment, age and sex (**Table 11**). In young flies (11 days) there is no difference in NMDS values between any of the groups (**Figure 19**), but in old flies (49 days) there is a clear separation between the groups (**Figure 20**). Grouped males are most different to single females, and the two remaining groups, single males and grouped females cluster closely together in between.

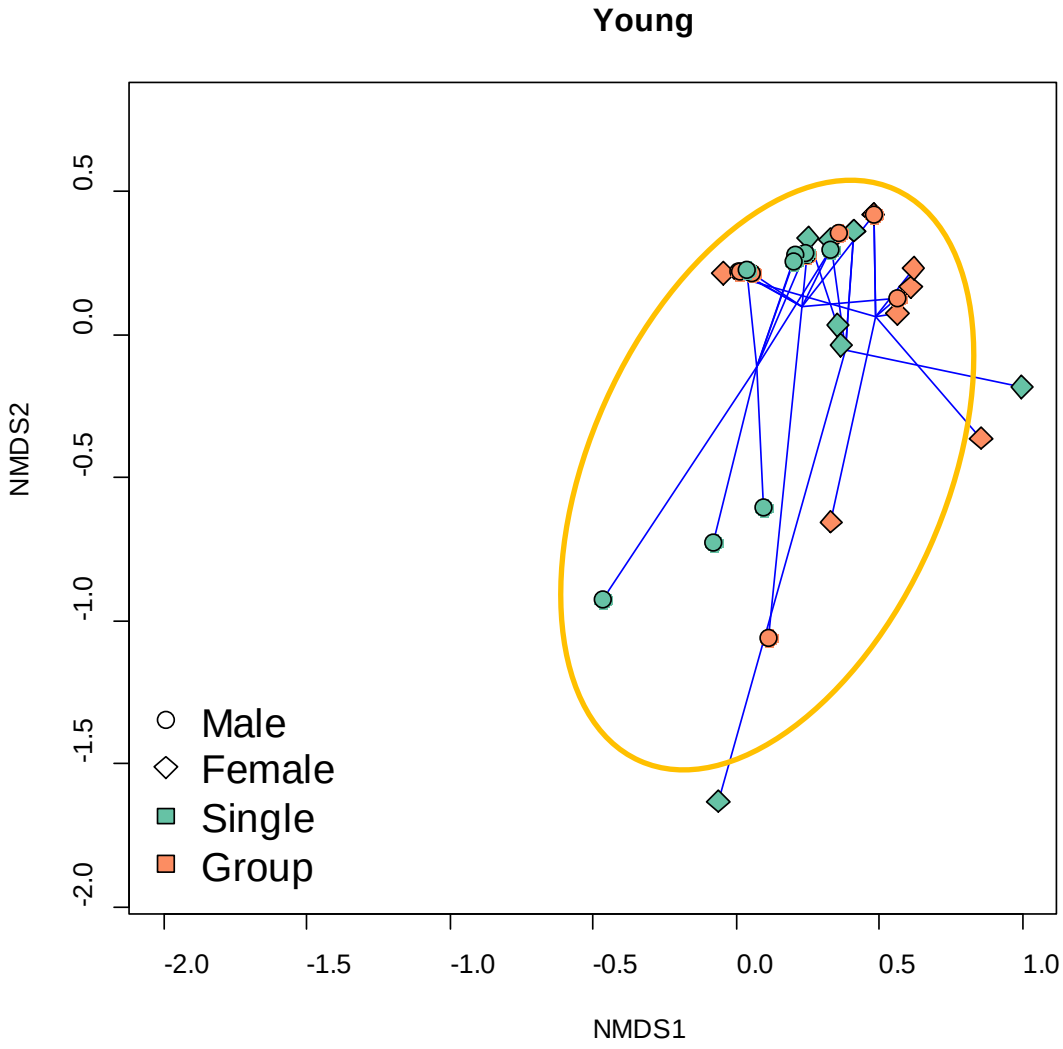

Figure 19 NMDS plot for young male and female flies kept alone or in groups of 10. All points fall within a single distinct area indicating the little variation observed cannot be explained by sex or social environment.

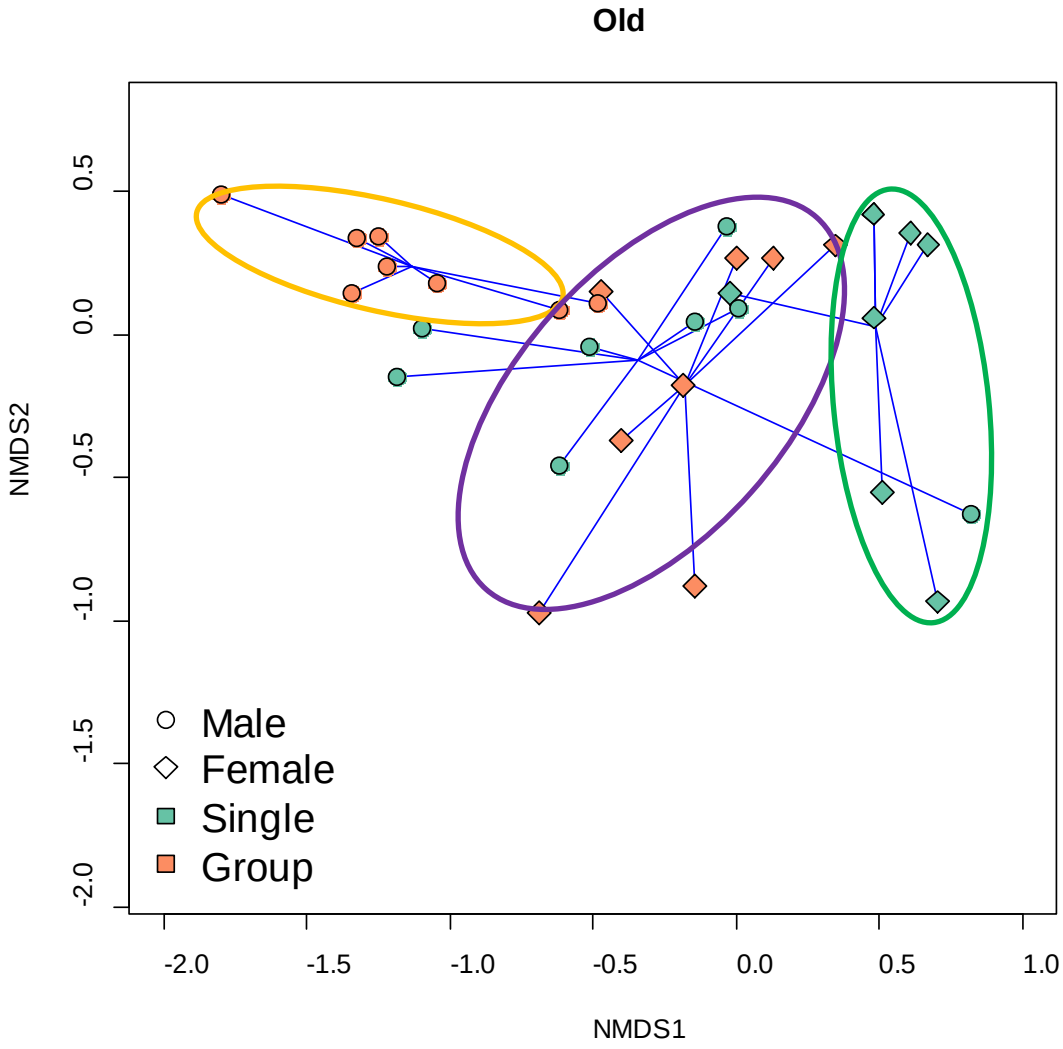

Figure 20 NMDS plot for old male and female flies kept alone or in groups of 10. The points fall within 3 distinct areas denoted by the yellow (grouped males), purple (single males and grouped females) and green circles (single females).

Table 11 PERMANOVA results to assess beta diversity for singles flies and groups. Social environment, age and sex were entered as the explanatory variables.

Explanatory Variable	df	F	*P* value
Social environment	1, 63	15.868	**<0.0001**
Sex	1, 63	68.659	**<0.0001**
Age	1, 63	79.189	**<0.0001**
Social environment*Sex	1, 63	10.14	**<0.0001**
Social environment*Age	1, 63	15.322	**<0.0001**
Sex*Age	1, 63	66.467	**<0.0001**
Social environment*Sex*Age	1, 63	10.769	**<0.004**

4.4.3 Mixed age groups alpha diversity

4.4.3.1 Alpha Diversity mixed age groups versus young flies

Since babysitter flies can be considered intermediates in terms of age (they are biologically old flies, but have lived with constantly young cohabitants), it is necessary to compare them to both the grouped young flies, and the grouped old flies from the previous experiment (all flies came from the same cohort). When compared to grouped young flies there is no effect of social environment or sex (**Table 12**), and alpha diversity appears to be quite low, with little variance between replicates (**Figure 21**).

