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The Effect Of Age On Social Behaviour In Drosophila melanogaster And The Progeny of Aged Parents

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Abstract

Recent studies have linked neuropsychiatric disorders to older parents. These disorders often include changes in social behaviours like the social spacing between neighbouring individuals, which can be modeled in organisms such as *Drosophila melanogaster*. I investigated the effects of aging on the social space between neighbouring *D. melanogaster* and how aging impacts the next generation. To achieve this, I used the social space assay and found that individuals become less social with age and that this effect is passed on to the first generation only. Additionally, accelerating the physiological process of aging via increased rearing temperatures or exposure to oxidative stress resulted in individuals and their progeny that were less social. Finally, I found that only male progeny of old fathers were less social. Although it is unclear how aging affects gametes leading to changes in social behaviours, the powerful model system of *Drosophila* will allow us to identify the underlying mechanisms.

Keywords

Aging

Biological aging

Caloric restriction

Chronological aging

Drosophila melanogaster

Fecundity

Germline mutation

Longevity

Maternal aging

Methyl viologen (Paraquat)

Paternal aging

Reactive oxygen species

Senescence

Social avoidance

Social behaviour

Social space

Survival

Trans-generational

Abbreviations

AF- All flies

AMA- advanced maternal age

APA- advanced paternal age

ASD- Autism Spectrum Disorders

CR- caloric restriction

Cs- Canton S

GSC- Germline Stem Cell

NN- nearest neighbour

ROS- reactive oxygen species

XY radius- number of flies within each body length radius away from that fly

Co-Authorship Statement

This thesis includes two manuscripts. I am the principle author and will share authorship with my supervisor Dr. Anne Simon. I have performed all the experiments and I will write each manuscript. The manuscripts are entitled "Aged *Drosophila* And Their Progeny Are Less Social" (Chapter 3), "Manipulations of the Aging Process Affect Social Behaviour of Parents and Progeny" (Chapter 4). Both manuscripts will be submitted as one article to PNAS, Nature Communications, Current Biology, or PLOS Biology. If I am unsuccessful with submitting the manuscripts together, I will submit each manuscript individually.

Dr. Anne Simon has provided laboratory space and resources, support and guidance and will aide in preparing the manuscripts for publication. Shirley Long will be co-author for chapter 3 as she helped with collecting the data for the survival and fecundity curves.

Acknowledgments

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Chapter 1 – Literature Review

Overview

In this thesis, I explore how aging affects the social behaviour of *Drosophila melanogaster* and its progeny. It is known that some behaviours, including social behaviour, changes with aging. However, certain social behaviours such as the social spacing between individuals have not been yet been investigated. My question is: how does social spacing change with aging and can this effect be passed on to the next generation? This question is especially important as certain neuropsychiatric disorders like autism and schizophrenia, which each have a social component, have been linked to older parents. To better understand the context and significance of this work, I first review the basic definition of aging and several theories that attempt to explain how and why aging occurs. I then define social behaviour, the evolution of behaviour in groups, and how we can assess social behaviours in the lab. I connect these two concepts by discussing how social behaviours may change with age and how this effect may be transmitted to the next generation. Finally, I discuss the model organism, *Drosophila melanogaster*, and explain how *Drosophila* can be used to study social behaviour and aging due to its biological similarity and relevance to humans.

1 Introduction to Aging and Social Behaviour

1.1 Aging

1.1.1 Aging And Senescence

In this section, I provide basic definitions for the concepts of aging, longevity, and cellular senescence and explain the differences between these occasionally overlapping ideas. Aging is an almost universal phenomenon for living organisms, although studying aging in the wild is difficult because of environmental factors like starvation, drought, and predation (Bowles, 1998). In its broadest definition, aging describes any change in an

organism over time following development (Austad, 2004). A more narrowed definition of aging is the progressive deterioration of physiological function and fertility accompanied by an increased susceptibility to death (Hayflick, 1998; Kirkwood and Austad, 2000). Some have more strictly defined aging as the increase in molecular disorder after reproduction, which might not be determined by the genome alone (Hayflick, 1998). This increase in molecular disorder is thought to be due to excess energy reserves following reproduction that are unable to be replenished at the same rate as prior to reproductive maturity and therefore molecular disorder ensues (Hayflick, 1998).

Longevity is the length of time an organism can live, and as suggested by Leonard Hayflick, is distinguished from aging because longevity is influenced or is indirectly linked to the genome, whereas aging is not (Hayflick, 1998). Hayflick argues that throughout evolution, organisms have developed better avoidance techniques to evade predators, which was accompanied by the expansion of energy reserves to be used in case of emergency for wound healing, disease survival, and as a heightened response to sensory information. A greater reserve of physiological capacity (energy) results in a higher chance for organismal survival and successful reproduction. Following reproduction, any excess of this physiological capacity may be used towards increased longevity. This capacity to live after reproduction is known as longevity determination. Therefore, genes that enable the organism to maintain these reserves and reach sexual maturity to reproduce have the potential for longevity. Because these reserves do not replenish, molecular disorder can still occur and thus lead to a progressive decline in physiological function.

Chronological aging is defined as the number of years an organism has lived and is mostly determined by heritable genes (Iliadi et al., 2012). Biological aging, however, is influenced by both genetic and environmental factors and is sometimes referred to as biological senescence, or simply senescence (Iliadi et al., 2012). Senescence may be genetically programmed to either limit cell population growth or increase population turnover following reproduction to allow faster adaptation to the environment (Kirkwood

and Austad, 2000). Evolutionary theorists state that senescence is actually a consequence of a decline in natural selection for genes acting in later life. Kern et al (2001) expanded this idea and thought that older parents who have begun to senesce should therefore have children that are less viable. They showed that older *Drosophila melanogaster* had a reduction in overall egg-to-adult viability (how many adults survive compared to the number of eggs laid) (Kern et al., 2001). From this we can also include the quality of the offspring into definitions of senescence as this captures the impact of the tradeoff between aging and reproduction.

Cellular senescence is different from aging, as senescence refers to a state in which cells will be following a certain sequence of events, such as a chemical or morphological change (Dröge, 2002). These changes can be due to oxidative stress, repeated replication events that shorten telomeres (replicative senescence), double stranded breaks, and improper repair (Lou and Chen, 2006). Each of these cellular changes contributes to the aged phenotype as they result in a decline in cells' ability to divide or perform other normal functions (van Deursen, 2014). Senescence is due to an accumulation of cellular damage due to factors such as telomere erosion, DNA lesions, activated oncogenes, and increased heterochromatic DNA (van Deursen, 2014; De Loof, 2011). Some have also suggested that senescence is mainly due to reactive oxygen species (ROS) damage. However there is some critique of this as the overexpression of antioxidant enzymes in several model organisms has not been able to ameliorate the effects of ROS damage (Back et al., 2010; Doonan et al., 2008).

1.1.2 Theories Of Aging: Evolutionary And Oxidative Stress

Several theories aim to explain why and how aging occurs. Here, I discuss several evolutionary theories that explain aging over a longer time scale and other theories that focus on the genome or molecular events within the cell (Table 1-1). It is important to understand the evolutionary theories, which may not be mutually exclusive, before the cellular theories, as they shed light on how aging evolved as a result of damage, error, or

small changes over time. Alternatively, the oxidative stress theory may explain how an individual will develop a visible aged phenotype.

Table 1-1 Several theories of aging attempt to explain how or why aging occurs.

