Anticipatory plasticity: how early life environmental cues affect development and behaviour

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Abstract

In this thesis, I examine how social conditions experienced during early life stages can have effects into adulthood. I use the fruit fly *Drosophila melanogaster* as a model species to explore this; using larval social conditions that produce an anticipatory response to reproductive competition in adult males, namely high density and the presence of adult males. I demonstrate that the type of social conditions experienced by the larvae can have distinct effects on aspects of cognition, stress, immunity and the microbiome.

First, I demonstrate that manipulating larval density can influence adult learning ability and relative expression of a synaptic growth gene. I show that adult lifespan is negatively affected by the presence of adult males during larval stages, and that development time is longer for those reared at high larval density, but a number of adult female reproductive traits are not influenced by these conditions. I find type of larval social conditions and sex-specific responses to specific stressors in young adults. In particular, females from adult presence larval conditions show increased cold and desiccation stress tolerance, but decreased post-infection lifespan after bacterial injection with *Bacillus thuringiensis*, suggesting a trade-off may be occurring. I also examine the effect of these conditions on microbiome composition, finding distinct effects of pupal versus adult stages, and an increased microbial diversity in adult presence pupae. Overall, my results suggest that there are a number of factors in adult fruit flies that can be affected by larval social environments. These responses are often dependent on the type of social conditions experienced, the adult trait examined, and the sex of the fly. This study highlights early life conditions can have important and long-lasting consequences.

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Chapter 1

General Introduction

1.1 Phenotypic Plasticity

Phenotypic plasticity is the ability of a genotype to form different phenotypes under variable environmental conditions, and encompasses a wide range of responses, including behavioural, physiological and morphological traits (Auld et al., 2010). Under heterogeneous conditions, malleability in such traits can allow an individual to follow environmental fluctuations and more closely match strategies to them. The extent of plasticity can vary from complete canalization, where there is no change in trait under different circumstances, to complete plasticity (Flatt, 2005). The benefits of fine-tuning of phenotype to environment are evident where a failure to effectively match the conditions results in significant fitness costs (Auld et al., 2010). Subsequently, where plastic responses result in fitness benefits, they can be considered adaptive (Fordyce, 2006). However, non-adaptive plasticity, where there is a response to environmental conditions that do not have fitness advantages (Ghalambor et al., 2007), can also occur. It is, therefore, important to understand how individuals respond to differences in environmental conditions and the subsequent effects of this response. Both are factors which could vary depending on the type of plasticity shown.

Phenotypic plasticity can be broadly categorised into either activational or developmental plasticity. Activational plasticity involves distinct stimulation of an established underlying system, and is usually a rapid, flexible and reversible response to changes in the environment that

should be particularly advantageous under fine-grained environmental variation (Snell-Rood, 2013). Alternately, developmental plasticity refers to the emergence of different phenotypic trajectories under different environmental conditions (Snell-Rood, 2013). Developmental plasticity is therefore expected to be a slower process, and could have major influences on successive plastic responses later in life (DeWitt et al., 1998; Snell-Rood, 2013). This form of plasticity is predicted to be beneficial where environmental variation is coarsely-grained, changing more between than within generations (Snell-Rood, 2013). Furthermore, whilst activational plasticity is usually reversible, developmental plasticity is expected to be less so (Kasumovic, 2013). Further, developmental changes could have large effects on future plastic response, perhaps directing or constraining them (Kasumovic, 2013; Taborsky, 2017). Additionally, as there are potential time lags between receiving and processing cues and the phenotypic response, this may require a significant level of information (Taborsky, 2017). Developmental plasticity often occurs over longer timescales, which may also provide a greater period in which an individual can assess environmental cues, to ensure an overall better match (Snell-Rood, 2013). However, plastic responses may occur that are non-adaptive, for example, in response to novel stimuli not previously encountered in the species' evolutionary history, for example, larval salamanders, *Ambystoma maculatum*, show morphological differences in head and tail shape when induced by herbicide-exposure (Levis et al., 2016). Mismatches between phenotype and environment could also occur where there are time-lags between gathering cues and producing phenotypic responses (Wennersten and Forsman, 2012). This may be particularly pertinent for developmental plasticity which is associated with longer time lags (Snell-Rood, 2013), or where plastic responses occur in prediction of future environmental conditions that do not then manifest (Taborsky, 2017). Additionally, despite the clear advantage of matching phenotype to environmental conditions, phenotypic plasticity is not shown by organisms under all situations, indicating that there are costs associated with it (DeWitt et al., 1998).

1.1.1 Benefits and Costs of Phenotypic Plasticity

Under heterogeneous conditions, the ability to alter traits to match the environment could be very beneficial, for example, it could provide an enhanced ability to survive toxic conditions (Li et al., 2002), evade predation (Van Buskirk et al., 1997) or alter reproductive investment (Bretman et al., 2009). However, despite the benefits of phenotypic plasticity, there are associated costs (DeWitt et al., 1998). Costs of plasticity are defined as reductions in fitness of an individual displaying a plastic response when compared to a non-plastic one expressing the same phenotype under a specific set of conditions (DeWitt et al., 1998). These costs include those associated with gathering the information and those that occur from production of the response (DeWitt et al., 1998). In the previous examples, allocation to the production of enzymes by the corn earworm, *Helicoverpa zea* (Li et al., 2002) or alterations in morphology by tadpoles, *Pseudacris triseriata* (Van Buskirk et al., 1997) could require significant investments of energy. However, as these individuals display phenotypic plasticity in these circumstances, it is expected that the benefits of the response outweigh such potential costs.

Another hypothesised cost occurs where there is a mismatch between response and environment, which could occur through a lack of information or time lags between these factors (DeWitt et al., 1998). This could be particularly true for developmental plasticity, for example, the gastropod *Physella virgata* reduces growth and produces rounder shells in response to both non-predatory and predatory sunfish (Langerhans and DeWitt, 2002). This shift in resource allocation under safe conditions could impact on available energy for other processes, make the individuals more vulnerable to different predators, and is not easily reversible (Langerhans and DeWitt, 2002). Additionally, energetic trade-offs could result in a reduction in functional ability of, for example, immune pathways (Gervasi and

Foufopoulos, 2008), or there may be negative effects from metabolic processes that indirectly affect other systems (Harshman and Zera, 2007). Furthermore, there may be costs involved in the extraction of information, such that individuals must be able to receive the relevant cues, and correctly interpret them. This could require distinct sensory and cognitive systems, and as neural tissue is expensive to produce (Niven and Laughlin, 2008), it could represent a particularly large cost. Therefore, it may be beneficial to reduce investment in cognitive structures where conditions are less variable and therefore there is a decreased requirement for complex, expensive neural architecture (Sol, 2009). However, in a dynamically changing environment, where there are potentially numerous different cues, investment in cognitive systems may be necessary to ensure a correct phenotype-environment match. In these circumstances, the costs involved with production and maintenance of cognition may be outweighed by the benefits of accurate assessment of cues, which allows for the production of a phenotype that better matches the conditions.

1.1.2 Phenotypic Plasticity and the Social Environment

Social contact describes any interaction between individuals (Fuller and Hahn, 1976), including both sexual and non-sexual contexts. This can often vary rapidly (Kasumovic et al., 2008), and as such, behavioural flexibility is often advantageous as it is usually quick and reversible (Mery and Burns, 2010; Bretman et al., 2011a). Factors such as temperature, photoperiod, food, and shelter availability could shape spatial and temporal aspects of the social environment (Kasumovic et al., 2008; Esperk et al., 2013), whilst the expansive effects of social conditions can themselves influence factors including pathogen spread, food availability, reproduction and aggressive interactions, with important fitness consequences. Indeed, in humans, social isolation has been linked to a number of health issues (Hawkley and Cacioppo, 2003). One meta-analysis demonstrated an association with an increased risk of mortality (Holt-Lunstad et al., 2015), whilst individuals

described as socially isolated have been shown to have an increased expression of pro-inflammatory cytokines (Cole et al., 2007).

Competition for resources is one aspect influenced by the social environment, for example, wood frog tadpoles, *Rana sylvatica*, have increased activity and slower growth as a result of limited resources under competitive conditions (Relyea, 2004). *Drosophila melanogaster* selected under larval crowding conditions develop an increased competitive ability at high density that allows them to increase developmental rates under conditions that can otherwise slow this growth (Joshi, 2001). Even when resources are not limited, other individuals could exert an influence through interfering with feeding (Goss-Custard, 2002). In sexually reproducing species, reproduction is inevitably influenced by the social environment too, and therefore, it can be beneficial for individuals to show plasticity in reproductive traits depending on social context. For example, female crickets, *Gryllus firmus*, show increased investment in ovary mass when exposed to male song (Conroy and Roff, 2018). Conversely, green swordtail fish males mature later in the presence of male competitors (Walling et al., 2007). Maturing early and reducing the time spent during vulnerable juvenile stages to quickly attain the opportunity to reproduce when competition is low is beneficial (Walling et al., 2007). Sperm competition, the competition between sperm for fertilization of an ova, can influence a number of phenotypic traits in males (Parker, 1970). Males can respond in a plastic manner to both the probability of sperm competition (the risk), or the level of sperm competition (the intensity) (Parker, 1970; Wedell et al., 2002). Such responses include varying sperm number, for example, mated male butterflies, *Pieris rapae*, transfer more sperm to females when sperm competition intensity is greater, in this case, when females had previously been mated to non-virgin males, that generally transfer more sperm to females than virgin males (Wedell and Cook, 1999). *D. melanogaster* males alter ejaculate composition in response to sperm competition risk (Wigby et al., 2009), and increase their mating duration following exposure to a competitor (Bretman et al., 2009). As there are costs associated with the

production of sperm and ejaculates, males should adjust these components in response to the competitive conditions in order to maximise fitness (Parker and Pizzari, 2010). Accurately gauging the number and quality of competitors or potential mates in the area can allow an individual to alter their reproductive traits in response (Parker and Pizzari, 2010). Under these circumstances, accurate and quick acquisition and processing of cues is advantageous to enable individuals to make quick and appropriate phenotypic responses (Pearce, 1997). For example, the increased mating duration response of *D. melanogaster* males to rivals is affected by the ability to detect multiple sensory cues (Rouse and Bretman, 2016), and requires memory (Rouse et al., 2018).

An increased cognitive capacity could enhance an individual's ability to respond to variable social contexts (Mery and Burns, 2010). For example, some fish species show differences in social learning abilities with regards to foraging activity, anti-predator behaviour, and migration (Laland et al., 2011). However, neural tissue functioning is energetically expensive to maintain, accounting for as much as 20% of basal oxygen consumption in adult men, with large costs associated with the maintenance of ion gradients across neuronal ion channels as well as the production of neurotransmitters (Clarke and Sokoloff, 1999; Niven and Laughlin, 2008). Therefore, investing in neural tissue when there is less need of it could represent an unnecessary expenditure. Displaying plasticity in cognition is therefore advantageous under these circumstances, and could involve changes in the development of brain structures or aspects of neural networks (Snell-Rood, 2013). For example, environmental enrichment increases synapse to neuron ratio in rats (Bhide and Bedi, 1984). Larger brain regions are often associated with an increased cognitive ability, for example, greater neocortex and striatum size of the brain has been linked with behavioural innovation and social learning in primates (Reader and Laland, 2002), however, there are often multiple parts of the brain involved in single behaviours and discrepancies arise between measurements of brain size (Healy and Rowe, 2007). Despite these issues, it is often predicted that larger brain structures enhance the

ability to manage variable conditions (the ‘cognitive buffer hypothesis’) (Sol, 2009). Additionally, due to the high costs involved in cognition (Niven and Laughlin, 2008), there may be trade-offs with other processes. In mammals, a reduced fertility rate is found in relatively larger-brained species, which suggests a trade-off between reproduction and cognition (Isler and van Schaik, 2009), whilst there is a negative relationship between brain and testes mass in bats (Pitnick et al., 2006).

Another factor that can be affected by the social environment is stress (Stefanski, 2001), which can generally be defined as occurring where a stimulus (or ‘stressor’) exerts a demand that exceeds the natural homeostatic state of the organism (Koolhaas et al., 2011). Stress responses are used to return to the homeostatic state or reduce damage (Koolhaas et al., 2011), but prolonged stressful conditions can reduce fitness or result in death (Lupien et al., 2009). Displaying plasticity in stress responses can allow an individual to reduce the damaging effects of stressors encountered, whilst decreasing the associated costs during periods when they are absent (Barnes and Siva-Jothy, 2000; Ardia et al., 2012). There are high metabolic costs associated with stress, such as in juvenile steelhead fish, *Salmo gairdneri*, that considerably increase their metabolic rate following a short period of stress (Barton and Schreck, 1987). The stress responses of animals often involve a number of different pathways, and activation of these may vary depending on the type of stressor experienced (Agaisse and Perrimon, 2004; Brun et al., 2006). Aggressive interactions between rats result in reductions in body mass and decreases in immune T cell activity (Stefanski, 2001), and in *D. melanogaster,* paired males have greater reductions in lifespan following injury versus isolated males than do paired females (Leech et al., 2017).

Immune systems may also display plasticity in response to the social environment. Microorganisms are ubiquitous in the environment, and as some of these can be harmful, the mechanisms used by an organism to

defend against pathogens are extremely important for survival (Lemaitre and Hoffmann, 2007). Once again, there are costs involved with immune activity, for example, induced immune activity is associated with a 25-28% increase in metabolic rate in some insect species (Ardia et al., 2012). As a result, it is beneficial to increase immunity following an immune challenge (Ardia et al., 2012) or when there is an increased likelihood of one occurring (Barnes and Siva-Jothy, 2000), but decrease this when risks are lower. The social environment is particularly important with regards to immunity as the presence of conspecifics can facilitate the spread of bacteria, with an increased risk of infection in larger groups (Reeson et al., 1998). For example, tick infestation is associated with the degree of social connectivity in the gidgee skink, *Egernia stokesii* (Godfrey et al., 2009). Individuals in, or likely to be in, high contact with other individuals could benefit from priming their immune system. This occurs in the mealworm beetle, *Tenebrio molitor*, which has an increased resistance to fungal infection when reared at a high larval density compared to those raised solitarily (Barnes and Siva-Jothy, 2000), and larvae of the moth, *Spodoptera exempta*, that exhibit greater resistance to viral infection when reared in groups than in isolation (Reeson et al., 1998). However, this density-dependent prophylaxis is not found in all cases. High larval density results in reduced immune activity in the butterfly, *Pieris napi*, possibly due to changes in resource allocation towards stress responses (Piesk et al., 2013).

Phenotypic responses to the social environment may be elicited by means other than the individual's own mechanisms. The microbiome, the combined genetic material of an organism and the associated microorganisms, is increasingly recognised as having a number of significant effects (Lee and Brey, 2013). The host can manipulate or keep in check, its microbiome through, for example, immune activity (Adair and Douglas, 2017). However, bacteria can also elicit changes in the host's immune system. For example, reductions of the microbiome via antibiotic treatment in mice can affect antiviral immunity, suggesting the presence of these bacteria can manipulate host antiviral responses (Abt et al., 2012).

Microbiome composition can be affected by the social environment through horizontal transfer, for example, wild baboons, *Papio cynocephalus*, transfer bacteria between individuals through grooming (Tung et al., 2015). However, it could also be altered indirectly through changes in host stress and immunity elicited by the social environment (Stefanski, 2001; Leech, 2017). For example, social stress in mice caused by conspecific aggression results in changes to the microbiota (Galley et al., 2014), and social isolation in rats gives rise to changes in microbiota composition that are associated with changes in cytokines in the hippocampus (Doherty et al., 2018). Microbiome changes could result in pathogens out-competing commensal bacteria (Kelly et al., 2005). Alternatively, there may be bacteria that, under homeostatic states, do not have negative effects on the host, but may have damaging effects under these conditions. This has been observed in fruit flies where the bacteria *Lactobacillus brevis* can induce inflammation under certain circumstances (Lee et al., 2013; Matos and Leulier, 2014). As such, alterations in the microbiome could have distinct effects on the host phenotype (Dinan and Cryan, 2017).

1.2 Early Life Effects

Plasticity shown during early life stages can have far-reaching effects (Relyea, 2001; Langerhans and DeWitt, 2002). Periods where the developmental phenotype is more strongly influenced by the environment compared to other stages are termed sensitive windows, and are affected by, for example, the amount and reliability of the cues received (Fawcett and Frankenhuis, 2015). Both of these factors are expected to increase with age, and therefore, uncertainty at early ages may promote increased plasticity at these stages (Fawcett and Frankenhuis, 2015). Hypotheses proposed to describe how responses made in early life will affect an individual later in life include the environmental matching hypothesis, which predicts that where early and later life conditions match, the individual should have greater

fitness (Taborsky, 2017). For example, Japanese quail, *Coturnix japonica*, show an exploratory activity that increases food acquisition after being stressed as eggs and then subjected to variable feeding conditions as chicks, enhancing their ability to find food under these conditions (Zimmer et al., 2013). Additionally, cross-fostered earwigs, *Forficula auricularia*, that develop at late juvenile stages in food conditions matching their mother's, have higher survival (Raveh et al., 2016). Included in this hypothesis are predictive adaptive responses that occur during development in preparation for the expected environmental conditions, and also expects greater fitness where these match (Monaghan, 2008). A second hypothesis, the silver spoon hypothesis, predicts that individuals benefit from good conditions early in life regardless of later conditions (Taborsky, 2017). For example, female roe deer, *Capreolus capreolus*, born in productive conditions have higher reproductive output than those from poorer conditions (Douhard et al., 2014), and in the burying beetle, *Nicrophorus vespilloides*, where the larval environment influences size (Hopwood et al., 2014). High resource availability increases beetle size, which, in turn, increases the chance of success in contests as adults (Hopwood et al., 2014), indicating that these individuals benefit later from good larval rearing conditions.

Early life stress has been identified as a factor that contributes to the risk of adverse health in adulthood in humans, known as the Developmental Origins of Health and Disease (DOHaD) (Hoffman et al., 2017). For example, decreased tolerance towards glucose, and therefore increased risk of Type II diabetes, has been observed in adults exposed to the 1944-45 Dutch famine during the gestation period compared to those born before or after (Ravelli et al., 1998). Other animals also show distinct effects of early life stress, for example, rats show greater anxiety in open field tests, higher corticosterone levels and changes in microbiota if stressed as pups (O'Mahony et al., 2009). Further, early life stress is often associated with immune dysregulation, including increased inflammation (Fagundes et al., 2013). Dysregulation of the immune system in early life has been linked to

changes in brain development, and has been implicated in disorders such as depression and schizophrenia (Bilbo and Schwarz, 2009).

Plasticity during early life can occur through direct sensing of the environment or indirectly in response to cues experienced by the parents, known as parental effects (Burton and Metcalfe, 2014). For example, *Drosophila melanogaster* females lay heavier eggs if raised on poor food as larvae, and their offspring eclose lighter and earlier on poor food (Vijendravarma et al., 2010). Epigenetic changes, alterations in gene expression not caused by changes in the DNA sequence, elicited from stressful environmental conditions can also be inherited. For example, DNA methylation changes in male rats subjected to maternal separation are found to persist in the next generation (Franklin et al., 2010). However, offspring environment is also likely to have effects (Tregenza et al., 2003). Under fine-grained environmental variation, where parental and offspring environmental conditions are less likely to match, such parental effects may be less advantageous (Burton and Metcalfe, 2014), and phenotypic plasticity in the offspring themselves may be favourable.

Structural developmental changes by individuals during early life could have important influences on future morphology. For instance, the wood frog, *Rana sylvatica*, develops larger limbs if reared with predator cues than those reared without, a change that could influence adult movement (Relyea, 2001). In addition, adjustments in brain structure could influence receptivity to hormonal change (Dufty et al., 2002), and structural changes in neural circuits may also have important consequences for processing of environmental cues (Snell-Rood, 2013). Developmental plasticity is also expected to result in differential resource allocation (Snell-Rood, 2012). Under larval food limitation, the butterfly *Bicyclus anynana*, alters resource allocation to increase their thorax ratio, measured as the ratio of dry thorax mass to total dry mass, which is associated with an enhanced ability to cope with forced flight (Saastamoinen et al., 2010). However, juveniles may show

compensatory growth following poor early conditions once food availability increases (Fisher et al., 2006). Such compensatory growth has been observed in, for example, swordtail fish, *Xiphophorus helleri* (Royle et al., 2005) and zebra finches, *Taeniopygia guttata* (Fisher et al., 2006). However, differential resource allocation towards this could negatively impact on other factors, for example, it appears to affect the learning ability of adult zebra finches (Fisher et al., 2006). Such trade-offs could have lasting effects on other traits (Buchanan et al., 2013).

Negative fitness effects may also occur if the response does not match the environmental conditions experienced later (DeWitt et al., 1998). Under these conditions, showing reversibility of traits could reduce costs (Relyea, 2003), but may be difficult where there are distinct morphological changes during development (Langerhans and DeWitt, 2002). As such, the timing of cues is likely to be extremely important for developmental plasticity. In one example, the calls of mother superb fairy-wrens, *Malurus cyaneus*, are learned in part by chicks in the egg and used by the chicks post-hatch, which could help the mother to discriminate between her own offspring and cuckoos that have not had time to learn the call (Colombelli-Négrel et al., 2012). In this example, the cue given by the mother is learned by the developing chick but not used until after they have hatched, giving a distinct time lag between the gathering of information and the response. Longer gathering periods could allow more cues to be collected or establish their consistency over a longer timescale, both of which could be beneficial in establishing cue reliability in order to produce an appropriate response (Fawcett and Frankenhuis, 2015).

In early life, the ability to assimilate information about the environment may be particularly affected by the development of the sensory systems (Romagny et al., 2012), a lack of prior experience (Dukas, 1998), as well as the frequency and number of cues available (Fawcett and Frankenhuis, 2015). An individual that has had less experience of the environment may

be more likely to make choices that reduce fitness (Frankenhuis and Panchanathan, 2011). Such erroneous decisions could include, for example, feeding on a low quality or pathogen-infected food source that results in increased stress, disease or even death. These potential costs of naivety (Dukas, 1998) could be reduced with an increased ability to process cues quickly and with accuracy (Fawcett and Frankenhuis, 2015). However, as neural structures are energetically costly to produce (Niven and Laughlin, 2008), it is beneficial for individuals in early life to optimise the energy spent on neural development, including reducing it or differentially channelling it when conditions are less heterogeneous (Durisko and Dukas, 2013b).

During early life, cognitive structures may not be fully developed (Armstrong et al., 1998). Therefore it seems intuitive that the development of brain structures could be affected by the environmental conditions during these periods, but that individuals may require investment in cognitive structures in order to respond to these conditions in a plastic manner. For example, enrichment of enclosures during early life in pheasants, *Phasianus colchicus*, enhances spatial memory (Whiteside et al., 2016). Early social conditions have also been shown to influence cognitive ability, for instance, isolating honey bees, *Apis mellifera*, during the first few days after emergence reduces their ability to associate an odour with a sucrose reward (Ichikawa and Sasaki, 2003), and early life social isolation in prairie voles, *Microtus ochrogaster*, has been shown to negatively impact on social discrimination ability in a socio-spatial task, a factor which appears to be rescued by group-housing at later stages (Prounis et al., 2015). However, it appears that social enrichment does not necessarily result in an increase in all aspects of cognition, for example, rats subjected to maternal deprivation show reduced social, but not spatial learning compared to those that received social stimulation as pups (Lévy et al., 2003), suggesting the type of stimulus has specific effects on different elements of cognition. This may be important where there is variation in the requirement for different aspects of cognition across different life stages. For example, the learning ability of jack mackerel, *Trachurus japonicus*, changes in a stimulus-specific manner in

accordance with ontogenetic shifts in life history (Takahashi et al., 2012). During early life, therefore, one form of cognitive ability may be less required at later stages, or vice versa, and these stages may be particularly sensitive to changes in environmental cues where sensory or neurological systems are still developing or where there is a lack of experience that could result in incorrect decision-making (Dukas, 1998).

Juveniles may also have less developed immune systems or stress responses, and therefore may be particularly vulnerable to the effects of stressful or harmful conditions (Dukas, 1998; Lupien et al., 2009). However, even juveniles with developed stress responses can be severely affected by stressful early life conditions. Infant rhesus monkeys, *Macaca mulatta*, subject to stress display large increases in cortisol and stress-associated behaviours (Bailey and Coe, 1999). Whilst high quantities of stress experienced during early life are expected to have negative effects, more mildly stressful conditions could improve later stress tolerance, a response known as hormesis (Costantini et al., 2012). This priming response has been found in zebra finches, *T. guttata*, that improve adult heat stress tolerance if subjected to a mild exposure as juveniles (Costantini et al., 2012), and in fruit flies, *D. melanogaster*, where mild heat stress improves resistance to higher heat stress (Hercus et al., 2003). Similarly, priming of immune responses has been shown for flour beetles, *Tribolium castaneum*, that following a heat-killed dose of *Bacillus thuringiensis* as larvae, subsequently increase adult post-infection survival when challenged with a live strain of the bacterium (Khan et al., 2016).

Social conditions experienced during early life stages can have distinct effects on stress responses, for example, female baboons, *Papio cynocephalus*, that are more socially incorporated, have infants with higher survival than those that are less socially involved in the group, which has been attributed to factors including reduced stress (Silk et al., 2003). Rats undergoing maternal separation stress show differences in long-term

potentiation of hippocampal synapses following a shock stimulus compared to those that had not been maternally separated (Derks et al., 2016). These rats also have reduced cell proliferation in part of the hippocampus, suggesting effects of this stress on brain development and functioning (Mirescu et al., 2004). Further, rhesus monkeys, that are maternally separated have a disrupted microbiota, with reductions in the number of *Lactobacillus* species found in faecal samples, and show an increase in stress-associated behaviours compared to non-stressed individuals (Bailey and Coe, 1999). Therefore, an additional component arising from early life stressful conditions, with potential long-term implications for health, are changes in the bacterial community of the gut (Crouzet et al., 2013).

For some animals, the early life microbiome can be relatively unstable or compositionally different compared to adult stages (Wong et al., 2011; Arrieta et al., 2014). Given the range of effects that the microbiome can have on an individual (Lee and Brey, 2013), early shaping of these bacterial communities could be directly influenced by diet (Snijders et al., 2016), contact (Tung et al., 2015), or indirectly influenced through the induction of stress and immune responses (Buchon et al., 2009; Galley et al., 2014; Leech, 2017), and could have significant effects on the host. Higher corticosterone levels have been found in germ-free mice, which can be reversed by adding *Bifidobacterium infantis*, but only during early life stages, suggesting that this bacterium could help protect against negative effects of elevated stress levels (Sudo et al., 2004). Furthermore, germ-free mice have increased hippocampal neurogenesis as adults compared to those with conventionally-colonised microbiomes (Ogbonnaya et al., 2015). This effect is not changed by adding bacteria after weaning, suggesting that these effects are established during early life stages (Ogbonnaya et al., 2015). Human babies delivered by Caesarean section have a bacterial composition that is more similar to adult skin compared to those born naturally which have a bacterial composition resembling the mother's vaginal microbiome (Dominguez-Bello et al., 2010). The differences arising from these changes have been linked to asthma and obesity in later life (Arrieta et al., 2014). In

young mice, obesity has been specifically linked to changes in relative abundance of Firmicutes and Bacteroidetes bacteria (Turnbaugh et al., 2006), although more work must be done to establish these connections. Furthermore, as immune systems may not necessarily be established at these young stages (Dominguez-Bello et al., 2010), there may be an increased likelihood for dysbiotic microbial compositions or pathogenic infection that could have distinct effects on host immune activity, neurodevelopment and stress (Sudo et al., 2004; Lee and Brey, 2013; Ogbonnaya et al., 2015). Therefore, the microbiome could be both an element that is affected by early life conditions, and an effector of change in other factors.

1.2.1 Anticipatory Plasticity

Anticipatory plasticity is a specific form of developmental plasticity whereby the individual responds plastically to cues that indicate aspects of the future environment (Kasumovic, 2013). Anticipatory plasticity differs from reactive plasticity in that there is generally more time available before the future conditions are met to gather and process information, and to ultimately refine the phenotype (Kasumovic, 2013). One potential constraint to this form of plasticity is the requirement for current cues to give an indication of future conditions, as variations could result in future phenotype-environment mismatch if these are not reliable indicators or due to incorrect assessment of them (Kasumovic and Brooks, 2011). Anticipatory plasticity may therefore be expected to occur more readily in shorter-lived species where conditions are more predictable within an individual's lifetime (Douhard et al., 2014). However, it has been observed both in some short-lived species such as crickets and fruit flies (Bailey et al., 2010; Bretman et al., 2016), and species that live relatively longer such as bank voles, *Myodes glareolus* (Lemaître et al., 2011). Conditions that could be predicted in this manner include prey availability and predation risk. For example, the cuttlefish, *Sepia officinalis*, increase their uniform background matching camouflage ability as juveniles

following exposure to predator cues as embryos (Darmaillacq et al., 2014) and can shift prey preference after hatching following exposure to specific prey types whilst inside the egg (Darmaillacq et al., 2008).

Another element of the environment that individuals could respond to in an anticipatory manner are social conditions. Conspecific presence can be determined through acoustic (Bailey et al., 2010), tactile (Gage, 1995), visual (Rosenqvist and Houde, 1997) or chemical cues (Kasumovic et al., 2009), and could indicate future competition or mating opportunities (Kasumovic and Brooks, 2011). For example, male zebra finches reared in mixed-sex groups have lower aggression and courtship as adults than those kept with a single female, with reduced aggression likely to be beneficial in a competitive context (Ruploh et al., 2013). As previously mentioned, sperm competition can be an important determinant of male fitness, and optimising reproductive traits during development in anticipation of competition could be valuable in yielding an individual equipped to deal with a competitive environment (Gage, 1995; Bretman et al., 2016), or directing energy towards other traits when sperm competition is low (Relyea, 2004). For example, bank voles, *M. glareolus*, when reared in the presence of odours indicating rival male presence, develop larger seminal vesicles (Lemaître et al., 2011); male moths, *Plodia interpunctella*, from high larval densities develop relatively larger testes than those from low densities (Gage, 1995), and crickets, *Teleogryllus oceanicus*, develop larger reproductive tissues and display different reproductive tactics if reared as juveniles with the calls of conspecific males than those reared without acoustic cues (Bailey et al., 2010). In addition, male moths, *Uraba lugens*, reared at a low larval density develop larger antennae and wing lengths, which could help with mate searching where female density is low (Johnson et al., 2017). In addition, those reared with a greater number of other individuals have larger testes, which is beneficial where high reproductive competition is predicted (Johnson et al., 2017). This example highlights that both reproductive traits (larger testes) and other morphological traits not directly associated with

reproduction (wing length and antennae size) can show developmental plasticity in response to social cues, and can occur simultaneously.

Despite the fact that the social environment could show substantial variation, both spatially and temporally (Kasumovic et al., 2008), there must be some predictability or consistency required for anticipatory plasticity to occur (Kasumovic and Brooks, 2011). Cues about the future social environment could be gathered from individuals of the same age (Gage, 1995; Bretman et al., 2016) or from a different age cohort (Kasumovic et al., 2013; Bretman et al., 2016). Thus, how generations overlap is likely to be an important factor in what cues are present, and how an individual then responds (Kasumovic and Brooks, 2011). Development time will also have an effect, through influencing time to process information, respond, and ultimately, what social environmental conditions are met (Kasumovic and Brooks, 2011). For example, juvenile crickets, *Teleogryllus commodus*, reared in perceived low competition conditions develop quicker and at a smaller size than those from high competition environments (Kasumovic et al., 2011). This allows the opportunity to reproduce under less competitive conditions, and likely also reduces the chance of the conditions varying between stages (Kasumovic et al., 2011). As with other forms of plasticity, cognitive processing is important for producing an appropriate anticipatory response, and will be heavily influenced by the underlying neural structures (Snell-Rood, 2013), and prior experience, or perhaps the lack thereof, in early life (Dukas, 1998). The response should also not be significantly costly to the individual during juvenile stages (Monaghan, 2008), and the overall benefits of anticipatory plasticity, whereby future environmental conditions are correctly predicted, must outweigh the costs for this to be adaptive (DeWitt et al., 1998).

1.3 Study System

1.3.1 *Drosophila melanogaster*

This study uses the established model species, the fruit fly *Drosophila melanogaster* to investigate the effects of early life social conditions during larval stages on development and adult behaviour and physiology. This species has been used to examine phenotypic plasticity under a large number of environmental conditions (Chippindale et al., 2004; Bretman et al., 2009), making it an ideal species to study these effects. Under laboratory conditions, *D. melanogaster* develops from egg to adult in approximately 10 days at 25°C (Joshi, 2001). Eggs hatch into larvae after approximately 1 day, then continue through three instar stages before pupating. During metamorphosis, the pupa undergoes major morphological changes before eclosion as an adult (Robertson, 1936).

In particular, this species has been shown to exhibit plasticity in response to the social environment, for example, male *D. melanogaster* increase their mating duration following exposure to a rival male; this improves their paternity share (Bretman et al., 2009); is reversible following rival removal for 3 days (Bretman et al., 2012), and involves a greater transfer of seminal fluid proteins which are produced in the accessory glands and contribute in a variety of ways to male fitness (Gillott, 2003; Wigby et al., 2009). To respond in this manner requires a combination of two cues from smell, touch and sound (Bretman et al., 2011b). However, this response is connected to an overall decrease in later life mating opportunities (Bretman et al., 2013b). Social conditions are also important for fruit fly larvae (Durisko and Dukas, 2013a). For example, larvae are attracted to food previously occupied by others (Durisko and Dukas, 2013a), and can benefit from burrowing in a social aggregation (Durisko et al., 2014b), which appears to require visual and some mechanosensory cues of conspecifics (Dombrovski et al., 2017). Additionally, the social environment has been shown to affect fruit flies in the wild (Wertheim et al., 2006). Individuals will aggregate on

substrates treated with the aggregation pheromone cVA, resulting in a large number of interactions between conspecifics, including interference of behaviour such as feeding (Wertheim et al., 2006). The increased numbers of females on the substrate then influences the numbers of eggs laid (Wertheim et al., 2006). This in turn, affects larval competition and offspring body size changes in a density-dependent manner, with the largest body sizes occurring in flies from intermediate larval densities (Wertheim et al., 2006). Additionally, substrates with increased aggregations of fruit flies have a higher risk of parasitism from parasitoid wasps, *Leptopilina* spp. (Wertheim et al., 2006).

Studies in laboratory reared *D. melanogaster* have found plasticity in learning ability (Rouse, 2016), neural structure (Heisenberg et al., 1995), and gene expression (Mohorianu et al., 2017) in response to the social environment. Furthermore, sexual selection has been demonstrated to affect cognition in *D. melanogaster*, such that males in monogamous lines show a reduced learning ability than those from polygamous lines (Hollis and Kawecki, 2014). However, flies that have been through a conditioning regime to form long-term memory, have a decreased starvation and desiccation resistance (Mery and Kawecki, 2005). Furthermore, the social environment could affect both stress and immunity in fruit flies (Rush et al., 2007; Leech et al., 2017). A number of different pathways are involved in stress responses of *D. melanogaster*, and may be differentially activated depending on the type of stress or the type of energy available (Gefen et al., 2006; Rush et al., 2007). In addition, fruit flies have an innate immune system that primarily consists of the Toll pathway, which is activated by Gram positive bacterial and fungal infections (Lemaitre and Hoffmann, 2007), and the immune deficiency or IMD pathway, which is activated by Gram negative bacteria (Lemaitre and Hoffmann, 2007). Housing males in pairs can significantly reduce lifespan following injury compared to those kept in isolation (Leech et al., 2017). In addition, the costs of exposure to males, both mating and non-mating related, can result in reduced lifespan in females (Partridge and Fowler, 1990), and the transfer of seminal fluid to

females during mating results in immune suppression (Short et al., 2012). In addition, aggressive encounters could also have effects, for example, by increasing the chance of injury (Nandy et al., 2016). Commensal bacteria too have been shown to affect host gene expression, including those involved in stress responses (Broderick et al., 2014), and the social environment has been found to influence the microbiome composition in older adult flies in a sex-specific manner (Leech, 2017). Changes in the microbiome could have significant effects on the host, for example, one common species of the fly microbiome, *Lactobacillus plantarum*, can increase larval growth under nutrient-limiting conditions by affecting the host's nutrient signalling pathways (Storelli et al., 2011). Early life exposure can be particularly important in developing these host-microbiome associations, for example, experience with *Acetobacter pomorum* results in a preference in larvae for this bacteria (Wong et al., 2017). Through direct or indirect means, microbiome composition could be affected by early life social conditions, which could subsequently have distinct effects on the host.

1.3.2 Anticipatory Plasticity in *D. melanogaster*

As mentioned, *Drosophila melanogaster* can show an anticipatory plastic response to future reproductive competition. In this case, rearing at a high larval density whilst providing a high concentration of food to reduce resource competition, or rearing with adult males present in the larval environment, results in an increase in male accessory gland size (Bretman et al., 2016). Increasing accessory gland size can benefit the fly through the production of seminal fluid proteins that can enhance male reproductive success (Wigby et al., 2009). Additionally, rearing flies under low larval densities, without manipulating food availability, results in larger males that transfer less seminal fluid proteins and larger females with a greater remating frequency than those reared at high larval densities, which could suggest condition-dependent resource allocation, and perhaps, also a response to cues of high sperm competition risk (Wigby et al., 2016).

Interestingly, Bretman et al. (2016) found no effect of larval density on testis size but these were smaller when reared in the presence of adult males, a response that was not associated with the increase in accessory gland size, suggesting that the two types of larval social environments have some differential effects. Neither high density nor presence of adult males affected the mating duration of males, suggesting that this behavioural response is plastic to the adult conditions. As the developmental social environment may not exactly match the adult conditions these individuals eventually meet, and if the adult social conditions are likely to be variable, then altering this response as adults is advantageous (Bretman et al., 2016). Further, whilst the definite developmental change in accessory gland size can have an obvious advantage under reproductive competition during the adult stage, this response, and indeed, these larval environments in general could have a wider range of interconnected influences on other traits, including cognition, stress and immunity. As such, total fitness will depend on the individual adopting its overall optimal phenotype for the conditions.

1.3.3 Thesis Outline

In this thesis, I use the fruit fly, *Drosophila melanogaster*, to investigate how larval social environments can influence a variety of factors in the adult fly. Anticipatory plasticity can allow an individual to prepare for the specific set of conditions it expects to face in the future, from prey availability (Darmaillacq et al., 2008) to reproductive competition (Gage, 1995). However, one ‘set’ of conditions is unlikely to have a single influence. From the research in our laboratory, we have observed distinct effects of the social environment on reproductive behaviour (Bretman et al., 2009), cognitive ability (Rouse, 2016), immunity, and the microbiome (Leech, 2017) in adult fruit flies. It is likely that social conditions can also influence individuals in early life. *D. melanogaster* larvae show a specific anticipatory response to future reproductive competition (Bretman et al., 2016). Through a variety of factors,

including differential resource allocation (Isler and van Schaik, 2006) or indirect physiological effects (Harshman and Zera, 2007), this anticipatory response could have influences on other traits, potentially producing a complicated network of connections as a consequence of this 'set' of conditions. Whilst each of these could have distinct effects during the stage when the conditions are first encountered, they also have the potential to affect (positively or negatively) at later life stages. I therefore alter larval social conditions, namely by manipulating larval density or by the presence/absence of adult males in the larval environment (Bretman et al., 2016) to examine a number of these factors, including cognition, stress, immunity and microbiome composition in adult flies.

First, I investigate how early life social conditions can affect cognition in adult fruit flies (Chapter 2). The processing of social cues requires cognitive processing ability and the provision of these cues could influence the development of cognition during larval stages. Previous work in our laboratory has found an increase in learning ability for adult males kept with rivals than for those kept singly (Rouse, 2016), and I expected that this would also be the case following changes in larval social environments. Alternatively, production of larger accessory glands could result in a trade-off that reduces cognitive ability in males from high density or adult presence larval environments. I use two learning paradigms to explore such effects in males, and attempt to elucidate potential underlying genetic changes that could influence cognitive ability by examining cognition-related gene expression using RT-qPCR.

Chapter 3 looks at the how these early life conditions can affect a number of life history traits in *D. melanogaster*. I explore the effects of larval social conditions on lifespan and development time. Development rate has previously been shown to be affected by larval density (Joshi, 2001; Horváth and Kalinka, 2016), but without controlling for competition for food under high density. As such, I use an increased food concentration to reduce these

effects. If the larval social conditions can also indicate the adult reproductive environment for females as they do for males, it could be hypothesised that females too would alter investment in reproduction, and therefore, I examine the effect of larval social conditions on a number of female reproductive traits. Following the results from Chapter 3, I next investigate how these early life social conditions may affect stress tolerance and immunity in adult fruit flies (Chapter 4). Through trade-offs with other functions, such as those investigated in the preceding chapters, these could be negatively affected by differing larval conditions. Alternatively, the larval conditions could act to prime immune and stress responses, resulting in increased tolerance at later stages. In this chapter, I subject adults to a variety of stressors, namely starvation, desiccation and extreme temperature stress, and examine post-infection lifespan, and appetite on an infected food source. In order to observe if there were potential changes in expression of immune or stress-related genes that could affect these responses, I carry out RT-qPCR on immune and stress-related genes previously found to be socially-sensitive in this species. Subsequently, Chapter 5 explores if early life social conditions can affect microbiome composition of fruit flies. The microbiome can have important effects on the host through changes in, for example, nutrient signalling pathways (Storelli et al., 2011), and could have indirect effects on factors explored in the previous chapters. As social conditions could affect the microbiome by horizontal transfer of bacteria, or indirectly through the differential activity of host stress and immune activity, I assess the microbial community associated with both pupal and adult stages for flies reared under different larval social conditions using 16S rRNA Sequencing.

Finally, I discuss the general findings of this research and the implications that it has for the understanding of the effects of early life on later stages (Chapter 6). This work demonstrates the breadth of responses that can be influenced by social experience in early life, and highlights the need for these critical periods of an organism's life history to be taken into account. I end with some ideas and questions that remain to be answered in future studies about the far-reaching consequences of early life conditions.

Appendix A.1 contains details of the fly food recipes for standard, concentrated and no-food containing media; and finally Appendix A.2 contains the primer sequences used to examine gene expression and for 16S rRNA sequencing.

Chapter 2

Effect of Early Life Social Conditions on Cognitive Responses in Young Adult Fruit Flies, *Drosophila melanogaster*

Thanks to James Rouse for help with data collection, Andrew Smith for use of the qPCR machine, and Elizabeth Duncan for help with RT-qPCR analysis.