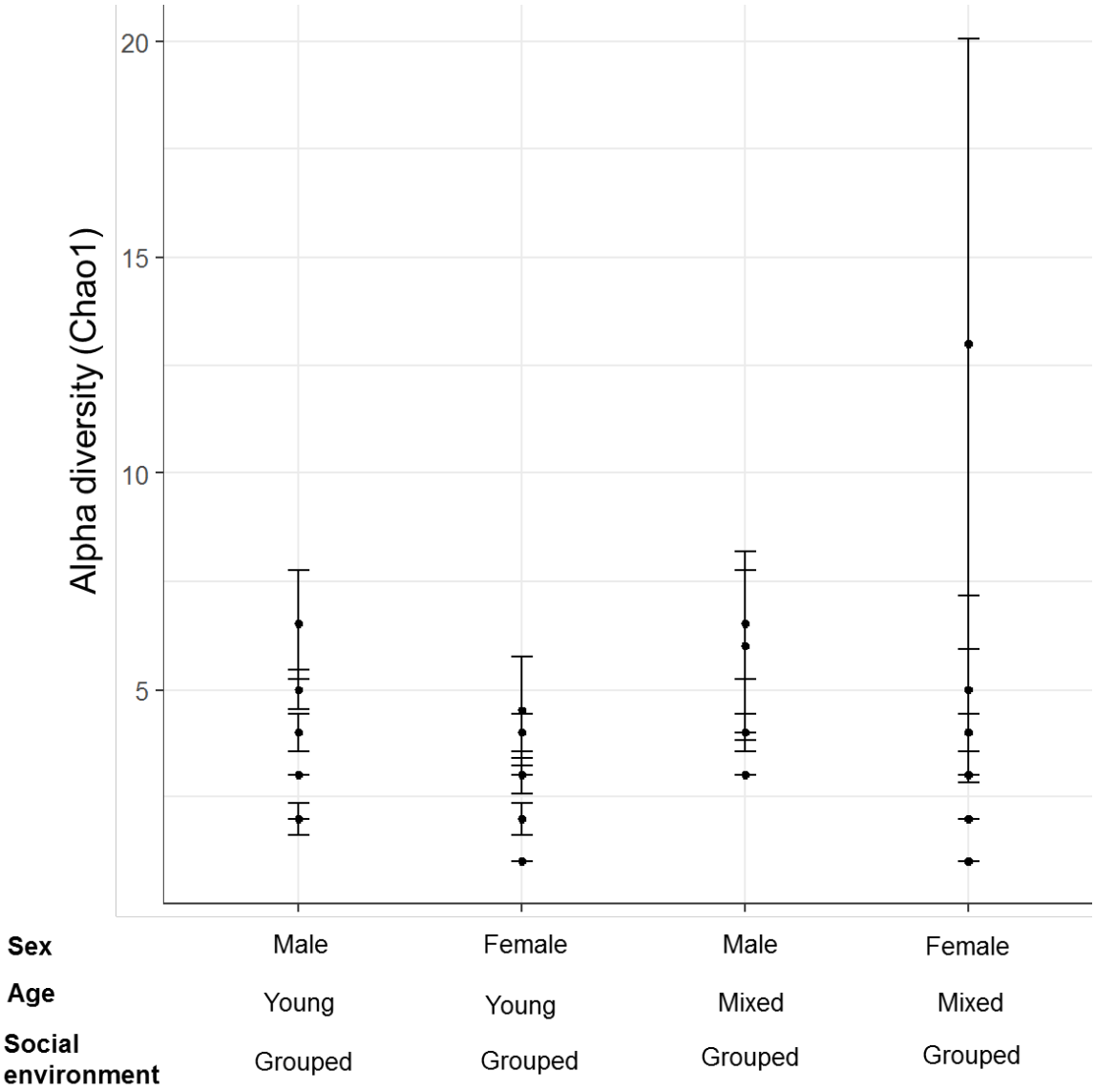

Figure 21 Chao1 Alpha diversity index scores for young (11 day old) male and females kept in groups of 10, compared to babysitter flies – flies aged to 49 days old in the presence of consistently young cohabitants.

Table 12 GLM AOD results of Chao1 values to assess alpha diversity for babysitter flies versus young flies. Social environment and sex were entered as fixed factors.

Explanatory Variable	**Test Statistic**	**df**	***P* value**
Social environment	2.41	-1, 31	0.131
Sex	0.419	1, 29	0.523
Social environment*Sex	0	-1, 29	1.000

4.4.3.2 Alpha diversity of mixed age groups flies versus old flies

In contrast to babysitter flies compared to young flies, when we compare babysitters to old grouped flies (that is to say flies of the same biological age), there is a significant effect of social environment (**Table 13** GLM AOD results of Chao1 values to assess alpha diversity for babysitter flies versus old flies. Social environment and sex were entered as fixed factors.**Table 13**) – flies cohoused with young non-focals display significantly lower alpha diversity than flies kept with similarly aged cohabitants (**Figure 22**). This effect is the same regardless of sex.

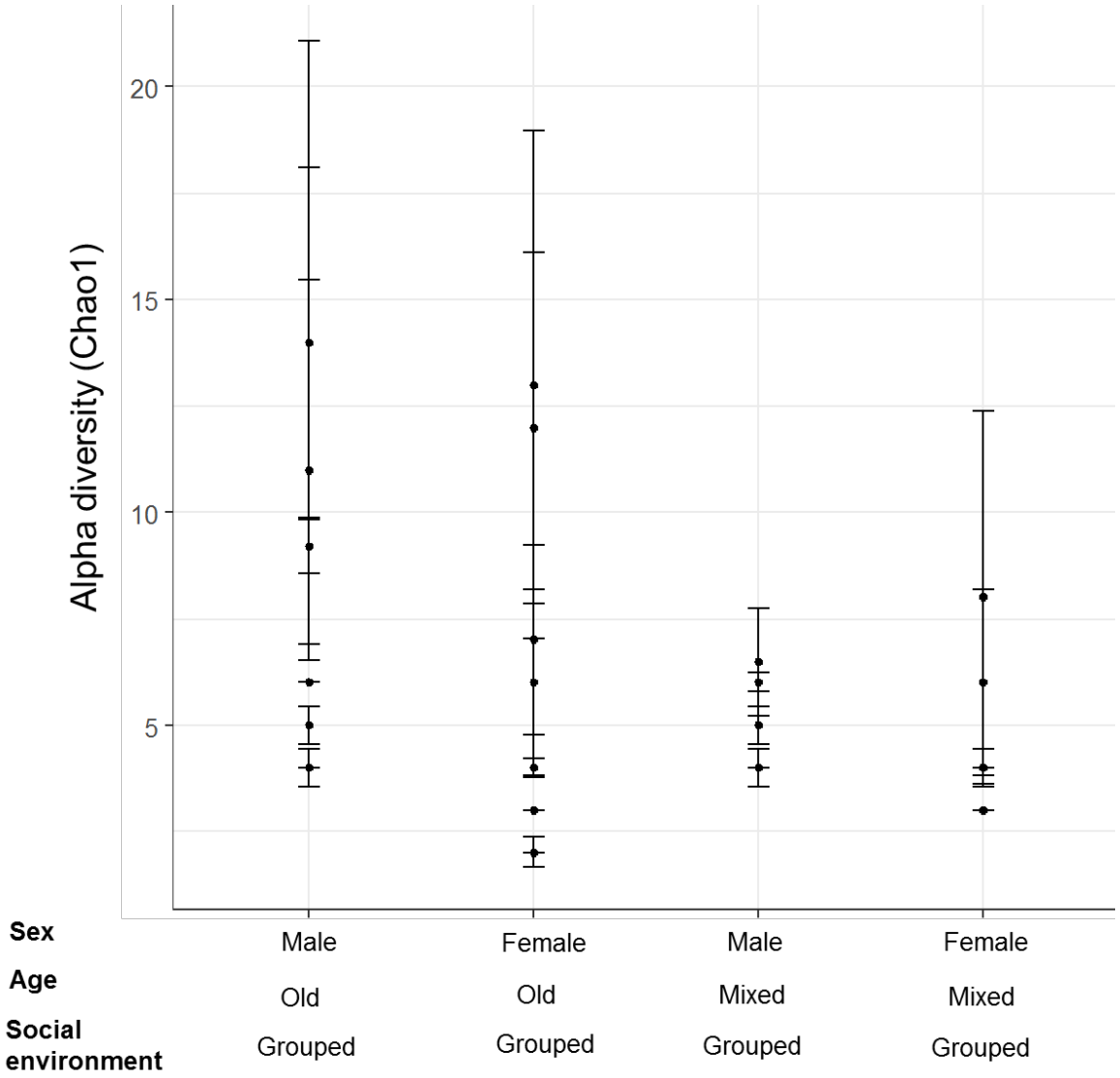

Figure 22 Chao1 Alpha diversity index scores for old (49 day old) male and females kept in groups of 10, compared to babysitter flies – flies aged to 49 days in the presence of 9 consistently young cohabitants.