Type of Theory	Name of Theory	Description	Reference
Evolutionary	Antagonistic Pleiotropy	Genes that are involved in development and early reproduction are pleiotropic and can become detrimental later in life	(Williams, 1957)
	Mutation Accumulation	Genes are beneficial in early life and possibly detrimental later, although these would be selected for during reproductive years	(Medawar, 1952)
	Programmed Death	There is an overall benefit to the group to remove older individuals to make resources for the collective group more available	(Weismann, 1882)
Error/Damage (Stochastic Theories)	Disposable Soma	Wear and tear of the soma over time at the expense of healthy gametes in early life	(Kirkwood, 1977)
	Wear and Tear	Normal damage accumulates over time that will lead to an aged phenotype (originally also proposed by August Weismann)	(Medawar, 1946)
ROS accumulation	Oxidative Stress Theory	Reactive oxygen species are detrimental to the individual and may lead to wear and tear of the soma	(Harman, 1956)
	Mitochondrial Theory	Revision of the oxidative stress theory: mitochondrial DNA is vulnerable to ROS as they are the site of respiration	(Harman, 1972)
Reliability theories		Aging results from an accumulation of very small defects	(Gavrilov and Gavrilova, 2001)
Telomere	Hayflick Limit Theory	Each cell has a defined replication point based on telomere length, at which point cells will become senescent	(Hayflick, 1998)

1.1.2.1 Antagonistic Pleiotropy Theory

The antagonistic pleiotropy theory of aging that was originally developed by George C. Williams states that many genes involved in development are also involved in the aging process (Williams, 1957). Therefore, aging is a timed mechanism and is not the result of decay. The timed mechanism is due to beneficial genes that are involved in early life processes like reproduction that have a pleiotropic function and become damaging in later life (Gavrilov and Gavrilova, 2002; Partridge and Gems, 2006). One caveat of this mechanism is that these pleiotropic genes that are harmful later in life cannot be selected against as the detrimental effects will only be phenotypically visible following reproduction (Kaplan and Robson, 2009).

Tradeoffs between longevity and reproduction have often been studied to determine if there is a direct link between reproduction and longevity. In male *Drosophila melanogaster*, decreases in sexual activity have been shown to lead to extensions in longevity (Partridge and Farquhar, 1981). As seen in female *Drosophila subobscura*, increased egg production can accelerate aging (Maynard-Smith, 1958). Mating and egg production are stressful for the female and can impact longevity. Therefore, antagonistic pleiotropy theory states that if the factors that increase egg production are absent, females will not lay eggs and will have increased longevity. It has even been suggested that if predation and starvation in the wild are eliminated, thus removing the main barrier to aging in the wild, animals will still not age to the lengths we have seen in humans as reproduction will be strongly selected for (Halle et al., 2015). Indeed, in order for wild animals to age (without the "interventions" we have available to us today), they will need to invest great amounts of energy into generating an efficient repair system, which is far more costly than reproduction (Hayflick, 1998). The cost of reproduction may also be due to generating germ cells, as seen in *Drosophila melanogaster* females who have an increased lifespan when they are unable to generate germ cells (Barnes et al., 2006).

1.1.2.2 Mutation Accumulation Theory

One alternate evolutionary theory of aging is called the mutation accumulation theory and was originally introduced by Peter Medawar (1952). This theory states that genes that reduce fitness early in life, such as a gene that would lead to death in children, are strongly selected against and are not passed on to the next generation (Gavrilov and Gavrilova, 2002). For example, individuals with Progeria experience premature aging and often do not live beyond the age of 12 and thus do not have the ability to pass on their genes. These cases could therefore only arise from specific gene mutations and are quickly eliminated from the population, as these individuals will not be able to reproduce. Alternatively, genes that would cause lethal effects in later life (post-reproductive age) are not selected against and remain in the population. These deleterious genes could also accumulate and lead to an aging phenotype. One question is therefore: would larger genomes be able to absorb mutations due to genomic redundancy and resist an accumulation of mutations and result in an individual who is unaffected? One study on

radiation inducing chromosomal damage on the lifespan of haploid and diploid male wasps (*Habrobracon juglandis*) revealed that both males had the same lifespan when exposed to radiation, which suggests that mutational load is not dependent on the number of gene copies (Clark and Rubin, 1961).

1.1.2.3　Disposable Soma Theory

The disposable soma theory states that throughout aging, damage randomly accumulates throughout the genome and, instead of repairing this damage with costly repair pathways, cells opt for repair with reduced accuracy or no repair at all (Gavrilov and Gavrilova, 2002). Throughout development, accelerated growth in somatic tissue is more favorable and the repair capacity is not high because this aligns with the needs to the rapidly dividing cells. However, this will become detrimental as it permits DNA damage to continue to new cell populations and will result in an accumulation of mutations. This damage may also have a lesser chance of being fixed because repair machinery depletes with age, which leads to higher incidences of chromosomal breaks and point mutations (Sloter et al., 2004).

Practical implications of this theory would mean that shorter-lived individuals will invest more energy into reproduction rather than somatic repair and will have more damage accumulating in the somatic genome rather than the gametes, as opposed to longer-lived individuals (Lucas and Keller, 2014). Longer-lived individuals would need to invest more energy into somatic repair, which reduces energy for reproduction and would therefore yield fewer offspring. Critiques of this theory have stated that the theory absolutely challenges evolutionary theory because cells would have to store energy and avoid somatic repair when resources are available in order to invest energy into reproduction later in life when resources may be more scarce (Blagosklonny, 2010). Intriguingly, the disposable soma theory suggests that menopause in older women serves the function of investing energy and resources for grandchildren, as opposed to menopause simply acting as a byproduct of aging, as suggested by others (Kachel et al., 2011; Kim et al., 2012). Benefit to the future generations by grandparents can only happen if the benefit of helping to rear reproductively successful grandchildren offsets the cost of preventing

mortality and reproduction in later life, which can be a very big strain on resources (Kachel et al., 2011). Interestingly, this likelihood of rearing successful grand-offspring increases in social groups where there is resource sharing (Kachel et al., 2011). Overall, the disposable soma theory cannot be tested accurately in the lab and can be viewed as a more refined version of other evolutionary theories of aging.

1.1.2.4   Oxidative Stress Hypothesis

The oxidative stress hypothesis originally proposed by Denham Harman states that the rate of aging depends on the rate of oxidative damage, which is often mediated by reactive oxygen species (ROS) (Harman, 1956). ROS accumulates in cellular components and can have deleterious effects leading to an aged phenotype, thus linking oxidative damage and the regulation of lifespan (Harman, 1956). Biologically, oxidative phosphorylation generates ROS as a by-product and causes damage by accumulating on lipids, proteins, and nucleic acids that can then alter cellular function (Baraibar et al., 2012; Speakman and Selman, 2011). For example, residues such as methionine and cysteine are highly reactive to ROS, thus affecting protein structure or function. If this ROS-induced change is irreversible, it will cause functional impairment. If this ROS-induced damage is reversible, such as ROS used for signaling purposes, or if it can be repaired, it will not lead to damage (Baraibar et al., 2012). For example, the aquatic salamander *Proteus anguinus* is able to live upwards of 68 years due to its ability to tolerate very high levels of anoxia and food deprivation thereby preventing the damaging effects of ROS (Speakman and Selman, 2011).