2.1 Summary

Organisms are constantly encountering cues from their environment that could inform them about, for example, competitors, food or mates. The ability to process and use this information is therefore extremely important for their fitness across life stages, and changes in cognition occurring during developmental stages could have significant effects later in life. The presence of conspecifics is expected to provide a wealth of new information, and it may be predicted that such cognitive stimulation would lead to an increase in general cognitive ability. Early life stages are likely to provide a source of many novel cues, which could also influence development of neural structures and could be important periods for influencing general cognitive capacity. In this chapter, I explored the effects of larval social conditions on the cognitive ability of young adult *Drosophila melanogaster*. Larvae were reared at high or low density, or with or without adult males in the environment. Males kept at high larval densities and in the presence of adults have been shown to produce an anticipatory response to future reproductive competition, and must gather and process information about conspecifics in order to respond. I measured the expression of a number of genes known to be involved in learning and synapse formation and function

that respond to the social environment in adult males. Males raised at low density as larvae had higher expression of the gene *Neurexin-1*, which can increase the formation of synapses. To investigate the phenotypic consequences of this, I then used two established learning assays to test for possible effects on adult male learning ability. The presence of adult males in the larval environment had no significant effects on learning ability of these young adults. Males raised at low density as larvae showed an increased ability in the sexual-context learning assay. These results suggest that larval social conditions could have important effects on synaptic plasticity, with potential influences on cognition in adults.

2.2 Introduction

Cognition, the ability to gather, process and retain information, can vary depending on environmental conditions (Pearce, 1997; Morand-Ferron and Quinn, 2015). Locating food and mates, for example, requires cognitive processing, the results of which could have important consequences on an individual's fitness, such that failure to accurately gather this information and process it could, for example, decrease reproductive success or decrease the availability of energy for other processes (Buchanan et al., 2013). Environmental enrichment has been linked to increases in learning and memory in animals including mice, *Mus musculus* (Kempermann et al., 1997), pheasants, *Phasianus colchicus* (Whiteside et al., 2016), crickets, *Acheta domesticus* (Mallory et al., 2016), and fruit flies, *Drosophila melanogaster* (Dukas and Mooers, 2003). Additionally, environmental factors could stimulate or activate the development and function of neural structures (Buchanan et al., 2013), such as the number of neurons (Kempermann et al., 1997) or increase synapse to neuron ratio (Bhide and Bedi, 1984), and increased development of these structures could increase the cognitive capability of an individual. As neural tissue is energetically expensive (Niven and Laughlin, 2008), the costs of investing in the

production and maintenance of such neural structures may outweigh the benefits if not required, such as under stable environmental conditions (Sol, 2009). During early life, there are costs to naïve decision-making (Dukas, 1998). For example, selecting a poor quality food source, not avoiding a predator or failing to respond to a competitor could have significant effects. Under these circumstances, it may be beneficial to invest in cognition, and this could be particularly true when conditions are variable (Sol, 2009). One type of condition that is likely to vary is the social environment (Kasumovic et al., 2008). The presence of conspecifics could be an abundant source of novel information about, for example, the availability of mates (Kasumovic and Andrade, 2006) or rivals (Bretman et al., 2009). Indeed, social isolation during early life has been implicated in a number of human disorders, including schizophrenia, suggesting social cues are paramount for normal brain function (Ellenbroek and Cools, 1998). Likewise in young rodents, isolation reduces performance in conditioning tasks (Fone and Porkess, 2008), whilst the deprivation of young honeybees to social stimuli results in a decreased learning ability that reduces in line with increasing isolation time (Ichikawa and Sasaki, 2003). Using this paradigm, learning could be reversed following later social isolation even after successful acquisition of the task, suggesting that social cues are necessary for the maintenance of learning ability (Ichikawa and Sasaki, 2003).

Often such experiments have tested the cognitive ability of individuals following social deprivation at one stage, for example, during post-weaning stages in rodents (Fone and Porkess, 2008), and post-emergence in bees (Ichikawa and Sasaki, 2003). However, experiments that add a social element at one stage and compare at another are less common. Social isolation during juvenile stages reduces the synapse to neuron ratio in rats tested 30 days later (Turner and Greenough, 1985). Additionally, rearing jumping spiders, *Marpissa muscosa*, in socially enriched conditions enhances their learning ability and reduces aggression compared to those reared in isolation (Liedtke and Schneider, 2017). Thus, social contact during rearing results in increased learning and social competence through a

reduced propensity to engage in damaging encounters with conspecifics (Liedtke and Schneider, 2017). Conversely, *Lymnaea stagnalis* snails kept in social isolation do not perform differently in a memory task to those kept in groups, except under low calcium stress when they actually show an improvement in long-term memory ability (Dalesman and Lukowiak, 2011). Under these social conditions, increasing cognition may not be beneficial to these snails, but under low calcium, reductions in reproductive activity could affect energy availability for cognition (Dalesman and Lukowiak, 2011). This indicates that social stimulation does not always result in an increase in cognitive ability, and can depend on the environmental context.

Social conditions are an important indicator to males with regards to the levels of sperm competition they may face within an environment (Parker, 1970). The need for males to gauge the level of reproductive competition may represent a particular requirement for increased cognitive capacity in this sex. The fruit fly, *Drosophila melanogaster*, has been established as a suitable invertebrate model for studying sperm competition (Bretman et al., 2009), and has also been used in studies of learning (Dukas, 2008), despite possessing a seemingly simple brain (Greenspan and van Swinderen, 2004). For example, adult male *Drosophila melanogaster* improve general learning ability in response to conspecific but not heterospecific *Drosophila virilis* males (Rouse et al., *in prep*), demonstrating that this is a response to reproductive competitors. An improved ability to process information about rivals would be beneficial in allowing males to plastically adjust sperm competition responses appropriately, such as altering their mating duration (Bretman et al., 2009). Social conditions have previously been shown to affect learning ability in adult male fruit flies, for example, evolutionary lines held under monogamous conditions for over 100 generations reduce their performance in learning tasks compared to those under polygamous conditions (Hollis and Kawecki, 2014). Male *D. melanogaster* exposed to rivals increase their mating duration (Bretman et al., 2009), and gene expression changes recorded in the heads and thorax of these males suggest these could be important underlying mechanisms of the phenotypic

changes associated with this response (Mohorianu et al., 2017). In addition to the improvement in learning ability in *D. melanogaster* males in response to conspecific rivals, our laboratory has also found changes in the expression of genes involved in synaptic growth and plasticity in these rival-exposed males (Rouse, 2016).

In insect brains, the mushroom bodies (MBs) are structures important for olfactory learning and memory (de Belle and Heisenberg, 1994). These structures may be influenced by social interactions, for example, honey bees, *Apis mellifera*, show a slowed growth of MBs when reared in social isolation compared to those in grouped conditions (Maleszka et al., 2009). These individuals also perform worse at an associative learning task (Maleszka et al., 2009). Increases in MBs have been found in social reproductive sweat bees, *Megalopta genalis*, compared to solitary individuals (Smith et al., 2010). Additionally, *D. melanogaster* mushroom bodies can also vary in size in response to the fly's social context (Heisenberg et al., 1995). During early adult stages, social conditions may affect the structure of, or gene expression in, the fruit fly brain (Heisenberg et al., 1995). For example, social enrichment in 11 day old adult flies induces growth in synapses of lateral ventral neurons, with a corresponding increase in sleep that is associated with dopaminergic signalling (Donlea et al., 2014). Decreased synaptic plasticity has been linked to age-related reductions in human memory formation (Grady and Craik, 2000), and therefore, these changes could have important effects on cognition later in life (Donlea et al., 2014). The capability for specific memories to endure through metamorphosis in *Drosophila* has been debated (Tully et al., 1994; Barron and Corbet, 1999). Whilst Tully et al. (1994) found evidence for long-lasting memory formation in larvae in an associative odour learning task when tested as adults 8 days later, another study found no such effect (Barron and Corbet, 1999), and suggested previous studies may have been confounded by odour contamination of the pupae (Barron and Corbet, 1999). Despite this disagreement, there is evidence in other species, such as houseflies, *Musca domestica* (Ray, 1999); moths, *Manduca sexta* (Blackiston et al., 2008); and

parasitic wasps, *Hyssopus pallidus* (Gandolfi et al., 2003). During *D. melanogaster* metamorphosis, many neurons present in the larval mushroom bodies degenerate, but others persist into adult stages (Lee et al., 1999), possibly representing a method by which memories formed during early life could endure. Additionally, genes known to be involved in learning in adult flies are also similarly expressed in larval mushroom bodies (Crittenden et al., 1998). Thus, changes in gene expression or neuron development in response to larval social conditions could affect the cognitive processing capabilities of adults.

In this chapter, the effect of early life social conditions on cognitive responses of young adult fruit flies was investigated by manipulating larval density or by the presence of adult males in larval vials. These manipulations change the larval social conditions in a manner that indicates to males an increase in future reproductive competition, resulting in larger accessory gland growth once they reach the adult stage (Bretman et al., 2016). These conditions involve changes both within-age cohort (larval density) and between cohorts (adult presence), and could, therefore, involve the assimilation of different types of cues. As males require cognitive ability to assess the level of reproductive competition in the environment, and as adult males increase learning ability under conditions of reproductive competition (Rouse, 2016), it may be expected that males exposed to these environments as larvae may show an increase in cognition that could persist into the adult stage (Gandolfi et al., 2003). Enhanced social stimulation during development may lead to an increase in cognitive ability through changes in underlying neural mechanisms, such as synaptic plasticity. Additionally, as both the presence of adult males and higher larval densities can indicate reproductive competition at later stages and result in an anticipatory response of increased accessory gland growth (Bretman et al., 2016), any increase in cognitive capacity could be beneficial for adult males, possibly allowing for an increased ability to process information about such competitive conditions once they are met. Improving cognition in response to these early life conditions could, in this way, prime cognitive processing at

later life stages. Alternatively, increased growth of accessory glands could result in a trade-off between reproduction and cognition (Pitnick et al., 2006). Both reproductive and brain tissues represent energetic costs (Isler and van Schaik, 2006; Niven and Laughlin, 2008), and therefore, investment into one could negatively affect energy availability for the other factor. To elucidate possible underlying mechanisms of cognitive ability that could be affected by these conditions, I examined changes in the expression of genes linked to cognitive ability. These genes had previously been shown to change in adult males presented with a rival male (Mohorianu et al., 2017). I predicted that, if increased stimulation through high density or adult presence resulted in greater cognitive ability, there would be an increased relative expression in these genes, indicative of increased efficiency or stimulation of learning pathways or changes in the underlying neural structures in both sexes. However, if there are distinct requirements for cognition between the sexes, for example, from the need to assess sperm competition in males, then there may be differences in gene expression in males but not in females from these different larval conditions. Changes were found in males from different larval densities only for expression of the gene *Neurexin-1*, involved in synaptic plasticity. As such, I assessed cognition proximately by testing learning ability in the sexual environment and in an associative learning task (Hollis and Kawecki, 2014; Rouse, 2016) in young adult males from the different larval social environments. The first of these tested olfactory learning in a non-social task. The second examined learning in a sexual context by testing the ability of a male to find and court a virgin female in a group of mated females (Hollis and Kawecki, 2014).

2.3 Methods

2.3.1 Fly Husbandry

All flies used were wild type Dahomey strain *Drosophila melanogaster*. They were reared in a humidified room at 25°C, under a 12:12 light dark cycle on a

sugar-yeast diet. Adults were provided with purple grape juice agar plates to lay eggs, with larvae harvested approximately 24 hours later and transferred to treatment groups in plastic vials with 7ml of fresh medium, supplemented with live yeast paste. Adults were collected under ice anaesthesia.

2.3.2 Larval Social Treatments

Larvae were haphazardly allocated to treatment groups. For density treatments, larvae were either kept at 200 larvae per vial (high density) or 20 larvae per vial (low density). These larvae were kept on 150% concentrated food medium to prevent food being a limiting factor at high densities (Bretman et al., 2016) (Appendix A.1).

Adult presence treatments consisted of 100 larvae per vial with or without 20 added adult males aspirated in after larval collection. These adults were approximately 5 days old, and had been reared at a standard lab larval density of 100 per vial, then collected on the day of eclosion, anaesthetized on ice and sexed. The adults were removed from the adult presence vials the day before pupae eclosed. Adults from the larval treatment vials were also collected as above before use in the assays.

2.3.3 Gene Expression

Adult flies from both treatments were snap frozen in liquid nitrogen at 1 day old and sorted on dry ice. Approximately 50 flies per treatment were transferred into an Eppendorf on dry ice before being tapped several times to remove the heads, that were then stored at -80°C. In total, 43-50 heads were used per group, repeated over 7 weeks. From these samples, RNA was extracted by grinding the heads using a micropestle, and using the Direct-zol ™ RNA miniprep kits, following the manufacturer's protocol. The

RNA was checked on a Nanodrop and run on a 1% agarose gel to check for degradation (Figure 2.3.3-1). RNA was converted to cDNA using the First Strand cDNA synthesis kit (Thermo Fisher) following the manufacturer's protocol. Samples were amplified by PCR using *Actin* (*Act5c*) primers and run on a gel to check there was no contamination (Figure 2.3.3-1).

A total of 5 genes were studied for changes in expression, and were identified as differing in expression with the presence of adult rival males (Mohorianu et al., 2017), and had also been used in another study to examine expression responses in adult males to rival presence (Rouse, 2016). These include genes involved in the production of learning and memory, namely *dikar* which is involved in long-term memory formation (Akalal et al., 2011), and *dunce* which produces an enzyme involved in learning (Gervasi et al., 2010). Additionally, *bruchpilot*, a cytoskeletal protein gene involved in neurotransmitter release (Kittel et al., 2006), and *futsch* and *Neurexin-1*, that are associated with structural formation and growth of synapses (Roos et al., 2000; Zeng et al., 2007), were examined. Briefly, primers were designed with a melting temperature of 60°C ± 1°C and a CG content of 20-80% (Appendix A.2). Efficiency was determined using a 10 times dilution series. Primers were accepted if this efficiency was between 90 and 110% for dilutions, and if the pipetting accuracy (R^2) was greater than 0.99. Two housekeeper genes were used to normalise gene expression and had previously been identified as candidate reference genes, namely *Ef1* and *Rap21* (Ling and Salvaterra, 2011). Each sample was run on the qPCR plate in triplicate. Average Cq values were obtained for each sample. Triplicates which had extreme values (a difference in value of greater than 0.5) were excluded. Relative quantity was obtained by subtracting the Cq of each sample from the Cq of a selected reference sample (in all cases, this was the first sample in the dataset) and raising the efficiency of the primer to this number. Normalized expression for each gene of interest was calculated by dividing the RQ of each sample by the housekeeper geometric mean.

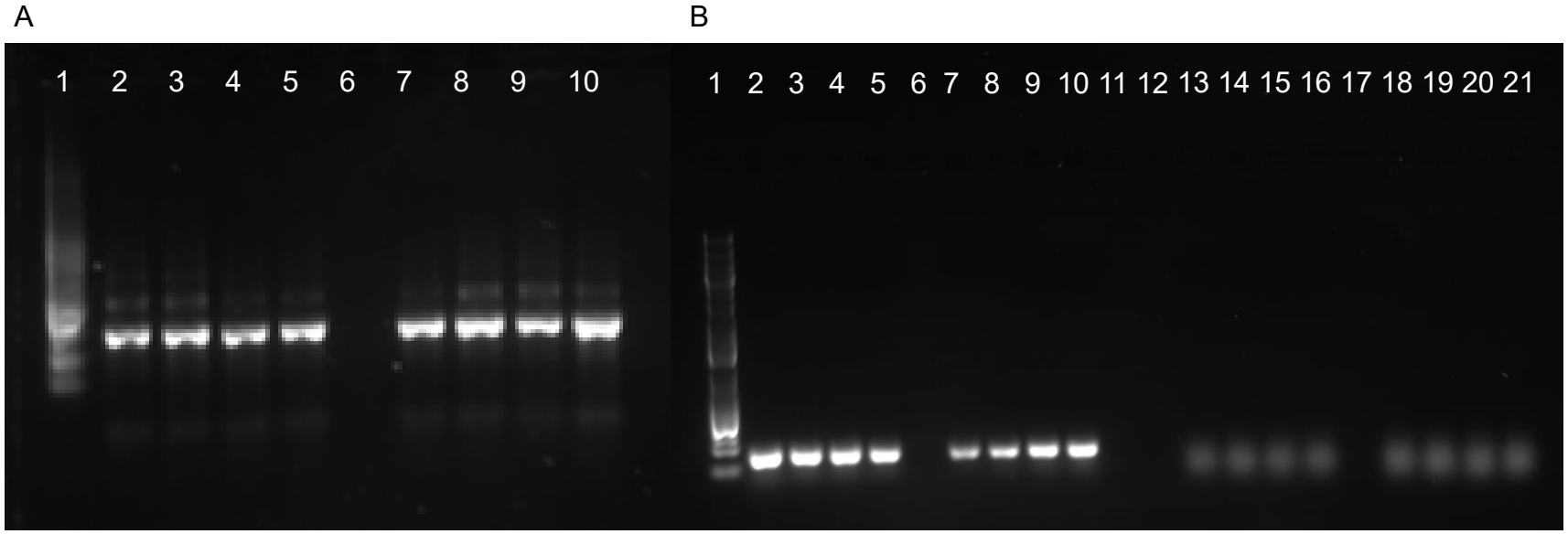


Figure 2.3.3-1 Example Electrophoresis Gels for RNA Extractions (A) and cDNA synthesis (B). A: Lane 1 contains ssRNA ladder; Lanes 2-5 contain RNA from Low Density males, Low Density females, High Density males and High Density females; Lanes 7-10 contain RNA from Adult Absence males, Adult Absence females, Adult Presence males, and Adult Presence females. In insects, the 28S ribosomal RNA is made up of two fragments that are disrupted and migrate in a similar manner to the 18S rRNA as seen above (Winnebeck et al., 2010). B: Lane 1 contains 100bp DNA ladder; Lanes 2-10 are groups ordered as before; Lanes 13-16 are negative Reverse Transcriptase (RT) controls for density groups; Lanes 18-21 are negative RT controls for adult presence groups. Markings around the bands occur through buffer degradation.

2.3.4 Virgin Finding

The virgin finding assay tests the ability of a male to find and court a virgin female in a group of mated females (Hollis and Kawecki, 2014). Female flies are unreceptive following mating due to the transfer of seminal fluid proteins (Wolfner, 1997), and therefore, males should learn which females have been mated and focus courtship on the virgin female over time. Males were collected on the day of eclosion and held in single sex groups of 10. As adult learning ability increases during the first week (Guo et al., 1996), and some males can take up to 3 days to be sexually mature, males were held until 5-6 days old for this assay (Eastwood and Burnet, 1977). Virgin females were collected from separate vials and the day before the virgin finding assay were anaesthetized using CO_2, and wing-clipped using a scalpel to allow their identification during the assay. For mated females, 10 adult males were aspirated into the vial and were left overnight. These females were considered mated for the following day. The assay was carried out at 25°C. Four mated females and one virgin female were aspirated into a new vial. The females were left for 5 minutes to acclimate, and then a single male originating from one of the larval social treatments was added. Each vial was scanned for approximately 6 seconds, during which the male was scored on whether he was displaying courtship behaviour towards a female (O'Dell, 2003), and if so, which type of female was being courted. Each vial was scanned once per minute for a total of 20 minutes. Males and virgin females were removed after each experimental run. Mated females were renewed every second run, and the order of treatment groups was reversed such that the groups added first during the initial run were then added second to reduce the possibility for timing effects on female behaviour. This was independently replicated three times, with 30 males per treatment in each replicate.

2.3.5 Associative Learning

Learning to avoid an odour paired with an aversive stimulus is a conditioning paradigm that has been used to study learning ability in fruit flies (Claridge-Chang et al., 2009; Hollis and Kawecki, 2014) (Figure 2.3.5-1). Flies were individually tested for their ability to associate an aversive mechanical shock with an odour in a T-maze. This assay was carried out at 23 ± 2°C and under red light to reduce the effects of visual cues. Odours used were 3-octanol (OCT) and 4-methylcyclohexanol (MCH), and have previously been used in associative learning assays with *D. melanogaster* (Hollis and Kawecki, 2014; Rouse, 2016). The odours were used at concentrations of 27µl OCT and 10µl MCH diluted in 10ml of light mineral oil. These concentrations had been balanced prior to the start of the assay by testing groups of flies at various concentrations until there was an approximate even split in numbers dispersing towards each side, in order to ensure one odour was not more influential on fly behaviour than the other. A vacuum pump was used to pull the odours through the T-maze, and a vortex was used to administer a mechanical shock as the aversive stimulus. The experimenter was blind as to which group belonged to each treatment during the assay. The flies were tested for their initial preference of each odour by measuring the amount of time spent in each arm of the T-maze over 2 minutes. To ensure that the fly was not simply responding to its preferred odour, this odour was then paired with the shock during the training period. Training cycles consisted of subjecting the fly to 1 minute of odour plus mechanical shock for 1s every 5s. This was followed by 30s of air only. The fly then received 1 minute of the alternate odour without shock, then another 30s of air. The training cycle was carried out twice in total. Following the training, the fly was immediately tested with a choice between both odours in the T-maze for 2 minutes. The amount of time spent in each arm was recorded. Therefore, individuals that had learnt to associate shock with the paired odour should avoid the arm with that odour. Treatment groups were tested alternately, and the side of the odours was changed after every second fly. Odours were renewed approximately every 10 trials. Preliminary power

analysis was carried out in R v3.3.3, calculated from a sample size of 10 individuals from adult presence and absence groups, with an effect size of 0.34. This suggested that a sample size of 35 was required to obtain a power of 0.8. Larval density flies were tested at 1 day old and at 4-8 days old, and adult presence flies were tested both at 1 day and 5 days old. Although I expected that any differences would be detectable at 1 day old, testing a few days later could potentially also reveal differences between these groups as learning ability has been shown to increase during the first week of adulthood (Guo et al., 1996).

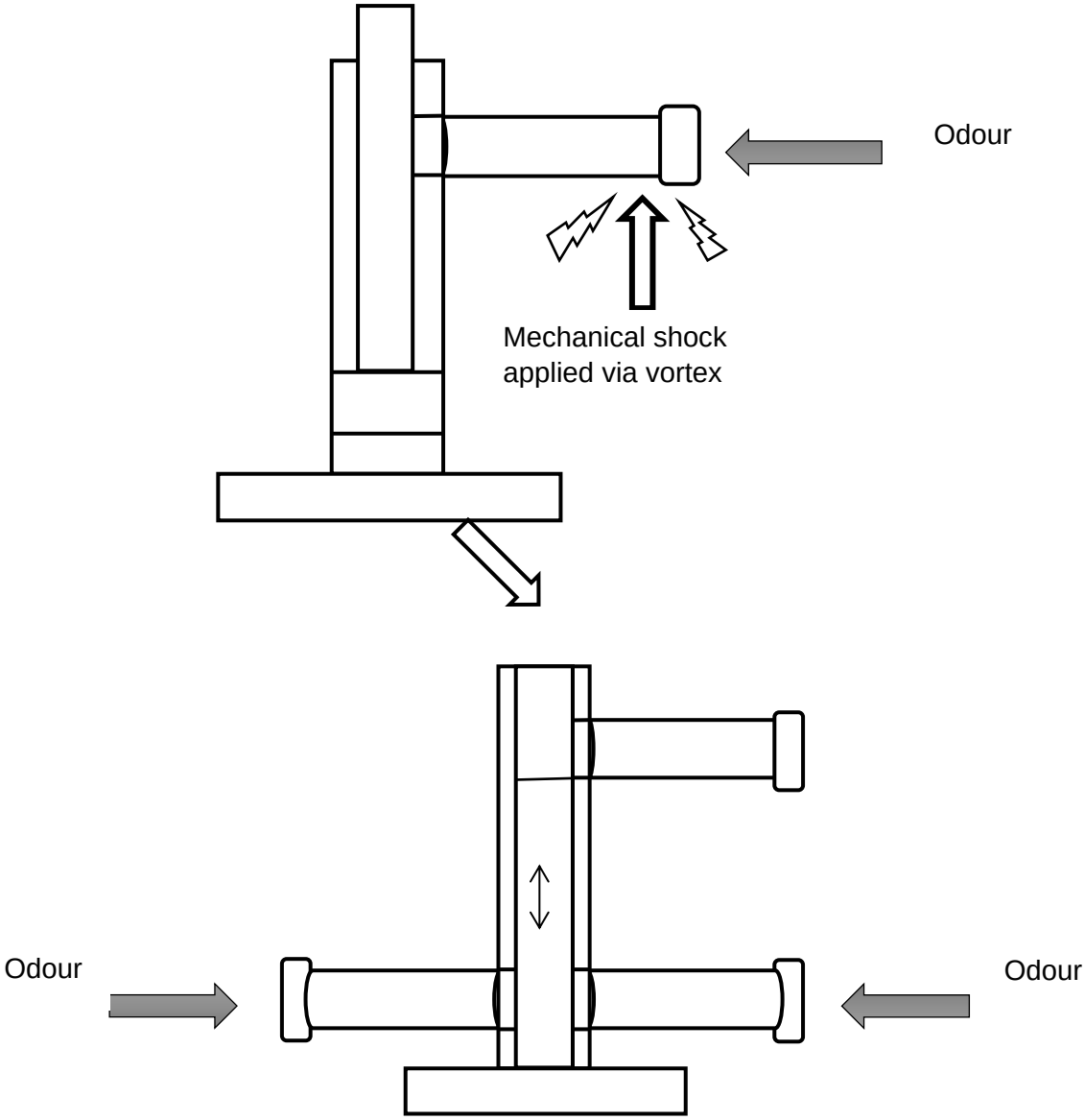


Figure 2.3.5-1 T-maze during training (top) and testing (bottom) phases. Flies were subjected to a mechanical shock paired with an odour for 1 minute and another odour without shock for an additional minute. Two training cycles were completed before the fly was tested for preference between the two odours.

2.3.6 Statistical Analysis

The data was analysed using R v.3.3.3 and SPSS v21. Gene expression for each gene of interest was analysed using Generalised Linear Models, with social treatment and sex as fixed factors, and a quasi-Poisson distribution to account for underdispersion. Models were reduced and compared by Analysis of Deviance.

For the virgin finding assay, the number of males courting and the number of males courting the correct female (virgin female) were totalled per minute for each repeat. Individuals that mated during the assay were removed from the remainder of the analysis, so numbers were always calculated from the total number of flies still available to court. Generalised Linear Models were carried out with social treatment and time as fixed factors, and repeat as a random effect, with a Binomial distribution (courting or not courting; correct female or incorrect female). Models were simplified using Analysis of Deviance. Overall correct courting, removing time as a factor, was analysed with Mann Whitney U tests. The number of individuals that mated or did not mate during the 20 minute test period were compared with Chi-square tests.

Learning index scores for the associative learning assay were calculated by dividing the total time spent in the correct tube during the testing period by the total time in this and the tube now associated with the shock. Any flies that did not make a choice were subsequently removed from the analysis. Learning indices were not normally distributed and were compared with Mann Whitney U tests. In order to test if variance differed between larval density treatments due to potential variability in these environments, Levene's tests were carried out on gene expression, the percent of males correctly courting the virgin female in the virgin finding assay, and the learning indices from the associative learning assay.

2.4 Results

2.4.1 Effect of Larval Density on cognition

No significant effect of larval density on normalized gene expression was found for *bruchpilot*, *dikar*, *dunce* or *futsch* (Table 2.4.1-1; Figure 2.4.1-1). There was a significant interaction between sex and density treatment for gene expression of *Neurexin-1* (Table 2.4.1-1). Analysing the sexes separately found no effect of density on females (t=-1.484; df= 12; p=0.164), but low density males had greater normalized expression relative to high density males (t=2.899; df =11; p=0.014) (Figure 2.4.1-1 E).

Table 2.4.1-1 Generalised Linear Model output for Gene Expression in the heads of 1 day old flies reared at high or low larval density.

Gene	Factor	F	df	p-value
bruchpilot	Density*Sex	0.246	1, 24	0.624
	Density	2.339	1, 26	0.139
	Sex	<0.001	1, 27	0.981
dikar	Density*Sex	0.283	1, 24	0.560
	Density	3.817	1, 27	0.062
	Sex	0.507	1, 26	0.483
dunce	Density*Sex	0.096	1, 24	0.758
	Density	2.576	1, 27	0.121
	Sex	0.233	1, 26	0.633
futsch	Density*Sex	3.290	1, 24	0.082
	Density	2.449	1, 26	0.130
	Sex	5.639	1, 27	0.025
Neurexin-1	Density*Sex	8.902	1, 24	0.007

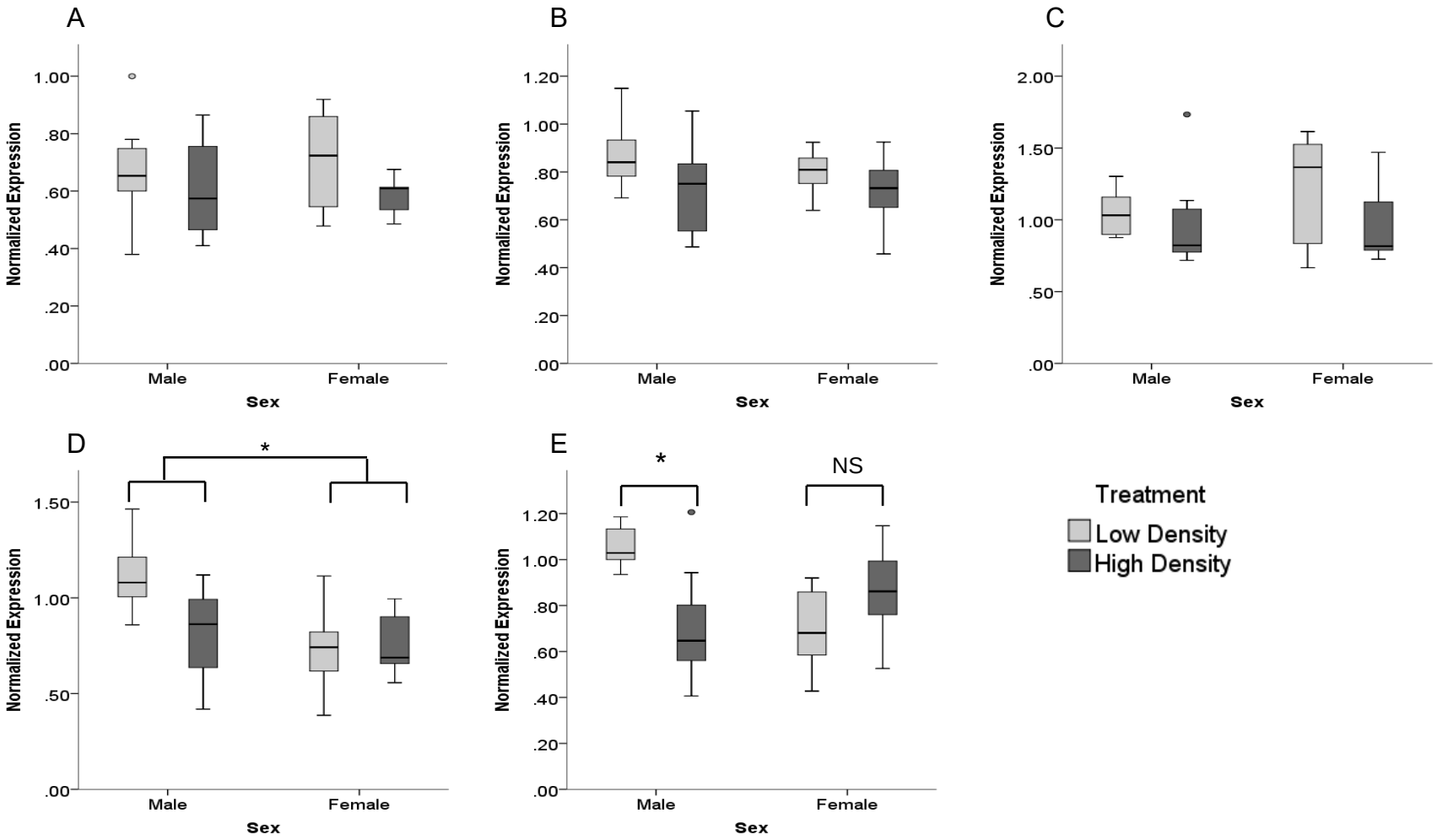


Figure 2.4.1-1 Median Normalized Expression for genes in the heads of adult flies reared at low or high larval density. A= *bruchpilot*; B=*dikar*; C= *dunce*; D=*futsch*; E=*Neurexin-1*. Extreme outliers (more than 1.5x Interquartile Range) were removed. * denotes significant differences ($p<0.05$); NS denotes non-significance.

There was no significant interaction between larval density and time for the number of males courting in the virgin finding assay (Analysis of Deviance χ^2=0.628; df= 1, 5; p=0.428). There was also no effect of larval density on the number of males courting (AOD χ^2=2.508; df = 1, 4; p = 0.113). There was a general increase in the percentage of flies courting over time (χ^2= 162.22; df= 1, 3; p<0.001) (Figure 2.4.1-2). There was no interaction between time and larval density for the number of males courting the correct (virgin) female (χ^2 = 0.001; df= 1, 5; p=0.973). However, there was a significant effect of time (χ^2=13.877; df= 1, 4; p<0.001), and a significant effect of larval social treatment for correct courting (χ^2 =4.853; df= 1, 4; p=0.028). This largely appears to be a result of a greater percentage of males correctly courting in the low density group during the middle period of the assay (Figure 2.4.1-2). However, overall correct courting, without time as a factor, was also significantly greater in low density males (U=1388, N=60, p=0.030). However, there was no significant effect of larval density on the numbers that mated during the assay (χ^2=1.168; df= 1; p=0.280).

No significant effect of larval density was found for learning index in the associative learning assay for 1 day old males (U=738, N=78, p=0.801) or 4-8 day old flies (U=501, N=69, p=0.231) (Figure 2.4.1-3). There was no significant difference in variance between flies from low or high larval densities for *bruchpilot* (F=2.222; p=0.112), *dikar* (F=1.233; p=0.320), *dunce* (F=2.627; p=0.075), *futsch* (F=0.510; p=0.679) or *Neurexin-1* expression (F=1.430; p=0.260); associative learning indices at 1 day (F=1.008; p=0.319) or 5 days old (F=0.099; p=0.754), or the percent of males correctly courting the virgin female in the virgin finding assay (F=0.024; p=0.878).

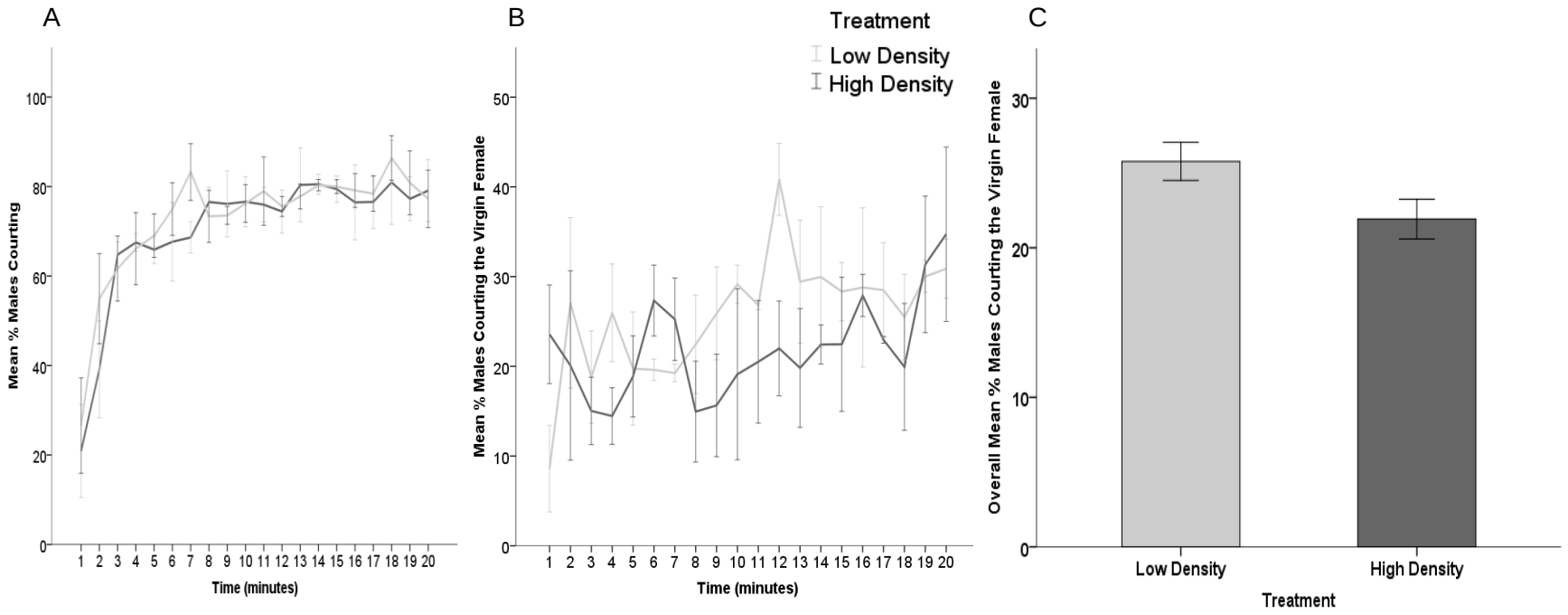


Figure 2.4.1-2 Effect of larval density on mean % of males courting a female during the virgin finding assay at each minute (A) and mean % males courting the virgin female at each minute (B) following male introduction, and overall mean % males courting the virgin female (C). Males were reared as larvae at high or low density and tested at day 5 post-eclosion. Error bars represent ± 1 S.E.

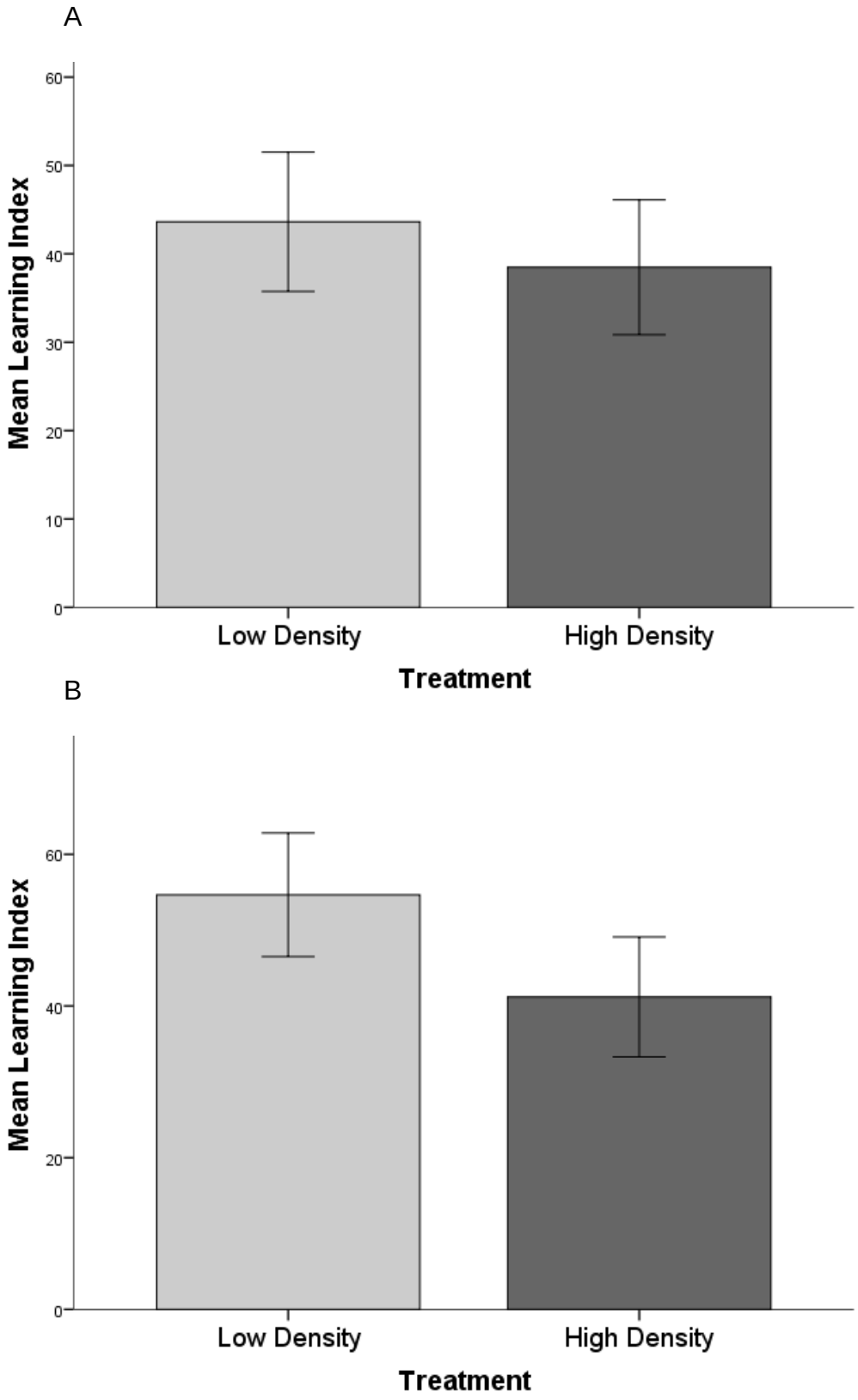


Figure 2.4.1-3 Mean Learning Indices in associative learning assay for adult males from high and low larval density treatments. A= 1 day old adult males (Low Density n=39, High Density n=39); B= 4-8 day old adult males (Low Density n=33, High Density n=36). Error bars represent ± 1 S.E.

2.4.2 Effect of Adult Presence on cognition

No significant effect of adult presence was found for normalized gene expression in any of the genes examined (Table 2.4.2-1; Figure 2.4.2-1).

Table 2.4.2-1 Generalised Linear Model output for Gene Expression in the heads of 1 day old flies reared as larvae with or without adult males present.