Table 13 GLM AOD results of Chao1 values to assess alpha diversity for babysitter flies versus old flies. Social environment and sex were entered as fixed factors.

Explanatory Variable	Test Statistic	df	*P* value
Social environment	5.3342	1, 31	**0.028**
Sex	1.801	1, 29	0.190
Social environment*Sex	0.449	1, 29	0.508

4.4.4 Mixed age groups flies beta diversity

4.4.4.1 Beta diversity of mixed age groups flies vs young flies

When compared to young flies, there is a significant interaction between social environment and sex (**Table 15**). This interaction is explained by the clear clustering of babysitter males separately from the female babysitter flies and both young groups (**Figure 23**).

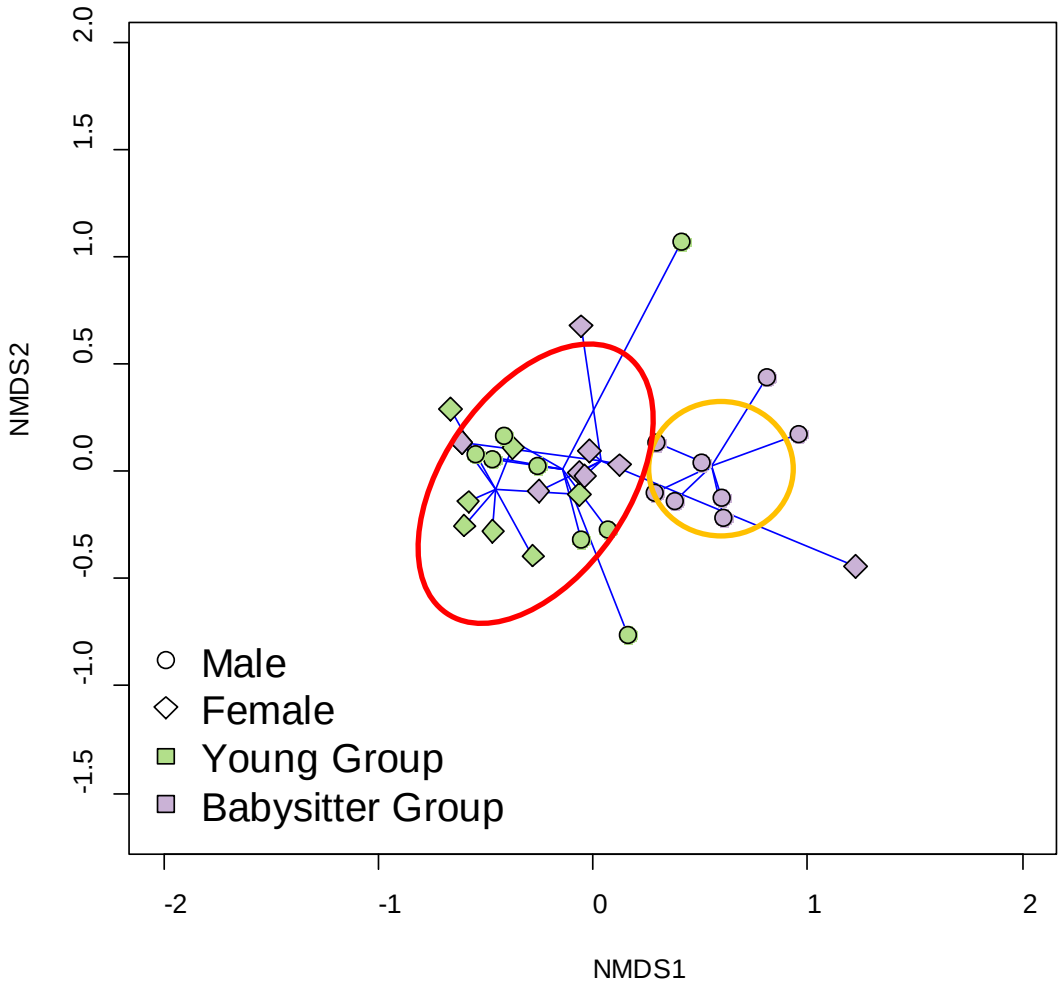

Figure 23 NMDS plot for young (10 days old) male and females kept in groups of 10, compared to babysitter flies – flies aged to 49 days in the presence of 9 consistently young cohabitants. Babysitter males are circled in yellow, whilst the babysitter females, and young male and female flies all appear similar.

Table 14 PERMANOVA results to assess beta diversity for babysitter flies and young (grouped) flies. Social environment (babysitter or not) and sex were entered as explanatory variables.

Explanatory Variable	**df**	**F**	***P* value**
Sex	1, 31	8.1557	**<0.002**
Social environment	1, 31	22.7141	**<0.0001**
Social environment*Sex	1, 31	7.5333	**<0.007**

4.4.4.2 Beta diversity of mixed age groups flies vs old flies

A similar pattern emerges when babysitter flies are compared to old flies – there is a significant interaction between social environment and sex, however, this interaction is explained by the old males falling separately from the other groups, rather than the babysitter males (**Figure 24** and **Table 16**).

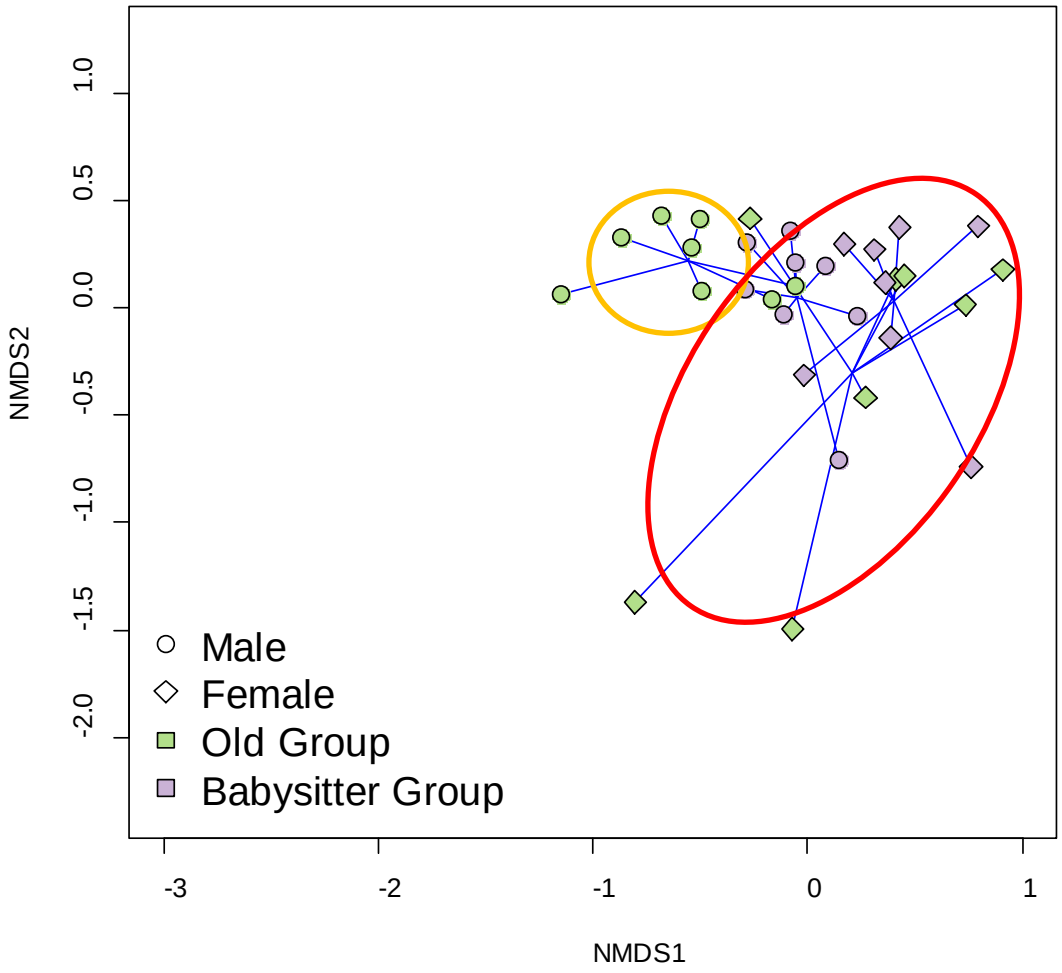

Figure 24 NMDS plot for old (49 day old) male and females kept in groups of 10, compared to babysitter flies – flies aged to 49 days in the presence of 9 consistently young cohabitants. Old males fall separately to the rest of the groups (yellow circle), indicating a distinct difference in microbial composition, whilst the others are more variable but less different to each other (red circle).