Although antioxidants naturally found in cells work to combat ROS stress, they can lessen in both quality and quantity over time. This leads to cells that are more vulnerable to ROS, which then accumulate and cause an aged phenotype. For example, a reduction in the antioxidant manganese superoxide dismutase (MnSOD or SOD2) in *Drosophila* mutants led to reduced longevity, accelerated senescence, increased neuronal degeneration, and double stranded breaks in the DNA of neurons (Paul et al., 2007). Interestingly, this model did not work in all organisms, as *C. elegans* that were experimentally engineered to express five different superoxide dismutase isoforms

individually did not lead to enhanced longevity (Doonan et al., 2008; Orr and Sohal, 1994). However, longevity was extended in *Drosophila* expressing three versions of the antioxidant simultaneously (Doonan et al., 2008; Orr and Sohal, 1994).

There is also a link between changes in organ function as a result of age and oxidative stress. For example, Cook-Weins and Grotwiel (2002) tested *Drosophila melanogaster* with behavioural assays and found an age-related decline in olfactory avoidance and motor behaviour but no change in both their ability to avoid aversive stimuli such as shock, and in their orientation toward attractive stimuli such as light (Cook-Wiens and Grotewiel, 2002). Interestingly, this change in behaviour through aging was observed in both control flies and the long-lived *methuselah* (*mth*) flies that have enhanced resistance to oxidative stress. Therefore, the mechanism by which these flies resist stress does not reverse the effects of aging on all behaviours. Others found a dissociation between resistance to stressors and longevity because removal of a key substrate in the insulin/insulin growth factor signaling pathway resulted in increased lifespan but did not affect stress resistance (Clancy, 2001). As such, the relationship between increased stress resistance and senescence remains unclear.

Mitochondria are the primary target of this oxidative stress because they contain the main site of respiration, the electron transport chain, which is a key generator of ROS. Additionally, mitochondrial DNA (mtDNA) do not have histones that are present in nuclear DNA that can act to shield DNA from ROS (Amaral and Ramalho-Santos, 2009). Therefore mitochondria are both the source and target of ROS damage. The mitochondrial theory of aging links ROS generated by mitochondria and the aging phenotype because there is an increase in the amount of ROS modified proteins and lipids with age, however the exact mechanism of this process has been debated (Amaral and Ramalho-Santos, 2009; Baraibar et al., 2012). Additionally, one study looked at the link between ROS (hydrogen peroxide) generated by mitochondria in long-lived (>15 years) and short-lived (<10 years) colubrid snakes (family Colubridae) and found that long lived snakes had reduced peroxide levels, possibly contributing to their enhanced longevity (Robert et al., 2007).

1.1.3 The Cellular Aging Process

Some of the many cellular changes associated with aging include changes to the genome, telomere shortening, changes in epigenetic marks as a result of interaction with the environment, and changes to protein stability (López-Otín et al., 2013). Many of these macromolecules, pathways, or processes contribute to the aged phenotype in many organisms. To limit what could be a very long section, I am choosing to focus on those pathways that have been previously linked to an increase or decrease in lifespan of an organism and can be targeted in the lab.

One of the main pathways involved in the aging process is the Insulin/ Insulin Growth Factor (IGF) signaling pathway. This essential pathway, that can cause diabetes in knockout mice, also contributes to aging in several model organisms. Indeed, specific manipulations within the pathway can lead to enhanced longevity through changes in gene expression by changing different Forkhead box O (FOXO) transcription factors and heat shock transcription factors (HSF-1) (Garigan et al., 2002). These transcription factors further up-regulate or down-regulate stress response genes, genes encoding microbial peptides, chaperons, and lipases that work in complex pathways to lead to the aged phenotype (Kenyon, 2010). For example, *C. elegans* that is deficient in the FOXO ortholog daf-2 can only enhance longevity when autophagy, the recycling of organelles, is possible (Meléndez et al., 2003). Other proteins, such as NAD+ -dependent protein deacetylases known as sirtuins are involved in longevity can also act through this pathway to extend longevity, as seen in the overexpression of sir-2.1 in *C. elegans* (Berdichevsky et al., 2006). Increased longevity through this pathway has even been seen in humans who have a variant of AKT and FOXO3A (Pawlikowska et al., 2009).

Target of rapamycin (TOR) kinases are downstream of the insulin receptor. TOR kinases sense amino acids and nutrients and are involved in stimulating growth and blocking pathways like autophagy when there is enough food (Kenyon, 2010). TOR works by regulating gene expression and can increase translation when activated by different nutrients. TOR can also inhibit translational inhibitors and activate the ribosomal subunit

S6 kinase. The inhibition of the targets of TOR have been shown to extend lifespan in *C. elegans* (Vellai et al., 2003), mice (Miller et al., 2014) and *Drosophila* (Kapahi et al., 2004), each through pathways distinct from the Insulin/IGF pathway.

Another pathway involves AMP activated protein kinase (AMPK) that has been linked to extension of lifespan by sensing energy levels, blocking energy consumption, and activating energy production (Ostojić et al., 2009). Overexpression of AMPK has been shown to extend lifespan in *C. elegans* and activation of this pathway through drugs such as metformin, used in the treatment of diabetes, can increase longevity in mice (Anisimov et al., 2008; Apfeld et al., 2004).

In addition, the electron transport chain has been implicated in lifespan extension in several model organisms (Copeland et al., 2009; Rea et al., 2007). It has been shown that the RNAi knockdown of respiratory complexes I, III, IV and V each have led to the extension of lifespan in *Drosophila*, where just the knockdown of complex I was sufficient to cause longer life (Copeland et al., 2009). Interestingly, the RNAi knockdown of Complex I increased longevity without reducing fertility, increasing resistance to other stressors, or reducing ATP production. It is thought that lifespan extension through respiratory inhibition is due to the activation of genes that up-regulate substitute energy production pathways when mitochondria are impaired and is due to improved oxidative stress response (Cristina et al., 2009). These alternate pathways suggest conserved underlying mechanisms of energy production in different organisms that are present to compensate for the loss of energy production in one pathway.

Finally, although telomeres are often first thought of when discussing cellular aging processes, telomeres cannot extend longevity in somatic tissues, as the enzyme telomerase is not present. Some authors even suggest that if telomerase were present in these tissues, cells would most likely develop cancer as opposed to demonstrating extension of longevity and thus lifespan would only increase in organisms that could resist cancer (Kenyon, 2010). Additionally, and importantly for this project, the structure of *Drosophila* telomeres is different from eukaryotic telomeres and they are maintained

by a unique transposition mechanism and thus cannot be used as a measure of aging or be attributed to damage associated with aging (Louis, 2002).

1.2 Social behaviour

The purpose of this section is to define social behaviour and review the literature regarding the evolution of social behaviour. In later sections I will bridge the concept of aging with social behaviour by describing what is known regarding changes to social behaviour with age. For the context of this project, I then describe what is currently known about the inheritance of the aging process and how behavioural changes can also be transmitted to the next generation.

1.2.1 What Is Social Behaviour?

Behaviour can be defined as a coordinated response of an individual or group to a stimulus, whether internal or external, that cannot be attributed to developmental stages of life (Levitis et al., 2009). Thus, social behaviours are responses to specific stimuli. Evolutionarily, working in groups was beneficial to both the individual and the entire group and thus specific social behaviours arose (Levitis et al., 2009). As such, cooperative individuals in a group were more likely to survive and reproduce, which then began the social behavioural network in response to specific cues from other individuals in that group or in the surrounding environment (Kokko et al., 2001). These group interactions often involve members of the same species and thus the social group is composed of like animals.

Social cues are contextual information generated by one member of a group that inform the behaviour of other members of the group (Kacsoh et al., 2015; Raven et al., 2005). For example, animals that were not exposed to a predator would adjust their behaviour as though they were exposed by observing and learning from others who have been exposed to the predator (Kacsoh et al., 2015). Cues may also be in the form of a chemical signal. For example, in *Drosophila,* the transfer of the hydrocarbon cis-vaccenyl acetate (cVA) from males to females during copulation (social behaviour) can act as a long-range attractant to promote aggregation of a group (social behaviour) (Bartelt et al., 1985).