Gene	Factor	F	df	p-value
bruchpilot	Adult presence*Sex	0.868	1, 24	0.361
	Adult Presence	0.085	1, 26	0.773
	Sex	9.578	1, 27	0.005
dikar	Adult Presence*Sex	2.053	1, 23	0.165
	Adult Presence	1.009	1, 25	0.325
	Sex	2.099	1, 26	0.160
dunce	Adult Presence*Sex	0.076	1, 24	0.785
	Adult Presence	0.138	1, 26	0.714
	Sex	2.56	1, 27	0.121
futsch	Adult Presence*Sex	0.362	1, 24	0.553
	Adult Presence	1.562	1, 26	0.223
	Sex	7.184	1, 27	0.013
Neurexin-1	Adult Presence*Sex	0.212	1, 24	0.650
	Adult Presence	0.080	1, 26	0.779
	Sex	3.215	1, 27	0.085

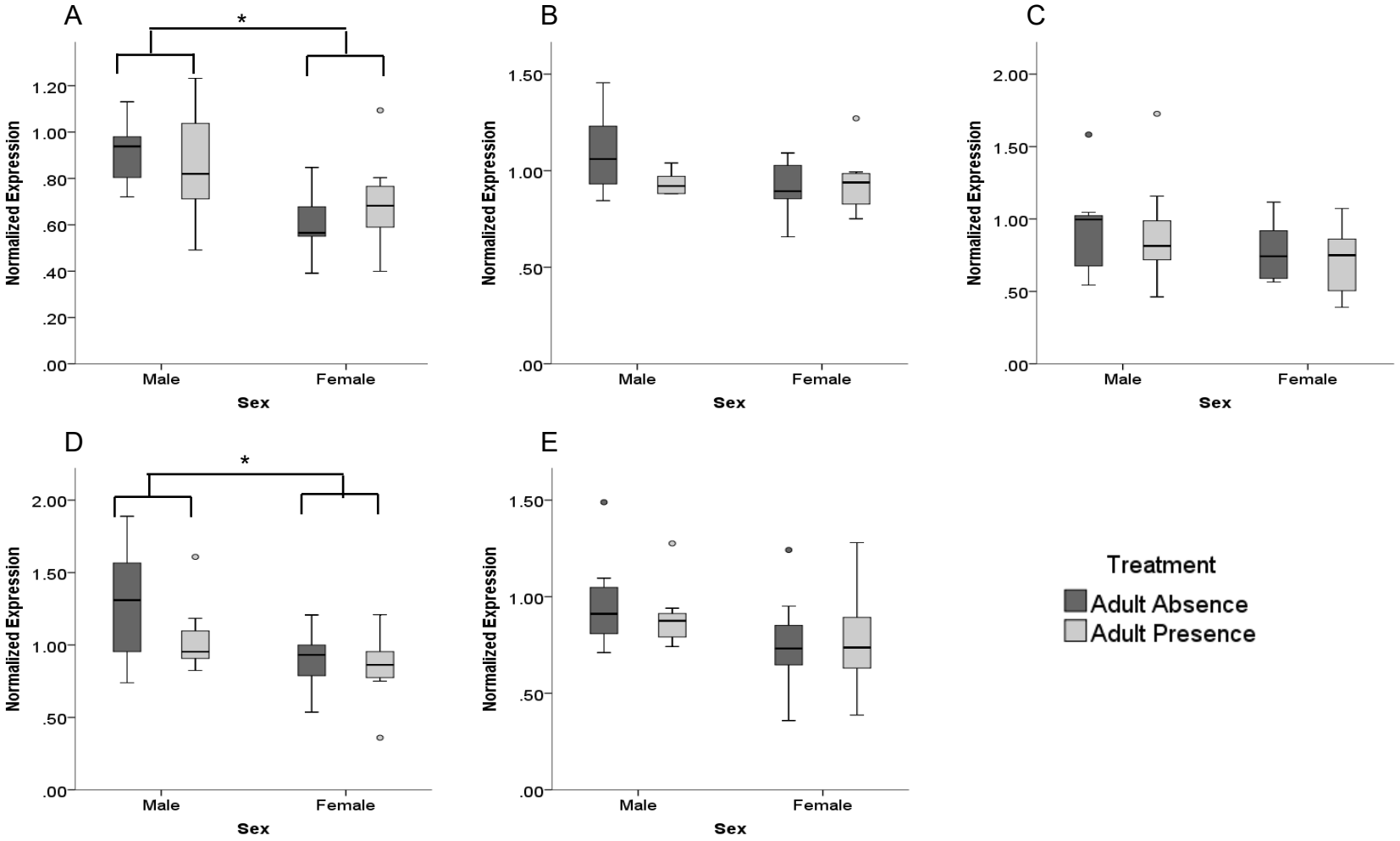


Figure 2.4.2-1 Median Normalized Expression for genes in the heads of adult flies reared as larvae with or without adult males present. A= *bruchpilot*; B=*dikar*; C= *dunce*; D=*futsch*; E=*Neurexin-1*. Extreme outliers (more than 1.5x Interquartile Range) were removed. * denotes significant differences ($p<0.05$).

There was no significant interaction between time and adult presence for the number of males courting in the virgin finding assay (Analysis of Deviance χ^2=0.022; df= 1, 5; p=0.881), although time was significant (χ^2=191.46; df=1, 3; p<0.001). The number of males courting was not affected by the presence of adult males during larval stages (χ^2=0.338; df= 1, 4; p=0.561). There was no significant interaction between time and adult presence for the number of males correctly courting (χ^2=0.133; df= 1, 5; p=0.716), but time was significant (χ^2= 11.534; df= 1, 3; p<0.001). Correct courting was not affected by adult presence (χ^2=0.681; df= 1, 4; p=0.409). Both responses showed a general increase over time (Figure 2.4.2-2). Overall correct courting, without the time factor, was also not significant between the groups (U=1570, N=60, p=0.227). There was no effect of adult presence on the numbers that mated during the assay (χ^2=0.806; df= 1; p=0.369).

There was no significant effect of adult presence on the associative learning task at 1 day (U=985, N=95, p=0.250) or 5 days old (U=1954.5, N=133, p=0.221) (Figure 2.4.2-3). Although, there was a general trend for adult presence males to have higher average learning indices than adult absence males at both ages.

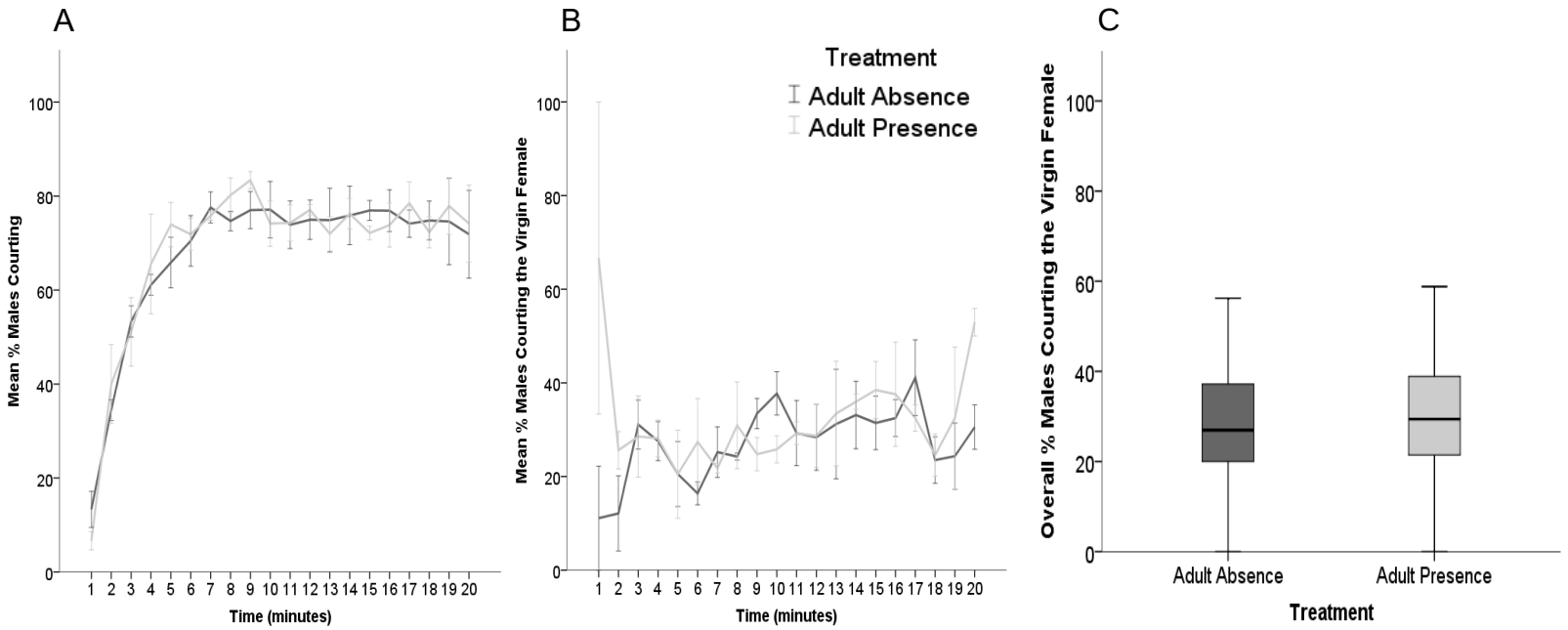


Figure 2.4.2-2 Mean % of males courting a female during the virgin finding assay at each minute (A) and mean % males courting the virgin female at each minute following male introduction (B), and overall median % males courting the virgin female (C). Males were reared as larvae with or without adult males present, and tested at 5 days post-eclosion. Error bars represent ± 1 S.E.

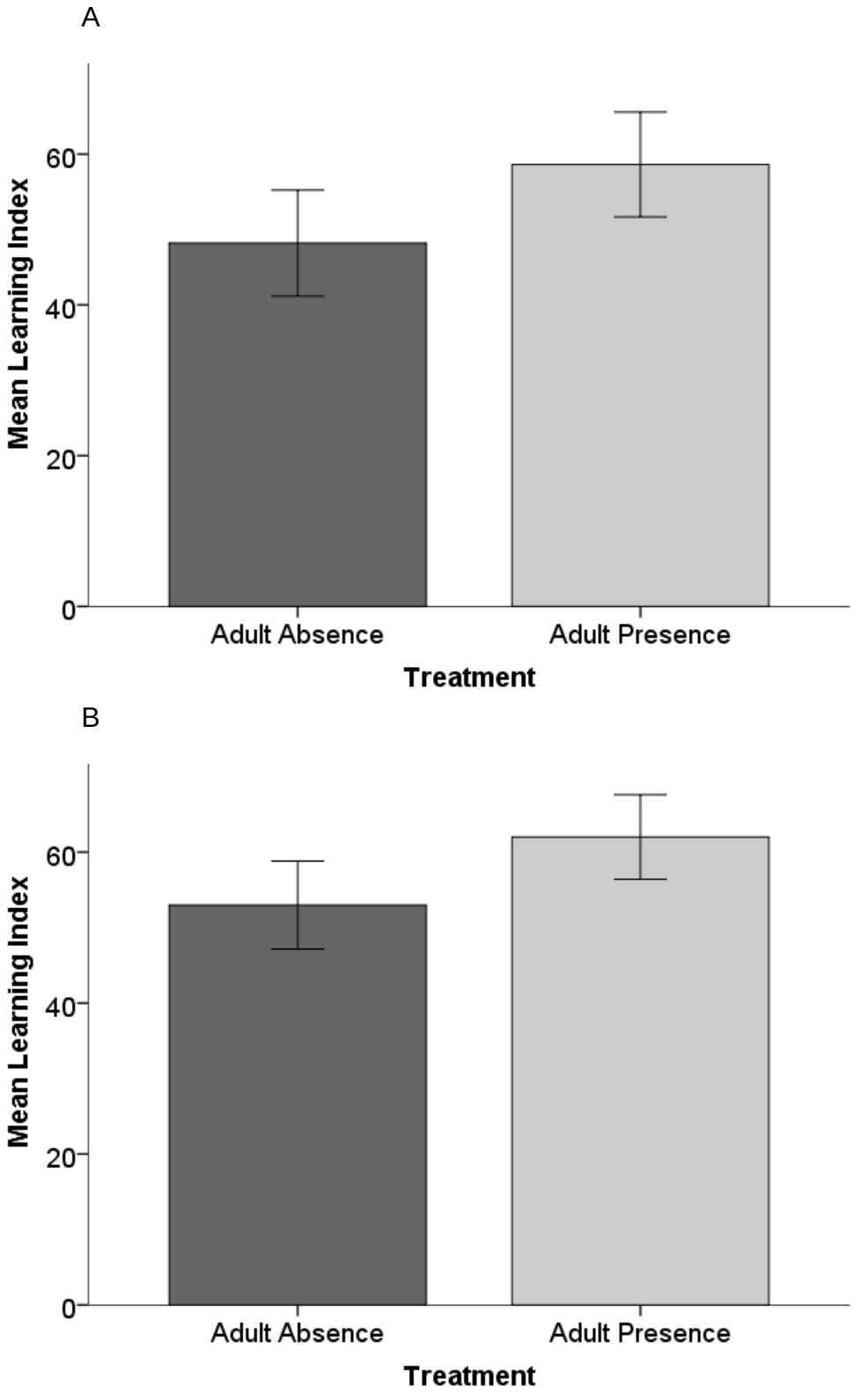


Figure 2.4.2-3 Mean Learning Indices in associative learning assay for adult males reared as larvae in the absence of adults and in the presence of adults. A= 1 day old adult males (Adult Absence n=48, Adult Presence n=47); B= 5 day old adult males (Adult Absence n=65, Adult Presence n=68). Error bars represent ± 1 S.E.

2.5 Discussion

In this chapter, I explored whether larval social environments can affect the expression of cognition-related genes in adults of both sexes, and whether these conditions can also affect the cognitive performance of adult male *Drosophila melanogaster*. An increase in social stimulation may be predicted to influence cognitive development, and as high larval density and adult presence during the larval stage result in an anticipatory response to future reproductive competition in males, it could be predicted that males from these environments would show an increase in learning ability. Alternatively, trade-offs between investment in reproduction and cognition could negatively affect cognition in these individuals. There was no effect of social conditions found for gene expression in females, however, the expression of *Neurexin-1* was relatively higher in males from low density larval conditions than in males from high larval densities. This suggests that larval social conditions can impact certain aspects of cognition in adult males. Overall, there was no effect of the presence of adults during the larval period on adult male learning ability. However, larval rearing densities had some effects. Males from low larval densities had overall increased correct courting in the virgin finding assay compared to high density males.

2.5.1 Larval Density has an effect on learning ability

Of the genes examined, only *Neurexin-1* showed significant sensitivity in expression to larval density, with higher normalized expression in 1 day old low density males than in high density males. Neurexin-1 is a synaptic molecule which has an important role in the formation of synapses in larvae and synaptic functioning (Zeng et al., 2007). Enhanced synaptic plasticity through increased *Neurexin-1* expression in low density males could link to the overall increased correct courting level also observed in the virgin finding assay. Decreased synaptic plasticity is associated with age-related declines

of memory in humans (Grady and Craik, 2000), whilst enhanced synaptic plasticity is associated with increased learning ability in mice (van Praag et al., 1999). Our laboratory previously found an increase in *Neurexin-1* expression in male adult *D. melanogaster* that had been kept with a rival compared to those kept singly (Rouse, 2016), suggesting it is socially responsive. Additionally, although the mechanisms still require further investigation, sleep appears to be associated with synaptic plasticity and is a socially responsive behaviour (Ganguly-Fitzgerald et al., 2006), that can also be affected by *Neurexin-1* expression (Larkin et al., 2015), and it would be interesting to explore this connection further. In addition, *futsch*, a synaptic cytoskeletal-associated protein important for synaptic growth (Roos et al., 2000), also increased in those kept with a rival (Rouse, 2016). Despite no significant effect of larval density on its expression, it is interesting to note that males in the low density group also appear to have a trend for higher expression than high density males in this gene. No effects were found for learning and memory genes *bruchpilot*, *dunce* and *dikar*, which resemble the results found in adults for these genes (Rouse, 2016). This suggests that the genes involved in synaptic plasticity may be more socially responsive in both larvae and adults than the genes involved in the learning process. Unlike the results obtained for the expression of *Neurexin-1* in males, there was no effect of larval density on gene expression of females, and this suggested that there would not be a difference in female learning ability. Combined with our previous result that found no effect of conspecific presence in adult female ability in the associative learning assay (Rouse et al., *in prep*), there was no indication that there would be an effect of larval density on adult female learning, and therefore this was not explored further. Further, as *Neurexin-1* expression changed in males from different larval densities, but not in females, this could indicate a difference in cognitive requirements between the sexes, which may be related to the need to process cues related to sperm competition in males.

The finding that low density males performed better at courting a virgin female than high density flies is not likely due to resource competition

limiting energy available for investment in neural structures, as food concentrations were increased in the density treatments to avoid this (Bretman et al., 2016). The largest difference between density groups occurred during the middle of the assay, occurring approximately between the 8th and 16th minutes. This could indicate that low density males can reach this level of correct courting more quickly than high density males. This is further emphasised with the overall correct courting, without taking time into account, which was significantly higher for low density than high density males. Notably, the numbers that mated during the assay did not significantly differ between groups, suggesting that the improvement in correct courting did not lead to greater mating success in the low density group during this time. However, as the assay was limited to only 20 minutes, it is possible that this could vary under longer timescales. Intriguingly, low density flies also had slightly higher averages in the associative learning task during the first week of adulthood. This trend was not significantly different from the high density group, but was consistent across assay sessions. Previously, Wang et al. (2018) found no effect of larval crowding on the learning ability of 3-6 day old adults in the T-maze assay, in accord with the results obtained here. In the Wang et al. (2018) study, however, there was also a trend for flies from intermediate larval densities (150-300 larvae in 8ml medium) to do better than either low (50) or high density (450 larvae) flies, which are higher densities than used here. However, the reasons for the differences observed between these studies are not entirely clear, but could implicate the level of competition for food. Indeed, the intermediate densities are close to the high density treatment in this study, and our larvae were maintained on a higher quantity of food to prevent its limitation at high density (Bretman et al., 2016). Furthermore, even in the presence of large quantities of food, larvae at high density could interfere with the feeding of others which could, for example, increase stress or reduce food consumption (Goss-Custard, 2002). In this study, however, the body mass of newly eclosed adults has been found to not significantly differ between these groups (see Chapter 3). Competition for food could result in a differential allocation of resources for other uses, such as immune activity and stress responses, and these social conditions could be

differentially stressful (Stefanski, 2001) or indicate a requirement for investment into immune activity (Barnes and Siva-Jothy, 2000). Indeed, Wang et al. (2018) suggest that the intermediate densities could provide an optimal balance between the negative effects of crowding such as food competition and stress, but also an amount of social stimuli that together encourage an increase in cognitive performance. It is possible that the results obtained here similarly involve such factors. Unfortunately, due to the differences in experimental design, it is difficult to establish the mechanisms behind these observed differences.

Taken together, the results indicate that there may be effects of larval density on cognition in young adult male fruit flies. Adult males previously kept at low density as larvae were more accomplished in a social learning and memory task requiring a male to learn the mating status of females. This increase in learning ability was paired with an increase in expression levels of a gene known to control synaptic plasticity. It may have been expected that the difference, if any, would have been greater in high density males as a result of increased social enrichment during the larval stage. In adults, the presence of a rival male increases learning ability in the T-maze and virgin finding assay, and increases *Neurexin-1* and *futsch* expression (Rouse, 2016). In this case, cues of a rival indicate an increased sperm competition threat and there is an enhancement in individual learning ability (Rouse, 2016). The larvae in the high density treatment react in an anticipatory manner to future reproductive competition by increasing accessory gland size (Bretman et al., 2016). However, they do not alter male mating duration or their ability to increase this in response to a rival compared to those from low larval densities, suggesting this response to sperm competition is affected by the adult social conditions (Bretman et al., 2016), and may indicate that these cues during the larval stages are not affecting the cognitive ability to differentially express this particular behaviour. Given that flies from high larval densities respond in an anticipatory manner to future reproductive competition by altering an aspect of their morphology, it is interesting that the provision of these cues do not also result in an increased

cognitive ability. Instead, there could be a subtle increase in the low density groups. Conceivably, this could be in response to inconsistent, fluctuating cues (Fawcett and Frankenhuis, 2015). Whereas, high density larvae are likely to be in more or less constant contact with one another, the low density larvae may not. Variability in this frequency of contact with other individuals could potentially result in an increased requirement for neural plasticity (Fawcett and Frankenhuis, 2015), for example, in synaptic growth or function, which could also be indicated by the increased *Neurexin-1* expression in low density males. Indeed, investment in cognition is predicted to be beneficial under more variable conditions (Sol, 2009). However, there is not a similar increase in expression in low density females, which again may represent a difference in the requirement of cognitive processing of the social conditions between the sexes. Furthermore, where there are variable cues of reproductive competition, it may be more beneficial for low density males to refrain from increasing accessory gland size which could be costly and may not confer benefits if competition is low, and instead, invest in cognition to process fluctuating cues. However, there was no increased variance in the response variables of flies from low larval densities. It is possible that variation in cues results in the increased responses observed, without necessarily resulting in greater variance between individuals. Additionally, there could also be greater variation in other factors that have not been investigated here.

Alternatively, the production of larger accessory glands could directly trade-off with cognition, in which case, poorer learning scores in the high density group would be expected. Negative relationships between brain size and testes mass have been observed in bats (Pitnick et al., 2006), whilst decreased fecundity is linked to an increased learning ability in butterflies, *Pieris rapae* (Snell-Rood et al., 2011). Therefore, the results I have observed here could indicate a trade-off is occurring in the high density males. Increasing their accessory gland size could direct resources towards this reproductive trait and reduce energy availability for investment into cognition, resulting in the decreased ability in the virgin finding assay, and

perhaps also the decreased relative expression of *Neurexin-1*. Further, the high density group could be more stressful, and production of stress responses could affect cognition (Lupien et al., 2009). In addition to this, there could be other trade-offs occurring, such as towards the immune system (Buchanan et al., 2013). For example, immune-challenged bees are less able to learn an associative task than those non-challenged (Mallon et al., 2003), and there is evidence that formation of long-term memory in fruit flies results in a decreased tolerance to certain stressors (Mery and Kawecki, 2005), and a further study found that lines selected for learning ability had shorter lifespans (Burger et al., 2008). As high larval density could increase the risk of infection (Reeson et al., 1998), it seems possible that the high density group could be allocating resources to factors other than cognition.

2.5.2 Adult Presence does not affect learning ability

No effects of adult presence were observed in either of the assays used to assess learning ability, or for the expression of any of the genes examined. I had predicted that, as the presence of adult males during larval development results in the increase in accessory gland size, that this group would show an increased cognitive ability as a result of these social conditions, similar to those observed in adults (Rouse, 2016). However, if there was a trade-off between production of larger accessory glands and other factors, there might be a reduction in learning ability in this group. There was a consistent non-significant trend for increased learning scores in the adult presence groups at both one and five days old across assay sessions, but no direct evidence of the presence of adults during larval stages influencing cognition. The presence of adults may also provide a consistent cue, and as with high density flies, a relatively stable level of information provision, and reduced requirement for synaptic plasticity (Fawcett and Frankenhuis, 2015). Thus, these individuals may not invest into synaptic plasticity during the larval stage as I may have expected.

Previously, another study found no increase in learning ability of *D. melanogaster* larvae reared in complex feeding environments when tested as either larvae or young adults (Durisko and Dukas, 2013b). Durisko and Dukas (2013b) suggest a number of reasons for this result, including that the type of cues they were testing may not be indicative of those required by the larvae for an increase in learning ability. It is possible that the type and consistency of cues, as well as how learning is measured could influence the results obtained here as well. As group burrowing in larvae appears to require both vision and mechanosensation (Dombrovski et al.), these may be important cues for larvae, and as our learning paradigms largely involve olfactory learning, it could be speculated that there may be differences in, for example, visual learning, that would not be picked up in these assays. There is also variability in the setups or types of cues used between laboratories for learning assays. For example, I used an aversive mechanical shock (Hollis and Kawecki (2014), whereas others have used appetitive stimuli (Durisko and Dukas (2013b) or electrical shock (Tully and Quinn, 1985). This variation makes comparisons difficult, and these setups may differ in their power to detect differences in learning. Additionally, the virgin finding may represent a more natural situation than the associative learning task, and therefore, may be the more relevant of the two assays.

The associative learning task was carried out at 1 day old and then again later in the first week of adulthood. Adult *D. melanogaster* usually increase their learning ability during the first week post-eclosion (Guo et al., 1996). If there was an effect of larval environment on adults, I envisaged that it would be detectable at 1 day old, and hence the use of this age in the associative learning task, but the flies were also tested at a slightly later stage when learning ability is expected to have increased (Guo et al., 1996). This also brings the age in line with the virgin finding assay. However, no significant differences were found for either density or adult presence groups at either age in the associative learning task. Additionally, the power analysis from initial trials suggested a sample size of 35 would be sufficient to detect a difference with a power of 0.8, however, despite consistent trends

across assay sessions, increasing sample sizes beyond this number did not find a significant difference.

Finally, as previously mentioned, no significant effect on learning ability has been found for adult female *D. melanogaster* kept with a female conspecific (Rouse et al., *in prep*). Considering this result; that there was no significant effect of adult presence on learning ability of males, and, that there was no effect of adult presence on female gene expression, it seems unlikely that any difference in learning ability would be found in females either. However, there were sex effects in gene expression for *bruchpilot* and *futsch* in adult presence and absence groups observed, with males having relatively higher expression of both genes compared to females. Similarly, *futsch* was significantly higher in males for density groups. The reasons for this are unclear, but sex differences in learning and memory have been found in other animals (Mizuno and Giese, 2010). It may be that there are differences in the general requirement of males for learning and synaptic plasticity in the integration of cues, possibly related to the need to assess multiple cues involved in reproduction (Bretman et al., 2011b). Alternatively, there may be other aspects which differ between the sexes that are influencing the expression of these genes, such as transcription factors (Mizuno and Giese, 2010).

The results in this chapter suggest that larval density can affect cognition in adult male flies through the results observed in the virgin finding assay and *Neurexin-1* expression, and it would be interesting to investigate if the increased expression of *Neurexin-1* results in increased synapse formation. These results may indicate a trade-off between the production of larger accessory glands in high density males and cognition, or an increase in low density males, possibly through the variability of cues under these conditions. Tentatively, it could be interesting to consider whether individuals of the same age cohort (larval density) are better predictors of future reproductive competition than those from an earlier cohort (adult presence),

resulting in differences in cognitive requirements that could differentially influence its development. This may be difficult to disentangle from changes arising from differences between types of cues, and the larval density and adult presence groups cannot be compared due to differences in the experimental set-up. Furthermore, whilst there could be advantages to altering aspects of cognition during larval stages, a change that results in a mismatch at later stages could be costly (DeWitt et al., 1998; Niven and Laughlin, 2008). As brains continue to show plasticity in adulthood (Donlea et al., 2014), it may be important for changes in cognition to be affected at this stage. As male competition appears to be an important influence on cognition (Rouse, 2016), and as the social environment can be variable, plasticity at sexual maturity could potentially represent a route that is less likely to result in mismatches for males responding to these types of cues. However, it should also be noted that the learning assays used here are proximate measures of cognition, and do not necessarily represent overall cognitive ability. Additionally, social conditions can have other effects, including affecting reproductive morphology and immune activity (Bretman et al., 2016; Leech et al., 2017). It is, therefore, possible that trade-offs between these factors could play a role in mediating cognitive ability, particularly as the production of neural structures is costly (Niven and Laughlin, 2008). However, as incorrect information processing could result in poor decision-making, and potentially have negative effects on immunity and stress, investment in cognition is also beneficial to these factors, and this could be especially true during early life where inexperience is prevalent (Dukas, 1998).

Chapter 3

Effect of Early Life Social Conditions on Life History Traits of Fruit Flies, *Drosophila melanogaster*

Thanks to Nicola Saville, Kayleigh Farrow, and Sophie Heath-Whyte for their help with data collection.

3.1 Summary

An individual's life history is affected by a wide range of factors occurring at different life stages. The conditions experienced during early life have been shown to have particular effects on traits including body size, stress responsiveness, and health in a variety of species. The complex interactions between these factors can have considerable consequences on adult fitness and survival. The social environment is one factor that can affect a suite of these responses, such as influencing competition for resources and the availability of mates. Manipulating the larval social environment of fruit flies, by increasing larval density or having adult males present, induces the growth of larger accessory glands in males, a response to future reproductive competition. Resource allocation towards this trait could affect the energy available for other factors, such as growth. It is unknown whether the cues of future reproductive environment affect female reproductive traits. In this chapter, I investigate how these larval social conditions impact on a number of life history traits in adult fruit flies, *Drosophila melanogaster*. Larvae were kept at high or low density; with or without adult males present. Effects of these larval environments on development time and adult lifespan were examined. I also tested the effect of these larval conditions on

reproductive traits in adult females, namely latency to mate, number of eggs laid and offspring counts. Lifespan was negatively affected by the presence of adults, but was not affected by larval density. Conversely, development time was slower for those reared at high larval density compared to low density, but not affected by adult presence. Both the larval density and adult absence groups had a greater proportion of females that eclosed than males. Neither manipulation of the larval social environment had an effect on any of the female reproductive traits examined, suggesting these may be more influenced by adult social conditions.

3.2 Introduction

An organism's life history is determined by a host of genetic and environmental factors. Individuals are faced with many challenges that could decrease fitness or lead to mortality. These include, for example, food availability (Skorupa et al., 2008), pathogenic infection (Reeson et al., 1998), and stress (Vermeulen and Loeschcke, 2007). Immune systems and stress responses are used by the individual to prevent or reduce damage caused by pathogens and stressful conditions (Lemaitre and Hoffmann, 2007; Vermeulen and Loeschcke, 2007), however, the provision of energy to these factors could divert energy from other processes, including reproduction. For example, selecting *D. melanogaster* lines for resistance to infection by the bacterium *Pseudomonas aeruginosa* increases survival post-infection, but decreases egg viability (Ye et al., 2009). Furthermore, there is the possibility that these not only affect the discrete part of the life cycle during which they are encountered, but also influence subsequent stages, for example, stressful childhood conditions have been found to affect inflammation in adults (Danese et al., 2007). Indeed, early life stages may be particularly important in this regard, and in some cases represent sensitive windows where effects may be accentuated or carried over to other stages (Taborsky, 2017). Stressful conditions experienced during early life in humans are also

connected to a variety of other health issues in adulthood (Godfrey et al., 2007). However, stress during development can have opposing effects, such that high stress can reduce adult lifespan (Monaghan et al., 2012), but sometimes, milder stress exposure can actually extend this (Hercus et al., 2003). Furthermore, predator presence during larval development can affect limb length in wood frog tadpoles, *Rana sylvatica*, with potential effects on adult limbs (Relyea, 2001), and acoustic signals during the juvenile period in field crickets, *Teleogryllus oceanicus*, can influence adult mating behaviour (Bailey et al., 2010). Additionally, larval density can impact on reproductive investment in moths, *Plodia interpunctella*, which develop relatively larger testes when reared at high density (Gage, 1995). However, despite achieving a similar body mass to those from lower densities, these individuals also had longer development times, which is possibly related to competition for resources at high density (Gage, 1995). Similarly, fruit flies, *Drosophila melanogaster*, reared at high density, but with increased food concentrations to reduce such competitive effects, also display an increased investment in reproductive tissue (Bretman et al., 2016). Therefore, early life conditions can have important influences on resource allocation into later life stages.

Development time is another factor which can be greatly affected by the environmental conditions experienced. For example, diets rich in yeast can influence larval development rate in *D. melanogaster* (Rodrigues et al., 2015), increase egg production in females, and reduce adult lifespan (Skorupa et al., 2008). Predator presence can both delay and advance hatching in some amphibians, depending on the type of predation threat (Sih and Moore, 1993; Warkentin, 2005), and temperature affects the rate of development in many species, including larval mosquitoes, *Anopheles gambiae* (Bayoh and Lindsay, 2007). Furthermore, male green swordtails, *Xiphophorus helleri*, delay maturation in the presence of cues from rival males, that indicate high reproductive competition (Walling et al., 2007). Larval growth can also be influenced by the associated bacterial community, known as the microbiome (Storelli et al., 2011; Newell and Douglas, 2014).

For example, *Lactobacillus plantarum* can increase growth under nutrient poor conditions by affecting nutrient signalling pathways in *D. melanogaster* (Storelli et al., 2011). The rate of development could have distinct implications on later stages, for example, under poor conditions, changes in this rate could result in modifications in adult body mass or size, which could have implications in aggressive encounters (Kasumovic and Andrade, 2009) or mating success (Partridge et al., 1987b).

The social environment has been found to affect a wide range of traits, from reproductive investment, to stress and immune activity (Bartolomucci, 2007; Bretman et al., 2010; Leech et al., 2017) (also Chapter 4). For example, subordinate lab rats that have experienced aggressive encounters with conspecifics have a decreased body mass and reduced immune T cells (Stefanski, 2001). Female guppies, *Poecilia reticulata*, display more 'choosy' behaviour when there are more males (Jirotkul, 1999), and increased reproductive investment in response to rival males has been observed in *D. melanogaster* males, which increase their mating duration under these circumstances (Bretman et al., 2009). In addition, *D. melanogaster* female egg production changes with, amongst other factors, larval density under certain conditions (Edward and Chapman, 2012). Further, adult females reared in cages with high larval density and overlapping generations, have smaller body sizes, greater latencies to mate and reduced egg production than those reared in larvae-only vial conditions at determined densities (Edward and Chapman, 2012). This could be connected to the increased offspring production of larger females (Lefranc and Bundgaard, 2004), with which males prefer to mate (Byrne and Rice, 2006). Additionally, effects of larval social conditions can also be modified by those experienced as adults in *D. melanogaster* (Morimoto et al., 2017). Specifically, courtship levels have been shown to increase in adults from low larval densities, compared to adults from high larval densities or mixed groups, however, increases in offspring mass with time were slower when males, but not females, originated from mixed larval densities (Morimoto et

al., 2017), indicating that the combination of larval and adult social settings can have significant fitness effects.

As the social conditions experienced as adults can affect, for example, mating duration (Bretman et al., 2009) and immunity (Leech et al., 2017) in *D. melanogaster*, it could also have distinct effects on larvae. Indeed, the larval social environment has been shown to increase the size of accessory glands in adult males reared at high larval density or with adult males present (Bretman et al., 2016). Differences in resource allocation, for example, towards reproductive traits, stress or immunity, could divert energy away from other factors, such as growth during these stages. Additionally, as these environments are already providing cues relating to future reproductive conditions to males (Bretman et al., 2016), they could inform investment into other factors, such as immunity in response to increased infection risk (Reeson et al., 1998) or reproductive traits through cues relating to mating opportunity for females (Jirotkul, 1999). As such, these larval social conditions have the potential to influence a number of life history traits. To investigate some of these effects, I examined adult lifespan, development time and female reproductive traits in *D. melanogaster* from different larval social conditions, by manipulating larval density or by the presence of adult males. I predicted that males producing larger accessory glands in response to these conditions would have a shorter lifespan due to a trade-off in resource allocation, but expected no such effect in females. Furthermore, as *D. melanogaster* pupate once they attain a critical weight (De Moed et al., 1999), this may result in a slower development time in high density or adult presence flies that are investing in accessory gland growth, or, alternatively, these individuals could delay their development in order to wait for a reduction in reproductive competition. Larval density has previously been shown to slow development rate in *D. melanogaster* (Horváth and Kalinka, 2016), but in order to remove confounding effects of competition for food, I increased the food concentration in larval density groups. The body mass of 1 day old adults was measured to assess whether this was successful in removing competition for food. As female

reproductive traits had not previously been examined (Bretman et al., 2016), I measured latency to mate, egg and offspring counts. Females do not have control of mating duration, but are able to exert an influence on latency primarily through rejection behaviour (Bretman et al., 2013a). It could be predicted, due to the increased number of males expected in the high density and adult presence groups, and therefore the increased potential opportunity for mating, that these females may exhibit more 'choosy' behaviour and have greater latency to mate (Jirotkul, 1999). Additionally, if these conditions are stressful for females, this could result in stress responses that reduce the energy allocation towards a number of reproductive traits. Under these circumstances, egg and offspring counts would be expected to decrease in females from high larval densities and adult presence groups.

3.3 Methods

3.3.1 Fly Husbandry

All flies were wild type *Drosophila melanogaster* of the Dahomey strain, reared in a humidified room at 25°C, under a 12:12 light:dark cycle on a sugar-yeast diet. Adults laid eggs on purple grape juice agar plates; larvae were harvested approximately 24 hours later and transferred to treatment groups in plastic vials with 7ml of fresh medium, supplemented with live yeast paste.

3.3.2 Larval Social Treatments

Larvae were haphazardly allocated to density or adult presence treatment groups. Density treatments consisted of 200 larvae per vial (high) or 20 larvae per vial (low), and were kept on concentrated food medium to prevent this being a limiting factor at high densities (Bretman et al., 2016) (Appendix

A.1). Adult presence treatments consisted of 100 larvae per vial with or without 20 added males. These adult males were aspirated in after larval collection, were approximately 5 days old, and had been reared at a standard lab larval density of 100 per vial. The adult males were removed from the adult presence vials the day before pupae eclosed. All experimental adult flies were used in assays at 1 day old, unless stated.

3.3.3 Body Mass

As an approximate check that competition for food at high density was not negatively impacting on body mass of these individuals, the body mass of 1 day old adult flies from the different larval social environments was measured. Body mass has been shown to significantly correlate with other measures of body size in the literature, including with thorax length (r=0.98) (Starmer and Wolf, 1989), and wing length (r=0.82) (Barnes et al., 1998). As such, it was determined to be a suitable proximate measurement for this body size check. Flies were frozen in single-sex groups of 10 at -20°C. The frozen flies were then transferred to 25°C to thaw for approximately 30 minutes before weighing. The wet weight of flies was measured three times and the average taken.

3.3.4 Lifespan and Development Time

To examine adult lifespan, flies were collected from larval social environments as they eclosed, and kept in single sex groups of 10 on fresh yeast-sugar medium. Each day, the number of mortalities was recorded. These were then removed weekly, when the surviving flies were transferred onto fresh food.

To investigate development time, purple agar plates were added to the population cages for 2 hours to allow females time to lay eggs and to enable calculation of development time from this period. Larvae were then collected 24 hours later, within the same 2 hour window, into their larval social conditions and held at 25 °C throughout this period. The adult flies were collected, sexed and counted at specified intervals as they eclosed approximately 10 days later. Four times points were used on the first day of eclosion at 9am, 12pm, 3pm and 6pm; 9am, 12pm and 3pm on the second and third days, and one final check at 10am on the fourth day of eclosion.

3.3.5 Female Reproductive Traits

To investigate if the larval social environments can influence reproductive traits in young adult females, mating latency, egg and offspring counts were recorded from adults originating from these larval conditions. Virgin females were collected after eclosion from the different larval treatments and kept singly for approximately 1 day. Adult males were also collected as virgins from standard vials and kept in groups of 10 until the assay. The males were then individually introduced by aspiration into the female vials and the time to start mating was recorded. The assay was censored after 7 hours. Following mating, the females were transferred via aspiration to new standard vials and left for 24 hours to lay eggs. After this, they were removed and the eggs were subsequently counted. Offspring counts were taken following eclosion from these vials 12 days later.

3.3.6 Statistical Analysis

All statistical analyses were carried out in R v.3.3.3 and SPSS v.21. Average body masses between larval densities for each sex were analysed by Mann Whitney U tests as the data was not normally distributed. For adult absence and presence groups, which were normally distributed, T-tests were used to

compare body mass within sexes. Generalised Linear Models were used to analyse the effect of larval social environments on adult lifespan, with treatment and sex as fixed factors, and vial and fly ID as random factors, with a Poisson distribution. The sexes were then analysed separately for adult presence groups. Male lifespan was normally distributed, and was analysed using an independent T-test, and female lifespan was not normally distributed, and was analysed using a Mann-Whitney U test. Survival analysis of lifespan was also carried out, using Cox Proportional Hazards models, with treatment and sex as fixed factors, and Analysis of Deviance to compare.

For development time, Generalised Linear Models were carried out with sex and treatment as fixed factors and vial and fly ID as random effects, with a Poisson distribution. Models were compared with Analysis of Deviance. The proportion of males and females from the total number of flies that eclosed were also compared within and between treatments. The data was normally distributed and compared using T-tests.

Offspring counts were calculated as the percentage of the eggs laid by each female that eclosed. All female reproductive traits were analysed using Generalised Linear Models, with treatment as a fixed factor and fly ID as a random effect, with a Poisson distribution. Models were reduced by Analysis of Deviance. To test for differences in variance between low and high density groups due to possible variability between these environments, Levene's tests were carried out on all response variables.

3.4 Results

3.4.1 Effect of Larval Density on life history traits

There was no significant difference in body mass between low and high density males (U=1744.5; n=60; p=0.892), and no difference between low and high density females (U=1712.0; n=60; p=0.644; Figure 3.4.1-1). There was no significant effect of larval rearing density on fly lifespan (χ^2=1.224; df = 1, 5; p=0.269). Adult females survived significantly longer than males (χ^2=24.702; df= 1, 4; p<0.001; Figure 3.4.1-2). The survival analysis found no interaction between treatment and sex (χ^2= 0.184; df= 1; p=0.668), no effect of larval density (χ^2=1.302; df= 1; p=0.254), but a significant effect of sex (χ^2= 105.85; df= 1; p<0.001). There was no difference in variance in body mass between low and high density groups for males (F=1.212; p=0.273) or females (F=0.367; p=0.546), and no difference in variance of lifespan for larval density males (F=0.904; p=0.344) or females (F=2.579; p=0.111).

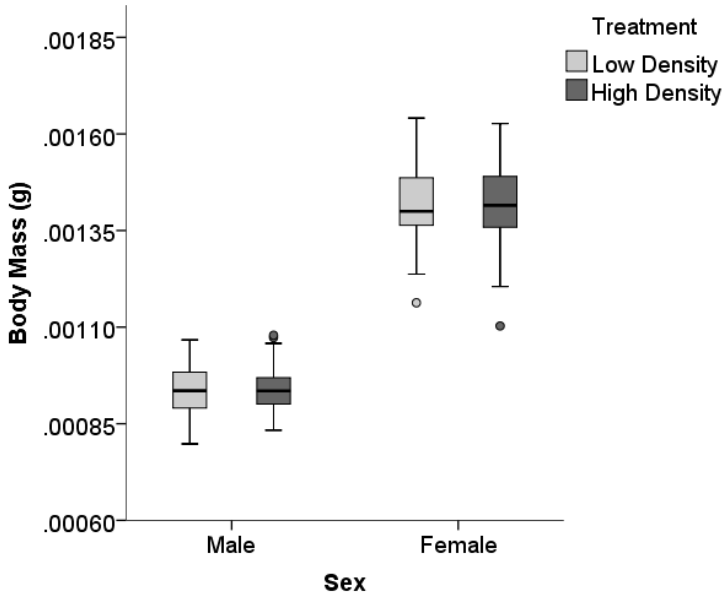


Figure 3.4.1-1 Median body mass (g) of 1 day old adult flies from different larval densities. Larvae were reared at high or low density (n=60) on a concentrated food source to prevent food competition effects at high density. Flies were weighed three times and the average mass taken.