Table 15 PERMANOVA results to assess beta diversity for babysitter flies and old (grouped) flies. Social environment (babysitter or not) and sex were entered as explanatory variables.

Explanatory Variable	df	F	*P* value
Sex	1, 31	93.473	**<0.0001**
Social environment	1, 31	36.746	**<0.0001**
Social environment*Sex	1, 31	39.959	**<0.0001**

4.4.5 Relative bacterial abundance

4.4.5.1 Singles vs groups

Once quality controlled, there were about 14 different OTUs detected. When comparing relative levels of individual species of bacteria for the single and grouped flies there was no significant effect of social environment – grouping flies did not result significant changes in specific bacterial species.

4.4.5.2 Effect of sex

There were, however, significant effects of sex in old flies regardless of social environment (**Table 16**). Old grouped females have significantly lower levels of *Lactobacillus plantarum* compared to males and the same is true of old single females, who also have lower levels of *Lactobacillus brevis* (*L. brevis*) compared to males.

Table 16 Relative abundance of bacterial species as identified by DESeq2 analysis, looking at the effect of sex by comparing males and females of the same age and social environment. Table only shows species exhibiting significant changes in abundance.

Comparison	**Species**	**Log2 Fold Change**	**Adjusted *p* value**
Female Group Old vs Male Group Old	*Lactobacillus plantarum*	-4.693649	**0.026**
Female Single Old vs Male Single Old	*Lactobacillus plantarum*	-6.436006	**0.0001**
	Lactobacillus brevis	-4.067376	**0.036**

4.4.5.3 Effect of age

Effects of age were only observed in males, for both single and grouped flies. The effects were more severe for grouped males, where young flies had significantly less *L. plantarum* and *L. brevis* than old flies of the same social environment. Single flies exhibit the same pattern, but to a lesser extent (**Table 17**).

Table 17 Relative abundance of bacterial species as identified by DESeq2 analysis, looking at the effect of age by comparing young and old flies of the same sex and social environment. Table only shows significant results.

Comparison	**Species**	**Log2 Fold Change**	**Adjusted *p* value**
Male Group Young vs Male Group Old	*Lactobacillus plantarum*	-6.138006	**0.0001**
	Lactobacillus brevis	-5.784371	**0.0001**
Male Single Young vs Male Single Old	*Lactobacillus plantarum*	-4.681618	**0.013**
	Lactobacillus brevis	-4.395021	**0.013**

4.4.5.4 Mixed age groups

For babysitter flies, only when comparing them to young flies were there any significant differential levels of specific bacteria between the groups. Babysitter flies, both male and female, exhibited higher levels of *L. brevis* and for males, so too *L. plantarum* (**Table 18**).

Table 18 Relative abundance showing the log2 fold change in specific bacterial species between groups, looking at the effect of social environment by comparing babysitter flies to both young and old grouped flies.

Comparison	Species	Log2 Fold Change	Adjusted *p* value
Female Babysitter vs Female Group Young	*Lactobacillus brevis*	4.035	0.009
Male Babysitter vs Male Group Young	*Lactobacillus brevis*	4.477	0.002
	Lactobacillus plantarum	4.101	0.009

4.4.6 Oral infection assay

Paired males were significantly less likely to survive infection with *P. fluorescens* than those that were kept alone (X^2 = 8.294, df = 1, p = 0.004), but there was no effect of social environment for females (X^2 = 0.699, df = 1, p = 0.403) (**Figure 25**). The CAFE feeding assay data were first analysed using a GLM; there was a significant interaction between sex and treatment (F= 6.997, df= 1, 134, p<0.01) which indicates that pairing acts to increase the amount of food eaten in females but not males (**Figure 26**). Groups were subsequently divided by sex and analysed using a Mann-Whitney U test to confirm the results of the GLM. Indeed, for males, pairing did not change the amount of food consumed (X^2= 14.312, df= 1, p=0.852), however in females paired females ate more (X^2= 25.375, df= 1, p=0.044). Therefore a difference in the bacterial dose ingested does not account for why paired males were significantly worse at surviving an oral infection.

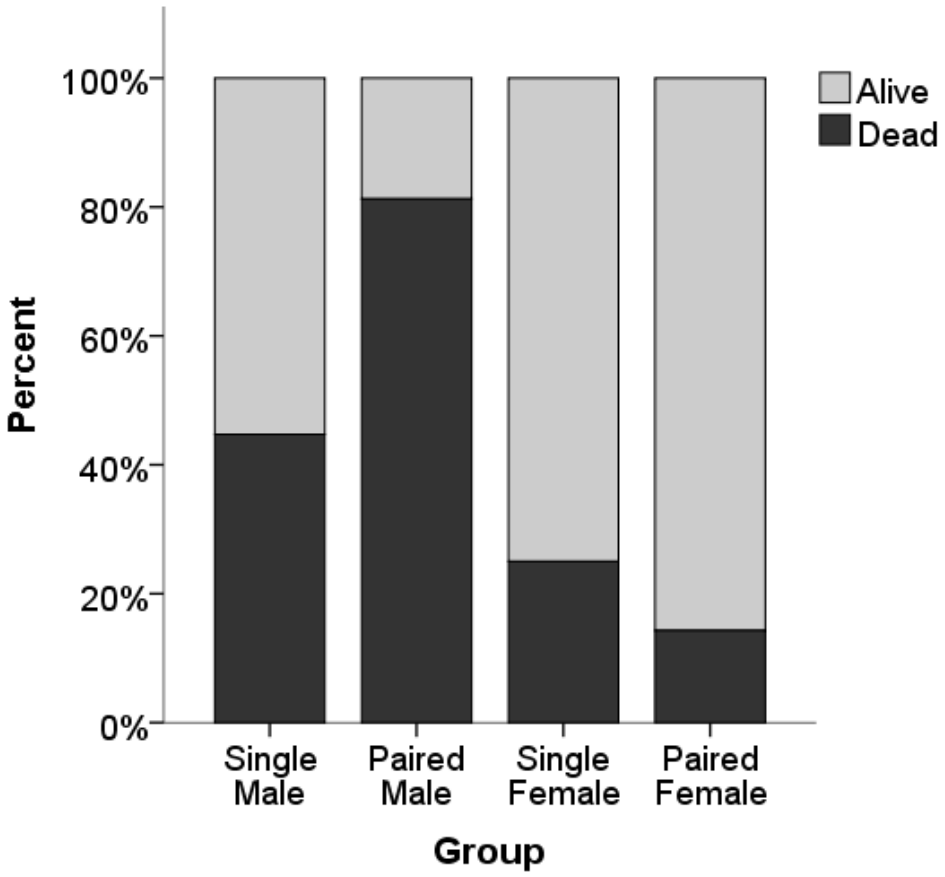

Figure 25 Percentage of flies that were alive or dead at the end of a one week period, after oral infection with *P. fluorescens*. Flies were kept either alone or in same sex pairs and were 52 days old at the time of infection.