These social cues must then be integrated with internal physiological information in order to generate a behavioural output (Rubenstein and Hofmann, 2015). While the behavioural outputs may be different among animal groups, the underlying biological function (and metabolic need) that drives the behaviour is shared among different species (Figure 1.1). For example, the *egr1* gene is important for song recognition in the zebra finch (*Taeniopygia guttata)* but is needed for dominance behaviour in cichlid fish (*Astatotilapia burtoni*) (Robinson et al., 2008). Because there are conserved underlying pathways of social behaviour, we can use model systems to study basic social behaviours in order to understand these networks and mechanism to inform the behaviour of more complex animals. In short, while the behaviours may not be translatable, the underlying mechanism of the pathways determining social behaviour might be conserved.

Some examples of commonly studied social behaviours include foraging behaviour (Tinette et al., 2004), aggression among males (Zwarts et al., 2012), courtship (Chandra et al., 2001), and divisions of labour (Robinson, 1992). Many of these behaviours require a neural decision-making network in order to interpret environmental social cues and generate a proper social response (Pasquaretta et al., 2016; Sokolowski, 2010). One of the most commonly observed social behaviours, and of interest to this project, involves the interactions within aggregates of individuals, and their social spacing (Giuggioli et al., 2013; Simon et al., 2012). Some examples of aggregation include schools of fish, swarming insects, and flocks of birds (Emlen, 1952; Mogilner et al., 2003).
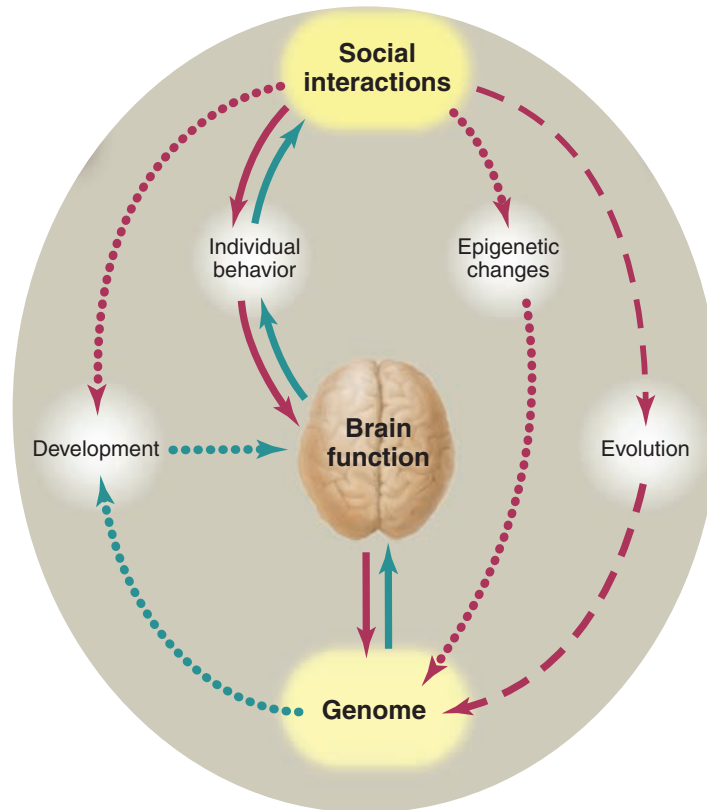
Figure 1.1 Social behaviours are fine responses to specific environmental stimuli that can vary based on species, the development of the brain, and epigenetic modifications to evolutionarily conserved pathways. The brain must integrate sensory information from its social environment and interpret this information in pathways influenced by the genome, epigenetics, and development to create an appropriate response (Robinson et al., 2008). This figure was adapted and used with permission granted from the author (personal communication with Dr. Gene Robinson, University of Illinois at Urbana-Champaign).

1.2.2 Social Versus Non-Social Behaviour

Sociality is a spectrum ranging from solitary to eusocial, the most social form of an animal. This is a spectrum because some animals can be exclusively solitary, meaning they do not engage in social behaviours other than those related to mating, or may oscillate between solitary and social, known as facultatively social. Some animals are rarely solitary, such as birds, but many mammals and amphibians are exclusively solitary (Reser, 2014). Solitary behaviour, in addition to various levels of social behaviour, have conserved underlying genetic pathways (Trivers, 1971). Interestingly, some researchers have compared the neural network of solitary animals to individuals with disorders that

have a social deficit, such as the autism spectrum disorders, as one possible way to understand the formation of neural networks in a brain that interprets social cues differently (Reser, 2014). Within animal sociality, eusocial species are the most social. Some examples of these include honeybees, ants and the naked mole rat (Keller and Genoud, 1997). A table outlining the spectrum of sociality can be found in Table 1-2.

Although insects are not typically described as social animals, both honeybee (*Apis mellifera*) and *Drosophila* have been used in social behavioural studies. Honeybees are described as a eusocial species and have been the prevailing model for social behaviour research. However, the parasocial genus *Drosophila* has recently started to be used in behavioural studies as well. For example, previously untrained *Drosophila* females will adjust their preference for different media for oviposition based on observing previously trained *Drosophila*'s specific preferences (Battesti et al., 2015). This display of social learning in a parasocial species indicates that different types of insects can be used as tools to study social interactions and the underlying internal mechanisms or pathways that generate social behaviours.

Table 1-2 Sociality can be divided into groups depending on the type of behaviour displayed

			Presocial			
				Parasocial		
Behaviour	Solitary	Subsocial	Communal	Quasisocial	Semisocial	Eusocial
Division of labour	✗	✗	✗	✗	✔	✔
Reproductive caste	✗	✗	✗	✗	✔	✔
Cooperative brood care	✗	✗	✗	✔	✔	✔
Occupy same space	✗	✗	✔	✔	✔	✔
Overlapping generations	✔	✗	✔	✔	✗	✔
Parental investment	✔	✔	✔	✔	✔	✔

Typical behaviours that are evaluated when categorized species as different forms of sociality ranging from solitary to eusocial, or purely social organisms. Each check mark (✔) indicates the presence of that behaviour in that type of social group where a cross (✗) indicates an absence of behaviour. The red check marks indicate that there is conflict regarding whether this behaviour is included in this type of social group. Presocial and parasocial are terms that describe several groups of sociality. Presocial and parasocial animals are broader terms for several social groups. *Drosophila* is in the presocial/parasocial category of species as they does not usually have cooperative brood care (Table adapted from the text of Gadagkar, 1987).

1.2.3 Evolution Of Social Behaviour In Groups

Animals evolved social cognitive frameworks in order to understand, evaluate, and respond to many social cues in the environment (Weitekamp and Hofmann, 2014). But how is the brain able to evolve novel structures to facilitate new behaviours that lead to the creation of the social brain? The complexity of the nervous system has been an integral factor in the evolution of the social network (Katz, 2011). It has also been suggested that certain behaviours exist in order to allow for novel brain patterns and behaviours to be introduced and included in the social network (Katz, 2011). Therefore the brain is considered to be a system with high evolvability, which allows for the nervous system, and thus behaviour, to adapt to the environment and increase in complexity.