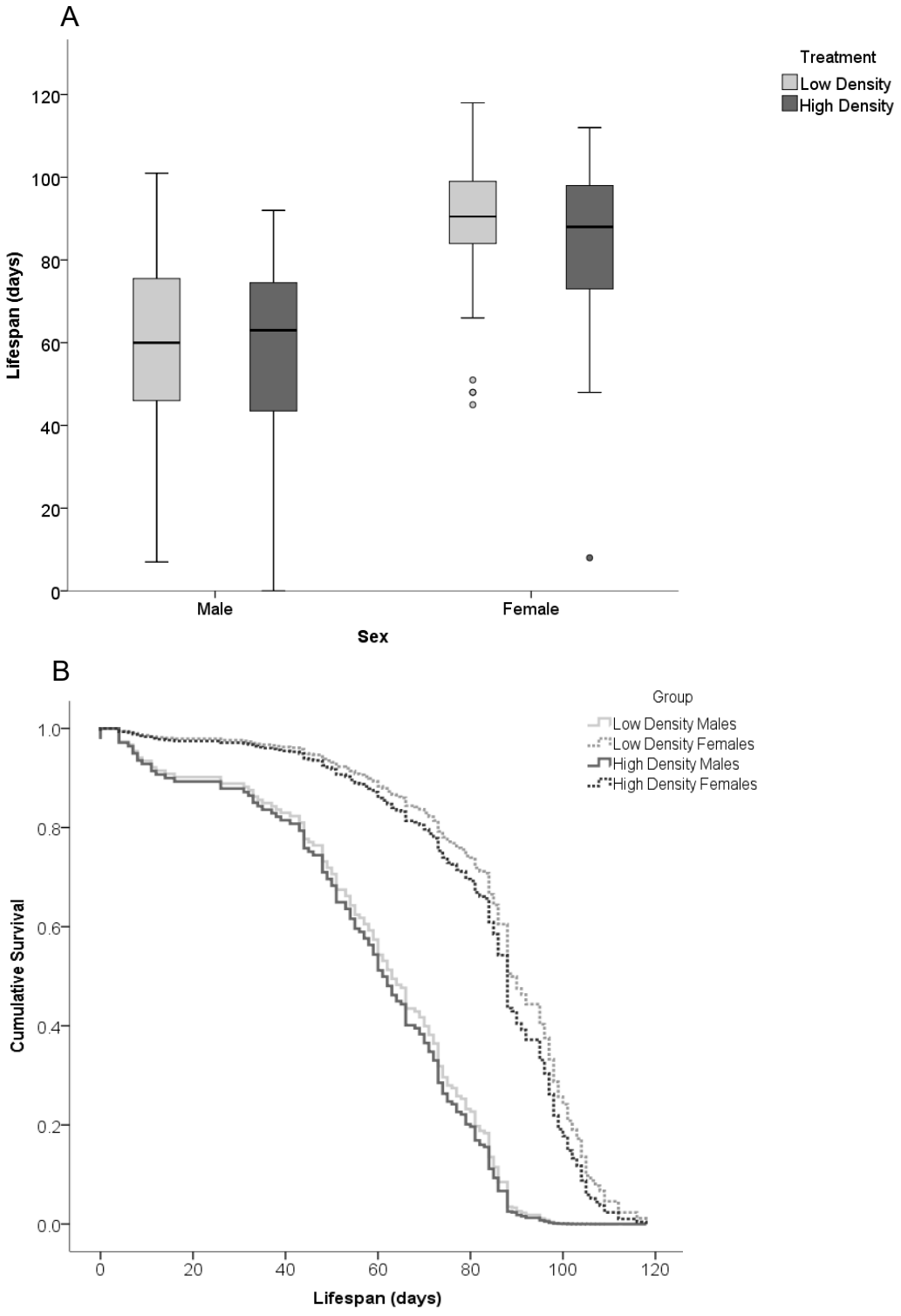
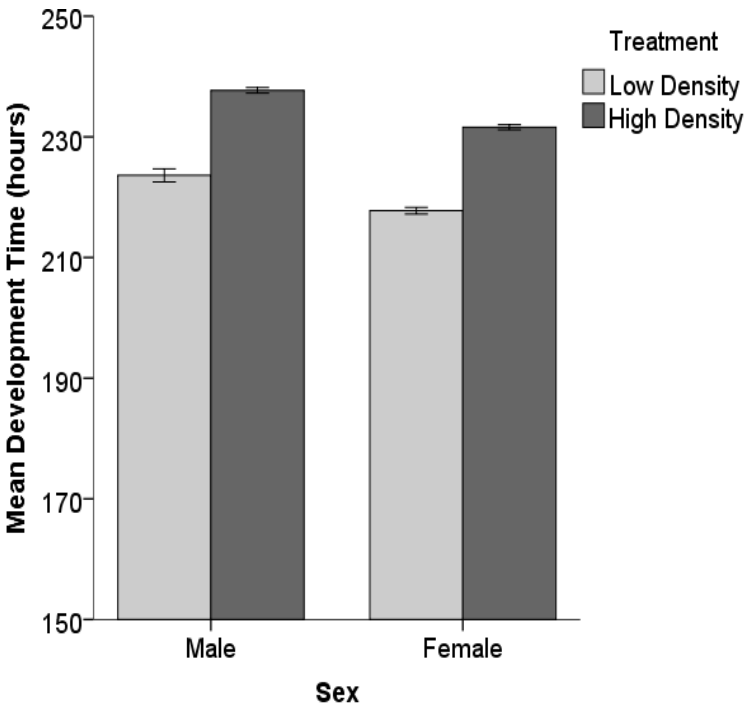


Figure 3.4.1-2 Lifespan of adult flies from different larval densities. Larvae were reared at high or low density (n=60), and maintained in single-sex groups of 10, and mortality recorded daily. A: Median lifespan (days). B: Cumulative survival using Cox Proportional Hazards model.

Larval density had a significant effect on development time. There was no interaction between larval density and sex (χ^2<0.001; df= 1, 6; p=0.991), but high density flies took significantly longer than low density ones to eclose (χ^2=41.361; df=1, 5; p<0.001) (Figure 3.4.1-3), and females were significantly quicker to eclose than males (χ^2= 95.981; df=1, 5; p<0.001). The proportion of males and females that eclosed from low density vials was significantly different, with a smaller proportion of males eclosing than females (t=-2.401; df= 22; p=0.025). This was also found in the high density vials (t=-5.649; df= 22; p<0.001). Comparing between larval densities found no significant effect in the proportion of males or the proportion of females that eclosed (males: t=-0.583; df = 12.638; p=0.570; females: t=0.583; df=12.638; p=0.570). There was a significant difference in variance between low and high larval density groups in development time for both males (F=12.011; p=0.001) and females (F=190.984; p<0.001), with greater variance found in low density groups. For female reproductive traits, no effect of larval density was found for mating latency (χ^2=0.014; df= 1, 3; p=0.906), number of eggs laid (χ^2=1.302; df=1, 3; p=0.254), or percent of eggs laid which eclosed (χ^2=0.031, df=1, 3, p=0.861; Figure 3.4.1-4). There was no significant difference in variance in mating latency (F=3.620; p=0.064), the number of eggs laid (F=3.726; p=0.060), or the percent of eggs laid which eclosed (F<0.001; p=0.994).

A



B

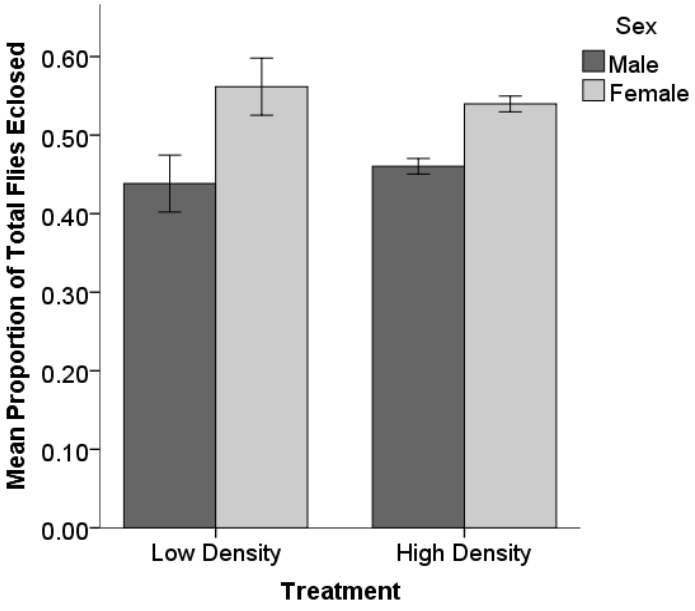


Figure 3.4.1-3 Development Time (hours) of flies reared under different larval densities. A: Mean development time (hours). Larvae were added to social treatment vials within a two hour window, and were counted and sexed as they eclosed as adults at specified time points. B: Mean proportion eclosed of each sex from total flies eclosed. Error bars represent ± 1 S.E.

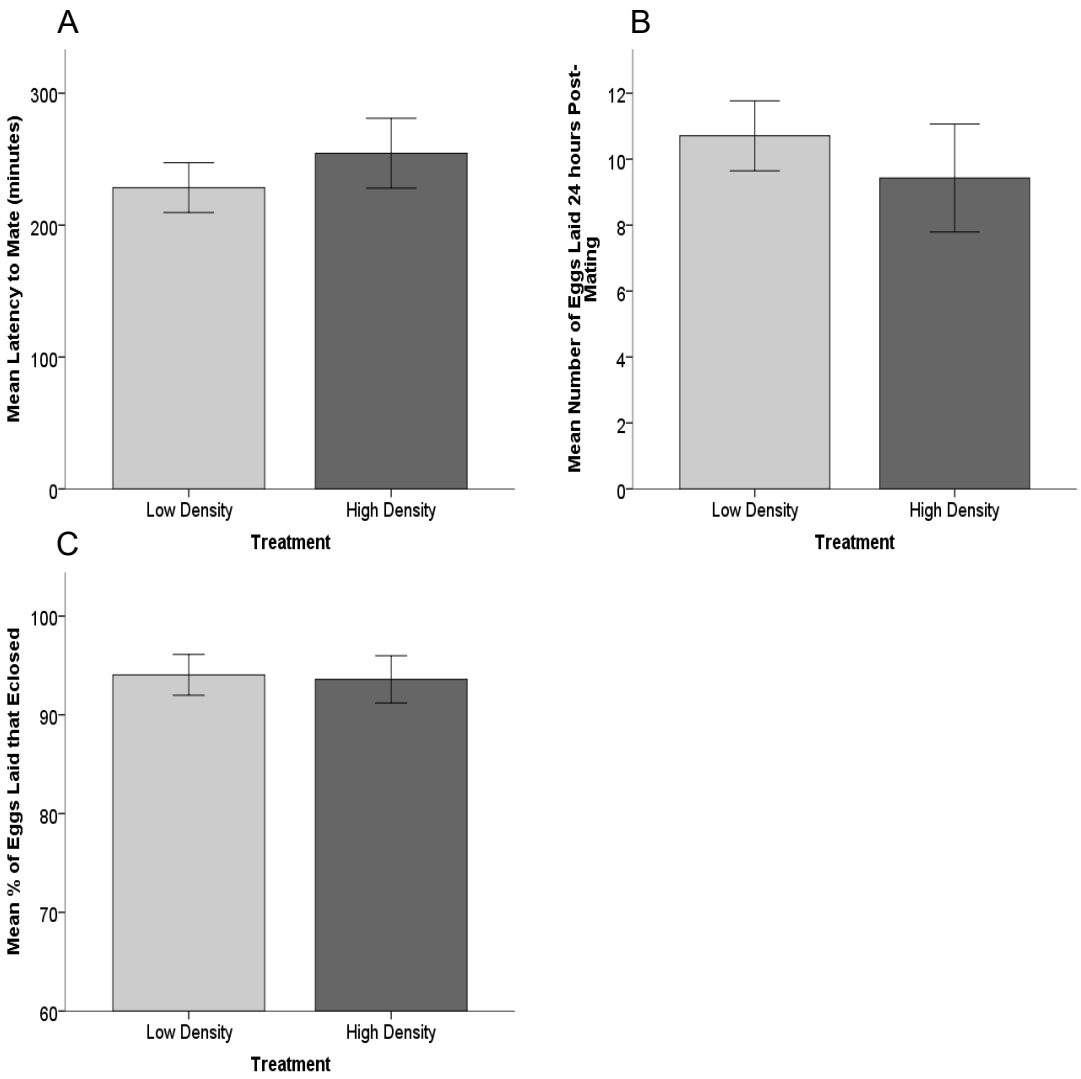


Figure 3.4.1-4 Mean Reproductive Traits of 1 day old adult females reared at different larval densities. A: Mean latency to mate (minutes) (Low Density females n=24; High Density females n=21). B: Mean number of eggs laid within 24 hours of mating. C: Mean percentage of offspring that eclosed from eggs laid. Error bars represent ± 1 S.E.

3.4.2 Effect of Adult Presence on life history traits

The presence of adults during development had no significant effect on body mass of adult males (t=1.008; df= 118; p=0.315) or females (t=-0.115; df=118; p=0.909; Figure 3.4.2-1). Adult presence significantly reduced lifespan (χ^2= 4.366; df= 1, 5; p=0.037). When analysed separately for each sex, males reared with adults in their environment lived significantly shorter than those reared without males (t=2.450; df= 113; p=0.016), but there was no significant difference for females (U=1405.500; N=119, p=0.052) (Figure 3.4.2-2). Again, females lived longer than males (χ^2=22.993; df= 1, 5; p<0.001). The survival analysis found no interaction between treatment and sex (χ^2= 0.025; df= 1; p=0.874), but a significant effect of sex (χ^2=110.04; df= 1; p<0.001), and a significant effect of adult presence (χ^2=5.687; df= 1; p=0.017).

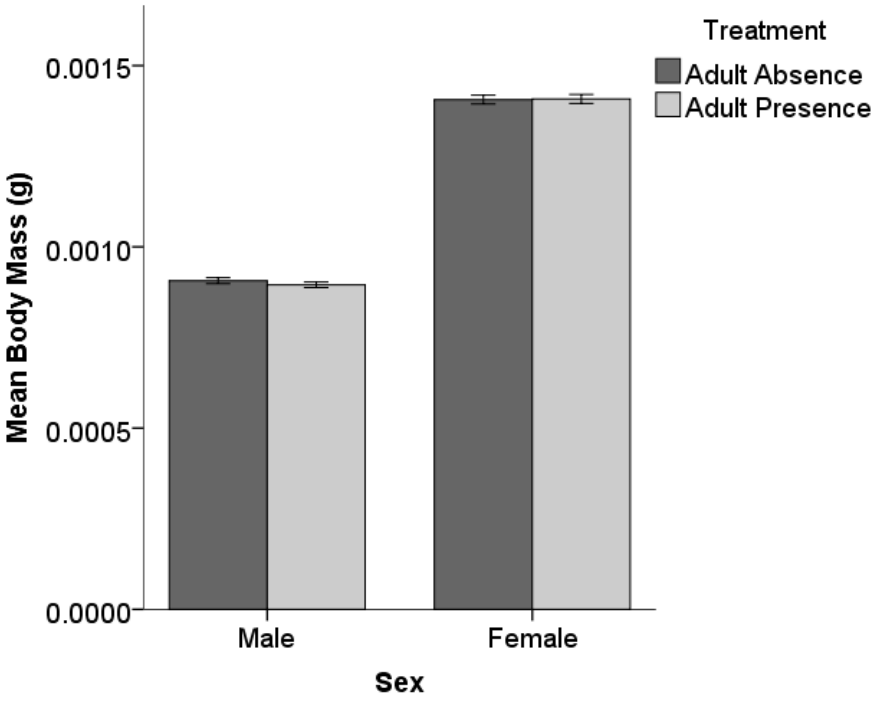


Figure 3.4.2-1 Mean body mass (g) of 1 day old adult flies reared with or without adult males in the larval environment (n=60). Flies were weighed three times and the average mass taken. Error bars represent ± 1 S.E.

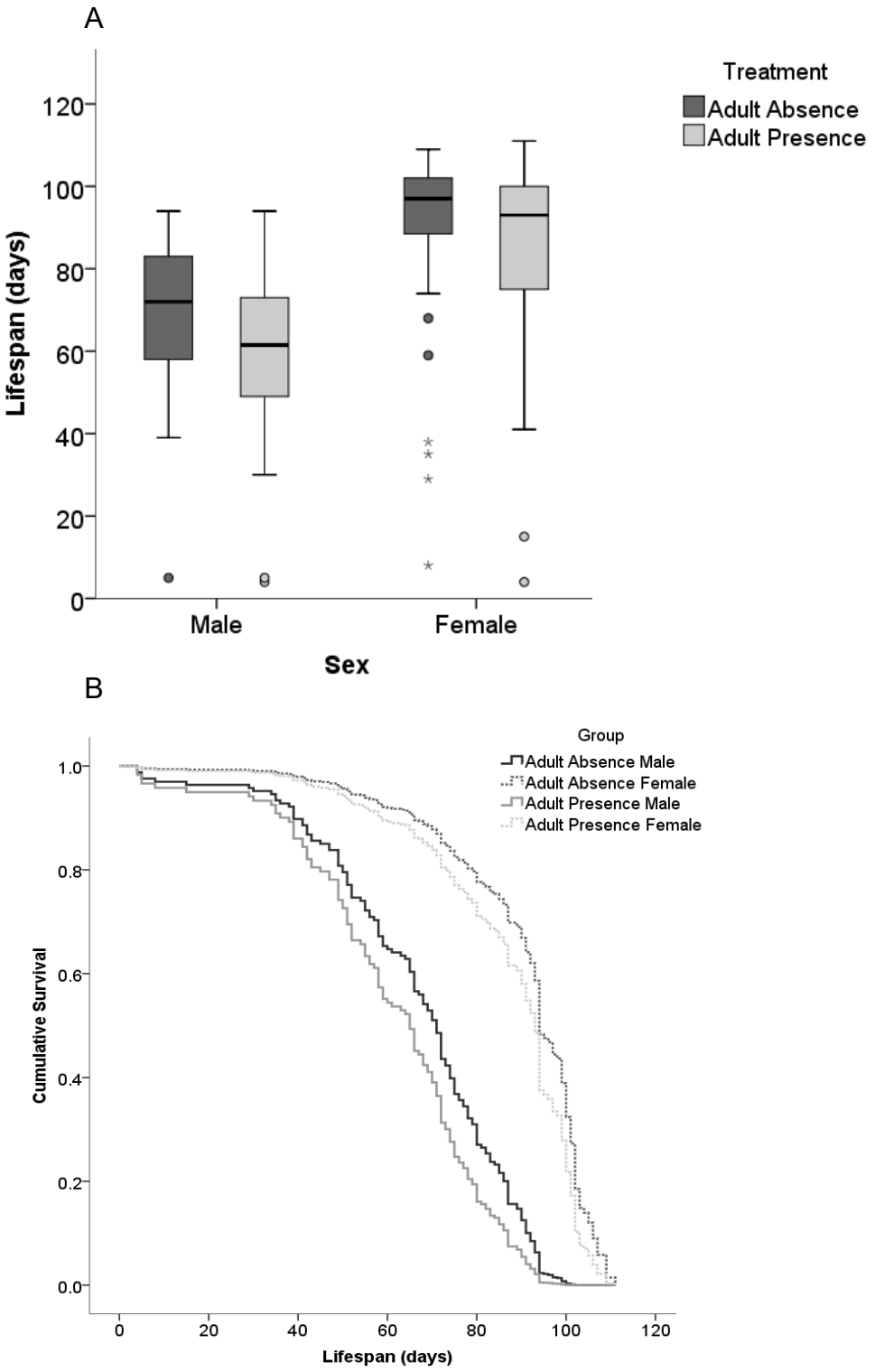


Figure 3.4.2-2 Lifespan of adult flies reared with or without adult males in the larval environment. Flies were maintained in single-sex groups of 10, and mortality recorded daily (Adult Absence males n= 57; Adult Absence females n=59; Adult Presence males n=58; Adult Presence females n=60). A: Median lifespan (days). B: Cumulative survival using Cox Proportional Hazards model.

Adult presence had no effect on development time. There was no interaction found between adult presence and sex (χ^2=0.494; df= 1, 6; p=0.482), and no effect of adult presence (χ^2= 0.406; df= 1, 5; p=0.524) (Figure 3.4.2-3). Females had a shorter development time than males (χ^2= 135.96; df= 1, 4; p<0.001). The proportion of males and females that eclosed in the adult absence vials was significantly different, with a smaller proportion of males eclosing than females (t=-4.705; df= 18; p<0.001). However, there was no difference between the sexes in the adult presence vials (t=-1.004; df= 18; p=0.389). Comparing between adult absence and presence treatments found no significant difference in the proportion of males or the proportion of females that eclosed (males: t=-1.458; df= 18; p=0.162; females: t=1.458; df= 18; p=0.162). As with larval density, no effect was found for the presence of adult males on mating latency (χ^2=0.584; df= 1, 3; p=0.445; Figure 3.4.2-4), the number of eggs laid (χ^2=0.040; df=1, 3; p=0.842) or the percent of offspring that eclosed from the eggs laid (χ^2=0.688, df=1, 3, p=0.407).

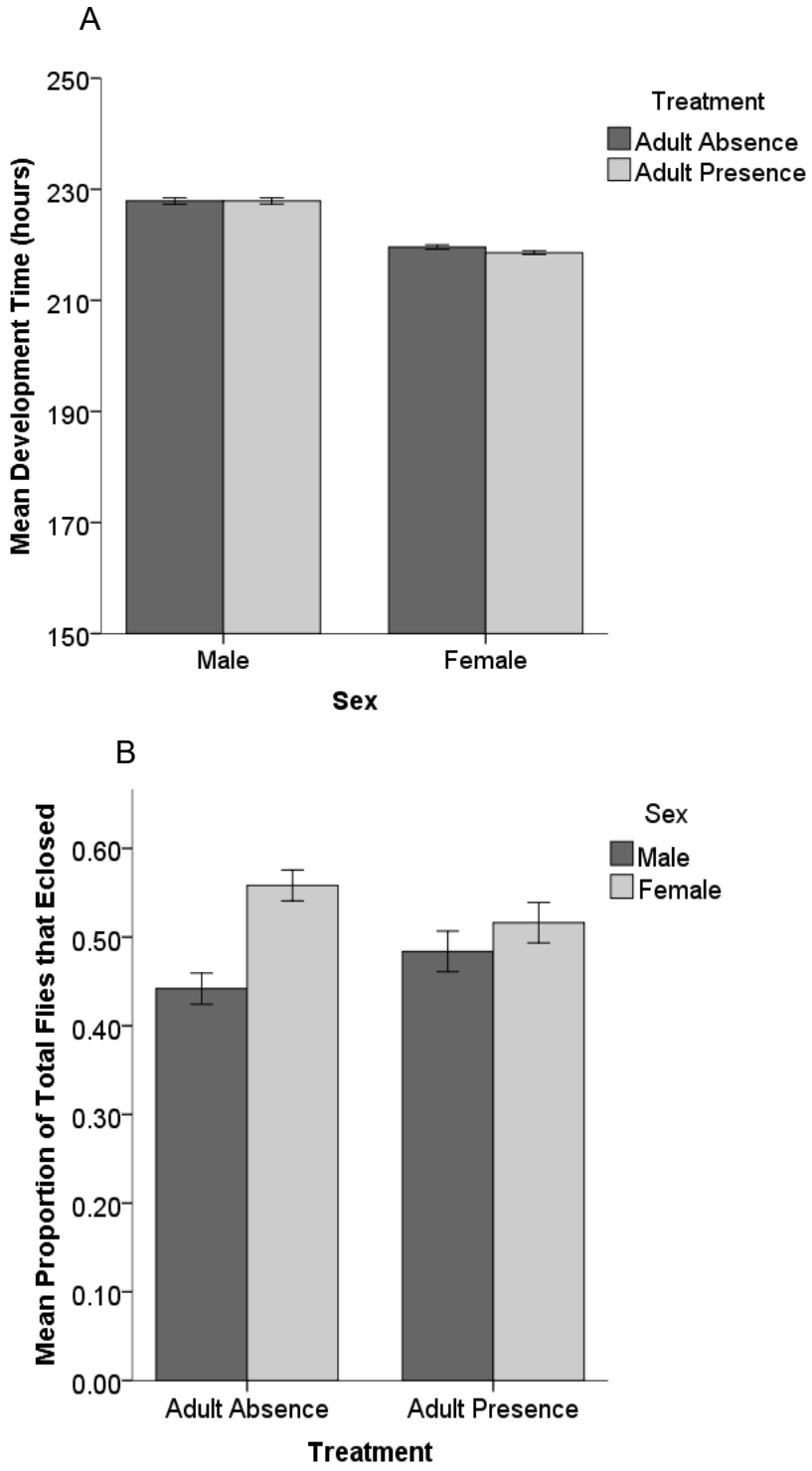


Figure 3.4.2-3 Development Time (hours) of flies reared with or without adults in the environment. A: Mean development time (hours). Larvae were added to social treatment vials within a two hour window, and were counted and sexed as they eclosed as adults at specified time points. B: Mean proportion eclosed of each sex from total flies eclosed. Error bars represent ± 1 S.E.

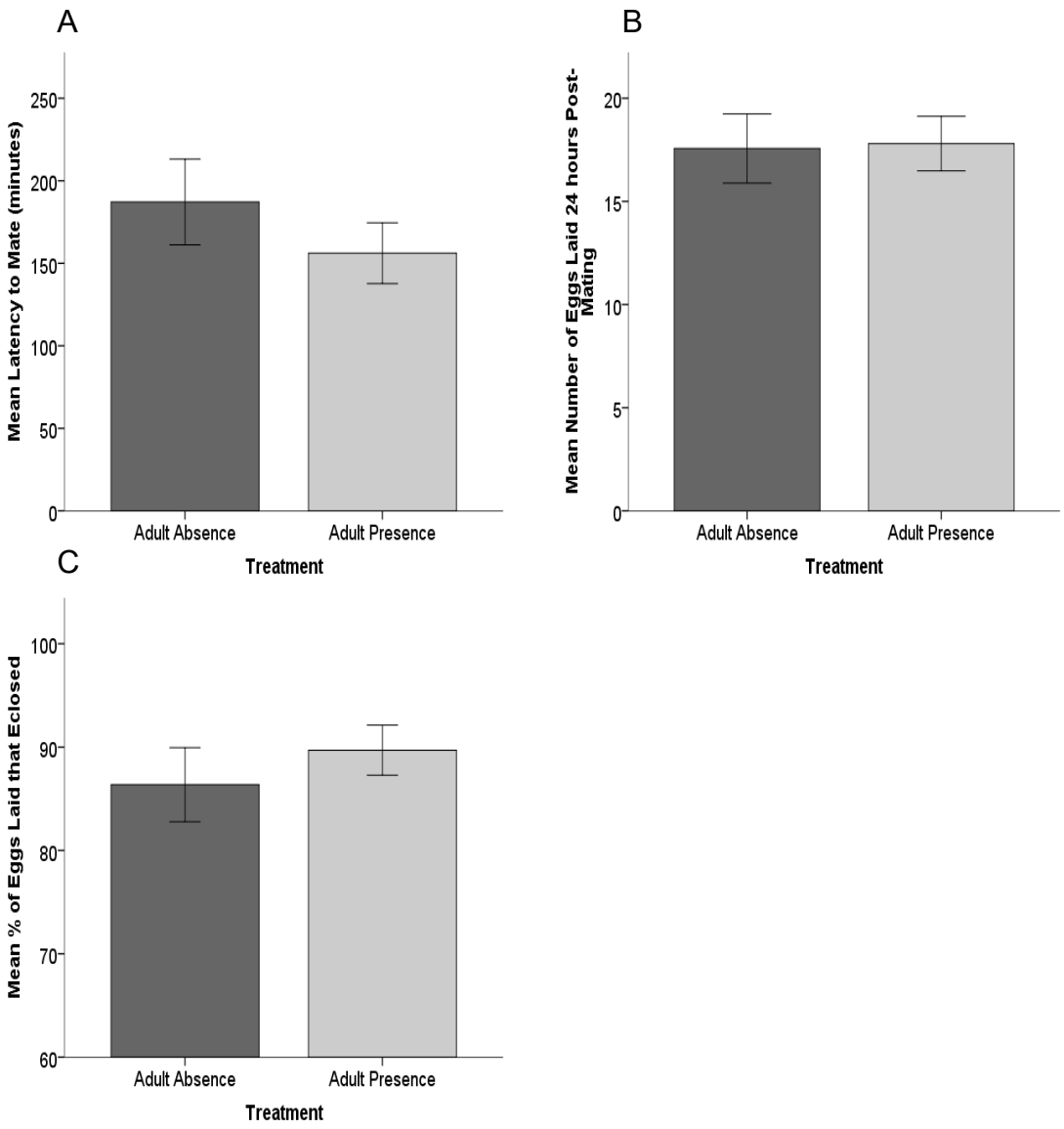


Figure 3.4.2-4 Mean Reproductive Traits for 1 day old adult females reared with or without adult males in the larval environment. A: Mean latency to mate (minutes) (Adult Absence females n=23; Adult Presence females n=30). B: Mean number of eggs laid within 24 hours of mating. C: Mean percentage of offspring that eclosed from eggs laid. Error bars represent ± 1 S.E.

3.5 Discussion

In this chapter, I investigated how larval social environments can affect a number of life history traits in fruit flies. There was no effect of larval density or adult presence on adult body mass. Rearing larvae at high density increased development times but there was no effect of larval rearing density on the proportions that eclosed of each sex. Both density groups had greater numbers of females that eclosed than males. Larval density also had no effect on lifespan, female latency to mate, egg or offspring counts. Larvae reared in the presence of adult males did not vary in development time or reproductive traits compared to those reared without adult males. However, males raised with adults present had a reduced lifespan, with a similar trend observed for females. Additionally, whilst no differences between adult presence and adult absence groups was found for the proportions of males and females that eclosed, increased proportions of females were found within adult absence vials, but not in adult presence ones. Overall, the results found here suggest a highly complex set of life history responses driven by these early life conditions.

3.5.1 Lifespan can be affected by larval social conditions

I hypothesised that differential energy allocation towards production of larger accessory glands (Bretman et al., 2016), would result in a decrease in lifespan of adult presence and high density males. However, only adult presence males showed a reduction in lifespan, with a similar trend observed in the females. There was no effect of larval density on adult lifespan. Due to the differences in diet, these groups cannot be directly compared, but from these results, it seems that there is not a simple direct shift in resource allocation, and there are other factors affecting this response. Reduced lifespan concurrent with an increased reproductive investment, or vice-versa, is a documented occurrence, for example, *D. melanogaster* lines selected for breeding at an older age increase their longevity compared to young-

breeding lines, but demonstrate a reduction in early life fecundity (Sgrò and Partridge, 1999). However, this connection between lifespan and reproduction can be separated (Edward and Chapman, 2011), for example, in *D. melanogaster* under dietary restriction conditions, there is an extension of lifespan and reduction of fecundity, but by adding the amino acid methionine to the diet, this can increase fecundity without a reduction in lifespan, suggesting that the balance of amino acids in the diet may be important here (Grandison et al., 2009). As such, the observed differences between lifespan and reproduction may be specifically affected by the type of resources available and not simply due to changes in total resource allocation (Grandison et al., 2009). This could be mediated by, for example, changes in signalling pathways (Edward and Chapman, 2011).

However, there could be other shifts in energy distribution that could impact on lifespan, including towards immunity. For example, as increased presence of conspecifics greatens the risk of infection (Reeson et al., 1998), an enhanced energy allocation towards immune activity could improve resistance to pathogens, but reduce energy availability for other processes (though see Chapter 4). Alternatively, there could be impairment or reductions of other functions, for example, oxidative damage through increased reactive oxygen species produced during reproductive metabolic activity (Harshman and Zera, 2007). *D. melanogaster* mutants for the antioxidant enzyme Cu\Zn superoxide dismutase, which is an important constituent of the antioxidant defence, display a reduced lifespan (Ruan and Wu, 2008). However, evidence for the effects of ROS on longevity is variable between studies (Speakman and Garratt, 2014). Additionally, there could be interference from physiological processes such as immune activity or stress responses, that could affect overall lifespan (Harshman and Zera, 2007), but attempts to observe stress effects on longevity have also often resulted in inconsistent results. For example, selection for stress tolerance in *D. melanogaster* has produced contrasting results, with both an increase (Rose et al., 1992), and no increase in longevity for flies selected for starvation tolerance (Harshman et al., 1999). Another study, not controlling

for food, found no effect of larval density on longevity, but influences on starvation resistance (Baldal et al., 2005). There are also costs on female longevity associated with the presence of males, including mating (Wigby and Chapman, 2005), and non-mating costs (Partridge and Fowler, 1990). In addition, the microbiome is able to exert a number of effects on its host, and could influence these results (Chapter 5). For example, *Lactobacilli* bacteria can alter nutrient signalling pathways in *D. melanogaster* (Storelli et al., 2011). Through this pathway, under nutrient poor conditions, *L. plantarum* can enhance protein assimilation, ultimately leading to a faster development rate (Storelli et al., 2011). Therefore, modifications to this signalling pathway, or others, could influence energy uptake, storage and usage by the host (Grewal, 2009; Storelli et al., 2011). Furthermore, the microbiome can also influence other host factors, such as immune activity (Snijders et al., 2016), and changes in immune activity could then affect lifespan. For example, immune-challenged field crickets, *Gryllus campestris*, have shorter lifespans than controls (Jacot et al., 2004). As such, there are a number of factors that could be influencing the observed differences in lifespan in adult presence flies found here.

3.5.2 Development time can be affected by Larval Density

There was no effect of adult presence on the development time of larvae, and this suggests that the production of larger accessory glands (Bretman et al., 2016) does not represent a shift in energy allocation in such a manner that results in a slower developmental period in this group. However, larval density did have an effect, with high density individuals taking longer to eclose than those from low density environments. Development time has previously been shown to be affected by larval density, with higher densities showing an increase in the time for development (Horváth and Kalinka, 2016). However, previous experiments have not controlled for competition for food (Miller, 1964; Horváth and Kalinka, 2016). As there is a minimum weight for pupation to occur in this species (De Moed et al., 1999), the

quantity of food is likely to have a large effect on development time. In the results presented here, this difference is not likely to be the result of competition for food as this was at a high concentration, and there was no effect of larval density on 1 day old adult body mass, although it cannot fully be dismissed. It could be theorised that these males slow their development to wait for a time when reproductive competition is reduced, such as is observed in male green swordtails, *Xiphophorus helleri* (Walling et al., 2007). Male praying mantids, *Pseudomantis albofimbriata*, also delay development when reared in a high sperm competition risk environment, which appears to be related to an increased investment in ejaculates (Allen et al., 2011). Other possible explanations may include the build-up of waste (Botella et al., 1985; Borash et al., 2000). In populations selected under high larval densities, faster development times have been connected to increased feeding rates and increased tolerance to waste build-up (Joshi and Mueller, 1988; Mueller et al., 1991; Joshi, 2001), but other studies have found increased development times in selected lines to be the result of increased food conversion efficiency, not increased feeding rate or waste tolerance, which may reflect differences in food availability between studies (Sarangi et al., 2016). Additionally, the presence of other individuals could interfere with feeding, and therefore, increase the amount of time needed to attain the critical weight for pupation to occur (Goss-Custard, 2002). Furthermore, there may be changes in hormonal responses, such as in juvenile hormone which has a wide-range of effects on, for example, development (Flatt et al., 2005). For example, if high larval densities are stressful (Chapter 4), this could impact on hormonal signalling that could then influence development time (Flatt et al., 2005). Furthermore, as juvenile hormone can influence stress resistance and lifespan, there could be additional effects of changes in its levels (Flatt et al., 2005). It is also worth noting that despite the higher median values in the proportion of larvae that eclosed in the low density groups, there was also significantly greater variation from these vials. These larvae may sometimes struggle due to a decreased capacity to burrow (Durisko et al., 2014b), and whilst I scored the surface of the food using a dissection needle to reduce this effect, it is possible that this could still affect the variability.

For both low and high larval density and adult absence groups, a greater proportion of females than males eclosed. This is not likely to be a result of a bias in the initial numbers of each sex added to the vials due to the haphazard nature of larval collection. The food provided could have such an effect if it is more suitable to the development of females, for example, more female *D. melanogaster* develop on protein-enriched larval media than carbohydrate-rich food (Andersen et al., 2010). However, the food used in this experiment is a standard laboratory medium for *D. melanogaster*, and contains both a sugar and protein source (yeast). Alternatively, there may be an effect due to the presence of the endosymbiont *Wolbachia*, which can have a number of effects on hosts, including cytoplasmic incompatibility and, in some species, feminization of males (McGraw and O'Neill, 2004) (Chapter 5). These larval conditions could potentially also represent differentially stressful environments and as extreme stress can decrease lifespan (Monaghan et al., 2012), this could potentially impact on the proportions that eclose. However, the exact reasons for this result remain undetermined. It is also unclear why there was no significant difference between the proportions of females and males that eclosed from adult presence vials, despite there being no significant differences in the proportions of each sex between these and the adult absence group.

3.5.3 No effect of larval social conditions on female reproductive traits

No effect of larval social environment was found for any of the female reproductive traits examined. Previously, no effect of larval social environment was found for male extended mating duration (Bretman et al., 2016). Though males have control over mating duration, females exert some control over mating latency (Bretman et al., 2013a). The flies used here were particularly young, and therefore, took a long time to start mating (Eastwood and Burnet, 1977), but there was no difference in the time to start mating between the groups. Another study by Edward and Chapman (2012),

in effect, combined both the presence of adults and high density larval environments in cage populations and compared to different larval densities in vials, and found distinct differences in body size, latency to mate and egg production in flies from cages compared to those from vials. However, when comparing differences in vial densities, there was also no evidence for changes in mating latency (Edward and Chapman, 2012), in agreement with these results. I hypothesised that due to the increased number of potential mates in the high density and adult presence groups, females may increase mating latency, exhibiting more 'choosiness' (Jirotkul, 1999). However, it may be more beneficial for females to respond behaviourally in accordance with the adult social environment, which could be variable, and may explain the lack of effect of larval social conditions on latency to mate, similar to the lack of response observed for male mating duration (Bretman et al., 2016). In another study, female crickets', *Teleogryllus oceani*cus, responses to male song is more influenced by adult exposure than juvenile exposure (Swanger and Zuk, 2015). Further, adult groups consisting of *D. melanogaste*r males and females from low larval densities have higher courtship levels and produced offspring with lower body mass compared to those from high larval densities or mixed larval density groups (Morimoto et al., 2017), demonstrating that the interaction between larval and adult social conditions is also important.

Egg and offspring number did not vary between the larval treatments in this study. As egg production is affected by female nutrition (Chapman and Partridge, 1996), food was standardised across corresponding larval treatments, including the increased concentrations for both high and low density larvae. Egg production in vial-reared females has previously been demonstrated to vary with larval density in *D. melanogaster*, increasing initially then decreasing at the highest densities used (greater than 400 larvae) (Edward and Chapman, 2012). The differences in food concentrations make these studies difficult to compare as high density is likely to affect competition for food, whereas this was controlled for here. Edward and Chapman (2012) also compared vial-reared flies with cage-

reared populations, but this does not allow the effects of high larval density and the presence of adults to be disentangled, and as our results suggest, these can have quite distinct effects on a number of life history traits, including lifespan. In addition, females selected for starvation resistance, have been found to decrease early age egg production (Wayne et al., 2006). It is possible that these larval social conditions could represent different levels of stress or potential infection risks (Reeson et al., 1998), and energy allocation to either of these factors may divert from reproductive investment, or alternatively, have indirect physiological effects on reproductive pathways (Flatt, 2011).

These results suggest that early life social conditions can have a number of effects on life history traits of fruit flies. Increased larval density delays development time, a response that is unlikely to be caused by competition for food. These results may implicate the build-up of wastes or trade-offs with other factors, such as stress, are occurring in individuals from the high density conditions. I had predicted that high density and adult presence males would show a reduced lifespan due to investment in increased accessory gland growth, but this does not appear to be the case, as there was no effect of larval density on lifespan. Adult presence during larval stages did not affect development time, but was found to reduce male lifespan, with a similar trend in females. No direct trade-off with reproduction was found for the female reproductive traits examined. Larval density also had no effect on these traits, which may suggest that the adult social environment may be more important in determining these responses (Swanger and Zuk, 2015). These results suggest that the increase in accessory gland growth in high density and adult presence males does not directly trade-off to reduce overall lifespan, but do not preclude the possibility that these individuals are investing instead in other responses which could be affected by the larval social environment, such as stress and immunity.

Chapter 4

Effect of Early Life Social Conditions on Stress Tolerance and Immunity of Adult Fruit Flies, *Drosophila melanogaster*

Thanks to Tom Leech for help with the injection assay, Zahra Nikakhtari and Sophie Heath-Whyte for help with data collection, and Andrew Smith for use of the qPCR machine.

4.1 Summary

Stress responses are critical for the survival of individuals under potentially harmful conditions to return an individual to its homeostatic state or reduce damage, however, extreme or extended periods of stress can have distinct costs. Infection or physical stress during early life, where stress response systems are not fully developed, or where naïve decision-making results in costly mistakes, could be especially important, and have consequences for later life. The social environment can affect the spread of infections, suggesting this could have important influences on immune activity. Furthermore, social conditions may be differentially stressful, such as increasing aggressive interactions or competition for resources. Alternatively, they could give rise to stress priming responses that increase stress responsiveness or efficiency later in life. In this chapter, I explore how larval social conditions can affect stress tolerance and immunity in young adult fruit flies, *Drosophila melanogaster*. To examine this, larvae were reared at low or high density, or with or without adult males in the environment. High density and adult presence conditions have been shown to increase the size of accessory glands in males, a response to future reproductive competition. The production of larger accessory glands could result in a trade-off with stress responses, or these conditions could

represent different stressful conditions. Previously, it has been shown that the social environment differentially affects adult flies when challenged with bacterial infection, and certain social conditions have been shown to elicit stress responses in rodents. As stressors could affect stress pathways in different ways, for example, through gene expression changes, I exposed young adults raised under these conditions to starvation, desiccation and temperature stress, and to bacterial infection. Overall, the only effect of larval social environment was that males reared at high density had higher cold tolerance than low density males, and for females, those reared in the presence of adult males had lower post-infection survival, but higher desiccation and cold stress tolerance than those reared without adults. To elucidate any potential underlying genetic mechanisms in these responses, I carried out RT-qPCR on flies from these larval environments for stress and immune-related gene expression. These genes were selected as they had previously been found to be socially sensitive and are known constituents of the major stress and immune pathways in *D. melanogaster*. Only density had an effect on the expression of the *Turandot* stress genes, with flies from low density environments showing higher expression than those from high density conditions. These results suggest that larval social conditions can affect stress responses, but there are particular differences depending on the type of conditions, stressor, and sex of the fly, that indicates an overall complex interplay of factors.

4.2 Introduction

Stress occurs when an individual is shifted away from its homeostatic state by a stimulus (Koolhaas et al., 2011). Individuals require stress responses to return to this state or to reduce damage caused by the stressor (Koolhaas et al., 2011), but high or prolonged stress can be costly (Lupien et al., 2009). Stressors can be abiotic, for example, extreme temperatures can damage proteins (Parsell and Lindquist, 1993). The production of certain heat shock proteins as part of the stress response can remove accumulations of these

damaged proteins (Parsell and Lindquist, 1993). Stressors can also be biotic, for example nutritional stress reduces the volume of the HVC region of the brain in song sparrows, *Melospiza melodia* (MacDonald et al., 2006). Differences in stress tolerance may exist between these different forms of stressors, and have been linked to changes in gene expression of those involved in the stress response (Ekengren and Hultmark, 2001) and changes in energy stores (Gefen et al., 2006). In vertebrates, the hypothalamus-pituitary-adrenal (HPA) axis is a crucial part of this stress response, influencing stress-related behaviour, but additionally, it can also affect development and energy utilisation (Denver, 2009). Early life stress has been implicated in a number of disorders in mammals, including humans (O'Mahony et al., 2009; Hoffman et al., 2017). For example, adults raised under adverse circumstances show an increased inflammatory response (Danese et al., 2007). Likewise, rats from mothers subjected to stress during pregnancy have longer periods of corticosterone secretion when stressed as adults and show higher levels of escape behaviours in novel environments than those from non-stressed females (Vallée et al., 1997). In mammals, it has been suggested that stress-induced activation of the HPA axis and the subsequent induction of glucocorticoids could impact on brain maturation (Lupien et al., 2009), and young rats maternally deprived show sex-specific differences in hippocampal synaptic plasticity (Derks et al., 2016). Similarly, invertebrate stress pathways can also influence elements of development, for example, the JNK pathway in fruit flies is linked to formation of tissue polarity as well as immune system regulation (Stronach and Perrimon, 1999; Lemaitre and Hoffmann, 2007).