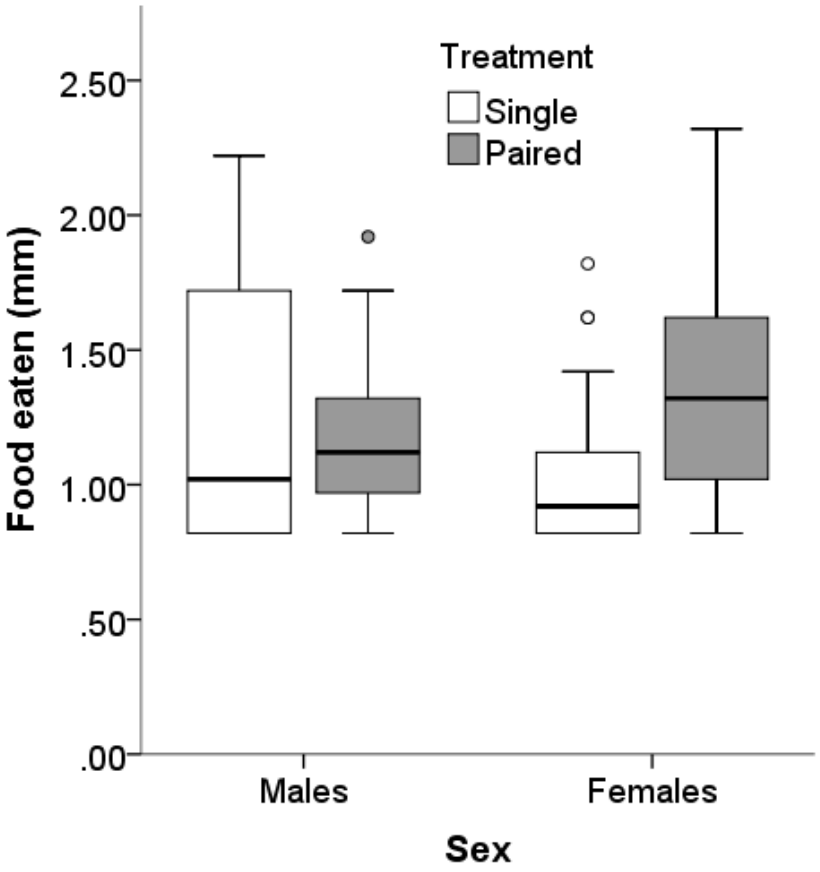

Figure 26 The amount of bacteria-infected food eaten by males and females kept singly and in same-sex pairs. Single flies are shown in white and pairs are in grey.

4.5 Discussion

The results from this study indicate that social environment can affect the microbiome of *D. melanogaster*, and that this effect is dependent on the flies age – only in older flies do these effects become apparent. The extent of the change is dependent on the sex of the fly, with males experiencing greater shifts in species richness and abundance than females, when grouped. I found

that older flies had greater species richness within samples, an effect that was exacerbated by grouping. An indication of the functional effect of these changes was provided by an oral infection assay which showed that grouped males are worse at surviving infection than males kept alone.

4.5.1 Social environment interacts with age to change the microbiome

An increase in microbial load with age has been described previously in *D. melanogaster* (Ren *et al.* 2007), although this study makes no mention of relative abundances or species richness, only indicating that both anaerobic (for example *Lactobacillus* species) and aerobic species (for example *Acetobacter*) proliferate with age (Ren *et al.* 2007), without a reduction in lifespan. Changes in abundance with age occur from mid-life, with all bacterial classes increasing significantly and resulting in distinct shifts in microbial community structure as the flies age (Clark *et al.* 2015).

Wong et al (2011) found little variation in number of OTU reads between 3 day old and 5 week old males and females, which mirrors my own alpha diversity results for flies kept singly. There was a shift in relative abundance from *Lactobacillus fructivorans* which dominated in 3-5 day old flies to *Acetobacter pomorum* once flies were aged to between 3 and 5 weeks, a result tentatively explained by the increasingly oxic environment of the ageing gut (Wong, Ng and Douglas 2011). My own study found that young males (regardless of social environment) had significantly lower amounts of *L. plantarum* and *L. brevis* compared to older males, in a similar manner to Wong et al (Wong, Ng and Douglas 2011), however I did not find a simultaneous increase in *Acetobacter* species. This may be due to differences in the structural integrity of the ageing gut between fly strains – different strains are known to display different senescence profiles (Ziehm, Piper and Thornton 2013) indicating that Dahomey flies may age slower than the Canton-S used by Wong et al (2011). This could also go some way to explaining why only males experience a significant change in *Lactobacillus* species compared to both sexes in work conducted by Wong *et al* (2011), since there is a known

interaction between sex, strain and ageing profile (Ziehm, Piper and Thornton 2013). It is, however, difficult to make any solid comparisons between these studies as no information is given as to the housing conditions of the flies used and only guts were subjected to 16SrRNA sequencing as opposed to whole flies in this study.

Changes in between-sample species richness and abundance are being driven by the three way interaction between sex, age and social environment. In the young group there are no effects of social environment or sex, but at 49 days old sex and social environment explain the majority of the observed variation. Males and females respond to social environment differently once aged, with grouped males experiencing more profound shifts in community structure. This therefore indicates that, the direct transfer of bacteria is less responsible for these changes, and indirect, internal mechanisms are the primary cause. One explanation for the lack of observed differences in young flies may be that immunosenescence is, at least in part, responsible for these changes, and they therefore are not apparent whilst the flies are young and healthy. As flies age, transcriptional control of many genes becomes deregulated (Grotewiel *et al.* 2005). *foxo* is a transcriptional activator that has been implicated specifically in immunosenescence of the gut (Guo *et al.* 2014; Rera, Clark and Walker 2012) and its chronic activation deregulates immune function in enterocytes, with the effect of increasing *Rel* expression and inhibiting PGRP-SC2 and PGRP-SC1a (Guo *et al.* 2014). PGRP-SC2 and PGRP-SC1a are peptidoglycan recognition proteins that sequester and catalyse peptidoglycan as well as acting as feedback inhibitors for the IMD/Rel immune pathways (Lemaitre and Hoffmann 2007). Inhibition of these genes therefore allows the unchecked proliferation of gut bacteria (Erkosar and Leulier 2014; Clark *et al.* 2015), which ultimately precedes and induces epithelial dysplasia (the unchecked proliferation of intestinal stem cells and associated accumulation of mis-differentiated polyploid cells) (Buchon *et al.* 2009a; Clark *et al.* 2015), eventually resulting in death. However, gene expression analysis of *foxo* in flies aged to 49 days and kept either singly or in same sex pairs did not differ between social environment (Chapter 3),

indicating that if social environment is altering immunosenescence of the gut, it is being mediated in a *foxo* independent manner (Chapter 3).

The interaction between sex and social environment indicates that, rather than changes in overall microbial community structure being caused simply by the horizontal transfer of bacteria between cohabitants (extrinsic factors), intrinsic physiological factors may be driving the disproportionately severe changes in the microbiome of grouped males. This pattern is mirrored by our own previous work which determined that lifespan was reduced drastically for paired males compared to those kept singly, but less so for females (Leech, Sait and Bretman 2017). Males respond to sexually competitive environments by increasing mating duration and therefore reproductive fitness (Bretman, Fricke and Chapman 2009). This response requires the integration of multiple sensory cues as well as complex learning and memory pathways (Rouse and Bretman 2016) in order to plastically alter behaviour (Bretman *et al.* 2011), but comes at the cost of lifespan and successful later-life mating attempts when compared to control males (Bretman *et al.* 2013). If reproduction-related investment trades-off with immunosenescence, the result could be quicker ageing and more severe microbial dysbiosis in grouped males. A study by Blum et al (Blum *et al.* 2013) highlights the contribution of external inputs, i.e. the replenishment of microorganisms via a food source, rather than internal maintenance mechanisms, in maintaining the microbiome, however it seems unlikely that these results are an artefact purely of the food-based microbial communities since all groups were fed identical diets.

Social stress may also play a key role in determining the structure of the microbiota. As mentioned previously, males and females likely find social isolation or stimulation differentially stressful. In mice, aggression between males affects colonic mucosa-associated bacterial communities in as little as 2 hours, reducing the relative abundance of key genera including *Lactobacillus* (Galley *et al.* 2014). The action of stress in this study is manipulating the microbiome in a different direction than my own work (older flies have more

Lactobacillus than younger flies and males have more than females, in line with lifespans), but this study does not used aged animals and therefore doesn't take senescence into account. In *D. melanogaster* males are more aggressive to each other than females (Nilsen *et al.* 2004), but the effect that this has on the microbiota is unknown. In mammals, the gut-brain axis (Montiel-Castro *et al.* 2013) links endocrine, immune, neural and gastrological functions in a multidirectional network of crosstalk mediated by neurotransmitters, endocrine hormones and cytokines (El Aidy, Dinan and Cryan 2014). Just as stress itself can manipulate the composition of the microbiome, the microbiome can, in a reciprocal manner, influence responses to stress (Foster, Rinaman and Cryan 2017). It is difficult to interpret my own results in the context of the gut-brain axis without further experiments to distinguish between the individual roles of the endocrine, immune and neurological systems, but examining the interplay between these systems would be likely be a fruitful avenue of future research.