Social behaviour may have also arisen through altruistic behaviours between kin. Investing resources and aiding in the survival of siblings' progeny would indirectly increase one's own fitness as shared genes between family members would still be transmitted to the next generation; therefore this system is beneficial even if the individual dies (Hamilton, 1964; Plomin et al., 2013). This may explain why an individual would engage in altruistic acts that do not directly benefit the individual and may further explain the occurrence of social behaviour networks in families and other groups. However, not all social groups engage in cooperative brood care, so an alternate explanation for the evolution of group formation may be due to the availability of resources when individuals of a group work together and the combined protection from predators in larger groups (Rohlfs and Hoffmeister, 2004).

Robert L. Trivers introduced a theory about the evolution of social behaviour between individuals known as reciprocal altruism (Trivers, 1971). This theory explains reciprocity between individuals of either the same or different species that are not related (Rankin and Taborsky, 2009; Trivers, 1971). Reciprocity often involves animals, mostly primates, sharing food and participating in grooming behaviour (Gomes et al., 2009). Through aiding non-relatives, individuals create a mutually beneficial social framework where resources are acquired as a group, rather than individuals expending energy to find

all their resources alone (Gomes et al., 2009; Taborsky, 2013). Individuals who were able to reciprocate with others were likely to be selected for and therefore the social network in the brain that allowed for cooperation was maintained.

One type of reciprocity, generalized reciprocity, is based on immediate previous experience and essentially involves helping others who have just helped you (Rankin and Taborsky, 2009). One example of generalized reciprocity has been seen in common vampire bats (*Desmodus rotundus*) who will share blood meals with non-relatives and have been shown to be more likely to receive a blood meal in return in the future from those with whom they have shared (Carter and Wilkinson, 2013). Alternatively, indirect reciprocity is reserved for animals with higher cognition who will remember previous encounters and will reciprocate only with those who have helped them (Rankin and Taborsky, 2009). Animals who engage in indirect reciprocity have been shown to invest in equal sharing over long periods of time (Rankin and Taborsky, 2009). Reciprocity can also give rise to cooperation, even in absence of advanced cognitive abilities, according to computer simulations (Barta et al., 2011). Reciprocity leads to cooperation in simple steps when the benefits far outweigh the costs in a decision-making framework (Barta et al., 2011).

1.2.4 Social Behaviours Can Be Assessed In Many Organisms

The study of social behaviour connects organisms with their environment and allows for the inclusion of many fields of biology, including neuroscience and physiology, as individuals must integrate external information and generate an appropriate response. Models can also be used to study simple or basic behaviours to understand the underlying behavioural mechanisms that can be found in animals with more complex nervous systems and more complex behaviours (Sokolowski, 2010). For example, behavioural responses to specific odorants helped to shed light on specific odor pathways within mice (Saraiva et al., 2016).

Various social behaviours observed in nature can be quantified by robust methods, allowing for the study of basic behaviours and manipulations of the environment to see

the impact on these behaviours. For example, the complex behaviour of bonding between individuals has been measured in the prairie vole (*Microtus ochrogaster*) by quantifying the attachment between partners with the partner preference test (McGraw and Young, 2011). Additionally, male dominance behaviour can be observed in species with hierarchical structures like birds or those with non-hierarchical structures like fish (Bayly et al., 2006; Desjardins et al., 2012). Recently, video tracking software of mice has allowed researchers to quantify various social behaviours in a cage at the same time, permitting rapid and robust measurements of social mice (Hong et al., 2015). These tools in the lab allow for quick and accurate accounts of social behaviours in animals with stereotyped behaviours in groups, which until recently have only been available in larger social animals.

Several basic social behaviours can be quantified in *Drosophila.* One of these behaviours, and of particular interest in this project, is the social spacing between individuals in a group (Simon et al., 2012). Social spacing must first take place prior to other behaviours such as aggression, courtship (Dankert et al., 2009), and social avoidance of stressed flies (Fernandez et al., 2014), all of which can be quantified. Often these measures involve the researcher or an automated process scanning for stereotyped behaviours. For example, the researcher can map the stages of courtship, count the number of physical interactions between males during aggression, or quantify the space between neighbouring individuals in social space. These methods are often robust and can assess the genetic and environmental contributions to test the effects on behaviour.

1.3 The Brain And Social Behaviours Change With Aging

1.3.1 Changes To The Brain With Age

As discussed above, aging causes many cellular changes. The cells of the brain are no exception, and thus aging could affect how individuals perceive and react to social situations (Morrison and Hof, 1997). It is important to note that the change to the brain associated with age is not due to neuronal death, as previously suggested, but to structural and morphological changes (Morrison and Hof, 1997). Neuronal changes with age can be accompanied by a reduction in the number of dendritic spines, as seen in non-human

primates with a reduction of 28-37%, in addition to reductions in spine density and loss of synapses (Duan et al., 2003; Page et al., 2002). Changes in neuronal morphology can lead to changes in communication ability between neurons resulting in a loss of integration between different parts of the brain over time and changes in communication to other tissues like muscle (Bishop et al., 2010; Yeoman and Faragher, 2001). This has been seen in aged rhesus macaques that show a reduction in the inhibitory neurotransmitter γ-aminobutyric acid (GABA) in the prefrontal cortex. Additionally, an analysis of transcriptomic changes with age in human and mouse revealed that a large number of genes that have altered expression patterns with age were found in three cell types in the brain: astrocytes, neurons, and oligodendrocytes (Loerch et al., 2008). However, the overall synapse number and neuron number was unchanged in the aged brain (Loerch et al., 2008). In *Drosophila*, researchers also found a reduced number of synaptic connections including fewer branches in 30-day-old flies (Corfas and Dudai, 1991). These changes to physical brain structures may be implicated in the changes in behaviour that are seen over time, perhaps as a programmed change in the brain due to functional senescence.

1.3.2 Changes To Social Behaviour With Age

Human social networks and behaviours have been shown to change with age. As we get older, we will often actively try and reduce the size of the group with which we interact. This is thought to be due to changing roles in society and family, and possibly due to physical limitations, with age (Charles and Carstensen, 2010). However, some researchers suggest that we actively try and reduce the size of our social network such that occasional and non-mutually beneficial relationships are eliminated and only interactions that will be meaningful and beneficial remain (Charles and Carstensen, 2010). These studies, however, only evaluate the social relationships among aging humans, and do not look at molecular changes associated with aging that may also impact social behaviours.

Animal models have been used to demonstrate the change in social behaviour with aging or when parents have been aged. For example, in *Drosophila*, it has been shown that 23% of genes change with aging and certain genes that affect lifespan such as *fragile X* and

SOD2 affect behaviours like climbing and olfactory avoidance (Iliadi and Boulianne, 2010). Social behaviours have also been shown to change over time. For example, honeybees have been shown to adjust their social behaviour inside of the hive (less than two weeks old) versus outside the hive (adults, greater than two weeks old; Ben-Shahar et al., 2002). These changes were accompanied by changes in gene expression, hormones, and brain chemistry to facilitate this timed change (Ben-Shahar et al., 2002). In *Drosophila*, the expression of courtship genes declines with aging (Ruedi and Hughes, 2009). Importantly, not all behaviours change with aging, as seen in *Drosophila* that continue to respond and avoid an electric shock when aged (Cook-Wiens and Grotewiel, 2002; Simon et al., 2006).

1.4 Inheritance of the Aging Process and Neuropsychiatric Disorders

Aging can physiologically change an individual and, if these changes occur at the level of the gamete, they can be passed on to the next generation. Epigenetic marks such as methylation of DNA or histones can be inherited from parents in humans if they are not erased during the two global demethylations that take place; one demethylation program occurs immediately following fertilization to remove any existing epigenetic marks that were not removed during fertilization and the other occurs in the germline, whereas parental marks are removed from somatic tissues (Heard and Martienssen, 2014). While epigenetic inheritance of methylation patterns are often paternally derived (Smith et al., 2009), other types of epigenetic inheritance can be in the form of non-coding RNA such as lncRNA, miRNA, siRNA and piRNA that are often, but not exclusively, inherited maternally (Heard and Martienssen, 2014). Each of these mechanisms can interfere with the regulation of transcription or transcription itself thus imposing maternally or paternally derived patterns.