Early life stress may have distinct implications for later life, for example, it has been associated with changes in hippocampal cell proliferation in brain development in rats (Mirescu et al., 2004), and inflammatory responses in humans (Danese et al., 2007). Exposure to stress in early life could reduce an organism’s lifespan, for example, artificially stimulating a stress response in young zebra finches, *Taeniopygia guttata*, decreases adult lifespan (Monaghan et al., 2012), but there is also

evidence that mild stress can actually extend longevity under later stress (Hercus et al., 2003). This has been observed in fruit flies exposed to a mild heat stress during young adult stages then challenged with a higher heat stress at an older age (Hercus et al., 2003). However, such hormetic effects are specific to the type, quantity and time they are experienced (Le Bourg et al., 2001; Costantini et al., 2010), and can interact with stressful conditions experienced at different life stages (Costantini et al., 2014). For example, zebra finches undergoing mild heat stress early in life increase survival when encountering heat stress in adulthood, but show reductions in survival if they do not encounter heat stress as adults, suggesting that costs are involved in producing this hormetic response (Costantini et al., 2014).

Another factor that can cause a change from the homeostatic state in an individual is an immune challenge. Stress and immune responses are closely connected, for example, rats subjected to social aggression have reduced immune functions (Stefanski, 2001). As animals are continually in contact with microorganisms in their surroundings, some of which could be harmful, they have evolved a number of mechanisms to deal with infections (Lemaitre and Hoffmann, 2007). The spread of bacteria can be facilitated by a number of factors, including through contact with an already infected individual. Consequently, larger group sizes can increase the likelihood of infection (Reeson et al., 1998). For example, infected zebra finches, *T. guttata*, can facilitate the spread of bacteria to co-housed individuals, likely through preening behaviour, as well as via sexual transfer to mates (Kulkarni and Heeb, 2007), and the mite *Coccipolipus* sp. is sexually transmitted between cocinellid beetle hosts, *Adalia bipunctata* (Hurst et al., 1995). Furthermore, the risk of infection is increased where there are aggregations of *D. melanogaster* in the wild, indicated by an increased incidence of parasitoid wasps, *Leptopilina* spp. (Wertheim et al., 2006). Under such circumstances, it could be beneficial to invest in heightened immune activity. One example of this has been observed in the mealworm beetle, *Tenebrio molitor*, which have an increased resistance to a pathogenic fungus, likely through higher melanization levels, if reared at high larval density than if

reared solitarily (Barnes and Siva-Jothy, 2000). Such density-dependent prophylaxis has also been found in butterfly pupae reared at high density that are able to destroy a larger number of parasitioids than those from low density environments (Rosa et al., 2017), and the moth, *Anticarsia gemmatalis*, that show higher encapsulation responses, higher haemocyte densities and are more resistant to viral infection when reared with other individuals (Silva et al., 2013). Furthermore, larvae of the beet webworm, *Loxostege sticticalis*, increase expression of a lysozyme-encoding gene when reared at high larval density (Kong et al., 2016). Lysozymes are important constituents of the immune response that digest bacterial cell walls, and increased production of these are likely to be beneficial under crowded conditions where the risk of infection is higher (Kong et al., 2016). In addition to pathogens, there are also important connections between commensal bacteria and the host immune system (Maillet et al., 2008; Broderick and Lemaitre, 2012) (Chapter 5).

The fruit fly *Drosophila melanogaster* is an established model organism to investigate stress responses and innate immune systems (Lemaitre and Hoffmann, 2007). The two primary immune pathways, namely the Toll and Immune deficiency (IMD) pathways, are well-characterised, and result in the production of antimicrobial peptides (AMPs) (Ferrandon et al., 2007). The Toll pathway is primarily activated in response to infection by Gram positive bacteria and fungi through cleaving of the ligand receptor Spätzle, which is initiated by protease cascades, including by the protease Persephone (Valanne et al., 2011). The IMD pathway is triggered in response to Gram negative bacteria. Peptidoglycan recognition proteins are used in the identification of bacteria, for example, the peptidoglycan recognition protein PGRP-LF is a negative regulator of the IMD pathway (Maillet et al., 2008). This interacts with the PGRP-LC protein that activates the IMD pathway cascade (Maillet et al., 2008; Broderick and Lemaitre, 2012). In addition to these two pathways, the JAK-STAT pathway, is another main factor in stress responses (Agaisse and Perrimon, 2004), and is involved in the expression of the *Turandot* genes (Lemaitre and Hoffmann,

2007; Ekengren and Hultmark, 2001; Ekengren et al., 2001). These genes have been shown to increase in expression following stress challenges, including heat shock and bacterial infection (Ekengren and Hultmark, 2001). Additionally, these pathways are closely linked, for example, both the IMD and JAK-STAT pathways are activated in response to septic injury (Brun et al., 2006). Furthermore, it has previously been demonstrated that social conditions can have important impacts on immune activity in adult *Drosophila melanogaster* (Leech et al., 2017; Leech, 2017). For example, living with another individual of the same sex significantly reduces the lifespan of injured flies, a response that that is more acute for males, suggesting differential extents of social stress for each sex (Leech et al., 2017).

In this chapter, the effect of early life social conditions on the ability of young adult fruit flies to cope with different stress challenges was investigated. Larger accessory gland growth in adult males has been found for larvae reared at high density or with adult males present (Bretman et al., 2016). To reduce the potential effects of competition for food at high density, high food concentrations were used in the larval density vials (Bretman et al., 2016). I have also found reductions in lifespan for males reared with adult males present, and slowed development times in flies reared at high larval density (Chapter 3). This could suggest that these conditions are differentially stressful or indicate future stressful conditions, and it could be predicted that greater activation of stress pathways in response to these social conditions could prime adult stress responses. Further to this hypothesis, these individuals could also potentially pre-empt conditions conducive to the spread of pathogens, and increase their immune activity, displaying a prophylactic response. Alternatively, differential resource allocation towards the accessory gland response could result in a decreased capacity in these individuals to cope with acute stress or infection. Differences in tolerance to different forms of stress have been found in *D. melanogaster* and have been associated with the type of energy stores available (Gefen et al., 2006; Rush et al., 2007). Thus, it is possible that individuals do not show a general change in stress tolerance, but this may be

specific to the type of stressor. Therefore, I investigated stress tolerance using a number of different acute stressors, both abiotic (heat and cold shock, and desiccation), and biotic (starvation and bacterial infection). For bacterial infection, three bacteria were injected that had been previously used to study the effect of adult social conditions on immunity (Leech, 2017). These included both a Gram positive bacterium, *Bacillus thuringiensis*, and Gram negative bacteria, *Pseudomonas aeruginosa* and *P. fluorescens*. However, as ingestion is a more natural route to infection (Vodovar et al., 2005), and these two routes can have different responses (Liehl et al., 2006), an oral infection assay was carried out using *P. fluorescens*. This assay also allowed a measure of appetite to be taken. As the production of larger accessory glands in high density and adult presence flies will require energy to produce the response, I expected there to be an increased appetite in males from these social treatments. As differences in stress responses could result from changes in gene expression, and to investigate this as a potential underlying mechanism, I used RT-qPCR to examine gene expression in stress and immune genes. I selected two stress-related genes, *Turandot A* and *Turandot C*, and two immune genes, *persephone* and *PGRP-LF*, that have been shown to be socially responsive in adult flies (Mohorianu et al., 2017). I expected that if these conditions are differentially stressful or can produce priming responses, that there would be differences in the expression of these genes in flies from different larval social environments. It might be expected that where there is greater stress or infection risk, gene expression may be increased. Alternatively, it could be predicted that these genes would show a decreased expression if the larval social conditions prime the stress and immune responses, except for *PGRP-LF*, which, as a negative regulator, may be expected to show the contrasting response.

4.3 Methods

4.3.1 Fly Husbandry

The flies used were wild type Dahomey strain *Drosophila melanogaster*, reared at 25°C, in a humidified room, under a 12:12 light dark cycle on a standard sugar-yeast diet. Adults were provided with purple grape juice agar plates upon which to lay eggs, and larvae were harvested approximately 24 hours later. Larvae were transferred to the treatment groups in plastic vials with 7ml of fresh medium, supplemented with live yeast paste.

4.3.2 Larval Social Treatments

Larvae were haphazardly allocated to treatment groups. Density treatments consisted of either 200 larvae per vial (high) or 20 larvae per vial (low). These larvae were kept on 150% concentrated food medium to prevent food becoming a limiting factor at high densities (Bretman et al., 2016) (Appendix A.1). Adult presence treatments consisted of 100 larvae per vial with or without 20 adult males. The adult males were aspirated into the vial after larval collection; were approximately 5 days old, and had been reared at a standard lab larval density of 100 per vial. The adult males were removed from adult presence vials the day before pupae eclosed. All experimental adult flies were assayed at 1 day old, unless stated.

4.3.3 Stress Tolerance

Adult flies from the different social environments were subjected to one of four stressors, namely starvation, desiccation, heat or cold shock. Flies were collected as they eclosed and held in single sex groups of 10 at 25°C. Starvation tolerance was measured by placing the flies in vials without a food source (i.e. containing an agar and water-only medium) (Appendix A.1).

Mortalities were recorded daily to measure lifespan under starvation conditions, and the medium was changed every 3-4 days to avoid flies becoming trapped due to the medium shrinking back from the vial. Flies that did become trapped were removed from the analysis, as were any flies that escaped during the media changeover. Desiccation tolerance was analysed by transferring the flies into empty vials with no food or moisture source. Any individuals that died in the first 8 hours under these conditions were excluded. Thereafter, flies were checked every 2 hours for the following 86 hours. It is possible that, as there is no food source, this assay also involves an element of starvation stress.

Extreme temperature tolerance was analysed separately using both cold and hot temperatures. For the experiment, 1 day old flies were kept in single-sex groups of 10 and subjected to either cold or heat shock. Temperatures and timings were determined through preliminary trials. For cold shock, this involved adding flies from standard larval rearing conditions (100 larvae per vial raised on standard food) into the freezer at -20°C across 5 minute intervals and checking after 0, 15, 30 and 60 minutes and 24 hours following removal from the freezer. This established that 15 minutes at this temperature was sufficient to induce chill coma in the flies (Andersen et al., 2015), but 60 minutes after this period at 25°C allowed for a number of flies to recover. Similarly, the period of time for heat shock was determined by testing for complete fall down of flies at temperatures between 39 and 42°C, and subsequent recovery at 25°C. From these trials, cold tolerance was assayed by holding the vials in a freezer at -20°C for 15 minutes, whilst heat tolerance was assayed by holding the vials in an incubator at 42 ± 0.5°C for 34 minutes. Following the temperature shocks, flies were transferred to a controlled temperature room at 25°C for 60 minutes, and the number of flies lying on their side or back were recorded after this time (Folk et al., 2006). This ‘fall down’ number included both dead individuals and those that, although alive, had not righted one hour after the temperature shock.

4.3.4 Assay of post-infection lifespan

Flies were injected with one of three bacteria using the Nanoliter 2010 (World Precision Instruments, Florida, United States). These were the Gram negative bacteria *Pseudomonas fluorescens* (DSMZ 50090), the Gram negative *Pseudomonas aeruginosa* (PAO1), or the Gram positive *Bacillus thuringiensis* (DSMZ 2046). *B. thuringiensis* and *P. aeruginosa* were cultured in 5ml of Lysogeny broth (LB) (Vodovar et al., 2005) at 37°C for 24 hours with 200rpm shaking, with 13.8nl of 10^{-1} or 9.2nl 10^{-2} respectively injected into the fly. *P. fluorescens* was grown at 25°C for 48 hours, with 13.8nl of 10^{-2} injected. Uninfected flies were injected with the same quantity of sterile $MgSO_4$ solution. These doses were used in the aforementioned study of adult social conditions in our laboratory (Leech, 2017), and were based on the protocol devised by Apidianakis and Rahme (2009). Flies were haphazardly selected for injection throughout. The glass needle was refilled every 10 injections to prevent the pooling of bacteria at the bottom, and flies were checked hourly for mortality.

4.3.5 Oral infection and appetite assay

As a more natural route of infection is through ingestion (Lemaitre and Hoffmann, 2007), an oral infection assay was also carried out using the CAFÉ assay methodology (Ja et al., 2007). This assay was originally intended to assess both oral post-infection lifespan and appetite on this infected food source. Flies were fed on a yeast and sugar solution containing *Pseudomonas fluorescens*, which had previously been used to study oral infection in adult flies (Leech, 2017), as well as in larvae (Olcott et al., 2010). Flies were 4 days old and were kept singly to ensure that the appetite of each individual could be measured. The bacteria was cultured in 5ml LB broth for 48 hours at 25°C as per the culture protocol for the injection assay. The culture was spun down to form a pellet which was re-suspended in a 10% yeast and sugar solution. Flies were transferred onto a medium

containing only agar and water for approximately 3 hours to ensure they had no food source with the aim of increasing hunger to induce feeding once the solution was added (Vodovar et al., 2005). The solution was added to a capillary tube (1mm in diameter) using capillary action. The beginning point of the solution was marked on the outside of the tube and mineral oil was added to the top of the tube to reduce losses from evaporation. This tube was placed inside a pipette tip which pierced through the parafilm that covered the vial. The flies were left for 3 days, with new cultures of *P. fluorescens*-containing solution added to the capillary each day. The measure of appetite was recorded by measuring the distance that the solution had moved down the tube each day. In order to control for evaporative losses that could influence movement of food in the capillaries, vials containing no flies were also set-up, and the quantity from the evaporation vials was subtracted by the quantity of food eaten for each fly per day. Mortality was recorded, but as only a few died during the assay, no effect of oral infection on mortality could be determined.

4.3.6 Gene Expression

To analyse gene expression in adults from the larval social environments, RT-qPCR was carried out. Two stress-related genes, *Turandot A* and *Turandot C* (Ekengren and Hultmark, 2001), were chosen as they have been shown to vary in expression in response to adult social conditions (Mohorianu et al., 2017), and in response to a number of different stressors (Ekengren and Hultmark, 2001). Additionally, the expression of two immune genes, namely *persephone* and *PGRP-LF*, was also investigated. These had been shown to vary in expression in adult males following exposure to a rival (Mohorianu et al., 2017), and in response to larval crowding (Zhou et al., 2012). As *persephone* is involved in activation of the Toll pathway, and *PGRP-LF* is a negative regulator of the IMD pathway, both of the major immune pathways were included. Liquid nitrogen was used to snap freeze flies at 1 day old. Twelve whole flies from each larval social treatment were

transferred into an Eppendorf, with 7 repeats. The flies were ground using a micropestle, and RNA extracted with the Direct-zol ™ RNA miniprep kits, following the manufacturer's protocol. The RNA was checked using a Nanodrop and a 1% agarose gel to check for degradation. RNA was then converted to cDNA using the First Strand cDNA synthesis kit (Thermo Fisher) following the manufacturer's protocol. Each sample also had a negative control containing no reverse transcriptase. Both positive and negative samples were then checked by PCR amplification with *Actin* (*Act5c*) primers and ran on a gel to check for contamination (Figure 4.3.6-1). Primers were designed with a melting temperature of 60°C ± 1°C and a CG content of 20-80%. Primer efficiency was determined using a 10 times dilution series, and were accepted if this efficiency was between 90 and 110% for dilutions, with a pipetting accuracy (R^2) greater than 0.99. Two housekeeper genes were used that had previously been identified as candidate reference genes, namely *Ef1* and *Actin* (Ling and Salvaterra, 2011). Each sample was run in triplicate. Average Cq values were obtained for each sample. Triplicates which had extreme values (a difference in value of greater than 0.5) were excluded. Relative quantity was obtained by subtracting the Cq of each sample from the Cq of a selected reference sample (in all cases, this was the first sample in the dataset) and raising the efficiency of the primer to this number. The geometric mean for the housekeeper genes was determined, and normalized expression for each gene of interest was calculated by dividing the RQ of each sample by the housekeeper geometric mean.

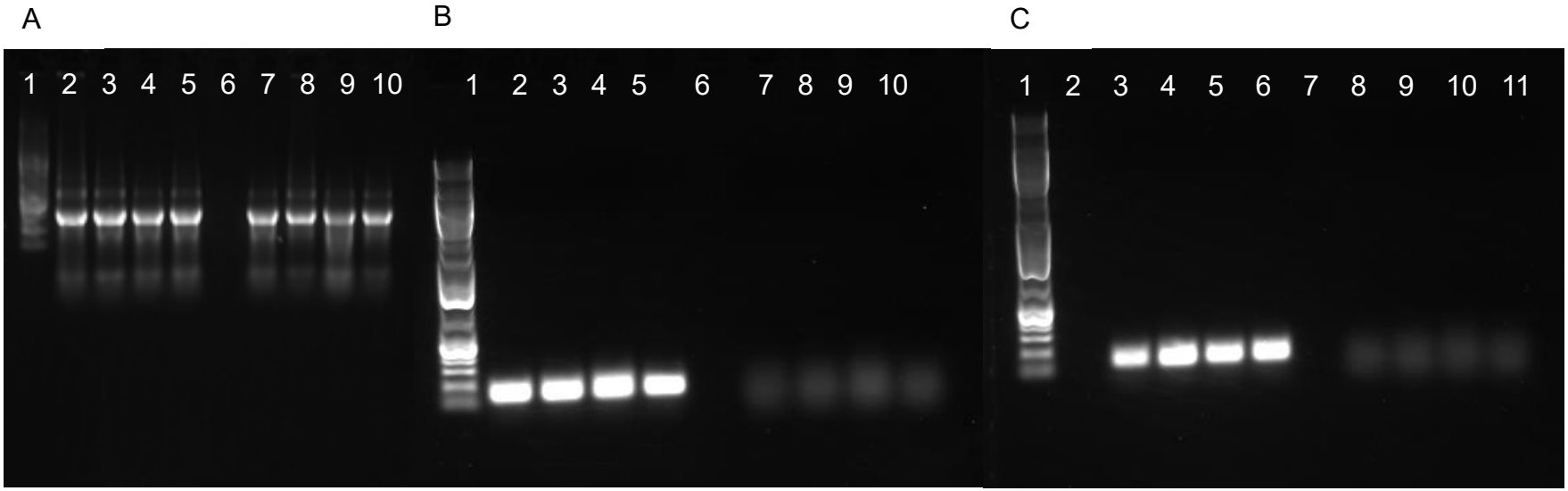


Figure 4.3.6-1 Example Electrophoresis Gels for RNA Extractions (A) and cDNA synthesis (B and C). A: Lane 1 contains ssRNA ladder; Lanes 2-5 contain RNA from Low Density males and females, High Density males and females; Lanes 7-10 contain RNA from Adult Absence males and females, Adult Presence males, and females. B: Lane 1 contains 100bp DNA ladder; Lanes 2-5 contain cDNA for Low Density males and females, High Density males and females; Lanes 7-10 contain negative Reverse Transcriptase (RT) controls for density groups. C: Lane 1 contains 100bp DNA ladder; Lanes 3-6 contain cDNA for Adult Absence males and females, Adult Presence males and females; Lanes 8-11 contain negative Reverse Transcriptase (RT) controls for adult presence groups. Markings around the bands indicate degradation of buffer.

4.3.7 Statistical Analysis

Both starvation and desiccation tolerance were analysed using Generalised Linear Models, with a Poisson distribution in R (v3.3.3). Extreme outliers, identified as 1.5x Interquartile Range, were removed. Both were analysed with social treatment and sex as fixed factors, and vial and fly ID as random effects, excluding adult presence group males and females that were assayed on different days for desiccation tolerance, and were therefore analysed separately. Heat and cold shock tolerance, measured as 'fall down' number, were analysed with Generalised Linear Models with treatment and sex as fixed effects, and vial as a random factor, with a Poisson distribution.

The lifespan post-*B. thuringiensis* and *P. fluorescens* infections were censored after 35 hours, and *P. aeruginosa* after 46 hours. There was no negative effect of sham-injection and so these were removed from analysis. The number of alive and dead individuals by the end-point for each group was compared using Chi-squared test in SPSSv21. Subsequently surviving individuals were removed from the analysis and time to death was analysed using Generalised Linear Models in R (v3.3.3), with treatment and sex as fixed factors, fly ID as a random effect, and a Poisson distribution. Models were reduced and compared with Analysis of Deviance. Survival analysis of post-infection lifespan was also carried out, using Cox Proportional Hazards models, with treatment and sex as fixed factors, and Analysis of Deviance to compare. The CAFÉ assay did not cause mortality of flies, so only data on appetite could be analysed. Unfortunately, as some evaporative losses were extreme, this resulted in negative values for a number of flies, so all values were made positive by adding 5.61. Finally, as a few outliers were having extreme significant effects and to reduce these impacts, the data was Winsorized (Barnett and Lewis, 1974) by limiting these outliers to the 5th and 95th percentiles. The quantity of food eaten was analysed using Generalised Linear Models, with treatment and sex as fixed factors, and day

and fly ID as random effects, with a Gamma distribution. Models were reduced and compared by Analysis of Deviance.

Normalized gene expression was analysed using Generalised Linear Models, with treatment and sex as fixed factors, and quasi-Poisson errors to account for underdispersion. Models were reduced and compared using Analysis of Deviance. To test for potential differences in variance between low and high larval densities, Levene's tests were carried out on response variables.

4.4 Results

4.4.1 Effect of Larval Density on stress and immune responses

There was no interaction between larval density and sex for starvation tolerance (χ^2=0.276; df= 1, 6; p=0.599). There was no effect of larval rearing density on the ability to tolerate starvation conditions (χ^2=1.842; df= 1, 5; p=0.175). There was a significant effect of sex (χ^2=5.752; df= 1, 4; p=0.017), with females generally tolerating these conditions better than males (Figure 4.4.1-1). There was no interaction between larval density and sex for desiccation tolerance (χ^2=0.201; df= 1, 6; p=0.654). Larval density had no effect on desiccation tolerance (χ^2=0.493; df= 1, 5; p=0.483) (Figure 4.4.1-2). Sex did have a significant effect (χ^2=17.529; df= 1, 4; p<0.001), with females generally having a higher tolerance than males to these conditions. There was no difference in the variance between low and high larval density groups for starvation (F=0.620; p=0.602) or desiccation tolerance (F=1.676; p=0.172).

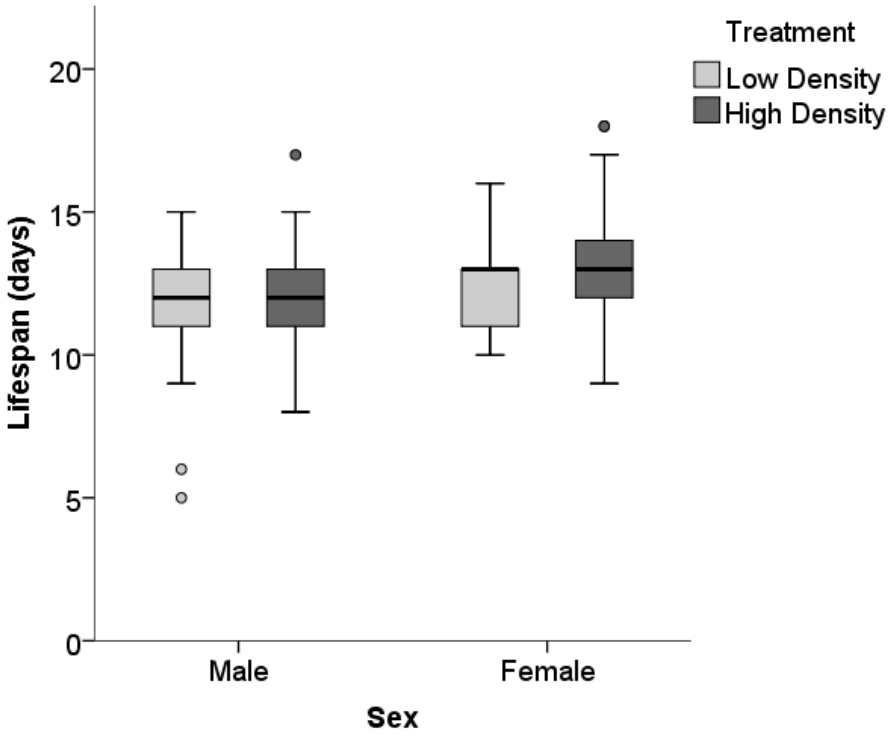


Figure 4.4.1-1 Median lifespan (days) for starvation tolerance of young adult flies from different larval densities. Low Density males (n=87) and High Density males (n=89), and Low Density females (n=89) and High Density females (n=94). Flies were kept on a medium without a food source and mortalities were recorded daily.

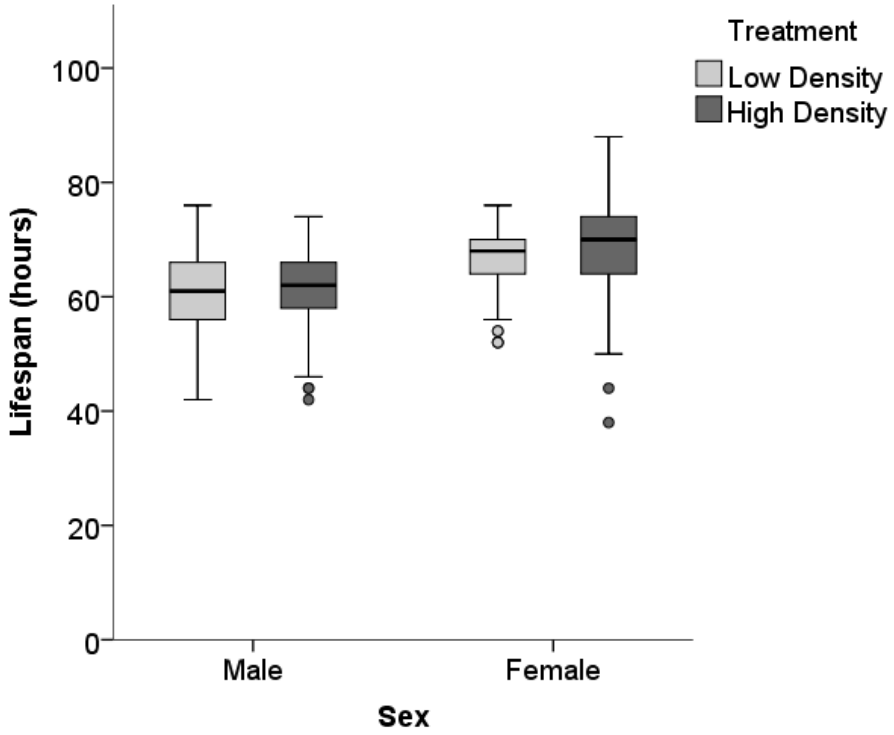


Figure 4.4.1-2 Median lifespan (hours) for desiccation tolerance of young adult flies from different larval densities. High and Low Density males (n=70), and High and Low Density females (LD n=70; HD n=71). Flies were kept in empty vials with no food and water, and mortality recorded every 2 hours.

For cold tolerance, there was a significant interaction between social treatment and sex for density group flies (χ^2=4.022; df= 1, 5; p=0.045; Figure 4.4.1-3). For females alone, there was no effect of larval rearing density (χ^2=0.956; df= 1, 3; p=0.328), but high density males recovered significantly better than low density males (χ^2=11.024; df=1, 3; p<0.001). For heat shock, there was no interaction between larval density and sex (χ^2=0.216; df= 1, 5; p=0.642). There was no effect of larval density (χ^2=0.952; df= 1, 4; p=0.329) or sex (χ^2=0.375; df=1, 3; p=0.540) (Figure 4.4.1-4). There was no effect of larval density on variance of heat shock tolerance (F=0.602; p=0.621) or cold shock tolerance (F=0.972; p=0.425).

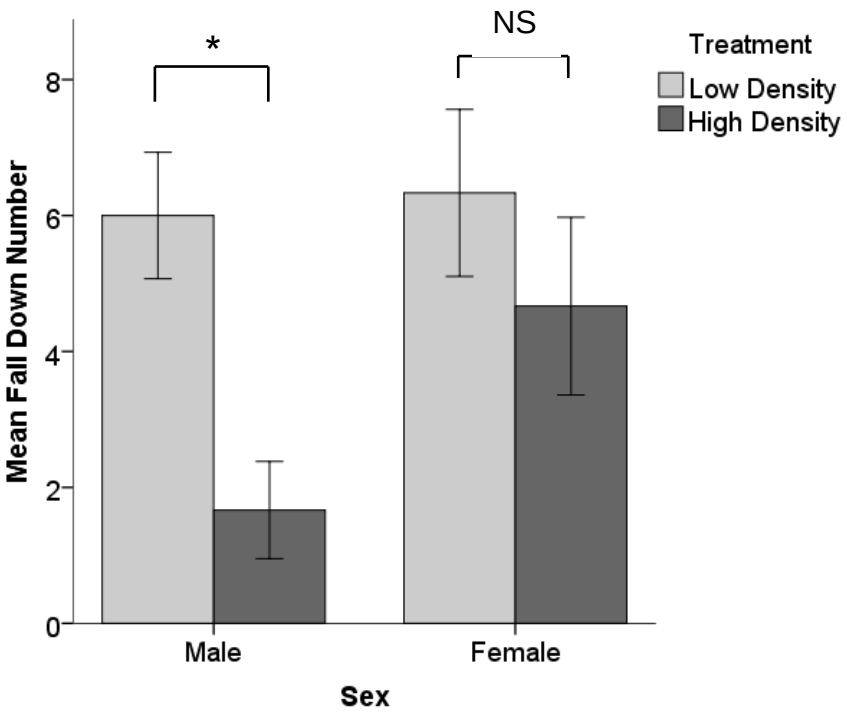


Figure 4.4.1-3 Mean fall down number for cold tolerance of young adult flies from different larval densities. Flies reared at high or low density (Error bars ± 1 S.E.). Flies were kept at -20°C for 15 minutes then at 25°C for 60 minutes (n=60). The number of flies still down was recorded as the 'fall down' number. * denotes a significant difference ($p<0.05$). NS denotes non-significance.

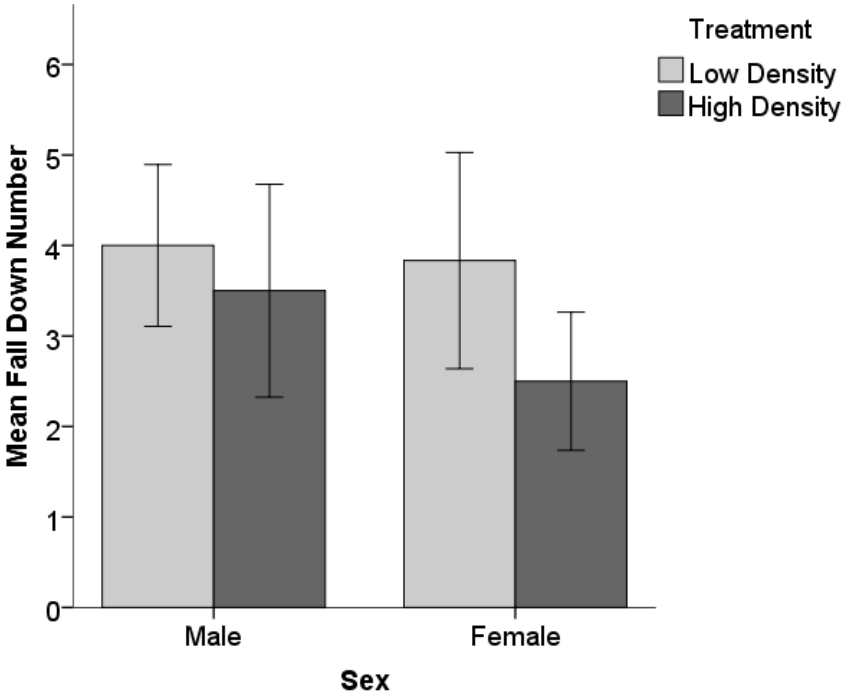


Figure 4.4.1-4 Mean fall down number for heat shock tolerance of young adult flies from different larval densities. Flies reared at high or low density (Error bars ± 1 S.E.). Flies were held at 42°C for 34 minutes then kept at 25°C for 60 minutes (n=60). The number of flies still down at this time was recorded as the 'fall down' number.

There was no effect of larval density on the numbers of individuals still alive by the censored time-point following injection with *Bacillus thuringiensis* (X^2= 3.482; df= 3; p=0.323); *Pseudomonas fluorescens* (X^2= 0.918; df= 3; p=0.821), or *Pseudomonas aeruginosa* (X^2= 1.187; df= 3; p=0.756). These individuals were subsequently removed from the analysis. There was no interaction between larval density and sex for post-*Bacillus thuringiensis* infection lifespan (χ^2= 2.781; df= 1, 5; p=0.095). There was a significant effect of sex for those infected with *B. thuringiensis* (χ^2=7.555; df= 1, 3; p=0.006), with females generally surviving better than males, but no significant effect of larval density (χ^2=0.073; df= 1, 4; p=0.787; Figure 4.4.1-5). Survival analysis found no interaction between treatment and sex (χ^2=2.007; df= 1; p=0.157), no effect of larval density (χ^2=0.031; df= 1; p=0.860), but no effect of sex (χ^2=3.371; df= 1; p=0.066) for post-infection lifespan following *B. thuringiensis* infection. There was no interaction between larval density and sex for lifespan following *P. fluorescens* injections (χ^2=1.922; df= 1, 5; p=0.166), no effect of sex (χ^2=1.453; df= 1, 4; p=0.228) or larval density (χ^2=2.433; df= 1, 3; p=0.119). Survival analysis found no interaction between larval density and sex following *P. fluorescens* injection (χ^2=1.024; df= 1; p=0.312), no effect of larval density (χ^2=0.898; df= 1; p=0.343), and no effect of sex (χ^2=0.004; df= 1; p=0.947). There was no interaction found following injection with *P. aeruginosa* (χ^2=0.873; df= 1, 5; p=0.350), no effect of sex (χ^2=3.230; df= 1, 3; p=0.072), or larval density (χ^2=0.741; df= 1, 4; p=0.389). Survival analysis also found no interaction between larval density and sex following *P. aeruginosa* injection (χ^2=0.251; df= 1; p=0.616), no effect of larval density (χ^2=0.175; df= 1; p=0.676), but an effect of sex (χ^2=5.144; df= 1; p=0.020). No difference in variance was found following *B. thuringiensis* (F=1.233; p=0.301) or *P. fluorescens* injection (F=2.382; p=0.072). There was a significant difference in variance following *P. aeruginosa* injection (F=3.921; p=0.010). Analysing the sexes separately found this was significant for females (F=8.762; p=0.005), but not males (F=0.005; p=0.942). There was no impact of *P. fluorescens* oral infection on mortality, however, high density flies ate significantly more than low density flies (χ^2=7.142; df= 1, 6; p=0.008) (Figure 4.4.1-6). Sex did not have an effect on appetite (χ^2=2.597; df= 1, 6; p=0.107).

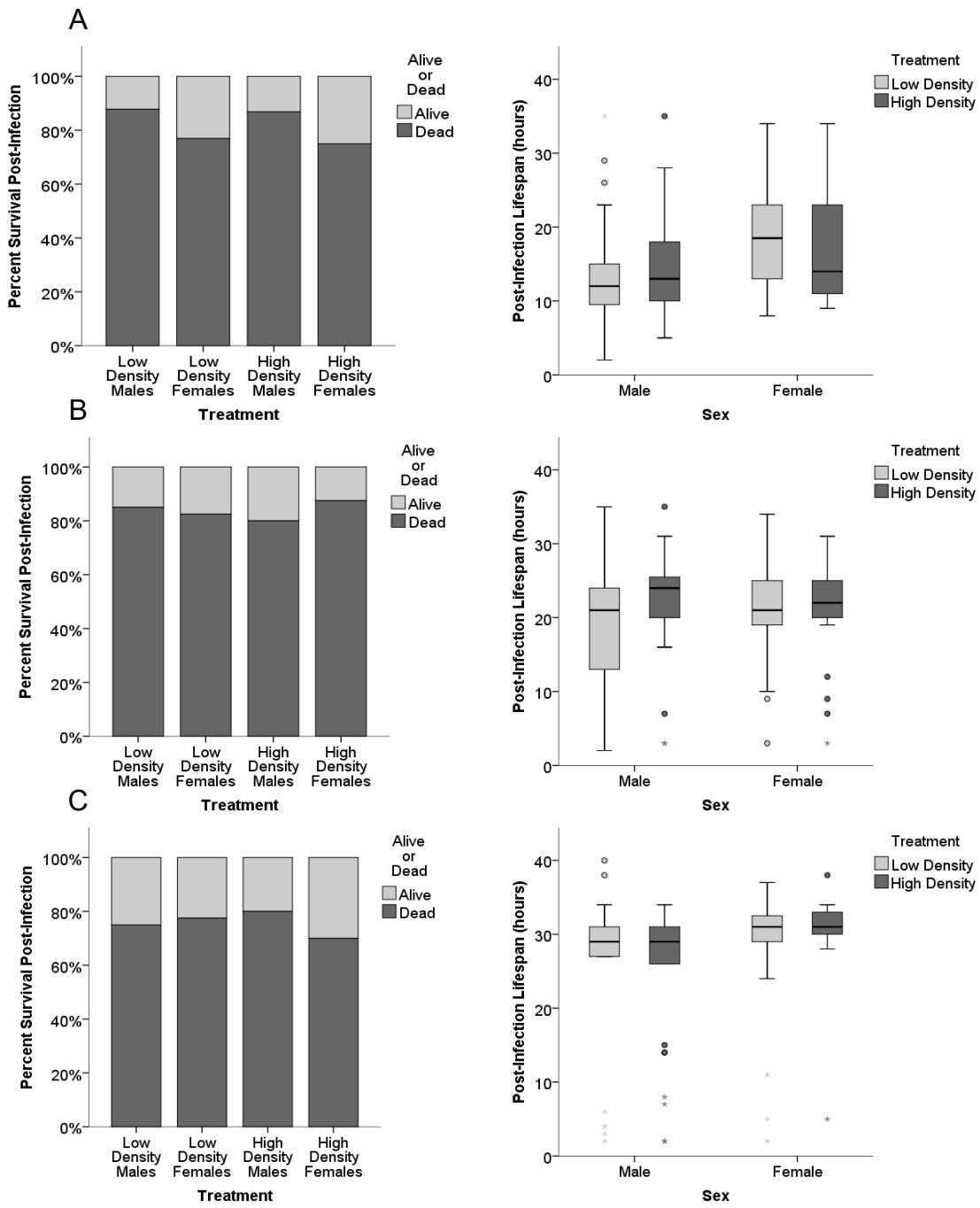


Figure 4.4.1-5 Percent survival of flies post-infection from different larval density environments at the censored time point (left) and median post-infection lifespan of those that died from infection (right). A= Low and High Larval Density groups injected with *B. thuringiensis* (LD males n= 36, females= 30; HD males= 33, females= 30); B= *P. fluorescens* (LD males n=34, females=33; HD males=32, females=35); C= *P. aeruginosa* (LD males n=30, females n=31; HD males n=32, HD females n=28).

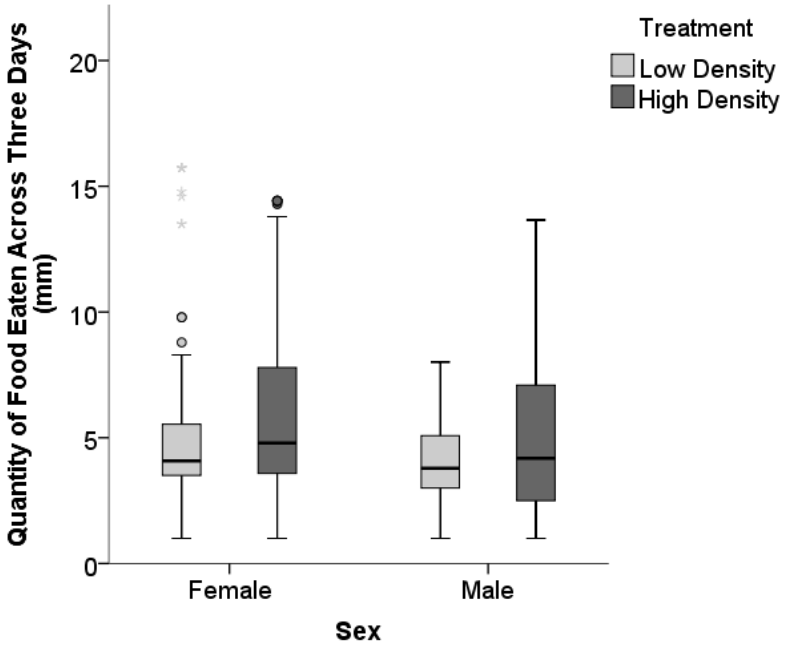


Figure 4.4.1-6 Median quantity of food eaten across three days (mm) in CAFÉ Assay for flies kept at different larval densities. Low Density males (n=38); Low Density females (n=38); High Density males (n=39) and High Density females (n=40).

There was no interaction between larval density and sex on the relative gene expression of *persephone* (F=3.239=; df=1, 24; p=0.085). There was no effect of larval density (F=0.253; df= 1, 25; p=0.620), but there was a significant effect of sex, with males having relatively greater expression than females *(*F=*70.411; df= 1, 26; p<0.001).* There was no interaction between larval density and sex on expression of *PGRP-LF* (F=0.850; df= 1, 24; p=0.366). There was no effect of larval density (F=0.320; df= 1, 25; p=0.576), but males had significantly greater relative expression than females (F=75.755; df= 1, 26; p<0.001). There was no interaction between larval density and sex on expression of *Turandot A* (F=1.868; df= 1, 24; p=0.184) or *Turandot C* (F=2.242; df= 1, 24; p=0.147). There was no effect of sex on *Turandot A* expression (F=0.128; df= 1, 25; p=0.724) or *Turandot C* (F=1.995; df=1, 25; p=0.170). However, there was a significant effect of larval density on *Turandot A* (F=16.282; df= 1, 26; p<0.001) and *Turandot C* expression (F=11.985; df=1, 26; p=0.002). Both of these genes had higher

relative expression in low density flies compared to high density flies (Figure 4.4.1-7). There was greater variance in normalized expression of *Turandot A* (F=5.160; p=0.007), *Turandot C* (F=7.123; p=0.001) and *persephone* (F=4.084; p=0.018) in low density flies compared to high density flies, but not for *PGRP-LF* expression (F=0.756; p=0.530).