4.5.2 Living with young cohabitants results in younger microbial profiles

As discussed, ageing results in the eventual dysbiosis of the microbiome via the dysregulation of key immunological transcriptional activators with age (Clark *et al.* 2015; Rera, Clark and Walker 2012). This work suggests that age-related dysbiosis can be offset, at least in part, by manipulating the age of cohabiting individuals within a social environment. In terms of alpha diversity, babysitter flies (focals aged with consistently young non-focals) were more similar to young flies (of the same sex) than to flies of the same biological age that were kept with other age-matched flies. In terms of beta diversity, we were unable to completely eradicate the effects of age in males, but this is unsurprising given the effect of grouping is strongest in these animals. For females, cohabitation with young flies appears to reduce the microbial variability that is associated with age and in terms of between group species richness and abundance, they are more similar to young flies. Taken together, this again indicates that some intrinsic level of control is responsible for shifts in community structure, rather than stochastic shifts based on physical contact (although this is also likely to play a part). Despite these interesting overall

patterns in alpha and beta diversity, on the level of individual species, babysitter flies still have more *Lactobacillus* than young flies of the same sex which means that the alpha and beta profiles are being driven by subtle shifts in overall composition rather than large changes in the “core” microbiome.

It is interesting to note that age did not affect relative abundance of *Lactobacilli* for females in the age-matched group assays, but did for babysitter females, who have more *Lactobacilli* than young grouped flies. Since a relative increase in these species seems to represent a lack of immune homeostasis (when matched to lifespan data), babysitter females may be more stressed than when kept with females of the same age, despite alpha and beta diversity profiles indicating little difference between them and young flies. Cohabitation with younger females may be considered a more sexually competitive environment, possibly potentiating the stress responses mentioned previously. Age of cohabiting individuals can affect lifespan extension in *D. melanogaster*, where younger cohabitants provide the biggest benefit (Ruan and Wu 2008) which demonstrates that social interaction with younger individuals can have tangible physiological advantages. In a broader context, elderly volunteers that spend time with younger individuals report improved mental health (Musick and Wilson 2003), although this study focusses on the psychological implications of mixed-age social interactions rather than the physiological.

Lactobacillus plantarum promotes larval growth rate in nutrient poor environments by increasing Ecdysone and Drosophila insulin-like peptide (dILPs) signalling (Storelli *et al.* 2011) and can have protective effects when orally infected (Blum *et al.* 2013). Control is exerted via the upregulation of the TOR kinase pathway prior to hormone release from the fat body and prothoracic gland respectively (Storelli *et al.* 2011). Suppression of TOR signalling has been linked to increased longevity and appears to ameliorate age-related damage in both *D. melanogaster* and a host of other organisms (Kenyon 2010; Partridge *et al.* 2011). The relative abundances of *L. plantarum* between my groups (in general, more in older flies than young, more in males than females, more in babysitter flies compared to young flies) and our own

previous lifespan data highlight the possibility that increased activation of TOR caused by increased amounts of this species is resulting in the accelerated ageing and death. Since there were not statistically significant differences for individual species between social environments, it is not possible to implicate social stimulation or the lack of it into this equation.

4.5.3 Functional implications of social environment related changes in the microbiome

The gut of *D. melanogaster* is in a delicate host-mutualist balance maintained by complex immune mechanisms that both regulates homeostasis of commensals (Ryu *et al.* 2008) and protects from pathogens (Kuraishi, Hori and Kurata 2013), since *D. melanogaster* ingests microorganisms when feeding on rotting fruit. As a healthy microbiome can help protect against a variety of oral infections (Sansone *et al.* 2015; Koch and Schmid-Hempel 2011; Blum *et al.* 2013), and with *L. plantarum* showing particular protective properties, I speculated that the dysbiosis associated with social stimulation in males is likely to negatively affect the ability of the fly to fight a naturally acquired oral infection. To test this, I investigated the ability of males and females exposed to different social environments to survive an oral infection.

In similarity to previous work (Chapter 2) and alpha and beta diversity measures, I found that socially stimulated males were more susceptible to oral infection than those kept alone, and that this effect was absent in females. These differences were not adequately explained by the quantity of infected food eaten, since paired males didn't eat more than singles, and whilst paired females did, there was no associated increased vulnerability to infection. The general trend presented in this work is for an increase in relative abundance of *Lactobacillus* species with age and in males. There were not, however, effects of social environment on the abundance of specific species, rather increases in overall alpha diversity (but only in grouped old flies) and shifts in beta diversity (more so for old grouped males). Previous studies have indicated that *L. plantarum* can be beneficial in terms of protection from oral infection in fruit flies

(Blum *et al.* 2013), and is frequently used as a probiotic (Mack *et al.* 1999) and to prevent pancreatitis (Olah *et al.* 2002) but the increase I observed did not provide any fitness benefit to orally infected flies. As discussed previously, the increase in these species is likely due to age-related dysbiosis and so is unlikely to provide any benefit. Shifts in the relative composition of host bacterial communities are well associated with increased susceptibility to infection, as seen in antibiotic perturbations of the microbiome (Theriot *et al.* 2014). In this case the overall changes in alpha and beta diversity induced by social environment likely led to the dysfunction of the gut epithelium (Buchon *et al.* 2009a; Clark *et al.* 2015) and associated immune dysfunction. In order to test this hypothesis, experimental manipulations of the microbiome could be used to examine susceptibility to a range of oral infections, histological studies accompanied with genetic mutants in key genes such as *foxo*, *PGRP-LC* (IMD activator) and *Toll10b* (Toll activator) could elucidate the role that epithelial and immunological dysregulation are playing and the commonly used Smurf assay (Rera, Clark and Walker 2012) would give an indicator of intestinal barrier integrity.

4.5.4 Conclusions

In conclusion, social stimulation acts to disproportionately affect the microbiome of males, but this is an affect that only becomes significant with age, raising the likelihood that immunosenescence is playing a key role in the process. Male flies kept with constant social stimulation are less able to cope with oral infection by a pathogenic bacteria indicating that shifts in the microbiome have specific fitness costs. By cohousing old flies with young flies it is possible to offset the changes in alpha and beta diversity that are associated with age, but I did not test the functional implications of these changes. Future work should focus on elucidating the link between socially induced changes in the microbiome and physiological changes in the gut epithelium and associated immune tissues.

Chapter 5 - General Discussion

Social contact often results in changes to physiology that can alter fitness. Immunity is one central aspect of physiology that is necessarily sensitive to social environment, but little is known about how social isolation or social contact ultimately influences the ability of an organism to fight infection. Even less is known about how this ability interacts with age and other aspects of physiology like the microbiome, or even how these responses differ by sex.

D. melanogaster has, for a number of years, been used as a successful model to study the four areas at the centre of this thesis, namely, the social environment, innate immunity, ageing and the microbiome. The work presented here has utilised this model, as well as appropriate functional and molecular techniques, to try and further our understanding of the biological implications of sociality on these key biological functions in *D. melanogaster*. **Figure 27** summarises the key findings of this thesis and where these findings sit in relation to each other.

In summary, this work has contributed to our understanding of the effects of social environment on immunity, ageing and the microbiome in a sex specific manner. I have also attempted to interpret the results on a finer scale by providing an insight into the mechanistic basis of the key findings, as well as providing a broader context for the work by analysing the conclusions in an evolutionary context and in relation to its wider implications.

5.1 Main findings

5.1.1 Males are more sensitive to social environment than females

Collectively, the results presented in this thesis indicate that males are disproportionally sensitive to the social environment compared to females. Initially, lifespan experiments using male and female flies kept alone or in