Aneuploidy is the addition or subtraction of the normal chromosome number and is a common age-related problem that can be inherited from both parents. Aneuploidy of the 21st chromosome in humans is most often inherited from the mother, but researchers have found up to 10% of trisomy 21 cases originating from the father (Sloter et al., 2004).

Interestingly, while both aged parents have higher incidence of trisomy or autosomal chromosomes, such as trisomy 22 that can be inherited from either parent, sex chromosome aneuploidy is often inherited from the father (Sloter et al., 2004).

Transmissible effects of maternal age have mostly been identified in terms of chromosomal aneuploidy on the autosomes and changes in crossover (Halle et al., 2015; Hunter et al., 2016; Marchetti and Wyrobek, 2005). Conversely, most *de novo* mutations have been found to originate from the father as males contribute to 3.9 times more single nucleotide mutations than females to their offspring and have been shown to contribute more copy number variants (CNV) than females (Kong et al., 2012). Other mutations include point mutations, structural rearrangements, sex chromosome aneuploidy, abnormal imprinting, single and double stranded breaks or DNA or protamine adducts (Marchetti and Wyrobek, 2005).

Advanced paternal age (APA) is one factor that has been associated with increased mutational load in humans (accumulation of mutations), as by age 40 spermategonia have undergone hundreds of divisions (200-660 divisions by age 40) (Malaspina, 2001; Saha et al., 2009; Sampino et al., 2014). Male germ cells are also more susceptible to the accumulation of mutations because their capacity to repair DNA declines in later (post-meiotic) spermiogenesis, so mutations introduced later in germ cell formation cannot be repaired (Marchetti et al., 2007). For example, Sanger sequencing revealed 4,933 *de novo* mutations introduced into the offspring from older fathers where 2.2 mutations per trio of nucleotides were deleterious (Keightley, 2012). The inability to repair damage introduced post-meiotically also makes sperm formation highly susceptible to repeated environmental stressors such as damage due to smoking which can then be passed to the next generation (Marchetti and Wyrobek, 2008). Interestingly, the connection between the accumulation of mutations with sperm divisions over time and reduced DNA repair capacity has been implicated in the fathers of children with Apert syndrome (Glaser et al., 2003). Younger males with affected children had a higher mutational frequency at younger ages that was then not fixed post-meiotically (Glaser et al., 2003). These unrepaired mutations in male germ cells may not persist upon fertilization of the egg as repair machinery within the egg has the capacity to repair some of these changes.

However, one study found that when chromosomal changes were induced within male germ cells, they persisted for several weeks as the cells went through the DNA repair deficient phase but, upon fertilization, the lesions were converted into chromosomal aberrations by the maternal DNA repair machinery in the zygote (Marchetti et al., 2015). Therefore, the damage may not be erased even when egg DNA repair machinery is present. Animal models should thus be used to investigate these questions of what is specifically affected in the gametes with age and how this affects the next generation.

APA has been implicated as one potential cause of several sporadic human autosomal dominant diseases such as Apert syndrome, Progeria, and Achondroplasia, in addition to neuropsychiatric disorders such as Autism Spectrum Disorders (ASD) and Schizophrenia (D'Onofrio et al., 2014; Malaspina et al., 2001). It is important to note, however, that advanced parental age is not the only source of these disorders, and is simply a proposed mechanism by which some of these disorders may manifest. In mice, progeny with old fathers (15 months) had communication problems, repetitive behaviours, social deficits, and anxiety; phenotypes which resemble those found in ASD (Sampino et al., 2014). Additionally, when male mice are aged to 12 months, their offspring were more social than offspring of two-month-old males when maternal age was kept constant (Janecka et al., 2015). Interestingly, in humans, grandpaternal age can negatively affect grandchildren even when the parents of the grandchild are young, however there are little data to date on how long this effect will be transmitted through the generations (Frans et al., 2013).

One explanation for why APA is mostly linked to certain types of neuropsychiatric disorders is that genes that form synapses in the brain are longer genes and therefore have increased probability of acquiring a mutation by chance with aging (King et al., 2013). Since these neuropsychiatric disorders are often disorders of the synapses, they are referred to as synaptopathies (Grant, 2012). For example, alteration of the post synaptic adhesion protein neuroligin or pre-synaptic adhesion protein neurexin will impair synaptic development and transmission, which in turn will affect the balance of excitatory and inhibitory signals in the brain. Changes to the balance of these signals in the brain can result in changes to social behaviours, including courtship songs and social

spacing in *Drosophila* (Hahn et al., 2013), or repetitive behaviours observed in mice (Blundell et al., 2010). Interestingly, the human homolog of the *Drosophila* neuroligin genes have been associated with disorders of social behaviour, such as ASD (Jamain et al., 2003). Additionally, a recent study found 27 recurrent double stranded break clusters located in the middle of long genes that encoded mostly synaptic proteins and cell adhesion molecules, where almost all genes were linked to ASD, Bipolar Disorder, Intellectual Disability, or Schizophrenia in either humans or mice (Wei et al., 2016). It is possible that reduced repair capacity with aging may be contributing to these double stranded breaks leading to neuropsychiatric disorders in the next generation. Finally, abnormal gene dosage or quantity of synaptic protein associated with changes with aging of the parent may also be contributing to the disease phenotype of ASD in the progeny (Toro et al., 2010).

1.5 *Drosophila* As A Model Organism For Aging And Social Behaviour

Drosophila melanogaster, commonly known as the fruit fly is a widely used model organism due to its fast generation time, its ability to be easily genetically manipulated, and its relative affordability to house (Lints and Soliman, 1988). The quick generation time is additionally beneficial because different generations may be studied in tandem. *Drosophila* also has very well defined genetic tools at our disposal for its genome of ~18,000 genes (Adams et al., 2000; Attrill et al., 2016). *Drosophila* has been used extensively in genetic research and has been the model involved in five Nobel Prizes in Physiology and Medicine (Jennings, 2011). The 1933 prize was awarded to Thomas Hunt Morgan for his work on the role of chromosomes in heredity. The 1946 prize was awarded to Hermann J Muller for his work with X-ray induced mutations. The 1995 prize was awarded to Edward B. Lewis, Christiane Nüsslein-Volhard and Eric F. Wieschaus for identifying important and conserved genetic mechanisms that control embryonic development. In 2004, the Nobel Prize was awarded to Richard Axel and Linda B. Buck for their identification of odorant receptors and how the olfactory system is constructed. And finally, in 2011, the prize was awarded to Bruce A. Beutler, Jules A. Hoffmann, and Ralph M. Steinman for their work on adaptive and innate immunity.

As many basic mechanisms and pathways have been conserved through evolution, a simple model organism such as *Drosophila* is used to study basic processes that are evolutionarily conserved throughout the animal kingdom, and even human mechanisms and the defects that result in disease. Indeed, almost 66% of disease genes in humans can be found in the form of a homolog in *Drosophila*, according to the Online Mendelian Inheritance in Man (OMIM) database (Lee et al., 2014; Okray and Hassan, 2013).