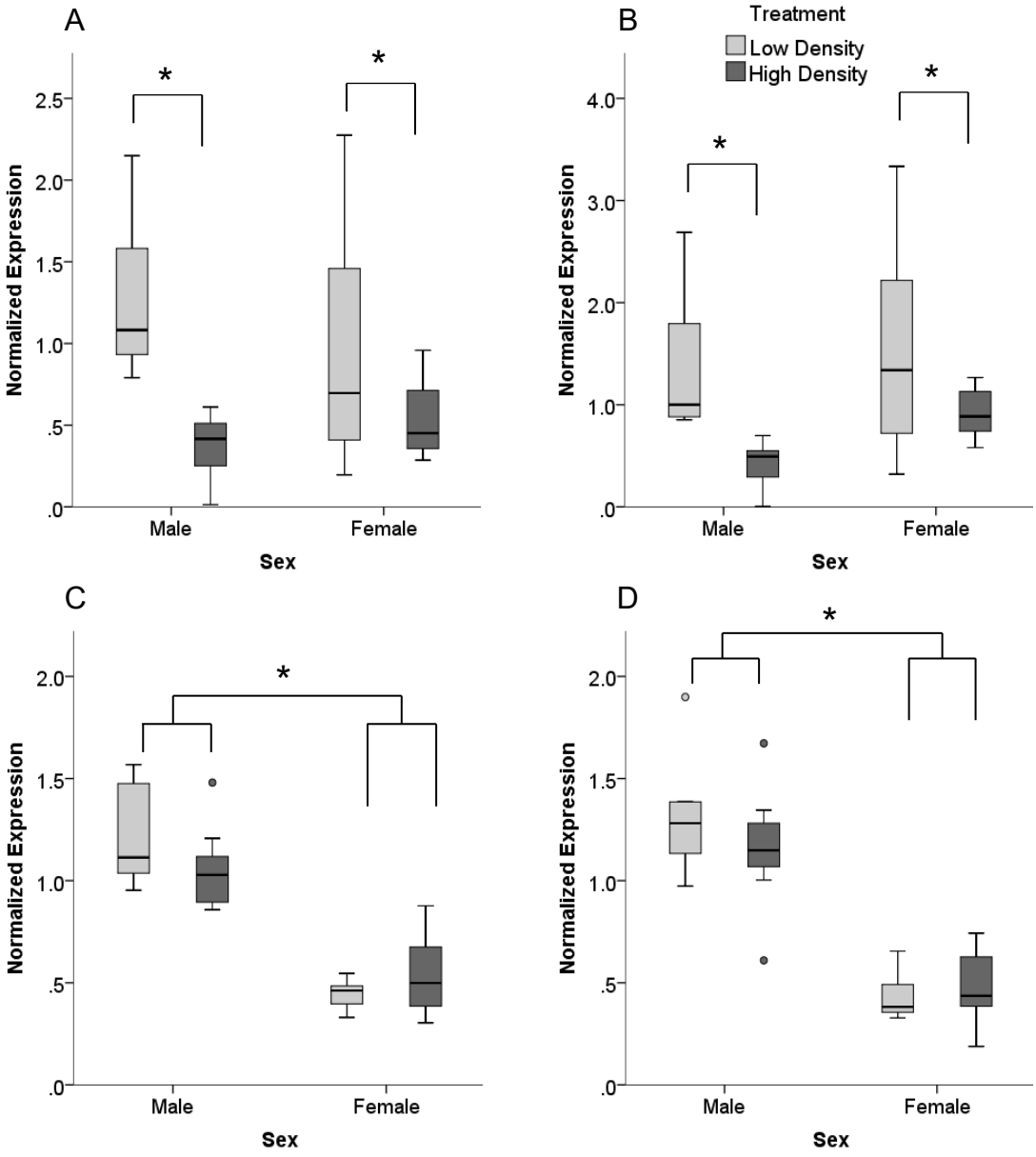


Figure 4.4.1-7 Median Normalized Expression for stress and immune genes of interest from 1 day old flies reared at low or high larval density. A= *Turandot A*; B=*Turandot C*; C=*Persephone*; D= *PGRP-LF.* Normalized expression for each gene was calculated by dividing the Relative Quantity of each sample by the housekeeper geometric mean. * denotes a significant difference ($p<0.05$).

4.4.2 Effect of Adult Presence on stress and immune responses

There was no interaction between adult presence and sex on starvation tolerance (χ^2= 0.565; df= 1, 6; p=0.452). There was no effect of adult presence on starvation tolerance (χ^2= 0.104; df= 1, 5; p=0.747), but there was a significant effect of sex (χ^2= 6.757; df=1, 4; p=0.009; Figure 4.4.2-1). There was no effect of larval environment for desiccation tolerance of adult presence males (χ^2=1.756; df= 1, 4; p=0.185). There was, however, a significant effect of larval conditions for desiccation tolerance of adult presence females (χ^2=7.186; df= 1, 3; p<0.001) (Figure 4.4.2-2), which largely tolerated the conditions better than adult absence females.

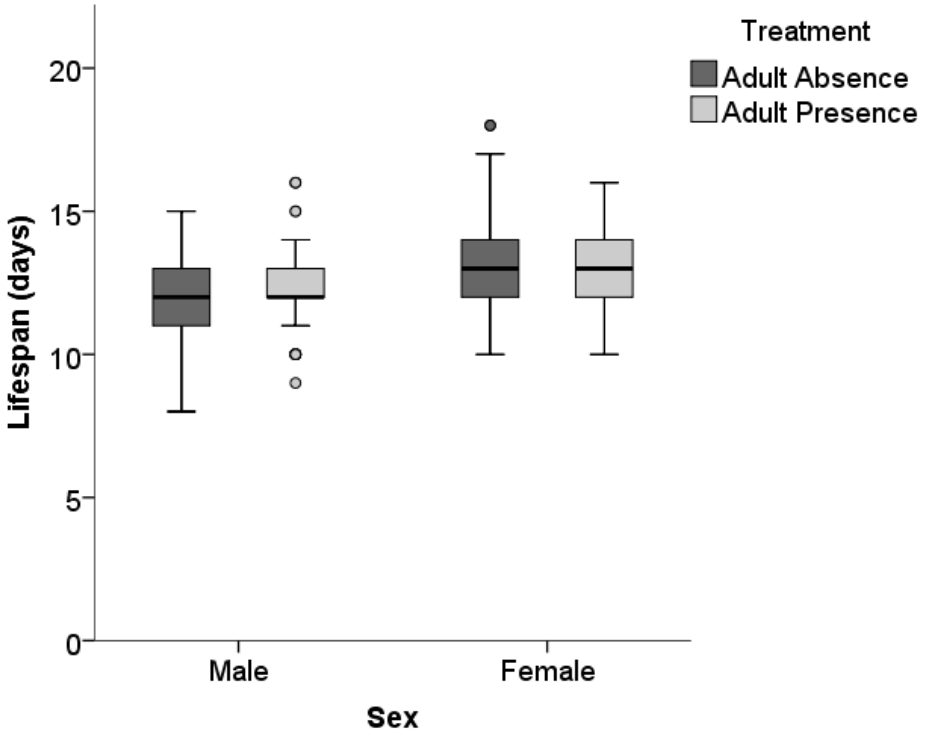


Figure 4.4.2-1 Median lifespan (days) for starvation tolerance of young adult flies reared with or without adults in the larval environment. Adult Absence (n=95) and Presence males (n=96), and Adult Absence (n=96) and Presence females (n=93). Flies were kept on a medium containing no food source and mortalities were recorded daily.

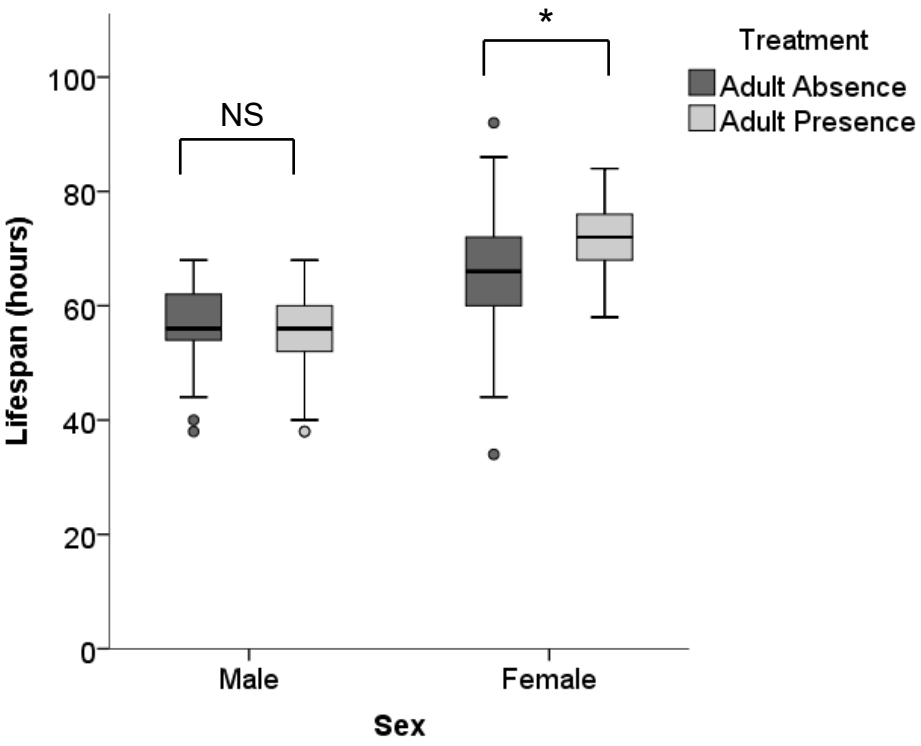


Figure 4.4.2-2 Median lifespan (hours) for desiccation tolerance of young adult flies reared with or without adult males in the larval environment. Adult Absence and Presence males (n=110), and Adult Absence and Presence females (n=100)**.** Flies were kept in empty vials with no food and water, and mortality recorded every 2 hours. * denotes a significant difference ($p<0.05$). NS indicates non-significance.

For cold shock, a significant interaction was found between social treatment and sex for adult presence groups (χ^2=15.102; df= 1, 5; p<0.001). Analysing the sexes separately found no effect of adult presence in the males (χ^2=0.982; df= 1, 3; p=0.322), but a significant effect in females (χ^2=15.885; df=1, 3; p<0.001), with a greater number of adult presence females recovering 60 minutes after cold shock than adult absence females (Figure 4.4.2-3). For heat shock, there was no interaction between adult presence and sex (χ^2=0.0004; df= 1, 5; p=0.984). No effect of adult presence was found for heat shock tolerance (χ^2=0.001; df= 1, 4; p=0.993). There was also no effect of sex (χ^2=0.813; df= 1, 3; p=0.367) (Figure 4.4.2-4).

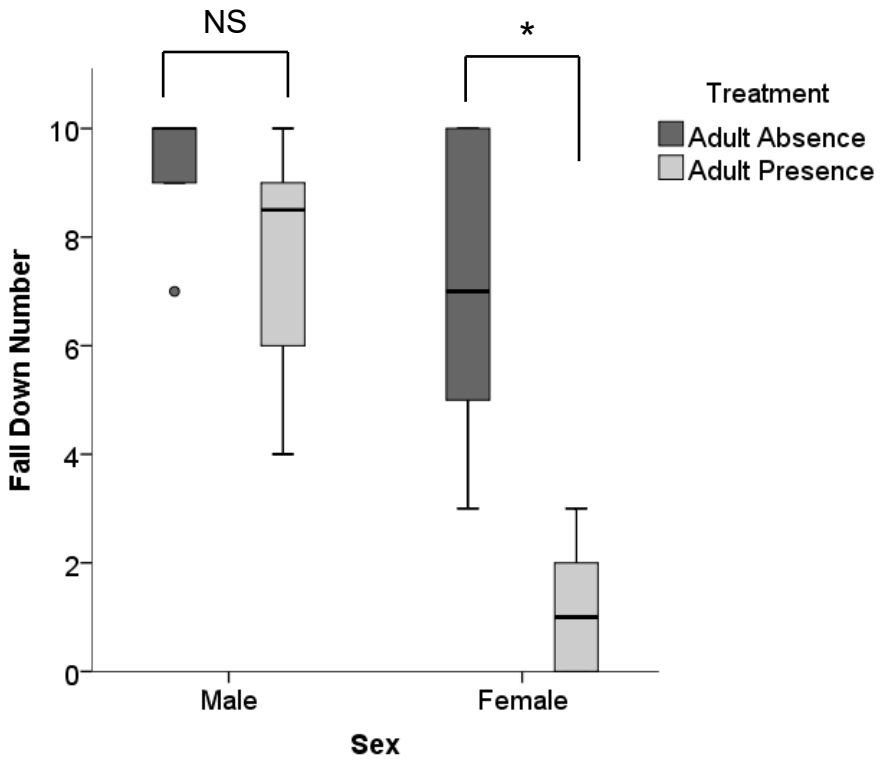


Figure 4.4.2-3 Median fall down number for cold tolerance of young adult flies reared with or without adult males in the environment. Flies were given a 15 minute cold shock at -20°C then kept at 25°C for 60 minutes (n=60). The number of flies still down at this time was recorded as the 'fall down' number. * denotes a significant difference (p<0.05). NS indicates non-significance.

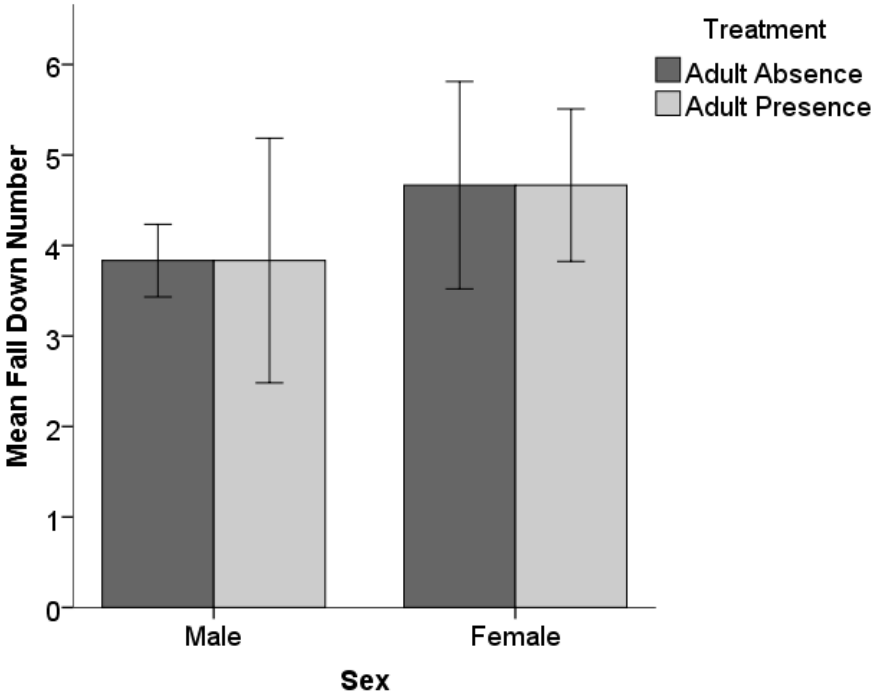


Figure 4.4.2-4 Mean fall down number for heat shock tolerance of young adult flies reared with or without adult males in the larval environment (Error bars ± 1 S.E.). Flies were held at 42°C for 34 minutes then at 25°C for 60 minutes (n=60). The number still down was recorded as the 'fall down' number.

There was no effect of adult presence on the numbers of individuals still alive by the censored time-point following injection with *Bacillus thuringiensis* (X^2=4.940; df= 3; p=0.176); *Pseudomonas fluorescens* (X^2=3.737; df=3; p=0.291), or *Pseudomonas aeruginosa* (X^2=0.964; df=3; p=0.964). These individuals were removed from the subsequent analysis. There was a significant interaction between treatment and sex on post-infection lifespan for those injected with *B. thuringiensis* (χ^2=4.904, df= 1, 5, p=0.027). Analysing the sexes separately found no effect of adult presence for males (χ^2=0.013, df= 1, 3, p=0.908), but a significant effect for females (χ^2=12.921, df=1, 3, p<0.001), with adult presence females dying significantly quicker than adult absence females (Figure 4.4.2-5). There was also a significant interaction found in the survival analysis (χ^2=4.505; df= 1; p=0.034). There was no interaction between adult presence and sex on lifespan following *P. fluorescens* injection (χ^2= 0.171; df =1, 5; p=0.679). There was no effect of adult presence following injection with *P. fluorescens* (χ^2= 0.738; df =1, 4; p=0.390), and no effect of sex (χ^2=2.417; df= 1, 3; p=0.120). The survival analysis found no interaction between adult presence and sex (χ^2=0.986; df= 1; p=0.321), no effect of adult presence (χ^2=0.216; df= 1; p=0.642), and no effect of sex (χ^2=0.380; df= 1; p=0.538). Similarly, there was no interaction following injection with *P. aeruginosa* (χ^2=0.433; df= 1, 5; p=0.510), no effect of adult presence (χ^2=1.721; df= 1, 3; p=0.190), and there was no effect of sex (χ^2=2.467; df =1, 4; p=0.116). Survival analysis also found no interaction (χ^2=1.098; df= 1; p=0.295), no effect of adult presence (χ^2=1.829; df= 1; p=0.176), and no effect of sex (χ^2=0.027; df= 1; p=0.868) following *P. aeruginosa* injection.

Adult presence did not significantly affect the quantity of food eaten in the CAFÉ Assay (χ^2=0.615; df= 1, 6; p=0.433), however, sex was significant for these groups (χ^2=13.272; df= 1, 5; p<0.001), with females generally eating more than males (Figure 4.4.2-6).

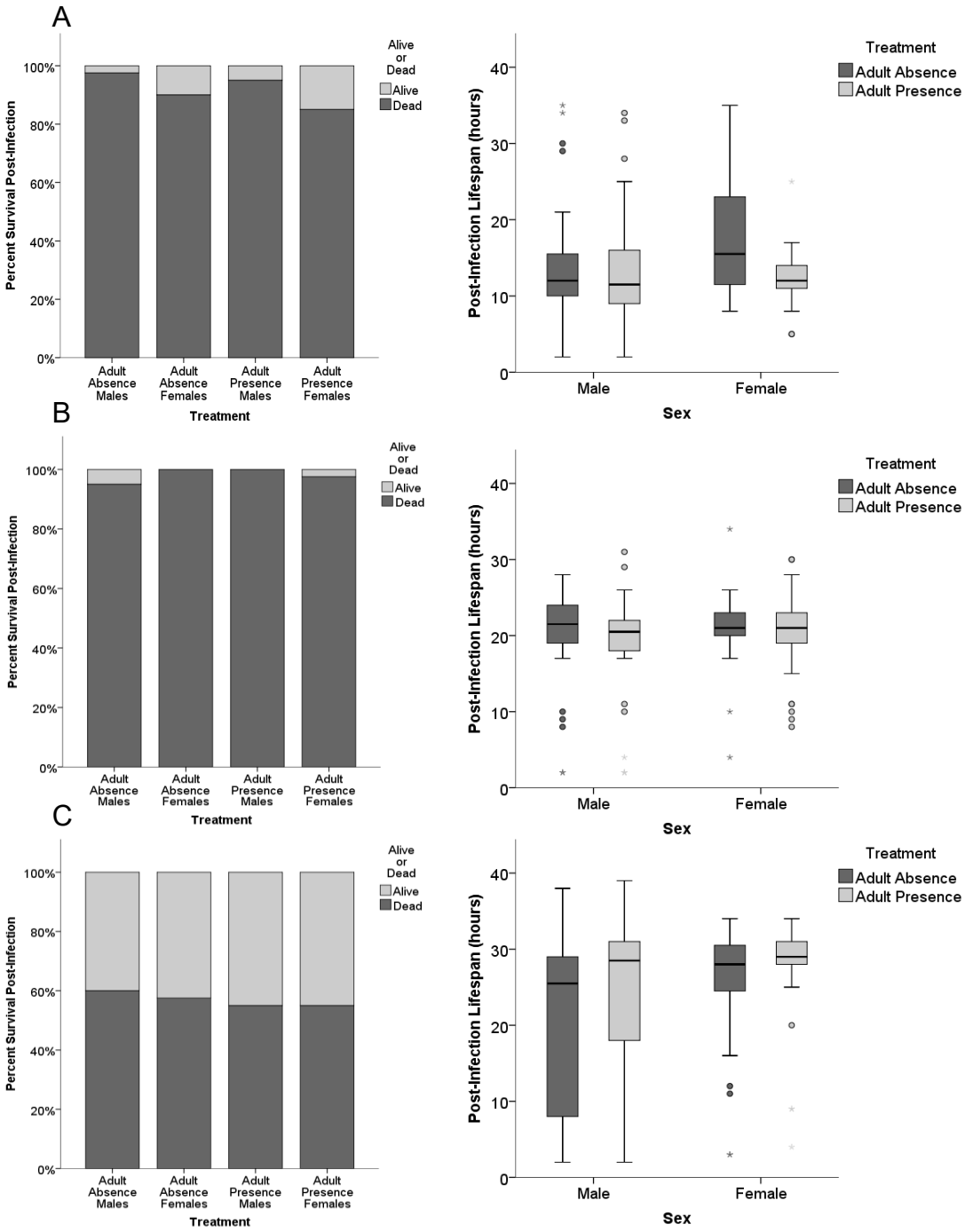


Figure 4.4.2-5 Percent survival of flies post-infection from adult absence and presence environments at the censored time point (left) and median post-infection lifespan of those that died from infection (right). A= Adult Absence (AA) and Adult Presence (AP) groups injected with *B. thuringiensis* (AA males n= 39, females= 36; AP males= 38, females= 34)*;* B= *P. fluorescens* (AA males n= 38, females= 40; AP males= 40, females= 39); C= *P. aeruginosa* (AA males n=24, females=23; AP males=22, females=22)*.*

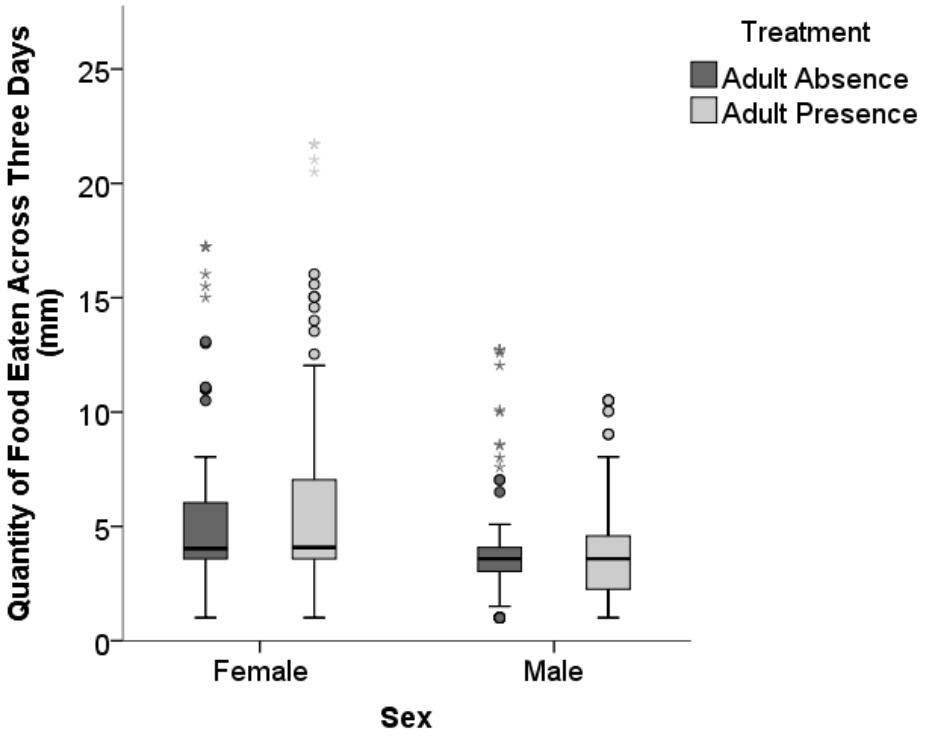


Figure 4.4.2-6 Median quantity of food eaten across three days (mm) in CAFÉ Assay for flies kept with or without adult males present. Adult Absence males (n=36); Adult Absence Females (n=36); Adult Presence males (n=37) and Adult Presence Females (n=39).

There was no interaction between adult presence and sex on gene expression of *persephone* (F=0.0008; df =1, 23; p=0.978). There was no effect of adult presence (F=2.979; df =1, 24; p=0.097). There was a significant effect of sex, with greater relative expression in males than in females (F=46.792; df= 1, 25; p<0.001). Similarly, there was no interaction between adult presence and sex on expression of *PGRP-LF* (F=0.437; df =1, 23; p=0.515). There was also no effect of adult presence (F=0.255; df= 1, 24; p=0.618), but males had higher relative expression than females (F=208.76; df= 1, 25; p<0.001; Figure 4.4.2-7). There was no interaction between adult presence and sex on expression of *Turandot A* (F=0.047; df= 1, 24; p=0.830). There was no effect of adult presence on expression of *Turandot A* (F<0.001; df= 1, 26; p=0.978), and no effect of sex (F=1.004; df=

1, 25; p=0.326). There was no interaction between adult presence and sex on expression of *Turandot C* (F=0.320; df= 1, 23; p=0.577). No effect of adult presence was found for expression of *Turandot C* (F=0.002; df= 1, 24; p=0.963), and there was no effect of sex (F=3.293; df= 1, 25; p=0.082).

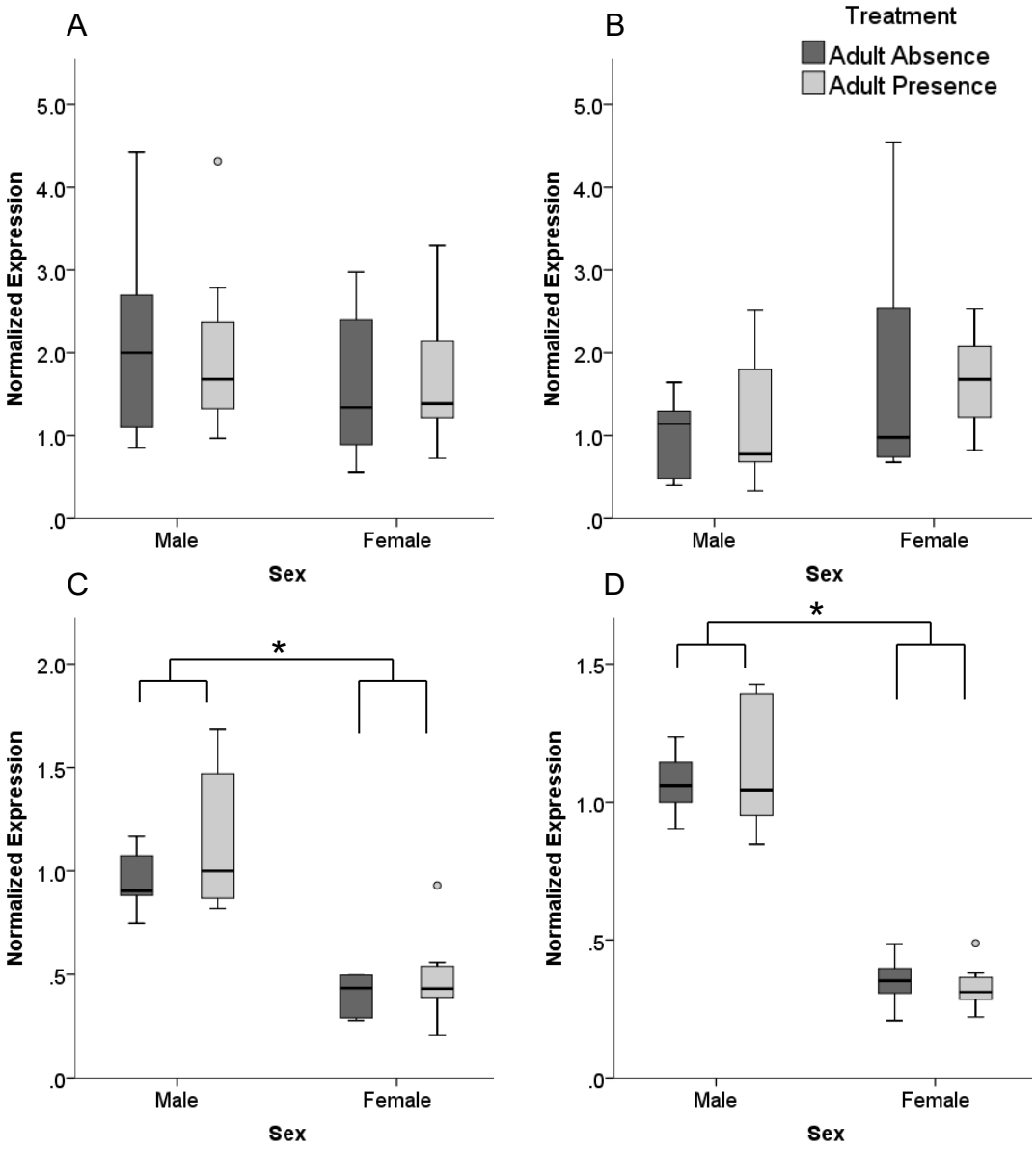


Figure 4.4.2-7 Median Normalized Expression for stress and immune genes in flies reared with or without adult males. A= *Turandot A*; B= *Turandot C*; C= *Persephone*; D= *PGRP-LF.* Extreme outliers (more than 1.5x Interquartile Range) were removed. Normalized expression for each gene was calculated by dividing the Relative Quantity of each sample by the housekeeper geometric mean. * denotes a significant difference (p<0.05).

4.5 Discussion

In this chapter, I investigated how early life social conditions can impact on the ability to tolerate a variety of stressors in young adult fruit flies. Whilst the larval social environment had some effects, these were not consistent across stressors or social conditions (see summary table in Chapter 6). High density affected cold tolerance in males, but not lifespan following bacteria injection. The oral infection assay failed to induce mortality, but flies from high larval densities had increased appetite on the infected food source. Adult presence increased desiccation and cold tolerance in females, but also decreased post-infection lifespan in females when injected with *Bacillus thuringiensis*. Stress-related *Turandot* gene expression was affected by larval density but not by adult presence, and the larval environments did not affect the expression of immune genes *persephone* or *PGRP-LF*. These results highlight that early life social conditions can have effects on these important components in adult flies, and highlights the complicated nature of these responses.

4.5.1 Stress tolerance is affected by larval social conditions in a type and sex-dependent manner

The presence of adults in the larval environment negatively affects male lifespan (Chapter 3). It is possible that different larval environments could have distinct impacts on stress responses, which could be connected to this result, although it is also possible that stressors elicit different responses (Gefen et al., 2006; Rush et al., 2007). I predicted that allocation to accessory gland growth in flies from high density and adult presence larval environments would decrease the energy available for stress responses, or, that greater stimulation of stress pathways due to the presence of conspecifics eliciting stress responses or indicating a stressful future environment, could prime these systems, resulting in greater stress tolerance at later stages. This may be expected particularly for biotic stressors, such

as infection and starvation that can be directly affected by the presence of conspecifics (Barnes and Siva-Jothy, 2000; MacDonald et al., 2006). However, no observable effect on tolerance of starvation, desiccation, cold or heat stressors was found for adult presence males compared to adult absence males, suggesting that there is no decreased investment towards responses to these stressors, despite the production of larger accessory glands or evidence for stress priming in these males. Conversely, adult presence females tolerated both desiccation and cold greater than adult absence females, although no effect was found for starvation resistance or heat shock. Perhaps, the presence of adult males represents a more stressful future environment for these females as there are costs to adult females through mating and exposure to males (Partridge and Fowler, 1990; Wigby and Chapman, 2005). It is possible that there is a priming of stress responses in preparation for these conditions. The results also suggest that there is no overall increase in stress in these groups, but the type of stressor is important. Both desiccation and cold shock are abiotic factors, and it is possible that differences in energy stores are occurring between these groups (Gefen et al., 2006). Alternatively, there may be trade-offs occurring with other factors influenced by these social conditions, such as immunity, and indeed, the adult presence females showed reduced post-infection lifespan when injected with *B. thuringiensis* compared to adult absence females. It is interesting that adult presence males did not show the same increases in stress tolerance to desiccation and cold shock that the females did, suggesting that these conditions are also having different effects on the sexes. Differences between the sexes have been found for heat shock responses in adult *D. melanogaster* from larval crowding conditions (Sørensen and Loeschcke, 2001), and in mammals, where differences in the hypothalamic-pituitary-adrenal (HPA) stress axis are generally the result of changes in gonadal hormones, but other factors are also likely to have effects (Bale and Epperson, 2015). For example, sex differences have been found in female rats that alter their behaviour in a conditioning task after early life stress exposure, a response not found in males (Fuentes et al., 2018).

Larval density only affected cold tolerance in males, with high density males showing a higher tolerance than low density males. It was critical for our study that differences between groups was the result of social experience and not food competition that could affect overall body mass, as quantity of stored energy could influence its availability for stress responses. At high density, competition between individuals could result in a deficiency in energy availability for stress responses, and interference of feeding behaviour at high density could also reduce accumulation of energy stores (Goss-Custard, 2002). The food concentration was increased to reduce potential food competition effects and I had previously checked overall body mass between social treatments and found no difference between high or low density individuals (Chapter 3). There was no effect of larval density on the tolerance of females to starvation, desiccation, heat or cold stress. Given that there was an effect of larval density on cold shock tolerance in males, this suggests a difference in this stress response between the two sexes. In *Drosophila*, the JAK-STAT pathway is involved in the stress response, regulating a number of genes, including the *Turandot* genes, with further involvement from the JNK and IMD pathways (Ekengren and Hultmark, 2001; Brun et al., 2006; Lemaitre and Hoffmann, 2007). It may be that, either the high density males may be primed for future stressful environments, or the low density males are more stressed initially. This seems possible given that there are benefits in cooperative burrowing in larval groups (Durisko et al., 2014b), which may be reduced at low density. However, as the females did not show the same increase in cold shock tolerance, this may not be a satisfactory explanation. Furthermore, I have found an increased relative expression in *Turandot A* and *C* in low density flies of both sexes, which could link to either of the preceding hypotheses.

In this study, the responses were dependent on the type of stressor used. There was no effect of larval social conditions on heat shock tolerance or starvation resistance. This may be surprising as an increased survival in heat shock assays have been found for flies reared at high larval density (Sørensen and Loeschcke, 2001; Arias et al., 2012). However, in these

studies, competition for food was not controlled for, and this may explain the differences observed between these results. A study in forest ants, *Aphaenogaster picea*, that combined heat and starvation stress, found a negative impact of starvation conditions on heat shock tolerance, but this was not observed to the same extent for those subjected to desiccation conditions (Nguyen et al., 2017). Therefore, combinations of different stressors may have specific implications. This may be particularly important in natural conditions where it is possible that more than one stressor is encountered simultaneously, for example, high heat could reduce prey availability (Nguyen et al., 2017). Furthermore, exposure to hot or cold temperatures in early life stages reduces activity in adult *D. melanogaster* (MacLean et al., 2017), and sexual status has been shown to affect starvation resistance in adult *D. melanogaster* (Rush et al., 2007). Mated females have an increased starvation resistance compared to virgin females, associated with fat stores, a reduced oxidative stress tolerance and shortened lifespan (Rush et al., 2007). Desiccation resistance, however, is linked to changes in carbohydrate and water content, and desiccation resistant fly lines also show increased developmental times (Gefen et al., 2006). Adult presence females showed an increased tolerance of desiccation than adult absence females. Whilst there was no difference in overall body mass observed between the groups, this does not exclude the possibility that various compounds are being stored differently between the groups, and could be contributing to these results. Selection for starvation resistance has been shown to correlate with resistance to other stresses, such as desiccation and cold shock (Bubliy and Loeschcke, 2005), and this link could be related to different energy stores (Chen and Walker, 1994). Alternatively, the stressors may activate stress pathways differentially (Rion and Kawecki, 2007). For example, heat-shock protein gene expression is increased following cold shock but not starvation or desiccation (Sinclair et al., 2007). Furthermore, another important effector of stress responses is the microbiome (Sudo et al., 2004), which could also potentially induce differences in these groups.

4.5.2 Stress-related gene expression and responses

In order to elucidate potential underlying mechanisms for the responses to the different stressors, I examined gene expression of two stress-associated genes. *Turandot A* and *C* are both stress-related genes previously shown to vary in response to adult social conditions (Mohorianu et al., 2017), as well as following bacterial infection (Ekengren and Hultmark, 2001). Their expression can also be influenced by the microbiome (Broderick et al., 2014). *Turandot C* is upregulated in females following conspecific male courtship song (Immonen and Ritchie, 2012), but was not shown to increase survival following a sexually transmitted fungal infection (Zhong et al., 2013). Both *Turandot A* and *C* are also upregulated following multiple or prolonged exposure to cold in females (Zhang et al., 2011), and increase in expression in selected lines of *D. simulans* under fluctuating temperature regimes (Manenti et al., 2018). Both *Turandot* genes had relatively higher expression in low density flies compared to high density ones in this study. High density males also tolerated cold shock better than low density males. Combining these results could suggest a couple of explanations. Firstly, it is possible that the low density treatment could represent a more 'stressful' condition for the larvae and, therefore, they have an increased level of stress even at 1 day post-eclosion to the high density flies (Durisko et al., 2014b). As groups of larvae exhibit cooperative burrowing behaviour (Durisko et al., 2014b), and this could be negatively impacted at low density, there may be increased stress in these individuals as a result of a decreased ability to burrow into the food. Secondly, high density could indicate a more 'stressful' environment will be met in the future, as high presence of males could represent costs to females (Partridge and Fowler, 1990) as well as increasing sperm competition for males (Bretman et al., 2016), and these individuals could prime their stress response, resulting in a lower expression of stress-related genes under standard conditions, but potentially with an increased efficiency once a stressor is encountered. Additionally, increased variance was found in gene expression in low density flies, which could be connected to

variability between these density conditions, however, there was no overall difference in variance for tolerance to the stressors investigated.

There was no effect of adult presence on the expression of the two *Turandot* genes. This is somewhat surprising given that our previous work had found an increased ability to tolerate both desiccation and cold stress in adult presence females, and it may have been predicted that these genes would show a difference in expression levels. As expression was measured under standard, or 'non-stressed' conditions, it may be that the expression of these genes in adult presence females differs only when stressed, and could indicate a greater efficiency in these individuals under 'stressful' conditions. Once again, however, the type of stressor is important, and therefore, other contributors to the stress response may be more significant (Agaisse and Perrimon, 2004).

4.5.3 Larval social environment can affect lifespan following infection

There were no clear effects of larval density on post-infection lifespan for any of the bacteria used. There was no difference in variance of post-infection lifespan between low and high density groups, except for females injected with *P. aeruginosa*. Though the reasons for this are unclear, overall the results suggest that there is not a significant effect of variability of environmental conditions between low and high larval densities in the variance of post-infection lifespan. There was, however, a significant effect of sex for post-infection lifespan of density group flies injected with *B. thuringiensis* when analysed with Generalised Linear Models. Females from these environments generally survived for longer after injection than males. This mirrors our other results which found that these females cope better with various stressors than males and tend to have longer lifespans. The increased post-infection lifespan of females has also been found in older flies injected with the same bacteria, although this was not the case in 7 day old

flies (Leech, 2017). This suggests that age is a critical factor in immune responses. Previous research has shown an increase in expression of a number of immune-related genes with age, in this case, from flies 4-7 days old to 40-43 days old, a response which can also be linked to changes in the microbiome (Broderick et al., 2014). During metamorphosis, the fly undergoes large morphological changes, and there is an increase in the expression of some immune genes prior to pupation that are related to these changes (Samakovlis et al., 1990). Thus, it is possible that the 1 day old adults used here show different responses when compared to week-old flies due to such changes in immune activity. This may also connect to the lack of mortality in the CAFÉ assay.

There was no effect of adult presence found for those injected with *P. fluorescens* or *P. aeruginosa*. Both have been shown to have lethal effects in fruit flies, and display cytotoxic effects on cultured epithelial cells (Pimenta et al., 2006). The third bacteria used, *Bacillus thuringiensis*, releases toxins which, when activated by proteases in insect guts, disrupt the epithelial membrane (Gill et al., 1992). There was a significant effect observed for adult presence females injected with *B. thuringiensis* that died quicker than adult absence females. Variation in bacterial virulence may account for the differences observed between these bacteria. Alternatively, as differential pathways are activated upon infection by distinct types of bacteria (both *Pseudomonas* species are Gram negative and *B. thuringiensis* is Gram positive), this may indicate reduced resistance specifically towards Gram positive infections in adult presence females, though the mechanisms behind this remain unclear. It may have been expected that both the high density and adult presence conditions would affect overall immune activity, either through the indication of an increased infection risk (Reeson et al., 1998) and subsequent increase, or a decrease as a result of differential resource allocation. That only adult presence females showed a response suggests that there are other important factors impacting these individuals as a result of the larval environment.

4.5.4 Larval Density can affect the appetite of young adults on an infected food source

P. fluorescens oral infection did not have an effect on fly mortality, despite impacting mortality when directly injected, and in a larval oral infection study (Olcott et al., 2010). This could suggest that the flies were able to mount an effective immune defence when the bacteria was ingested, possibly as a result of the application of smaller doses through ingestion, with this mode of infection representing a more natural route (Vodovar et al., 2005), or through increased immune activity associated with metamorphosis (Samakovlis et al., 1990). Although the flies did not die from this bacteria source, the assay provided data on appetite between the groups, albeit on an infected food source. Firstly, adult presence did not significantly affect the quantity of food eaten. However, females ate more of the food than males. This is unsurprising given the nutritional requirement for females in producing eggs (Partridge et al., 1987a). Overall, it appears that the appetite of young adult flies on an infected food source is not affected by the presence of adults during the larval period, and suggests there is no difference in the ability of these flies to detect pathogens in the food or that there is no obvious differences in nutritional requirements between these groups.

Flies reared at high larval density ate significantly more food than those reared at low density, but surprisingly there was no difference between the sexes. This suggests this difference is not due to a requirement for compensatory growth as a result of larger accessory gland production in males. Further, high density flies are not significantly different in body mass at 1 day post-eclosion, despite their larger accessory glands (Chapter 3). There may be differences in the type of energy stores that could be influencing these responses (Gefen et al., 2006). If the high density treatment represents an increased risk of infection, they could potentially prime aspects of their immune system (Hercus et al., 2003), and could increase their tolerance of an infected food source, and therefore are able to

consume more of it. Indeed, the high density flies did not show any increase in mortality as a result of this increased consumption of the infected food. Alternatively, if the low density represents a more stressful environment, there could be a decrease in the efficiency of the immune system as stress responses increase, and therefore, reducing intake would be necessary for survival. High density males also have higher cold tolerance than their low density counterparts, but this is not the case for females so there does not appear to be an obvious trade-off with stress responses either, and no differences were found for post-infection lifespan using the injection method with this bacteria. Reduced feeding has been found in flies exposed to oxidative stress-inducing compound paraquat (Ja et al., 2007). Furthermore, they have been shown to avoid toxic food sources (Stensmyr et al., 2012), and can learn to associate odours with pathogen infection (Babin et al., 2014). However, interestingly, naïve flies can show an initial attraction to the pathogen-infected food, likely attracted to factors produced by the bacteria (Babin et al., 2014). Tentatively, it seems possible then that the low density flies could have an increased ability to identify and then avoid the infected food source (Chapter 2). Such avoidance of infected food sources would be beneficial to reduce the risk of infection.