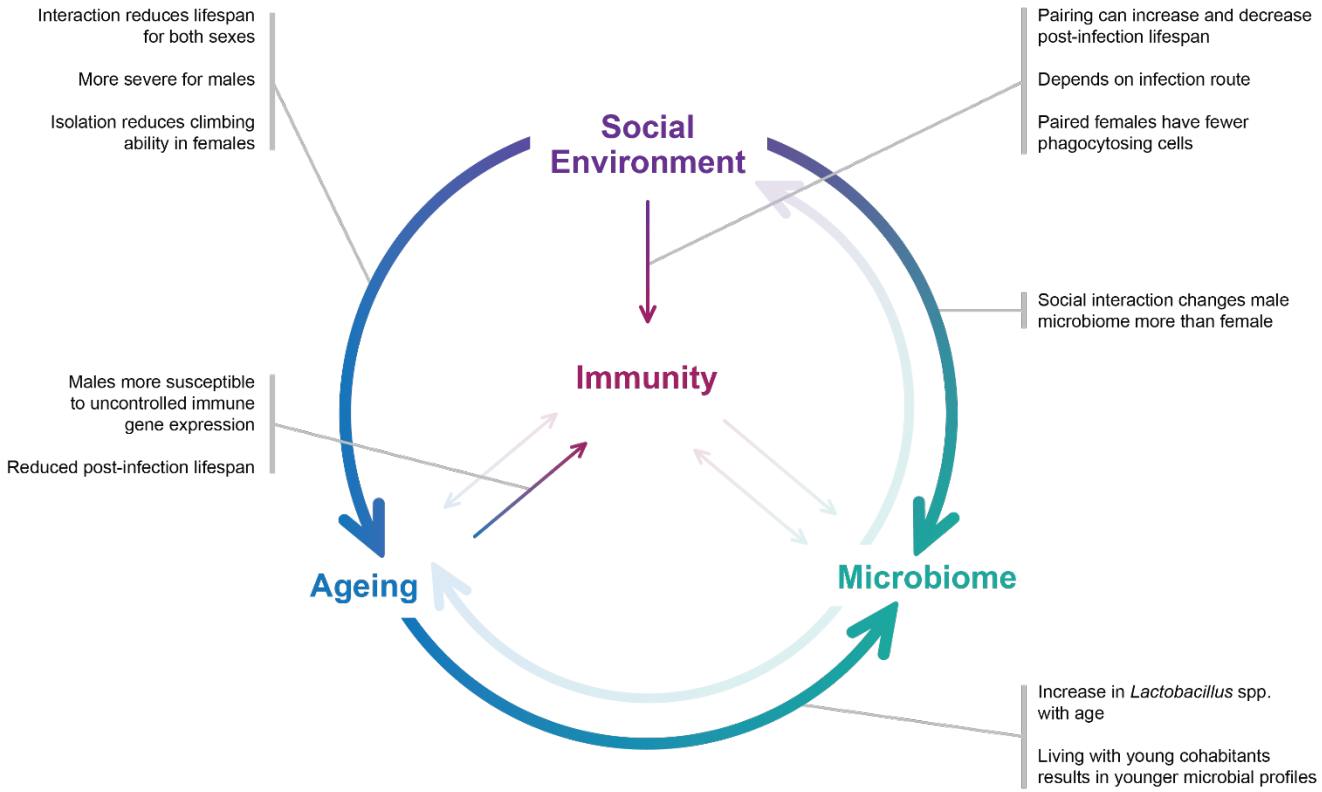

Figure 27 Social environment, immunity, ageing and the microbiome are intimately linked. This figure is adapted from Chapter 1 to highlight the interactions that take place between these components, and more specifically, my own results which have helped to clarify the nature of this relationship. Grey arrows represent factors between which there is the potential for interaction, and the directionality of this relationship, but which have not been examined experimentally in this thesis.

same-sex pairs and either injured or uninjured indicated that for males, the interactive effect of injury and pairing results in significantly greater reduction in lifespan than for females. Males were once again especially sensitive in assays that used an infected food source to asses lifespan post oral infection. Here, more males die when paired than when kept singly and females showed no noticeable sensitivity to social environment. Finally, in experiments to assess social environment associated changes to the microbiome, males kept in groups of 10 experienced more profound shifts in bacterial communities than females from the same social environment.

It is advantageous for males to be more sensitive to social environment in order to target specific females who are likely to be receptive to mating attempts (Bretman, Westmancoat and Chapman 2013). Males detect chemical and auditory cues which are subsequently integrated and processed in order to assess social environment and consequently mate successfully (Dickson 2008). From a neuro-mechanistic perspective, this raises the possibility that for males, this complex sexual response could trade-off against stress-resistance (Marshall and Sinclair 2010) potentially making them less able to cope with social stress. This explanation is not completely satisfactory though, since females pay a high price in order to reproduce, and egg production results in trade-offs with oxidative stress (Salmon, Marx and Harshman 2001). It may indicate, therefore, that males are predisposed to social-sensitivity, since they need to be in terms of their life-history strategy, and that this sensitivity is merely an unwanted side-effect of being socially-plastic in response to sexual competitors.

The observation that males and females respond differently to social environment may also indicate the involvement of hormones such as ecdysone and juvenile hormone (JH). These hormones are highly pleiotropic – as well as regulating key aspects of development, significantly, they are involved in longevity, immunity and stress resistance (Flatt, Tu and Tatar 2005). Whilst *D. melanogaster* does not have classical sex hormones in the vertebrate sense, it has been thought for some time that these two hormones can play a role in

some sex specific characteristics (De Loof and Huybrechts 1998). Ecdysone signalling modulates lifespan in a sex-specific fashion (Tu, Yin and Tatar 2002), despite titres of this hormone not differing between males and females (Handler 1982), indicating that ecdysone can work in fundamentally different ways for males and females. This is corroborated by the observation that ecdysone is key to the initiation of male specific traits such as courtship (Ganter *et al.* 2007), whilst clearly these behaviours are lacking in females. Consistent with this line of thinking, mutations to both the ligand and receptor responsible for ecdysone signalling result in sex-specific increases in lifespan and greater resistance to stress (Simon *et al.* 2003). Similarly, JH controls sex specific traits associated with reproduction (Kubli 2003; Bownes 1989) and acts to suppress both immunity and stress responses (Tatar, Chien and Priest 2001; Rantala, Vainikka and Kortet 2003).

These hormones in particular then, appear to be a promising mechanism by which social environment exerts different effects on males and females. This theory could be tested experimentally by manipulating social environment in male and female flies with mutations to genes critical to ecdysone and JH signalling as well as neurotransmitters such as dopamine, which can also exert sex specific effects in response to stress (Dalla *et al.* 2008). If the observed patterns are abolished, this would indicate that steroid hormones are responsible, at least partly, for sex differences in response to social environment. Significantly, this avenue of research may prove particularly fruitful in helping to understand activity of the major stress hormone axis in humans, the hypothalamus-pituitary-adrenal axis, which is directly and indirectly associated with the onset and propagation of sex-specific stress-related diseases (Dedovic *et al.* 2009).

5.1.2 Social environment and ageing often interact

A further finding central to this thesis is the observation that social environment and ageing often interact; that is to say the effect of social environment becomes more obvious with age. Initial evidence for this is provided by the lifespan experiments conducted in Chapter 2, where pairing ultimately reduces

lifespan, but this effect doesn't become apparent until the flies are about 50 days old. In light of this, subsequent experiments used two time-points to investigate the effect of social environment on post-infection lifespan. Once again, where there were effects of social environment they were only apparent at older ages, this time acting to improve post-infection lifespan or not affect it at all. Analysis of differential gene expression had similar results – although only older flies were tested, pairing reduces relative expression of stress genes *Turandot A* and *M* in females, as well as having a similar, although non-significant, effect on phagocytosis gene *eater*. Direct analysis of phagocytosis using flow cytometry found effects of social environment at both ages (pairing results in a significantly lower proportion of phagocytosing cells for females), but the effects were more pronounced in older flies. Finally, both alpha and beta bacterial diversity of the microbiome were explained by significant interactions between age and social environment. Once again, these indicated that social environment can have little effect at young ages, but in this instance, increases diversity with time.

Senescence results in the systematic decline in function of most physiological systems (Ricklefs 2008). Since social contact appears to be stressful to *D. melanogaster* (albeit to differing extents in males and females), it is likely that deterioration of the stress response, or factors affecting the stress response, are responsible for this observation. My results indicate that the flies experience immunosenescence and since the immune system, stress response and ageing are tightly linked in both *D. melanogaster* (Eleftherianos and Castillo 2012) and humans (Butcher and Lord 2004), this lends further weight to the idea that age reduces stress tolerance.

When young, it appears *D. melanogaster* are able to cope with negative effects of grouping in order to successfully mate, but as they age this ability is reduced, and in doing so conforms to two related evolutionary theories of ageing. Antagonistic pleiotropy (Williams 1957) posits that genes that are beneficial in youth can have detrimental effects with age, but are maintained because overall, they increase fitness and selection becomes weaker with age.

Similarly, the disposable soma theory (Kirkwood and Holliday 1979) suggests that there is a trade-off between a quality that is beneficial in an organism's youth (e.g. reproduction) and the maintenance of non-somatic traits which ultimately leads to individual senescence.