1.5.1 Life Cycle Of *Drosophila*

Drosophila has a four part life cycle beginning with the egg stage followed by larval, pupal, and finally the adult stage (Jennings, 2011; Hartwell et al., 2011). At a physiological temperature of 25°C, the fertilized egg laid on or near the surface of a food source will develop into the embryo for about 24 hours before becoming a larva. The larva will then undergo three molts as it eats the food source and grows for about five days before becoming a pupa. The process of pupation to metamorphosis, where embryonic and larval tissue is replaced with adult tissue, will take about four days. This process requires a total of ten days, but can be slowed by keeping stocks at a lower temperature of 18°C, where the life cycle will take up to 28 days, as opposed to around ten days at 25°C. As shown later in this thesis, and by others before, *Drosophila melanogaster* can live around three months at 25°C.

1.5.2 *Drosophila* Neurobiology

The common ancestry of *Drosophila* and humans had a similarly complex central nervous system, similar subdivisions of the brain (protocerebrum, deutocerebrum, and tritocerebrum in *Drosophila* are evolutionarily similar to the forebrain, midbrain, hindbrain of humans), similar neurotransmitters, and common behaviours (O'Kane, 2011). Although *Drosophila* has a fraction of the number of neurons as humans (10^5 vs. 10^{11}), *Drosophila* has many of the same cell types such as neurons and glia and exhibit the most basic behaviours required for survival, including sleep, courtship, response to stimuli and drugs, learning, memory, circadian behaviours, and social behaviours

(Jennings, 2011; O'Kane, 2011). Importantly, many molecular mechanisms underlying these behaviour are conserved between humans and *Drosophila* (Sokolowski, 2010).

1.5.3 Social Behaviour of *Drosophila*

Drosophila can be described as a social species according the definition that describes sociality as interactions with others and their kin (Gadagkar, 1987). As described above, *Drosophila* does not have a clear division of labour and parents do not participate in cooperative brood care and is therefore considered parasocial. *Drosophila* has a repertoire of behaviours that is affected by social experiences, and some direct measurements of socialization have been performed (Branson, 2009; Dankert, 2009; Fry, 2008; Schneider, 2012; Simon and Dickinson, 2010; Slawson, 2009; Wang, 2008). Most of the efforts to measure social behaviours in *Drosophila* have been focused on relatively complex social behaviours, such as aggressive interactions (Dankert, 2009; Wang, 2008), various aspects of courtship (Dankert, 2009; Ejima and Griffith, 2008; Mery, 2009; Miyamoto and Amrein, 2008; Montell, 2009; Villella, 2008), and how social experience affects other behaviours such as learning, or circadian rhythm (Billeter, 2009; Ganguly-Fitzgerald, 2006; Kent, 2008; Krupp, 2008; Levine, 2002). In the context of this project, I will study the spatial distancing that occurs during group formation and social spacing. One other behaviour I will study is the ability of *Drosophila* to avoid the aversive stimulus *Drosophila* stress odorant (dSO) in a binary choice assay.

 However, we do need to be aware of some limitations regarding *Drosophila* as a model for social behaviour. The main limitation is that *Drosophila* has species-specific behaviours and although the underlying circuits determining social response may be similar among different organisms, the behavioural output may vary. Therefore, a better understanding and characterization of *Drosophila*'s behaviour and ecology must be elucidated in order for it to be a well-rounded model for social behaviour. There must also be a greater effort put forth by the community to design new assays that are ecologically relevant and not limited by laboratory conditions, which will also inform why these behaviours exist in the wild.

1.5.4 Social Spacing In *Drosophila*

Our lab has previously studied factors affecting social space in *Drosophila*. For example, social experience was shown to affect social space, as socially isolated flies were more distal and therefore less social and this effect was more pronounced in females than in males (Simon et al., 2012). Additionally, the mating status of the fly also affected social space, as both virgin male and virgin females were less social (Simon et al., 2012). Factors unrelated to sociality also affected social space of groups. For example, a mutant for the *white* (*w*) gene that affects vision and the pigmentation of the *Drosophila* eye or a mutant for the phototranduction machinery (*trp*^{301}), were each shown to be less social, as well (Burg et al., 2013; Simon et al., 2012). However, some conditions were shown to not affect social space, as changes to classical odor sensing did not cause *Drosophila* to behave less socially (Simon et al., 2012).

Social space can also be affected by changes in synaptic genes that encode proteins located on either the pre- or post-synaptic membrane. Some of these genes that can cause changes to social space when altered have also been proposed as candidate genes for neuropsychiatric disorders (Figure 1.2). For example, the human gene *neurobeachin*, which encodes a large scaffolding protein, has been identified as a candidate gene for ASD and mutants of the *Drosophila* homolog of *neurobeachin,* called *Rugose*, were shown to be less social (Castermans et al., 2003; Wise et al., 2015). Additionally, a class of post-synaptic membrane proteins called neuroligin, which has homologs in human and *Drosophila*, have also been associated with changes in social space and have been proposed as candidate ASD genes (Hahn et al., 2013). Members of the dopamine pathway including the tyrosine hydroxylase, vesicular monoamine transporter and the neurotransmitters dopamine and acetylcholine have recently been implicated in causing changes to social space (Fernandez et al., under review).

Finally, social space can be affected by exposure to environmental toxins. For example, increasing concentrations of the chemical Bisphenol A (linked to neurodevelopmental disorders and other health effects) resulted in *Drosophila* that displayed abnormal social response and was more proximal, in a dose dependant manner (Kaur et al., 2015). Thus,

many factors affect *Drosophila*'s social spacing including mating status, social enrichment, genes, and environmental conditions. However, the effect of chronological or biological age of *Drosophila* has not yet been considered and will be addressed in Chapter 3.
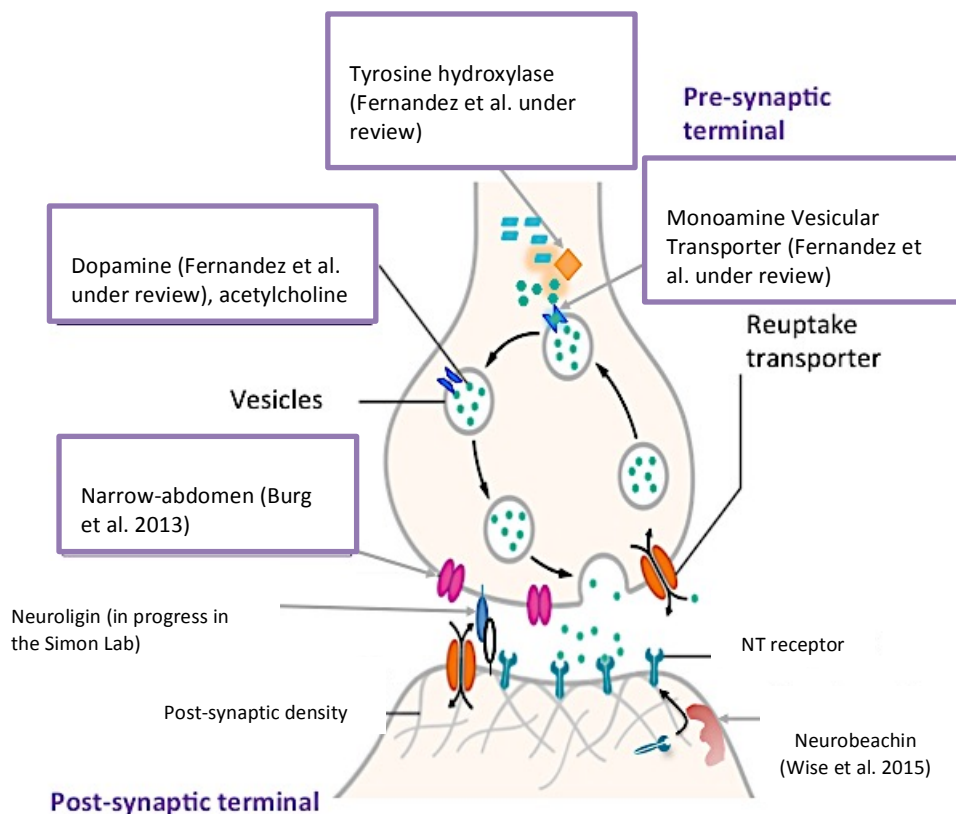
Figure 1.2 Several proteins and neurotransmitters have been implicated in causing changes to social spacing behaviour in *Drosophila*. These include the presynaptic proteins neuroligin, and narrow-abdomen (Burg et al., 2013), post synaptic scaffolding protein neurobeachin (Wise et al., 2015), and members of the dopamine pathway such as dopamine (neurotransmitter), VMAT (vesicular monoamine transporter), and tyrosine hydroxylase. Figure adapted from an original image by Thomas Splettstoesser (www.scistyle.com), permission granted on the website.