4.5.5 Immune gene expression

Following the results from the infection assay, I examined gene expression of immune-related genes as potential underlying mechanisms for the differences observed. No effect of larval density was observed for gene expression of *persephone* or *PGRP-LF*, though there was greater variance in expression of *persephone* in those from low larval densities, similar to the results found for *Turandot* genes. These genes were selected as both have been shown to vary under larval crowding conditions (Zhou et al., 2012), and to rival male presence in adults (Mohorianu et al., 2017). They are also components of each of the two main immune pathways in *D. melanogaster* (Werner et al., 2003; Lemaitre and Hoffmann, 2007). Ultimately, both of these pathways lead to the production of antimicrobial peptides (Lemaitre

and Hoffmann, 2007). The IMD pathway is activated in response to Gram negative bacterial infection (Lemaitre and Hoffmann, 2007). A group of peptidoglycan recognition proteins are involved in this response, one of which is PGRP-LF (Werner et al., 2000; Lemaitre and Hoffmann, 2007). Overexpression of *PGRP-LF* decreases antimicrobial peptide response to an immune challenge, demonstrating that it is a negative regulator of the IMD pathway (Werner et al., 2003; Persson et al., 2007), and it appears that this regulation occurs through interactions with the receptor PGRP-LC (Basbous et al., 2011). Suppression of immune responses following elimination of a pathogen could prevent damage or unnecessary energy expenditure (Persson et al., 2007), and therefore, negative regulation can be beneficial. Indeed, increasing the expression of *PGRP-LF* can lead to longer-lived flies (Paik et al., 2012), whilst its loss affects necessary apoptotic processes in some tissues during development (Tavignot et al., 2017). There was a significant difference between the sexes, with higher expression of *PGRP-LF* in males compared to females, which could suggest there is differences in activation of the pathway between the sexes, and perhaps a requirement for more dampening of this response in males (Lemaitre and Hoffmann, 2007). The situation is further complicated as the microbiome could affect immune pathways (Broderick and Lemaitre, 2012). Flies with reduced PGRP-LF show developmental defects in wing structure, and rearing these larvae on antibiotics show reductions in these developmental defects compared to those kept under normal laboratory conditions, associated with a reduced bacterial activation of the IMD pathway, despite decreased negative regulation by PGRP-LF (Maillet et al., 2008). As larval social conditions can affect the microbiome (Chapter 5), this could provide another mechanism to immune activity responses.

The other major immune pathway in *Drosophila* is the Toll pathway, which is stimulated primarily in response to fungi and Gram positive bacterial infection through activation of the Toll receptor, activated by the cleavage of the ligand Spätzle (Valanne et al., 2011). One of the routes to cleavage of Spätzle is mediated by the Persephone protease after pathogen recognition

(Ligoxygakis et al., 2002; Chamy et al., 2008). In this study, the expression of *persephone* was again relatively lower in females than in males. Interestingly, another study found that females infected with the Gram negative bacteria, *Providencia rettgeri*, survived less well than males, a difference associated with infection recognition via Persephone, as mutants for this gene removed any differences in resistance between the sexes (Duneau et al., 2017). However, in this experiment, males infected with *B. thuringiensis* reared under density treatments died significantly quicker than females, a result mirrored for adult absence and presence males compared with adult absence females (but not presence females), with no significant difference between sexes for the *Pseudomonas* species. Thus, the increased expression of *persephone* observed in males did not result in increased infection resistance in males, although the *persephone* expression was measured in uninfected flies, and may not reflect the ability to alter the magnitude of the post-infection response. One possibility for the observed increased expression of this gene in males could be as a result of males being more likely to encounter high intensity aggressive behaviour (Vrontou et al., 2006), which could result in wounding and increased infection risk. It may therefore be beneficial for them to increase immune activity in preparation for this. However, resistance to infection is likely to be dependent on the type of bacteria (Duneau et al., 2017), and further downstream responses of the host (Tzou et al., 2002), particularly as increased *persephone* expression may only increase ability to recognise and activate Spätzle cleavage, and therefore, may not indicate an actual increase in ability to fight infection.

As the presence of conspecifics could indicate an increased risk of infection, and if individuals were priming themselves for such an eventuality, it may have been expected that there would be a downregulation in high density and adult presence groups of *PGRP-LF*, and upregulation of *persephone*. Both of these genes have been shown to be responsive to adult social and larval crowding conditions (Zhou et al., 2012; Mohorianu et al., 2017), however, neither changed their expression in response to the

larval social conditions used here. This result echoes the post-infection lifespan results, where no significant differences were found across all bacteria for larval density, and post-*Pseudomonas* infection for adult presence groups. Furthermore, adult presence females did worse than adult absence females when injected with the Gram positive bacteria, *B. thuringiensis*. Whilst it is possible that these individuals have a decreased ability in particular aspects of immune activity (for example, in some factor involved in the Toll pathway), it is also possible that variability in responses could arise through differences in bacterial virulence. As the gene expression levels were measured in what could be described as a 'standard' or non-infected state, it is difficult to establish if there are differences in the functioning of this pathway in these females, for example, in their ability to change the magnitude of response following infection. It could be that, once activated, there is a decreased activity or function in the Toll pathway, but as there are a number of routes to Spätzle cleavage, this could prove difficult to pinpoint (Lemaitre and Hoffmann, 2007). Additionally, other factors could also be affecting this response, such as bacterial recognition or host antimicrobial peptide activity. Mating has also been shown to reduce female ability to fight infection (Short et al., 2012), but as the flies used in this study were 1 day old virgins, there is no effect of mating status in these individuals. Furthermore, it is also possible that trade-offs occur between other factors involved in immune and stress responses (Davies et al., 2012).

In this study, I investigated how larval social conditions can affect stress tolerance in young adult *D. melanogaster*. The responses were specific to the type of stressor, and sex of the fly. I had expected that either through trade-offs with increased accessory gland growth or other factor, or through priming of these responses, that high density and adult presence males would show differences in stress tolerance compared to the low density and adult absence counterparts. Adult presence females tolerated both desiccation and cold tolerance better than adult absence females, and when challenged with *B. thuringiensis* infection, the adult presence females died quicker than adult absence females. This could indicate a stress and

immunity trade-off in these females, however, there was no evidence for a trade-off in males, though high density males tolerated cold shock better than those from low density conditions. These differences may be connected to alterations in energy stores or particular aspects of stress pathways. Furthermore, expression of *Turandot A* and *C* were increased in low density flies of both sexes, suggesting density conditions could be differentially stressful. Overall, the results indicate that the larval social environment could have distinct effects on adult fitness. Both stress and immunity are intricately linked, and these results suggest that there a complex network of factors that influence the response to early life social conditions in fruit flies.

Chapter 5

Effect of Early Life Social Conditions on the Microbiome Composition of Fruit Flies, *Drosophila melanogaster*

Thanks to Xavier Harrison for help with data analysis; the Bretman lab for helping with sample collection, and Kevin Hopkins for the processing of samples post-extraction.

5.1 Summary

The complex interactions between animals and the bacterial communities associated with them can have large effects on host health and fitness. These microbiomes largely persist in the host's gut but also occur in other regions of the body. Investigations of the environmental conditions that determine microbiome composition have largely focussed on diet. The presence of conspecifics is another factor that could affect the microbial community structure, typically through interactions between individuals facilitating horizontal transfer. However, differences in stress or immune responses elicited by conspecifics could also influence its composition. Such effects could occur across the life cycle but early life conditions might have particularly profound effects, with substantial health effects associated with these perturbations at later ages. In this chapter, I used the fruit fly *Drosophila melanogaster* as a model to examine the effect of early life social conditions on the whole fly microbiome composition using 16S rRNA sequencing. These conditions could also affect individuals at the adult stage, by influencing traits such as resource allocation, stress and immunity, with potential effects on the microbiome from these changes. Larvae were reared at high or low density, or with or without adult males in the environment. High density and adult presence larval social environments are

known to produce an anticipatory response to future sperm competition in males at the adult stage. It was expected that these conditions could influence microbiome composition, either through horizontal transfer between individuals or through changes in stress or immunity. I found that the pupal stage of larvae reared in the presence of adult males had greater diversity in microbiome composition compared to those reared without adults. No effect was found for those reared at different larval densities or for 1 day old adults. Thus, it appears that the form of social environment is important in shaping the microbiome during early life, but the stage of the life cycle when these conditions are met is also important.

5.2 Introduction

The microbial community of animal hosts, known collectively as the microbiome, has been demonstrated to affect a wide range of factors from the processing of food, to immunity and health (Kau et al., 2011; Foster and McVey Neufeld, 2013; Cho and Blaser, 2012), including being implicated in a number of disorders such as autism and Parkinson's disease in humans (Dinan and Cryan, 2017). Interactions between the host and microbiome are complex, and microbiome composition can vary greatly between hosts (Adair and Douglas, 2017). A number of mechanisms appear to contribute to this variation. Diet has been shown to be an important factor in many species (Chandler et al., 2011; Claesson et al., 2012; O'Sullivan et al., 2013). Furthermore, immune activity can also be important, for example loss of immune pathways can lead to changes in the gut microbiome of mice (Kubinak et al., 2015). Another factor is the host's social environment. Changes in social conditions may alter the transmission of bacteria from one host to the other via horizontal transfer. Social interactions have been shown to affect the composition of bacterial communities in primates, for example, grooming interactions are thought to directly transfer bacteria between social group members in wild baboons, *Papio cynocephalus* (Tung et al., 2015),

and in chimpanzees (Moeller et al., 2016). The microbiota composition in ring-tailed lemurs, *Lemur catta*, similarly appears to be affected by interactions within social groups (Bennett et al., 2016). However, as host immune activity can affect microbiome composition (Kubinak et al., 2015) and immunity can be altered in response to social conditions (Leech, 2017), this could be a second route through which social contact affects microbial communities.

The term ‘gut-brain axis’ is used to refer to the bidirectional interactions that occur between the gut and brain that can influence a number of factors from behaviour to the functioning of the gastrointestinal system (Foster et al., 2017). More recently, this term has been expanded to include the microbiome, known as the ‘microbiota-gut-brain axis’, in acknowledgement of its influence on the host, and *vice versa* (Cryan and O’Mahony, 2011; Carabotti et al., 2015). Evidence of such connections have been identified using germ-free mice to examine the neuroendocrine system known as the hypothalamic-pituitary-adrenal (HPA) axis that is linked to stress responses (Sudo et al., 2004). Germ-free mice show higher levels of corticosterone hormone and adrenocorticotropic hormone in response to restraint stress compared to mice with a pathogen-free but otherwise full microbiome (Sudo et al., 2004). This elevated stress-induced response can be reversed by the addition of *Bifidobacterium infantis* (Sudo et al., 2004). Furthermore, reconstitution of germ-free mice with a complete microbiota can partly restore the stress response but only at a young age, suggesting that this effect of microbiota on the HPA axis is limited to early life (Sudo et al., 2004). Changes in the microbiota of older humans have been linked to measures of immunosenescence and health (Claesson et al., 2012), and exposure to social stress for as little as 2 hours can result in alterations of the intestinal microbiota of mice, *Mus musculus* (Galley et al., 2014). Additionally, rhesus monkey infants have a decrease in faecal *Lactobacilli* bacteria after maternal separation, which is accompanied by an increase in stress-associated behaviours, and a greater susceptibility to pathogen infection (Bailey and Coe, 1999). Likewise, the microbiome of the bumblebee, *Bombus terrestris*, can influence protection against certain

parasites, likely through direct interactions between the microbiome and the parasites (Koch and Schmid-Hempel, 2011), and tree frogs, *Osteopilus septentrionalis*, that have their microbiome disrupted through the use of antibiotics early in life, have an increased susceptibility to worm infections as adults (Knutie et al., 2017). Thus, the interactions between host and microbiome are complex, and could have long lasting effects across life stages. Such potential consequences have even been suggested for human babies (Arrieta et al., 2014), which, when delivered by Caesarean section, show a bacterial composition similar to adult skin, compared to those born naturally, which is dominated by species common to the mother's vaginal microbiome (Dominguez-Bello et al., 2010). These changes can be detectable in the infant even after 6 months (Grölund et al., 1999), and have been linked to increased risk of obesity and other health issues later in life for those delivered by Caesarean section (Arrieta et al., 2014). In particular, obesity appears to be linked to an increased ability for energy acquisition in mice connected to changes in the relative abundance of Firmicutes and Bacteroidetes bacteria (Turnbaugh et al., 2006).

The microbiome has already been shown to affect a suite of responses in *Drosophila melanogaster*, including modulating food choice behaviour in adults (Leitão-Gonçalves et al., 2017), and in larvae (Wong et al., 2017). In both laboratory and wild populations, microbiome communities are largely shaped by host diet (Chandler et al., 2011). Eggs collect bacteria from the substrate onto which they are laid, and from the female's faecal matter (Wong et al., 2015). The bacterial species richness in the gut increase as the larvae begin feeding (Bakula, 1969; Wong et al., 2011). Pupae undergo large-scale morphological reorganisation, and bacterial numbers decrease during this time, associated with a lack of feeding and an increase in immune response components, such as antimicrobial peptides (Bakula, 1969; Wong et al., 2011; Broderick, 2016). The microbiome of *D. melanogaster* is important in affecting the growth rate of larvae, which is increased in bacteria-associated flies compared to axenic individuals, and appears to be associated with microbiome-mediated changes in carbohydrate allocation (Bakula, 1969; Ridley et al., 2012). There is also

evidence that the microbiome can affect larval food choice, with larvae preferring food inoculated with the specific bacteria they have been seeded with as eggs (Wong et al., 2017). In addition, the presence of *Lactobacillus plantarum*, a bacteria common to the *D. melanogaster* microbiome, can reduce the mortality of germ-free flies when challenged with specific pathogenic bacteria (Blum et al., 2013). The microbiota also affects gut morphology through altering the epithelial renewal rate (Broderick et al., 2014). Such changes have been associated with alterations of immune gene activity and increased bacterial numbers in ageing flies (Broderick et al., 2014).

The social environment of fruit flies is known to affect a number of behavioural and physiological factors, including cognitive processing (Rouse, 2016), male reproductive morphology (Bretman et al., 2016), and gene expression changes (Mohorianu et al., 2017). There are also significant impacts of the social environment on the immune function of adult flies, with male flies that are kept in pairs showing a significant decrease in lifespan following wounding compared to those kept in isolation (Leech et al., 2017). Furthermore, the social environment has been shown to affect the microbiome composition in older adult flies (Leech, 2017). These results suggest that the social environment is important in immune activity and microbiome composition in adult fruit flies, and these factors could be linked. Here, I examine changes in the microbiome composition of pupae and 1 day old adult *Drosophila melanogaster* reared under different larval social environments by manipulating larval density or by the presence of adult males in larval culture vials. Both larval conditions have been shown to affect the size of accessory glands in adult males, an anticipatory response to future reproductive competition (Bretman et al., 2016). Microbial community composition could be affected by these social conditions through direct transfer. In this context, it may be predicted that those from different larval densities would display similar compositions as they are reared under the same standard food conditions, and that those reared in the presence of adult males would potentially have a decreased microbiome diversity similar to that found in adult flies (Wong et al., 2011). However, high larval densities

and the presence of adult males can also indicate an increased risk of infection (Reeson et al., 1998), and may require an increase in immune activity (Barnes and Siva-Jothy, 2000), that could indirectly impact on microbiome composition (Kubinak et al., 2015). They could also produce an increased stress response, which could similarly affect the microbiome (Bailey and Coe, 1999). I have previously found that larval social conditions can differentially impact on lifespan and tolerance of certain stressful conditions in young adults (Chapter 4), and that this can be sex-dependent. It is also possible that the increased investment towards larger accessory gland growth impacts on the energy availability of these responses. Therefore, alterations in immune activity or stress could impact on the microbiome composition during early life stages. If these conditions result in increases in immune activity or stress responses, this could potentially result in an increased microbial diversity such as those observed under conditions of immune dysregulation in ageing flies (Clark et al., 2015). Furthermore, as some bacteria can be held in the larval midgut through metamorphosis, it is possible that changes during larval stages could persist into adulthood (Broderick and Lemaitre, 2012). I predicted that high density or adult presence conditions would affect the microbiome composition either through direct transfer from other individuals or indirectly via alterations in immune or stress responses. I used 16S rRNA sequencing to investigate how the interactions between conspecifics during these early periods can impact on microbial community structure to improve our understanding of the complex connections occurring between conspecific individuals and their microbiome during early life stages.

5.3 Methods

5.3.1 Fly Husbandry

All flies used were wild type *Drosophila melanogaster* of the Dahomey strain, reared at 25ºC in a humidified room, under a 12:12 light dark cycle on a

standard sugar-yeast diet. Adults were provided with purple grape juice agar plates to lay eggs, from which larvae were harvested approximately 24 hours later, and transferred to plastic vials, containing 7ml of fresh food medium and supplemented with a live yeast paste. All adults were collected and sexed using ice anaesthesia.

5.3.2 Larval Social Treatments

Larval density treatments consisted of high (200) or low (20) numbers of larvae per vial reared on a concentrated medium to prevent food becoming a limiting factor for the high density group (Bretman et al., 2016) (Appendix A.1). Adult presence and adult absence groups were collected at a density of 100 larvae per vial. The adult absence group remained under these conditions. The adult presence group had 20 adult males aspirated into the vial. These adults had previously been reared at a standard density of 100 larvae per vial, and were collected the day of eclosion, anaesthetized on ice and sexed. The adult males were left for approximately 5 days before addition to the adult presence vials. They were removed from the vials the day before pupae were due to eclose.

5.3.3 Sample Collection

Pupae were collected the day before eclosion, and sexed by the presence of sex combs on the legs of males. Adults were collected from separate vials within 8 hours of eclosion, anaesthetized on iced and sexed, before being transferred singly to a vial containing fresh food and left for approximately 24 hours before freezing at -80°C. Each individual fly or pupa originated from a separate larval vial to reduce pseudoreplication. Eight individuals of a single sex were then pooled per sample and this was replicated 10 times per treatment group. All samples were frozen and stored at -80°C prior to DNA extraction.

5.3.4 DNA Extraction

Pooled pupae and adult fly samples were homogenized using a hand pestle, ensuring both extra- and intracellular bacterial DNA was extracted. Following this, the Mobio PowerSoil DNA Isolation Kit was used as per the manufacturer's protocol (PowerSoil ®, Mo Bio Laboratories Inc., California, United States). The concentration of DNA was quantified using NanoDrop (Thermo Fisher, Massachusetts, United States). Samples were then transported in dry ice to the Zoological Society of London for sequencing.

5.3.5 16S rRNA Sequencing

PCR amplification was carried out for the V4 region of the bacterial 16S rRNA gene using indexed primer sets to give each sample a unique combination of barcodes (Appendix A.2). Three replicates per sample were carried out using 5x HOT FIREPol® Blend Master Mix, with 2µl Master Mix and 4µl PCR-grade water per reaction. 3µl of the 2µM primers added to each well, and 1µl of gDNA. The PCR cycling conditions were as follows: 95°C for 15 mins, 95°C for 20s, 50°C for 1 min x 28 cycles, 72°C for 1 min, 72°C for 10 mins and held at 4°C. PCR products were checked on a 2% agarose gel stained with Gel Red. Replicates that failed this check were removed. Replicates were pooled and samples were cleaned using Ampure XP beads and ethanol. The DNA was assessed using Qubit for concentration determination, and TapeStation to check for primer dimers.

16S rRNA sequencing using paired end 250bp v2 chemistry was carried out using the Illumina MiSeq system, following the standard protocol (Illumina, Inc., California, United States). Forward and reverse sequences were aligned against the SILVA SEED database (v. 123, SILVA database, https://www.arb-silva.de/) using the mothur programme (mothur v1.38.1, https://mothur.org/). Any reads with ambiguous bases were removed, as

were chimeric and non-bacterial sequences. Taxonomic assignment of Operational Taxonomic Units (OTUs) was also carried out using the SILVA database.

5.3.6 Statistical Analysis

Analysis was carried out in R (v.3.3.3) using phyloseq (McMurdie and Holmes, 2013), vegan (Oksanen et al., 2017), ggplot2 (Wickham, 2009) and DESeq 2 (Love et al., 2014) packages. Bacteria that appeared in the negative controls, likely as a result of contamination by the Taq polymerase used, were removed from the dataset (Iulia et al., 2013). The data was loaded into R as a .biom file, and sequences were rarefied to standardise the library sizes. For larval density groups, the data was rarefied to 20,140 sequences, and for adult presence groups to 22,718 sequences. Alpha diversity, or species richness within samples, was measured using the Chao1 index, which estimates alpha diversity by taking low abundance OTUs, including those sequences that occur only once (singletons) into account, and is suitable for low count data as in these samples (Chao and Shen, 2003). Species richness was then compared by Generalised Linear Models using the lme4 (Bates et al., 2015) and lmerTest packages (Kuznetsova et al., 2016). Sex, treatment and age were included as fixed factors, and models were tested using Analysis of Deviance. Comparisons between samples (beta diversity) were plotted using the Bray-Curtis dissimilarity index which takes both presence and abundance of OTUs into account (Bray and Curtis, 1957), and using PERMANOVA (permutational analysis of variance) with the vegan adonis function, with 1000 permutations. Significant fold changes in abundance of OTUs between groups were identified using DESeq2. These sequences were then blasted against the Greengenes database to identify to species level, where possible (DeSantis et al., 2006).

5.4 Results

5.4.1 Effect of Larval Density on alpha diversity

There was no significant effect of larval density on the alpha diversity within samples using the Chao1 index (F=0.514, df =1, 76; p=0.475). There was also no effect of sex (F=2.696, df =1, 75; p=0.105). However, there was a significant effect of life stage (F=35.37, df =1, 77; p=<0.001), with pupae generally displaying a greater species richness than their 1 day old adult counterparts (Figure 5.4.1-1).

One outlier replicate was identified with an extreme species richness value compared to all other samples. This sample was of low density female pupae collected in week 3. It is unknown why this group was so distinct from all others, but possibly may have been contaminated at some stage of the process. It was removed from all analysis. The most prevalent OTU in all samples was *Wolbachia* sp, which appeared in all samples, with *Lactobacillus* (50 out of 79 samples) and *Staphylococcus* sp. (42 out of 79 samples) the next two most prevalent bacteria.

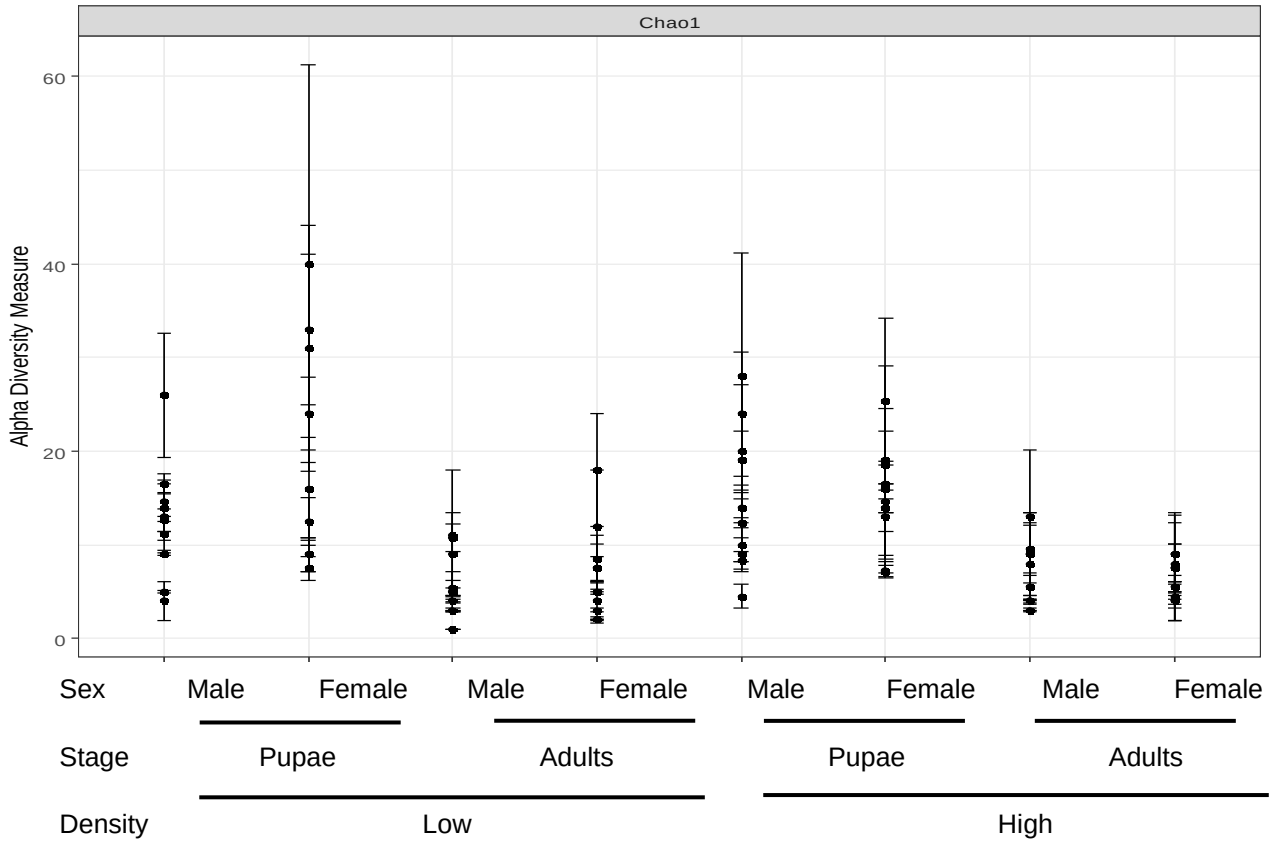


Figure 5.4.1-1 Alpha Diversity using the Chao1 Values for microbiome composition under different larval density treatments. Larvae were reared at high (200 larvae) or low (20 larvae) density; collected and sexed at pupal or 1 day old adult stages. Error bars represent standard error.

5.4.2 Effect of Larval Density on beta diversity

No effect of density was observed for beta diversity, with the NMDS plot displaying clustering between the pupal and 1 day old adult stages, but with no distinction between high and low density (Figure 5.4.2-1), indicating changes in diversity between the life stages but not density treatments. This corresponds to the PERMANOVA results, with a significant effect of life stage only ($F=4.52$, df =1, 78; $p=<0.001$). There was no effect of sex ($F=0.759$, df =1, 78; $p=0.756$) or treatment ($F=1.277$, df=1, 78; $p=0.154$).

From the DESeq2 analysis, both density groups had increased *Staphylococcus* sp., *Lactococcus* subsp. *lactis* and *Lactobacillus* sp. in the pupae compared to the adults (Table 5.4.2-1). Additionally, high density pupae also showed a decrease in *L. brevis* compared to the high density adults, which was not observed in the low density group. However, this difference did not have a significant effect on microbiome composition between low and high density groups.

Table 5.4.2-1 DESeq2 log2 fold change comparison of life stage for bacteria within density treatments. Comparing within treatments at both life stages. Positive values for log2 fold change indicate increases in pupae compared to adults. Where possible, bacteria were identified to species level using the Greengenes database. P-values are corrected for multiple testing using the Benjamini-Hochberg correction.

Groups	Bacteria	Log2 fold change	Adj. p-value
High Density Pupae versus High Density Adults	*Staphylococcus* sp.	3.462	<0.001
	Lactobacillus brevis	-3.567	<0.001
	Lactococcus subsp. *lactis*	4.773	<0.001
	Lactobacillus sp.	3.217	<0.001
Low Density Pupae versus Low Density Adults	*Staphylococcus* sp.	3.574	<0.001
	Lactococcus subsp. *lactis*	4.244	<0.001
	Lactobacillus sp.	4.320	<0.001

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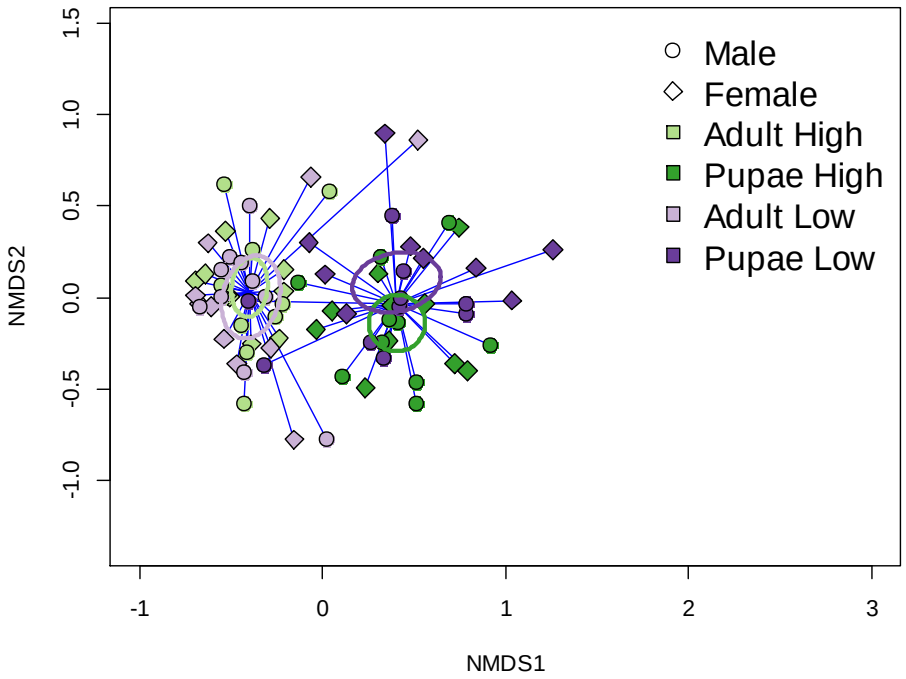

Figure 5.4.2-1 Beta Diversity NMDS Plot using Bray-Curtis Dissimilarity Index for pupae and 1 day old adults for larvae reared at high or low density. Larvae were reared with 200 per vial (high density) or 20 per vial (low density), collected and sexed as pupae (dark colours) or 1 day old adults (light colours). Ellipses represent 95% Confidence Intervals. Clustering occurs between pupae and adult stages but not between densities.

5.4.3 Effect of Adult Presence on alpha diversity

There was a marginally non-significant interaction between age and social treatment for alpha diversity in adult presence and absence groups ($F=3.677$; $df= 1, 75$; $p=0.059$). As with larval density, there was no effect of sex on the alpha diversity of the microbiome in adult absence and presence groups ($F=0.007$, $df= 1, 77$; $p=0.933$). However, significant effects of both adult presence ($F=4.648$, $df =1, 77$; $p=0.0342$) and life stage ($F=31.39$, $df=1, 78$; $p<0.001$) were observed. Generally, greater species richness was observed in pupae compared to adults and in adult presence compared to adult absence individuals (Figure 5.4.3-1). As observed with the density groups, the most prevalent OTU in all adult presence group samples was *Wolbachia* sp. (in all 80 samples). *Lactobacillus plantarum* (49 out of 80 samples) and *Lactobacillus brevis* (46 out of 80 samples) were the next two most prevalent OTUs in these groups.

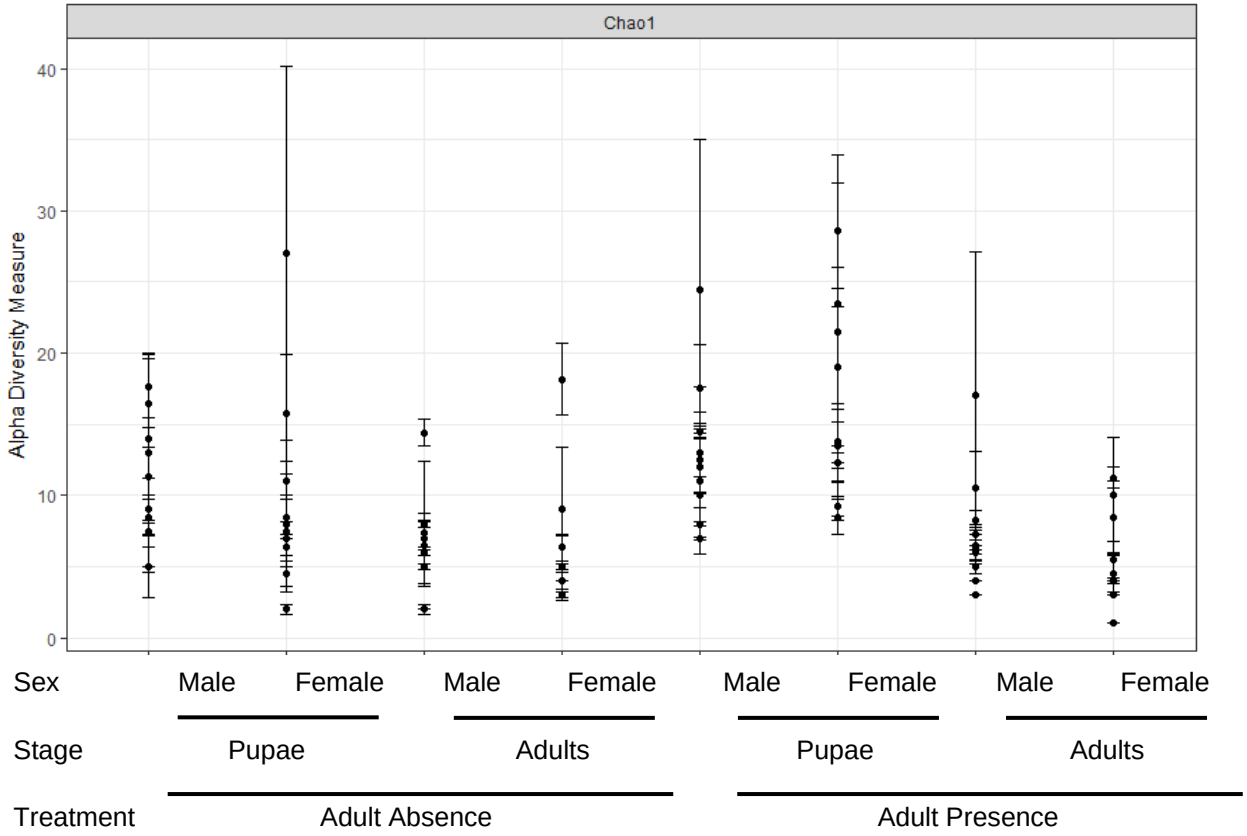

Figure 5.4.3-1 Alpha Diversity using the Chao1 Values for microbiome composition for larvae reared with or without adult males present. Larvae were reared in the presence or absence of adult males; collected and sexed as pupae or 1 day old adults. Error bars represent standard error.

5.4.4 Effect of Adult Presence on beta diversity

Significant differences between pupal and 1 day old adult stages were observed for adult presence and absence samples for beta diversity (Figure 5.4.4-1). Figure 5.4.4-1 displays a separation of adult presence and absence samples occurring for the pupae, but no distinction occurs between these treatments at the adult stage. This is supported by the PERMANOVA analysis that found a significant interaction between age and social treatment ($F=7.20$, df =1, 79; $p<0.001$). There was no effect of sex ($F=0.193$, df= 1, 79; $p=0.883$).

From the DESeq2 analysis, adult presence pupae had greater changes in *Lactobacillus plantarum*, *L. brevis* and *Corynebacterium* sp. compared to adult absence pupae; however, no significant differences were observed between the 1 day old adults (Table 5.4.4-1). Within larval treatments, both adult presence and absence pupae showed increased fold changes of *Lactococcus* subsp. *lactis* and *Lactobacillus* sp. compared to their corresponding adults. However, adult absence pupae also had reduced *L. brevis* compared to absence adults. This was actually increased in adult presence pupae compared to adult presence adults. In addition, these pupae also had an increase in *Corynebacterium* sp. compared to the 1 day old adults. Overall, more bacteria changed between the pupae and adults in the adult presence group.

Table 5.4.4-1 DESeq2 log2 fold change comparison of adult presence and absence groups. Comparing within treatment at different life stages and between treatments at both life stages. Where possible, bacteria were identified down to species level by blasting the sequence against the Greengenes database. P-values are corrected for multiple testing using the Benjamini-Hochberg correction.

Groups	Bacteria	Log2 fold change	Adj. p-value
Absence Pupae versus Absence Adults	*Lactobacillus brevis*	-3.503	0.021
	Lactococcus subsp. *lactis*	4.058	<0.001
	Lactobacillus sp.	3.495	0.003
Presence Pupae versus Presence Adults	*Lactobacillus plantarum*	6.241	<0.001
	Lactobacillus brevis	5.654	<0.001
	Lactococcus subsp. *lactis*	4.176	<0.001
	Lactobacillus sp.	3.914	<0.001
	Corynebacterium sp.	3.655	<0.001
Presence Pupae versus Absence Pupae	*Lactobacillus plantarum*	5.565	<0.001
	Lactobacillus brevis	10.681	<0.001
	Corynebacterium sp.	3.386	<0.001
Presence Adults versus Absence Adults	-	-	-

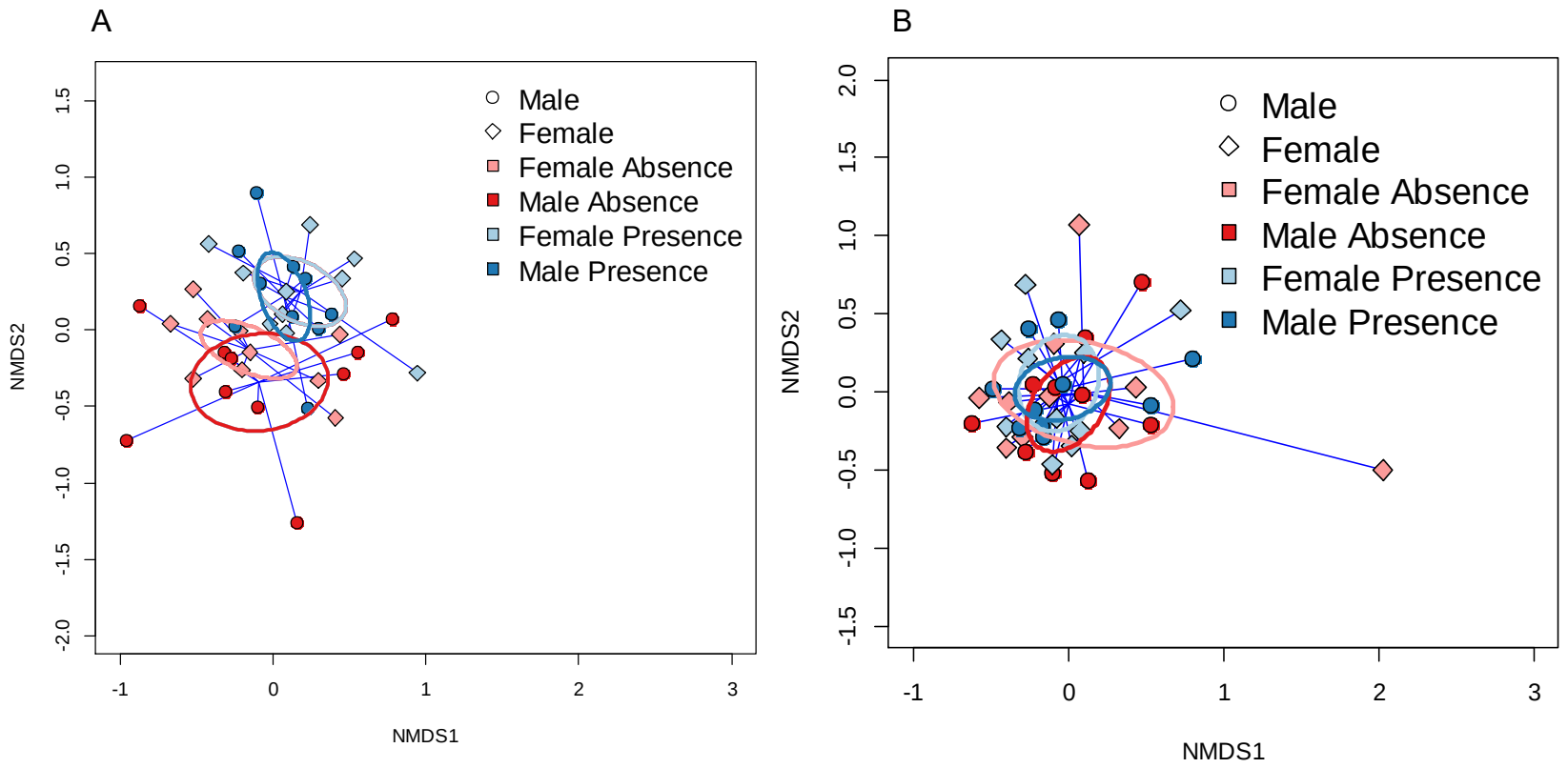

Figure 5.4.4-1 Beta Diversity NMDS Plot using Bray-Curtis Dissimilarity Index for pupae and 1 day old adults for larvae reared with or without adult males present. Larvae were reared in the presence of adult males or absence of adult males; collected and sexed at pupal (A) or 1 day old adult (B) stages. Ellipses represent 95% Confidence Intervals. Clustering between adult presence and absence treatments are visible during the pupal stage, but not for 1 day old adults.

5.5 Discussion

In this chapter, I demonstrate that early life social conditions can affect the microbiome of *Drosophila melanogaster*. These findings were, however, specific to the type of social conditions encountered by the developing flies, and there were no sex-specific effects. Broadly, pupae from both social conditions had more diverse microbiomes, and likewise those which were raised in the presence of adults. I had expected that either through horizontal transfer or changes in stress and immune activity as a result of the different social conditions, that the microbiome composition would be affected. Our previous results suggest that these social conditions may cause disparate changes in stress responses and immune activity that are specific to the type of stressor, type of social conditions and the sex of the individual (Chapter 4), which could differentially influence changes in the microbiome. No effect was found between those reared at high or low density. This suggests that there are no changes in immune or stress responses between these groups affecting the microbiome composition. However, adult presence pupae had significantly increased microbial diversity to those reared without adult males present. It may be predicted that direct transfer from adults would result in a decreased diversity, similar to that found in adults. As such, there may be indirect factors influencing this result, however, direct transfer could also be having an effect. As the microbiome can have large impacts on the host (Ridley et al., 2012; Broderick et al., 2014), these alterations in the microbial community could have important influences on later life stages.