5.1.3 Route of infection is important when considering the effects of sociality on immunity

Another interesting finding presented in this work is that social environment can affect post-infection lifespan in a different manner depending on how the infection is contracted. Work in Chapter 3 described how cohabitation of same sex flies could result in improvements in post-infection lifespan, dependent on the type of bacterial infection being used. Here, infections were performed by use of a microinjector system, introducing the bacteria directly into the body cavity of the fly. In Chapter 4, an alternative feeding based method was used to assess lifespan post oral infection. The results of this experiment differed from those when injected – here pairing reduced post-infection lifespan, but only for males, which is more in line with the other results presented in this thesis. For this experiment it should be noted that only a single bacteria (*P. fluorescens*) was used because *D. melanogaster* proved to be unsusceptible to *B. thuringiesis* and *P. aeruginosa* infection when dosed via an oral route.

There is a high level of genetic pleiotropy between genes which are responsible for wounding and injury repair and those which are responsible for immunity (Lemaitre and Hoffmann 2007). It is possible that by injecting the bacterial challenge (and thereby causing injury) recruitment of genes relating to wounding are co-opted by the immune response, ultimately resulting in either a faster or stronger response to bacterial challenge and subsequently, better post-infection lifespan. This is perhaps not a satisfactory explanation however, since both single and paired flies were injected in the same way, but did not respond in the same manner and injury actually lowers resistance to infection in *D. melanogaster* (Chambers *et al.* 2014). It remains more likely therefore that the differences observed are due to the evolutionarily distinct responses to

these type of infections and the interaction of the microbiome - microbiome composition can affect ability to survive oral infection (Blum *et al.* 2013) and is manipulated by the social environment (Chapter 4). It is also becoming increasingly clear that coordination of both the immune response and epithelial renewal mechanisms are needed to successfully overcome an oral infection (consistent with the idea that both resistance and tolerance to infection work in concert in order to survive infection) (Buchon *et al.* 2009b; Chatterjee and Ip 2009). Stress-inducing chemicals result in the proliferation and division of intestinal stem cells (Chatterjee and Ip 2009), a JNK-dependent effect that interacts with age and has severe pathological consequences (Biteau, Hochmuth and Jasper 2008). It is conceivable then that if environmental stress is in some way inhibiting the ability of intestinal stem cells to function appropriately, especially in older flies, one such consequence would be the inability of an individual to survive an oral pathogenic challenge.

5.1.4 The microbiome of *D. melanogaster* is modified by social environment in an internal manner, and interacts with age

Consistent with the combined findings of Chapters 2 and 3 which indicate social environment is able to alter fundamental biological processes such as ageing and immunity, analysis of the microbiome revealed that changes in *D. melanogaster* associated bacteria in relation to social environment are mediated, at least in part, internally. This conclusion was reached in light of the fact that changes in diversity are explained by an interaction between sex and social environment – males are subject to more profound changes in bacterial communities, indicating that these changes are not simply due to the transfer of bacteria between individuals. The age of cohabitants also affects microbiome composition in an internally mediated manner, since, once again, the sexes respond differently.

It is clear that the observed changes in microbiome are mediated at least in part in an internal manner due to the exacerbated effect of social environment in males. What remains unknown, however, is the cause and

effect nature of this relationship – does the social environment increase rate of ageing (as seen in data presented in Chapter 2) via alterations to the microbiome, or do the accelerated ageing patterns seen in paired flies cause the associated changes in microbiota? The use of axenic flies has the potential to illuminate this particular question, if flies grown without a resident microbiome removes the effect of microbiome on fitness. Similarly, by manipulating the microbiome of non-socially exposed flies to mirror that of flies exposed to social contact (a relatively easy task) and measuring lifespan this would provide a solid link between changes in microbiome structure and fitness. Finally, experiments manipulating the perception (i.e. mutants lacking olfactory, auditory or visual abilities) of social cues may also help to understand the relationship between social contact and the microbiome fully. Recent work suggests recolonizing the gut of middle-age killifish (*Nothobranchius furzeri*) with bacteria from young donors results in lifespan extension and delayed behavioural decline (Smith *et al.* 2017), a result that is especially intriguing in the light of my own observations that co-housing with younger individuals results in younger microbial profiles.

The observation that social environment can alter the microbiome in an intrinsic manner has potentially significant ramifications. Gut microbes both respond to and regulate the production of neurotransmitters (Yano *et al.* 2015; Dinan, Stanton and Cryan 2013) like dopamine, signalling of which is sensitive to social environment (Hall *et al.* 1998) and which responds in a sexually dimorphic manner to stress in *D. melanogaster* (Argue and Neckameyer 2013). In turn, changes to neurotransmitter signalling have the potential to affect an enormous amount of traits, including ageing and immunity (Omelyanchuk, Shaposhnikov and Moskalev 2015). These ramifications make this particular avenue of research particularly attractive in terms of translational medicinal applications in the future.

5.1.5 Implications and conclusions

Taken together this work indicates that social environment has the ability to alter the hosts response to infection, ageing rates and the microbiome, often in

a sex specific manner. An obvious implication of this work is that, given the variety of conditions *Drosophila* labs use to raise their flies in, and with experimental groups often kept at a variety of densities, this may inadvertently be affecting results, particularly where response variables include aspects relating to the immune system, rate of ageing or the microbiome.

The conclusions of this thesis also have implications beyond fundamental bioscience. As our ever-growing population begins to age, the burden of loneliness poses a health risk as potent as smoking and obesity (Pantell *et al.* 2013), but one that is comparatively overlooked. This work represents an insight into the physiological implications of socially stressed individuals and attempts to provide mechanistic understandings that may ultimately provide promising interventions for socially-mediated disease states; especially relevant therefore is the observation that social environment often interacts with age. My finding that the microbiome can be ‘rejuvenated’ by the presence of young individuals is an interesting one and one that warrants more research. The pervasive finding that males and females respond differently to social environment is one that could conceivably have useful clinical consequences and likewise, the studies pertaining to immunosenescence reiterate the importance of taking social environment into account when researching the effects of social contact on age and the immune system.

Appendix A

Sample sizes for Infection experiments

Bacteria	Age	Sex	Social Environment	Sample size (n)
P. aeruginosa	Young	Male	Single	35
			Paired	37
		Female	Single	38
			Paired	36
	Old	Male	Single	38
			Paired	37
		Female	Single	34
			Paired	37
P. fluorescens	Young	Male	Single	40
			Paired	38
		Female	Single	32
			Paired	35
	Old	Male	Single	29
			Paired	31
		Female	Single	31
			Paired	33
B. thuringiensis	Young	Male	Single	37
			Paired	39
		Female	Single	36
			Paired	38
	Old	Male	Single	36
			Paired	36
		Female	Single	36
			Paired	38

Primer sequences and RT-qPCR calculations

Gene	Forward Primer	Reverse Primer
Act5c	GTGGATACTCCTCCCGACAC	GCAGCAACTTCTTCGTCACA
E1f	GTCTGGAGGCAATGTGCTTT	AATATGATGTCGCCCTGGTT
Dro	GCCCGCCTAAAGATGTGTG	CGTGTGTTTATTGCTTACTGTTTGC
Eater	GGCAATAATAACCACCATGC	TAAAGCTCAGGCTCGAATGA
Vir 1	GAAGAACGCCAACACCACTT	CACCAAGCGGACCTTAAAGA
Tot A	GCTTCAGCGTTCCAAAAAGT	AGAGGACTAATCAGCAGCAGTG
Tot M	TTCGAGTTTGAAAGCCAAGC	AGCATTTACCTTTCCCAGCA
foxo	AGGCGCAGCCGATAGACGAA TTTA	TGCTGTTGACCAGGTTCGTGTTGA

Differential gene expression

By means of the average C_q of the technical replicates the relative quantity (ΔC_q) was calculated using the formula:

$$\text{Relative Quantity}_{\text{sample (GOI)}} = E_{\text{GOI}}^{\,(\text{Cq (control)} - \text{Cq (sample)})}$$

Where:

• E = Efficiency of primer. This efficiency is calculated with the formula:

(% Efficiency * 0.01) + 1, where 100% efficiency = 2

• Cq (control) = Average Cq for the control sample

• Cq (sample) = Average Cq for any samples with a GOI

• GOI = Gene of interest (one target)

When calculating gene expression changes a constant single sample was used to normalise against, within replicates. For example, to calculate the gene expression change for paired males, single females and paired females, these were all normalised against single males, within replicate.

Normalised relative quantity was then calculated using Hellemans method which allows for the use of more than one reference gene (Hellemans *et al.* 2007):

$$\text{Normalised relative quantity (fold change)} = \frac{(E_{GOI})\Delta C_T(GOI\ control - sample)}{r\sqrt{\prod(E_{ref\,gene})\,\Delta C_T(GOI\ ref\ gene\ control - sample)}}$$

The average of the replicates was then taken and Log_2 transformed (Hellemans and Vandesompele 2011).

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