5.5.1 The larval social environment can affect the microbiome of *Drosophila melanogaster*

Manipulating the larval rearing density, to alter social contact within an age cohort, had no significant effect on microbiome composition. As larvae gain advantages through an enhanced capability to burrow into food when in groups (Durisko et al., 2014b), it is possible that, as long as there is no competition for food or other negative factors, there is little or no change in stress responses or altered immune-related activity at high density as might have been expected. This also links to previous results whereby no significant effects of larval density were found on lifespan, desiccation tolerance or post-infection lifespan of young adult flies (Chapters 3 and 4). However, high density males have improved cold shock tolerance, and there are differences in the expression of stress-related *Turandot* genes between larval densities, which may suggest some changes in stress in response to these larval conditions. Despite these differences, however, it seems that these are not indirectly influencing the microbiome of individuals from different larval densities.

However, altering the social conditions via the presence of adult males did elicit changes in the microbiome. Adults from adult presence and absence groups had no OTUs that were significantly different between them. Intriguingly however, the adult presence pupae had increased *L. plantarum*, *L.brevis* and *Corynebacterium* sp. compared to the absence pupae, demonstrating that the adult males present during the larval stages affected pupal microbiome composition. These results also indicate that this effect did not carry over into the microbiome composition of 1 day old adults. This is perhaps unsurprising given that pupae undergo large modifications during metamorphosis before eclosion (Robertson, 1936), and the number of OTUs decreases (Wong et al., 2011). Correspondingly, there is an increase in expression of antimicrobial peptide genes, which may help to regulate bacteria at this point (Tryselius et al., 1992; Broderick and Lemaitre, 2012).

Bacteria can, however, be maintained in part of the larval midgut during this period and be transmitted to the adult stage following metamorphosis (Broderick and Lemaitre, 2012).

The observed microbiome changes could be affected by male adult presence in a couple of ways, namely by direct horizontal transfer or indirectly via changes in stress and immune activity (Tung et al., 2015; Broderick and Lemaitre, 2012). As the adult flies were free to move around the vial, and therefore able to contact the substrate and deposit faecal matter, it is possible that horizontal transfer could occur from the adults to pupae. Potentially, this could also occur in the opposite direction from pupae to adults. Our laboratory has shown that young adult flies kept singly or in groups until 11 days old do not have significantly different microbiomes from each other (Leech, 2017), and we might expect that the 5 day old adults used in the adult presence treatment would have a similar composition to these individuals, however as these adult males were not included as samples, this unfortunately cannot be determined. Transfer of bacteria in the direction of pupae to adults seems less likely, although not impossible, given that the larvae spend most of this period burrowed into the food (Durisko et al., 2014b). Notably, species richness was greater in the pupae compared to adults, regardless of whether adults were present. If adults were directly transferring bacteria to the pupae, it may be predicted that the pupal microbiome would then resemble the adults', with a reduced richness than was actually observed. As this does not appear to be the case, it is possible that there are other factors influencing this change.

The microbiome changes in adult presence pupae could perhaps suggest that this form of social interaction occurring between life stages results in a distinct immune response that is not found under the larval density conditions. Speculatively, the presence of adults could indicate an increased risk of infection (Perkins et al., 2009), with the potential for exposure to different pathogens which could be spread by adults upon

interacting with the younger individuals. Increasing immune activity under these conditions would be beneficial. Such density-dependent prophylactic responses have been observed in mealworms, *Tenebrio molitor* (Barnes and Siva-Jothy, 2000). Unfortunately, an obvious immune response that would provide evidence for this hypothesis has not been detected, however the different larval conditions could also be differentially stressful, and this may be suggested by previous results on stress tolerance (Chapter 4). Yet, these stress tolerance responses were also sex-specific, and as the microbiome changes were not, this does not offer a complete explanation for these results either. Alternatively, the larvae could be responding to the future reproductive environment in another discrete way from the larval density groups. Adult males show a reduced lifespan if they have been exposed to adult males during the larval period, a result not seen in the larval density flies (Chapter 3). This suggests that, despite both adult-exposed and high larval density males showing an increase in size of accessory glands, it may be that there are other physiological changes affecting these flies and this could be linked to the changes observed in the microbiome. Therefore, although different manipulations of the social environment produce equivalent phenotypes, it may be that this is achieved via different routes, an idea that requires further investigation. Examining changes in specific energy stores, for example, carbohydrate or lipid reserves, between these groups may offer some insight into potential differences in this regard. Although, this would require that both larval density and adult presence groups are raised on the same food source. Changes in gene expression of signalling pathways may offer another potential mechanism for such changes that could also be examined (DiAngelo et al., 2009).

5.5.2 Microbiome composition changes between pupae and 1 day old adults

Greater species richness was found in pupal samples compared to young adults, which is in line with the microbiota results observed by Wong et al.

(2011). Wong et al. (2011) found that the increase in richness during larval stages corresponded with feeding larvae, and the morphological changes and increased immune activity associated with metamorphosis corresponded to the subsequent reduction. Greater microbial diversity has also been found in infant chimpanzees, *Pan troglodytes schweinfurthii*, compared to adults (Degnan et al., 2012), which may be related to diet, interactions or immune activity. Differences between pupal and adult stages in larval density groups were driven by increased fold changes in *Lactococcus* subsp. *lactis*, *Lactobacillus* and *Staphylococcus* spp., whilst pupae of adult presence and absence groups had increased fold changes in *Lactococcus* subsp. *lactis*, and *Lactobacillus* sp. compared to adults. In the adult presence pupae, *L. plantarum*, *L.brevis* and *Corynebacterium* sp. were also increased in the pupae compared to adult presence adults, whilst adult absence pupae showed a decrease in *L. brevis* compared to their adults. Interestingly, the high density pupae also had a reduced fold change in *Lactobacillus brevis* compared to the high density adults, a change not observed in the low density treatment. This did not, however, affect the overall result, with larval density having no significant effect on the microbiome at either life stage.

5.5.3 Microbiome composition was not affected by sex of pupae or 1 day old adults

There were no observable differences between the sexes for any of the treatment groups. As larval conditions have been found to influence adult *D. melanogaster* reproductive traits in both sexes (Roper et al., 1996; Edward and Chapman, 2012; Bretman et al., 2016), and social conditions could be differentially stressful for the sexes (Partridge and Fowler, 1990; Leech et al., 2017), this could draw predictions that there would be some differences between males and females in stress or immunity. Changes in these factors could then affect the microbiome (Bailey and Coe, 1999; Kubinak et al., 2015). I have also found differences in stress tolerance between the sexes in young adults from these larval conditions (Chapter 4). There are also sex-

dependent effects of the social environment on adult fruit fly immunity, whereby paired adult males are more negatively affected by wounding than isolated males, an effect that is less severe in females (Leech et al., 2017). Social conditions have been shown to affect microbiome composition in older adults, with males in grouped conditions and isolated females most distinct from each other, and isolated males and grouped females having more similar microbiome compositions (Leech, 2017). This could suggest that group conditions may impose a greater stress on male flies, which could affect the microbiome. During later stages, when an individual is sexually mature, pressures elicited by specific social conditions, namely those of reproductive competition, could be enhanced in comparison to early life stages, resulting in physiological changes to immune activity and stress, which may be sex-specific, and this could partly account for the lack of sex effects observed at these young stages.

5.5.4 Specific bacterial changes between larval social conditions and possible functional effects

Examining the species that changed in these groups can reveal possible mechanisms by which the host and microbiome interacts. *Lactobacillus plantarum* is one of the bacterial species that was increased in the adult presence pupae compared to the absence pupae. *L. plantarum* is a common symbiont in laboratory strains of *D. melanogaster* (Wong et al., 2011; Wong et al., 2013). Under nutrient poor conditions, *L. plantarum* can increase larval growth through the nutrient signalling pathway which involves the promotion of the Ecdysone hormone in the prothoracic gland through TOR kinase activity, and via InR (insulin-like receptor) signalling in the fat body (Storelli et al., 2011). Axenic flies show increased development time and glucose content compared to flies with unmanipulated microbiomes, which can be restored in flies with mono-associations of bacteria such as *Lactobacillus* and *Acetobacter* spp. (Newell and Douglas, 2014), which may be connected to changes in nutrient pathways by the bacteria (Storelli et al., 2011). Although,

the larval rearing conditions used in this experiment would not be considered nutrient poor (Bretman et al., 2016), and I have not found an effect of adult presence on development time (Chapter 3), there is the possibility that the increased presence of *L. plantarum* could affect adult presence pupae in other ways, through alterations in these nutrient signalling pathways. *Lactobacillaceae* abundance is also associated with immune activity in mice, altering T-cell counts (Snijders et al., 2016), and the potential beneficial properties related to these effects have encouraged its use in human probiotic treatments (Matos and Leulier, 2014).

Another bacterium found elevated in adult presence pupae was *Lactobacillus brevis*. Under dysbiotic conditions, *L. brevis* can cause inflammation and cell death through the production of reactive oxygen species induced by bacterial uracil production (Lee et al., 2013). Increases in *L. brevis* have previously been linked to dysbiotic conditions associated with ageing (Erkosar and Leulier, 2014). Furthermore, interactions between *Acetobacter* sp. and *L. brevis* can reduce triglyceride levels in gnotobiotic flies to similar levels found in conventional flies via the promotion of *Acetobacter* abundance by *L. brevis* (Newell and Douglas, 2014). This contrasts with *L. plantarum* and *Acetobacter* which do not show this effect (Newell and Douglas, 2014). It seems, therefore, that these two *Lactobacillaceae* can have quite distinct effects on the host. Newell and Douglas (2014) also demonstrated that, despite *L. plantarum* and *L. brevis* both individually reducing development rate compared to axenic flies, no difference was found between flies with a mono-association of these two species. It has been suggested that the presence of genes involved in the production of bactericidal factors in *L. plantarum* and those that aid in nutrient acquisition in *L. brevis* may promote the survival of these bacteria in the gut, and could also affect their host (Petkau et al., 2016). How these two *Lactobacillus* species interact specifically with each other has been less explored, but they could potentially affect each other through competition or indirectly through their effects on immune activity (Matos and Leulier, 2014). Such potential interactions could have distinct implications for the adult

presence pupae in which both *L. plantarum* and *L. brevis* were increased compared to those reared without adults. Raising axenic larvae and associating them with these bacteria singly or combined, similar to experiments conducted with *Lactobacillaceae* and *Acetobacter* sp. (Newell and Douglas, 2014), could help to reveal these interactions in more detail.

The most prevalent OTU in these samples was *Wolbachia* sp., with a far greater number of reads than all other bacteria observed. This finding is unsurprising given the widespread establishment of this bacteria in arthropod species (Zug and Hammerstein, 2012), including laboratory strains (Clark et al., 2005) and up to 95% in some wild *Drosophila* populations (Hoffmann et al., 1994). Although known to be capable of feminization and cytoplasmic incompatibility, as shown by a reduced egg hatch rate (McGraw and O'Neill, 2004), the effects of *Wolbachia* on *Drosophila* hosts appears to be highly variable (Fry et al., 2004). The preservation of *Wolbachia* in such populations could be explained if there are fitness benefits to the host, but Harcombe and Hoffmann (2004) found no influence of *Wolbachia* infection on the time to emergence of larvae under nutrient poor conditions or on the wing size of emergent flies. Due to its prevalence in these samples, *Wolbachia* was not removed from the analysis. Furthermore, removal of *Wolbachia* by antibiotic treatment could have other implications on the flies (Li et al., 2014), for example, treatment with tetracycline has been shown to decrease survival in some laboratory strains compared to *Wolbachia*-infected flies (Fry et al., 2002). In fact, despite its dominant abundance in these samples, *Wolbachia* did not significantly differ between them, and therefore was not a driver for the changes observed between these groups.

The *D. melanogaster* microbiome is dominated by *Acetobacteraceae*, *Lactobacillales* and *Enterobacteriaceae* (Chandler et al., 2011; Wong et al., 2013), notwithstanding the variability between laboratory and wild flies, and between laboratories (Wong et al., 2013; Staubach et al., 2013). This is reflected in the results found here, with *Acetobacteraceae* relatively less

abundant than the *Lactobacillales* and the dominant *Wolbachia*. It is likely that diet plays an important role in the differences observed between studies (Chandler et al., 2011; Staubach et al., 2013), and particularly due to the requirement for consistent bacterial renewal that is needed for maintenance of the microbiota (Blum et al., 2013). *D. melanogaster* do not form stable social groups, but are known to aggregate, for example, larvae are attracted to food that has been occupied by others (Durisko and Dukas, 2013a). The presence of microbial volatiles in food could indicate substrates that have been occupied by other individuals, and potentially, also suitable microbiome species (Venu et al., 2014). In addition, it has been suggested that these bacteria could protect against harmful fungal growth on food sources (Rohlfs and Kürschner, 2010; Venu et al., 2014). Furthermore, the presence of adults on a substrate prior to, not during, larval rearing increases larval survival and reduces fungal growth (Wertheim et al., 2002). This effect can be linked to an increase in bacteria on the substrate leading to an enhanced survival of the larvae in the presence of fungal growth (Broderick and Lemaitre, 2012), and females are indeed attracted to lay eggs on sites that have been previously occupied by other mated females (Duménil et al., 2016).

The gut epithelium is an important protective barrier against pathogens (Lemaitre and Hoffmann, 2007), and its renewal is required to protect against damage from reactive oxygen species which are part of the immune response (Lemaitre and Hoffmann, 2007; Buchon et al., 2009). Increases in gut epithelial renewal rate can be affected by the microbiome (Buchon et al., 2009; Broderick et al., 2014). Additionally, alterations in immune pathways have already been shown to affect the microbiome of *Drosophila melanogaster*, for example, immune-deficient flies have an increased bacterial load (Buchon et al., 2009). Ageing flies have also been shown to have increased bacterial loads (Buchon et al., 2009; Erkosar et al., 2013; Erkosar and Leulier, 2014; Buchon et al., 2014). This dysbiosis has been linked to changes in immune activity (Martino et al., 2017). The microbiome has also been implicated in Irritable Bowel Syndrome (IBS)

(Carabotti et al., 2015; Crouzet et al., 2013). Transplantation of faecal microbiota from humans with IBS to germ-free rats resulted in increased sensitivity to colonic distension, a characteristic of IBS, compared to those that received a faecal transplant from healthy individuals (Crouzet et al., 2013). In *D. melanogaster*, Teixeira et al. (2008) found an increased resistance to *Drosophila* C virus in *Wolbachia*-infected flies. The expression of a number of metabolic activity genes can be influenced by the *Drosophila* microbiota through the immune deficiency (IMD) pathway (Combe et al., 2014), a pathway particularly known for its role in mediating antimicrobial peptide expression, amongst other processes (Myllymäki et al., 2014; Combe et al., 2014). Indeed, the microbiota has been shown to affect the basal expression of a number of immune genes through this pathway, which can in turn affect, for example, the composition of the bacterial community in the gut (Broderick et al., 2014). Therefore, it is possible that the changes observed in the microbiome of adult presence pupae can exert an influence beyond early life stages, even without the changes carrying onto the 1 day old adults. Such effects could arise through microbiome-mediated changes in nutrient allocation or immune function.

Although I found no effect of adult presence on development time (Chapter 3), the increase in *L. plantarum* in the adult presence pupae could have other important effects. The use of gnotobiotic flies could increase our understanding by looking at specific bacteria and, in turn, how these changes can affect the host. However, it is also important to understand how the microbiome as a whole is affected by these environments, under the normal laboratory conditions experienced by the flies. Understanding more of the cross-talk between host and microbiome will be important in future research of health and disease, and will further highlight effects of the social environment. The microbiome has the potential to instigate changes in the host through a variety of mechanisms (Broderick and Lemaitre, 2012), shaped by the specific bacterial species present (Newell and Douglas, 2014). In this study, there was no effect of larval density on the microbiome composition of pupae or young adult flies, however, the presence of adult

males did significantly affect microbial composition in pupae. The changes observed between the microbiome of pupae reared with adults in the environment compared to those without did not carry over into one day old adult flies. These results suggest that the type of social conditions encountered are important, with distinct effects on the microbiome of these early life stages, and may indicate differential stress or immune responses to these environments with subsequent effects on the microbiome, though this requires further investigation. The observed changes in the adult presence pupae could still have potentially far-reaching consequences for adult flies. Responses to early life conditions in hosts could both influence the microbiome, and be affected or modulated by the bacterial communities themselves.

Chapter 6

General Discussion

Developmental plasticity in response to early life conditions can have consequences for adult survival and fitness (Taborsky, 2017). Responding in an anticipatory manner to cues that indicate future conditions could help to prepare an individual, often providing time to gather information and refine the phenotype (Kasumovic, 2013). Yet, the changes made in response to these conditions could also result in trade-offs, or could have other indirect effects. It is also unlikely that the environmental conditions affect a single factor, for example, responding to stressful conditions in early life could have additional effects on immune activity (Freestone et al., 2008). The social environment is important for both reproduction and competition, and can vary spatially and temporally (Kasumovic and Brooks, 2011). Our laboratory has previously shown that the adult social environment can affect mating duration (Bretman et al., 2009), learning ability (Rouse et al., *in prep*) and immunity (Leech et al., 2017) in *Drosophila melanogaster*. However, the effects of early life social conditions on adult traits remained less explored. In this thesis, I examined these for early life conditions found to produce an anticipatory response to future reproductive competition in males, namely an increase in accessory gland growth when reared at high density or with adult males present in the larval environment (Bretman et al., 2016). I expected that these social conditions could have a number of additional effects, and that the energy allocation to this response could trade-off with other processes. I identified a number of potential traits that may be influenced, specifically aspects of cognition, stress, immunity and the microbiome. I demonstrate that the type of social conditions experienced by the larvae can have distinct effects on adult traits.

6.1 Main Findings

Most significantly, despite both the high density and adult presence males showing equivalent anticipatory responses to future reproductive competition, the two forms of social conditions have quite distinct effects on the adult fly that do not always appear to be the result of simple trade-offs (Table 6.1-1). These social environments have differing effects, and/or may indicate different future conditions to the larva, which can also be dependent on the sex of the individual. Differences between the types of cues experienced may exist between larval density and adult presence which could affect cognitive factors; be differentially stressful; or indicate differences in future stressful conditions or infection risk that could differ between the sexes, resulting in distinct developmental plasticity in these responses. Further, the information provided by these cues may be difficult to interpret. For example, whilst low larval densities and adult absence groups indicate a decreased risk of infection (Reeson et al., 1998), and therefore, a reduced need to invest in immune activity, they could speculatively also indicate less mating opportunities, and a potential requirement to invest into factors, including immunity, that would increase longevity long enough to find a mate (Shoemaker et al., 2006).

I expected that altering larval social conditions would provide a level of social stimulation that could affect cognition. Learning ability was found to increase for adult males reared under low larval densities compared to high larval densities in a sexual context assay. These males also had higher relative gene expression of the *Neurexin-1* gene, involved in synapse formation (Zeng et al., 2007). A requirement for the processing of fluctuating cues under low density could result in an increase in cognitive ability in these individuals (Fawcett and Frankenhuis, 2015). However, there was no evidence that the low density conditions resulted in an increased variance in the responses observed. Low density flies also showed a reduction in appetite on an infected food source which may be very tentatively connected

to an increased learning ability, although could also implicate differences in energy requirements between groups or increased tolerance of infected food in high density flies. Alternatively, this difference in learning ability could suggest a trade-off between reproduction (investment in accessory gland growth in high density males) and cognition, as both of these tissue types are energetically expensive (Pitnick et al., 2006; Isler and van Schaik, 2009). No significant effects of adult presence were found for learning ability or cognition-related gene expression changes. This could suggest that in these individuals, the presence of adult males does not require additional investment in cognition to process cues or there is no trade-off between these aspects of cognition and reproduction.

Rearing at high larval density increased development times compared to those kept at low density in both sexes. This could indicate that males are delaying maturation to increase reproductive investment in accessory gland size, for example, as found in the butterfly, *Bicyclus anynana* (Lewis et al., 2010), however, females also showed this response. Alternatively, a response to increased sperm competition risk, such as has been observed in green swordtails, *Xiphophorus hellerii* (Walling et al., 2007) could be occurring in males, with a similar response in females arising through an increased demand to reduce encounters at a high male density (Partridge and Fowler, 1990) or competition with other females for oviposition sites (Durisko et al., 2014a). The results could also, however, implicate a build-up of waste products (Borash et al., 2000), which would affect both sexes. Low density flies had increased relative gene expression of the stress-response genes, *Turandot A* and *Turandot C*, that have previously been shown to respond to adult social conditions (Mohorianu et al., 2017). This result could suggest either that the low density conditions are more stressful, which may be the case given that *D. melanogaster* larvae benefit from cooperative burrowing (Durisko et al., 2014b), or, that the high density conditions could be more stressful, and there is a priming of certain stress responses in these flies. That there was no effect on any stress tolerance, apart from an

increased cold tolerance in adult males from high larval densities, makes it difficult to determine which of these is more likely.

Adult presence significantly increased desiccation and cold shock tolerance in adult females. The presence of adult males may indicate a potentially stressful future environment for females, given the costs associated with adult exposure to males (Partridge et al., 1987a; Partridge and Fowler, 1990), and could suggest that these females may be priming their stress responses in anticipation of these conditions. Post-infection lifespan after injection with *Bacillus thuringiensis* was decreased in the adult presence females compared to adult absence females. These results could indicate a stress and immunity trade-off in this group. Adult presence also had an overall negative effect on adult lifespan. Whilst this could suggest a trade-off in this group between accessory gland growth and lifespan in males, the similar trend in females suggests there may be other effects involved, such as changes in stress responses. Overall, these results suggest that the sexes are responding differently to these conditions.

Table 6.1-1 Summary of main findings for traits investigated for flies reared at low vs high larval density or in the presence vs absence of adult males. Upwards/downwards arrows indicate an increase/decrease in the former of the two treatments.

Larval Environment	Sex	Cognition	Lifespan	Development Time	Desiccation Tolerance	Cold Shock Tolerance	Post-Infection Lifespan	Microbiome Diversity	
								Pupae	Adults
Low Density vs High Density	♂	↑	-	↓	-	↓	-	-	-
Low Density vs High Density	♀	NA	-	↓	-	-	-	-	-
Adult Presence vs Adult Absence	♂	-	↓	-	-	-	-	↑	-
Adult Presence vs Adult Absence	♀	NA	(trend ↓)	-	↑	↑	↓	↑	-

Unlike the results found in females, no effects of adult presence were observed in males for the stress or immune assays. That there was no effect on stress tolerance in adult presence males seems particularly surprising, given their anticipatory response to future reproductive competition (Bretman et al., 2016). It could be that the presence of adult males during larval stages does not necessarily indicate a future stressful environment for males, or at least, not to the extent that it does for females, or that males are investing in other areas, such as reproduction. Furthermore, cold shock tolerance was increased in adult males from high larval densities but not in females. In addition, despite the anticipatory response shown by males to future reproductive conditions, no effect of either larval density or adult presence was observed on female reproductive traits. This could suggest that, given the potential variability in social environments, it may be more beneficial for females to alter these traits at adult stages, when there may be more surety of the social conditions, similar to the results found for mating duration in males (Bretman et al., 2016). Generally, differences in the sexes may be related to differences in life history strategies, whereby females invest in traits that maximise longevity (Nunn et al., 2009) or the production of secondary sexual traits in males reduces energy availability for other traits (Zuk, 1990). Sex differences in immunity have been found to vary depending on the availability of specific resources required by each sex, for example, the immunity of female *D. melanogaster* is limited by yeast availability, and in males, by sexual activity (McKean and Nunney, 2005). Sex differences are widespread in the literature (McKean and Nunney, 2005; Leech et al., 2017), and my data suggests these can be triggered during early life stages, even before sexual maturity. It is therefore important for further research to investigate how and why these differences exist.

However, there were no sex effects found in microbiome compositions between the groups. This contrasts with the results found in older flies, in which social contact drives differences in male but not female microbiomes

(Leech, 2017). Microbiome composition could be affected by horizontal transfer or changes in stress and immune responses under different social conditions (Freestone et al., 2008), and dysbiosis has been associated with dysregulation of the immune system in ageing flies (Broderick et al., 2014). In this study, life stage was a significant factor, with greater microbial diversity found in pupae than in 1 day old adults, in accordance with previous research (Wong et al., 2011). Whilst no effect was found for those reared under different larval densities, pupae reared with adult males during larval development had increased microbial diversity, but this did not carry over into 1 day old adults, likely due to the major reorganisation of structures that occurs during metamorphosis.

Indeed, the distinct separation between larval and adult stages that occurs in holometabolous insects during metamorphosis, may be predicted to present a barrier to the transmission of responses between larval and adult stages. However, food limitation during larval stages can have significant effects on adult body size (Hopwood et al., 2014) and resource allocation (Saastamoinen et al., 2010). Furthermore, there is evidence in some insect species for maintenance of memory through metamorphosis (Gandolfi et al., 2003; Blackiston et al., 2008). In *D. melanogaster*, many neurons present in larval mushroom bodies persist into the adult stage (Lee et al., 1999), and learning-related genes expressed during adult stages are also expressed in larval mushroom bodies (Crittenden et al., 1998). Both of which could provide routes through which larval cognition affects adults. In addition, despite the structural reorganisation that occurs during metamorphosis, larval fat body cells also persist into the adult fly (Aguila et al., 2007), and there is some evidence for association of heat shock protein expression between life stages (Krebs et al., 1998). Following these studies, and the results obtained here, it appears that there are distinct effects of early life conditions that can persist through metamorphosis and into the adult fly.

Overall, the results suggest that larval social conditions have a number of effects on the adult fly. Early life stages may be particularly important where individuals have an increased uncertainty of the environment (Fawcett and Frankenhuis, 2015), and, as larvae have a limited ability to disperse, are less able to evade stressful conditions than adults (Feder et al., 1997). Under the environmental matching hypothesis, where early and later life conditions match, an increased fitness is expected. Conversely, the silver spoon hypothesis predicts that good early conditions benefit the individual regardless of later conditions. In this study, adults were kept under the same standard laboratory conditions and were usually only 1 day old when assayed. Therefore, to determine if these hypotheses can be applied to the results found, the adult conditions would also need to be manipulated and for a longer period of time. It may also be expected that under restricted resource conditions, trade-offs would become more evident or severe (French et al., 2007). The increased cognition found in low density males, increased stress tolerance in adult presence females and decreased expression of *Turandot* genes in high density flies (and increased cold tolerance in these males) are responses to early life conditions that appear to have beneficial effects at adult stages. Whether these are reactive or anticipatory developmental responses is not entirely clear, as the changes made to these traits during development could be in response to the immediate environment (i.e. reactive plasticity). For example, the conditions may be having direct influences on larval cognition. Increasing this at low density could allow an enhanced ability to process variable cues that could be useful for finding particularly suitable patches of substrate or other larvae that are beneficial for cooperative burrowing (Durisko et al., 2014b). However, these changes could also be in response to the expected future conditions (anticipatory plasticity), such that low density males could be investing into cognition in prediction of variable social conditions during adult stages. Similarly, the stress responses observed could correspond to changes in immediate or predicted environmental conditions, and in order to understand this more fully, further investigation into the connection between larval and adult environments is required. These results do, however, provide evidence for a trade-off between stress and immunity for females

reared in the presence of adult males. Examining the fitness effects of differing adult conditions on these individuals may help to elucidate these responses further, as will investigating the underlying mechanisms involved.

6.2 Potential underlying mechanisms for changes in response to larval social conditions

From these results, it is apparent that the social conditions experienced during larval stages can influence a number of factors in adult flies, but these responses are not likely to be mutually exclusive. For example, formation of long-term memory has been shown to reduce starvation and desiccation tolerance (Mery and Kawecki, 2005). Whilst simple trade-offs in resource allocation may partially underlie some of these connections, it also appears that there are other processes facilitating these responses. Trade-offs between reproduction and lifespan have been demonstrated (Sgrò and Partridge, 1999), but these can be uncoupled under certain conditions, for example, the provision of the amino acid methionine under dietary restriction can increase fecundity without decreasing lifespan (Grandison et al., 2009). Additionally, increased oxidative stress through the production of reactive oxygen species during reproductive metabolic activity has been implicated as a possible effector, but requires further investigation (Flatt, 2011). In particular, stress responses, immune activity and the microbiome can all be involved in mediation of the other responses (Stefanski, 2001; Freestone et al., 2008).

Regulation of a number of these responses could occur through changes in hormone levels (Flatt et al., 2005). One particular candidate for this is Juvenile Hormone (Flatt et al., 2005). This hormone affects a number of life history traits, and has been found to delay development time, increase reproduction, decrease lifespan, and reduce stress resistance and immunity in fruit flies (Flatt et al., 2005; Flatt and Kawecki, 2007). As a potential

mediator of some of these effects, it would be interesting to compare the levels of this hormone in individuals from the different larval social conditions. The neurotransmitter dopamine is also associated with a number of effects on, for example, learning, locomotion and sleep (Yamamoto and Seto, 2014), and therefore, could be another potential effector in some of the responses observed here. Additionally, energy stores could also exert an influence, specifically with regards to stress responses (Andersen et al., 2010), and particularly as larval fat body cells have been shown to persist into early adult stages (Aguila et al., 2007). Desiccation tolerance, for example, is especially affected by carbohydrate reserves (Andersen et al., 2010), whilst lipid stores are correlated to starvation resistance (Chippindale et al., 1996). Further, the acquisition of energy from these reserves can also be influenced by hormonal changes (Flatt et al., 2005). Differences in energy requirements, for example, those involved in egg production in females, may influence differences between the sexes (Magwere et al., 2004). Therefore, it may also be beneficial to measure specific types of energy stores in flies from different larval social conditions. For example, although overall body mass does not change, are there differences in the quantity of lipid or carbohydrates stored between treatments and sexes?

Additional underlying mechanisms for the differences observed may occur through differences in signalling pathways, for example, the insulin/insulin-like growth factor-like signalling (IIS) pathway is involved in energy storage and interacts with the stress-responsive JNK pathway (Wang et al., 2005). Under stressful conditions, activation of the JNK pathway and reduced signalling of the IIS pathway results in the translocation of the transcription factor Foxo to the nucleus where it affects the expression of genes, including those involved in stress responses, such as heat shock proteins (Wang et al., 2005). It also appears that, through this counteractive interaction with IIS, the JNK pathway can affect adult lifespan (Wang et al., 2003; Wang et al., 2005). Examining these pathways in more detail could therefore be a beneficial step to elucidate potential underlying mechanisms for the changes observed in flies from different larval social conditions. In

particular, IIS signalling appears to be a conserved pathway, found in both insects and mammals (Barbieri et al., 2003), and Foxo transcription factors have also been associated with oxidative stress in mammalian cells (Lehtinen et al., 2006). Therefore, understanding the mechanisms of these responses in fruit flies may help to understand the underlying responses to early life effects in other species.

Furthermore, the microbiome has been shown to affect host nutrient signalling (Storelli et al., 2011), with influences on energy storage and body mass, that can vary with host diet and be differentially affected in males and females (Wong et al., 2014). Although I found no differences in microbiome composition between larval densities, changes in microbial diversity for individuals reared in the presence of adult males during pupal stages could have distinct impacts on, for example, stress and nutrient signalling pathways. The use of gnotobiotic flies could allow these effects to be explored in more detail. For example, adding both *L. plantarum* and *L.brevis* to axenic larvae, and measuring aspects such as development time and lifespan could give some insight into the effects of this dual increase in the adult presence pupae. Further work also needs to be carried out to clarify specific effects that these bacteria have on, for example, the intestinal barrier (Buchon et al., 2009) and host signalling pathways, and similarly, to investigate the effects of the host on the microbiota.

Finally, some of the responses observed in individuals from different larval social environments may also derive from changes in the epigenome (Feil and Fraga, 2012). For example, desiccation stress is correlated to changes in epigenetic modifications in *D. melanogaster* (Sharma et al., 2017). Epigenetic changes in early life have been associated with human health, and may also be influenced by the microbiome (Indrio et al., 2017), for example, exposing immature human intestinal epithelial cell lines to either probiotics or pathogenic bacteria results in differential patterns of epigenetic modifications and this has been linked to changes in intestinal barriers, with

possible effects on later health (Cortese et al., 2016). As such, the analysis of epigenetic modifications in individuals from these larval conditions would be another constructive factor to investigate.

6.3 Conclusions and Implications

In this thesis, I have demonstrated that the social environment experienced during larval stages can impact on a number of traits in adult fruit flies, but there are significant factors entailed. Firstly, one 'set' of conditions (for example, the presence of adult males) can have effects on a variety of traits. It is also possible that these traits are connected, for example, by trade-offs. Secondly, different types of larval social conditions can elicit distinct effects, and thirdly, significant differences can occur between the sexes. Together, this work, combined with other studies from the laboratory, have demonstrated the importance of the social environment on, for example, reproductive behaviour (Bretman et al., 2009), learning ability (Rouse, 2016), and immunity (Leech, 2017). However, there is a need to recognise the multiplicative and connective effects of environmental conditions at different life stages. Therefore, it would also be constructive to examine how these early life and adult social conditions can interact to influence survival and fitness, such that if the cues of future reproductive competition to which the males respond do not materialise into adulthood, could there be additional costs of the production of this response? Furthermore, as it appears that cues from conspecifics of the same age cohort have specific effects compared to those from an earlier age cohort, suggesting that these could be providing distinct information about future social conditions, it would be interesting to investigate the particular cues involved to see which cues are used, and how these might vary under the different social environments.

Early life conditions have been implicated in a number of health issues in humans, for example, a study on individuals in New Zealand from 3 to 32

years of age found an association between maltreatment during childhood and increased inflammation in adults, as indicated by increasing levels of an inflammatory biomarker, even after controlling for confounding factors in adults (Danese et al., 2007). Additionally, adults exposed to famine during the gestation period in The Netherlands in 1944-45 show an increased risk of diabetes through a decreased glucose tolerance (Ravelli et al., 1998). These individuals also show epigenetic changes 60 years later of the insulin-like growth factor gene *IGF2* which is important for development (Heijmans et al., 2008), suggesting that epigenetic modifications may be important underlying factors for health issues later in life. Furthermore, changes in specific bacteria of the microbiome during infancy are associated with increased risk of asthma in children (Arrieta et al., 2015), and obesity in the first few years of life (Luoto et al., 2010). Thus, as environmental factors experienced during early life appear to be connected to a number of health problems in adults (Hoffman et al., 2017), understanding these effects and their mechanisms will be beneficial in interpretation and treatment, including for those associated with the social environment. Further, under the changing global environmental conditions occurring, there may be alterations in food production and availability, as well as changes in exposure to pathogens and air pollution that could have distinct effects on individuals following early life exposure (Raiten and Aimone, 2017). These changes could also affect the distribution of conspecifics, altering the social environment conditions, with knock-on effects in a number of factors. Whilst direct extrapolation of the results obtained here to other species is not possible, early life conditions have been shown to have important effects in a number of species (Gage, 1995; Taborsky et al., 2012; Danese et al., 2007). Therefore, the importance of early life conditions, including those associated with the social environment, is, and should be, increasingly recognised as having significant influences on individuals at later stages of life.

Appendix

A.1 Fly Media Recipes

Table A.1-1 Fly Food Recipes for 1 litre of Standard, Concentrated and No Food Containing Media

Ingredient	Standard	Concentrated	No Food
Water	970ml	945ml	970ml
Agar	15g	15g	15g
Sugar	50g	75g	-
Yeast	100g	100g	-
Nipagin Solution	30ml	30ml	30ml
Propionic Acid	3ml	3ml	3ml

A.2 RT-qPCR and 16S rRNA Primers

Table A.2-1 Forward and Reverse Primer Sequences for RT-qPCR genes with Efficiency values

Gene	Forward Sequence	Reverse Sequence	Efficiency
Actin 5C	GTGGATACTCCTCCCGACAC	GCAGCAACTTCTTCGTCACA	91.3
Ef1	GTCTGGAGGCAATGTGCTTT	AATATGATGTCGCCCTGGTT	106.4
Rap21	TTCACTTACGAACCATCAAACATT	GCTGGCTGACTTCCTTTCAC	107.4
bruchpilot	GACATCAAGGACCGCAAGAT	GCCATATCCACCTGGTTGTC	95.2
dikar	CATCTATAAAATCCCGCAGAGG	CGGTATCTCCCACCATGATT	99.8
dunce	TGTGGCATACACCATATTTCAG	GAAACGGATTGTCTTTGACG	97.8
futsch	ACGTTTCCGATTGTCACGTC	GCTGCTACCTCCTCATCGTC	99.6
Neurexin-1	GACAACAACTGGCACACGAT	TACTGTGGCGACCCAGAAT	98.8
Turandot A	GCTTCAGCGTTCCAAAAAGT	AGAGGACTAATCAGCAGCAGTG	98.1
Turandot C	CAGTTTGTCTTAAACCAGTGCTCT	CTCGTCAGAATAGCCCAAGC	102.3

persephone	TTGGGAGCTGTGAACATTGA	ACTGCGGATGGATCTTAACG	106.0
PGRP-LF	TTCGAGCTGATGCAAAATTG	CAACCAATAGGGTCGGGTAA	104.9

Table A.2-2 Forward Primer Sequences for 16S rRNA Sequencing (V4 Region)

Name	Sequence
SA501	AATGATACGGCGACCACCGAGATCTACACATCGTACGTATGGTAATTGTGTGCCAGCMGCCGCGGTAA
SA502	AATGATACGGCGACCACCGAGATCTACACACTATCTGTATGGTAATTGTGTGCCAGCMGCCGCGGTAA
SA503	AATGATACGGCGACCACCGAGATCTACACTAGCGAGTTATGGTAATTGTGTGCCAGCMGCCGCGGTAA
SA504	AATGATACGGCGACCACCGAGATCTACACCTGCGTGTTATGGTAATTGTGTGCCAGCMGCCGCGGTAA
SA505	AATGATACGGCGACCACCGAGATCTACACTCATCGAGTATGGTAATTGTGTGCCAGCMGCCGCGGTAA
SA506	AATGATACGGCGACCACCGAGATCTACACCGTGAGTGTATGGTAATTGTGTGCCAGCMGCCGCGGTAA
SA507	AATGATACGGCGACCACCGAGATCTACACGGATATCTTATGGTAATTGTGTGCCAGCMGCCGCGGTAA
SA508	AATGATACGGCGACCACCGAGATCTACACGACACCGTTATGGTAATTGTGTGCCAGCMGCCGCGGTAA
SB501	AATGATACGGCGACCACCGAGATCTACACCTACTATATATGGTAATTGTGTGCCAGCMGCCGCGGTAA
SB502	AATGATACGGCGACCACCGAGATCTACACCGTTACTATATGGTAATTGTGTGCCAGCMGCCGCGGTAA

SB503	AATGATACGGCGACCACCGAGATCTACACAGAGTCACTATGGTAATTGTGTGCCAGCMGCCGCGGTAA
SB504	AATGATACGGCGACCACCGAGATCTACACTACGAGACTATGGTAATTGTGTGCCAGCMGCCGCGGTAA
SB505	AATGATACGGCGACCACCGAGATCTACACACGTCTCGTATGGTAATTGTGTGCCAGCMGCCGCGGTAA
SB506	AATGATACGGCGACCACCGAGATCTACACTCGACGAGTATGGTAATTGTGTGCCAGCMGCCGCGGTAA
SB507	AATGATACGGCGACCACCGAGATCTACACGATCGTGTTATGGTAATTGTGTGCCAGCMGCCGCGGTAA
SB508	AATGATACGGCGACCACCGAGATCTACACGTCAGATATATGGTAATTGTGTGCCAGCMGCCGCGGTAA

Table A.2-3 Reverse Primer Sequences for 16S rRNA Sequencing (V4 Region)

Name	Sequence
SA701	CAAGCAGAAGACGGCATACGAGATAACTCTCGAGTCAGTCAGCCGGACTACHVGGGTWTCTAAT
SA702	CAAGCAGAAGACGGCATACGAGATACTATGTCAGTCAGTCAGCCGGACTACHVGGGTWTCTAAT
SA703	CAAGCAGAAGACGGCATACGAGATAGTAGCGTAGTCAGTCAGCCGGACTACHVGGGTWTCTAAT
SA704	CAAGCAGAAGACGGCATACGAGATCAGTGAGTAGTCAGTCAGCCGGACTACHVGGGTWTCTAAT
SA705	CAAGCAGAAGACGGCATACGAGATCGTACTCAAGTCAGTCAGCCGGACTACHVGGGTWTCTAAT
SA706	CAAGCAGAAGACGGCATACGAGATCTACGCAGAGTCAGTCAGCCGGACTACHVGGGTWTCTAAT
SA707	CAAGCAGAAGACGGCATACGAGATGGAGACTAAGTCAGTCAGCCGGACTACHVGGGTWTCTAAT

SA708	CAAGCAGAAGACGGCATACGAGATGTCGCTCGAGTCAGTCAGCCGGACTACHVGGGTWTCTAAT
SA709	CAAGCAGAAGACGGCATACGAGATGTCGTAGTAGTCAGTCAGCCGGACTACHVGGGTWTCTAAT
SA710	CAAGCAGAAGACGGCATACGAGATTAGCAGACAGTCAGTCAGCCGGACTACHVGGGTWTCTAAT
SA711	CAAGCAGAAGACGGCATACGAGATTCATAGACAGTCAGTCAGCCGGACTACHVGGGTWTCTAAT
SA712	CAAGCAGAAGACGGCATACGAGATTCGCTATAAGTCAGTCAGCCGGACTACHVGGGTWTCTAAT
SB701	CAAGCAGAAGACGGCATACGAGATAAGTCGAGAGTCAGTCAGCCGGACTACHVGGGTWTCTAAT
SB702	CAAGCAGAAGACGGCATACGAGATATACTTCGAGTCAGTCAGCCGGACTACHVGGGTWTCTAAT
SB703	CAAGCAGAAGACGGCATACGAGATAGCTGCTAAGTCAGTCAGCCGGACTACHVGGGTWTCTAAT
SB704	CAAGCAGAAGACGGCATACGAGATCATAGAGAAGTCAGTCAGCCGGACTACHVGGGTWTCTAAT
SB705	CAAGCAGAAGACGGCATACGAGATCGTAGATCAGTCAGTCAGCCGGACTACHVGGGTWTCTAAT
SB706	CAAGCAGAAGACGGCATACGAGATCTCGTTACAGTCAGTCAGCCGGACTACHVGGGTWTCTAAT
SB707	CAAGCAGAAGACGGCATACGAGATGCGCACGTAGTCAGTCAGCCGGACTACHVGGGTWTCTAAT
SB708	CAAGCAGAAGACGGCATACGAGATGGTACTATAGTCAGTCAGCCGGACTACHVGGGTWTCTAAT
SB709	CAAGCAGAAGACGGCATACGAGATGTATACGCAGTCAGTCAGCCGGACTACHVGGGTWTCTAAT
SB710	CAAGCAGAAGACGGCATACGAGATTACGAGCAAGTCAGTCAGCCGGACTACHVGGGTWTCTAAT
SB711	CAAGCAGAAGACGGCATACGAGATTCAGCGTTAGTCAGTCAGCCGGACTACHVGGGTWTCTAAT
SB712	CAAGCAGAAGACGGCATACGAGATTCGCTACGAGTCAGTCAGCCGGACTACHVGGGTWTCTAAT

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