1.5.5 *Drosophila* As A Model For Aging Studies

Drosophila, along with other invertebrates such as *C. elegans* and *Aplysia,* has been used as a model for normal aging. Many researchers suggest that aging should not be studied from diseases that accelerate aging or cause premature aging, as these are not processes that affect everyone. Conversely, aging is inevitable given enough time in every member of a species. One main advantage of using *Drosophila* for aging studies is the large number of genetic tools available to manipulate the genome and turn gene expression on or off in a spatiotemporal manner (He and Jasper, 2014). This allows for the identification of single gene mutations, in addition to how, when, and where aging is affected throughout the body and how tissue interactions affect aging in order to identify tissue-specific functional decline.

However there are several limitations to studying aging in *Drosophila*. For example, we cannot study telomeres and their relation to aging because *Drosophila* do not have true telomeres but two non-LTR retrotransposable elements (Louis, 2002). These elements are a modified version of telomeres that help maintain the ends of chromosomes to avoid erosion but they are not involved in senescence (Louis, 2002). One other limitation or large difference to consider with *Drosophila* is that male and female gametogenesis are very similar to each other in that eggs and sperm are continuously made, whereas in humans that is not the case and only sperm are continuously made (Panagopoulos, 2012). Therefore it is difficult to make inferences or draw conclusions regarding degradation within the egg since they are continuously made. Additionally there is little DNA methylation so epigenetic inheritance of methylation patterns cannot be studied (Lyko et al., 2000). And finally, as mentioned above, animals do not survive to old age in the wild so the aging studies may not be entirely ecologically relevant.

1.6 Significance And Statement Of Purpose

In humans, problems associated with advanced age have recently become public interest as advances in modern medicine and human intervention have allowed us to extend our lifespan. As no prehistoric human remains have been found to be over 50 years old, we

can conclude that aging is a modern issue, especially as the proportion of individuals over 60 in Canada has risen to 24% (Beard et al., 2015; Hayflick, 1998). With increases in our aged population and the use of assistive reproductive technologies for individuals at older ages, the effect of aging on the next generation is an important area of research.

I first examine, in chapter 2, different methods of analyzing the spacing between individuals and the group and different modes of representation to determine the most efficient way of communicating the results of the social space assay. My first goal is to characterize the effects of parental age on longevity, fertility and social behaviours such as social spacing and social avoidance in chapter 3. I hypothesize that aging will have an effect on both aged parents and this effect will be passed on to the progeny as other behaviours have been shown to change with age and affect the genome that can be passed on to the progeny. In the third chapter I also address the effect of having only aged fathers on the progeny in social spacing to determine if one aged parent is capable of recapitulating the effect of aged parents on the progeny. Here, I hypothesize that having just an aged father will result in a change in social spacing in the progeny. My second goal is to determine whether changes biological or chronological aging are necessary to cause a change in social behaviour by manipulating the aging process and testing the effects on social spacing in the parents and first generation (chapter 4). I hypothesize that manipulating the biological aging both of parents and their progeny will result in an early aged phenotype (when biological aging is accelerated, individuals and their progeny will be less social) or a delayed aged phenotype (when biological aging is decelerated, individuals and their progeny will be more social) and that this effect will be passed on to the next generation.

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Chapter 2 – Analyzing And Representing Social Spacing In *Drosophila*

2   Abstract

I examined several ways of analyzing the distances between pairs and groups of flies in the chamber of the social space assay and various modes of representation. Most recently, the results of the social space assay have been represented using box and whiskers, although this has not been the most effective way to communicate the results. Therefore, I proposed a way to transform non-parametric data to parametric data such that I may represent the social space data as the mean with standard error to the mean. The three main measures used to quantify the social space assay are distance to closest neighbour, distance to all flies, and the number of flies within each body length radius away from a given fly, which may or may not yield the same trend. In the case of aging, I showed that these three measures do show the same trend and I therefore continue to use only one measure for the rest of this thesis for simplicity.

2.1   Introduction

Social interactions between individuals are important for other complex behaviours to take place, like courtship and feeding (Rohlfs and Hoffmeister, 2004). One fundamental form of social interaction is local enhancement, also known as aggregation (Simon et al., 2012). Aggregates form groups via short range repulsive cues and long range attractive cues, as shown through simulations of social groups (Mogilner et al., 2003). One of these long-range cues is often resource availability, like food. *Drosophila* has been shown to move only short distances when there is an abundance of food, but will move farther if resource quantities are limited (Lefranc et al., 2001). Group formation is advantageous as grouping has been shown to both lower the risk of parasitism with increased larval

population and increase the probability of finding a mate and food (Lof et al., 2008; Rohlfs and Hoffmeister, 2004).

Factors that contribute to social spacing will depend on the nature of the interaction between individuals and the group. Whereas courtship would promote closeness or more proximal interactions, mate competition or male aggression may promote distance or more distal interactions (Bretman et al., 2013). Social spacing and social avoidance may be emergent behaviours that arise in groups when individuals try to avoid others in the same group (Giuggioli et al., 2013). This may be achieved through stigmergy, where individuals leave a mark on the environment that is interpreted by another individual in the group, which then influences spatial orientation and distance to their nearest neighbour that can feedback onto the whole population (Giuggioli et al., 2013). Additionally, grooming behaviours and heard immunity would promote distances based on the status of the individuals. For example, regarding heard immunity, individuals who are not infected would be closer to the group and others may be isolated or even quarantined (Cremer et al., 2007; Evans and Spivak, 2010). Social spacing has been quantified in many animals such as in herds of sheep (Sibbald and Hooper, 2003), tribes of goats (Vas and Andersen, 2015), flocks of birds (Emlen, 1952), schools of fish (Larkin and Walton, 1969), and swarms of insects, including *Drosophila melanogaster* (Parrish and Edelstein-Keshet, 1999; Sokolowski, 2010). Oftentimes, the measure of distance is reported in terms of body-length units (Mogilner et al., 2003).

In *Drosophila melanogaster,* social spacing can be easily quantified with the social space assay (Simon et al., 2012; McNeil et al., 2015), that allows measuring the distance of one fly to its nearest neighbour, a variable recently used in several other studies (Anderson et al., 2015; Burg et al., 2013; Hahn et al., 2013; Kaur et al., 2015; Wise et al., 2015). As described in detail in McNeil et al. (2015), the assay forces flies into a group and allows flies to decide how close or far to settle away from others along the glass-panes of a two-dimensional triangular vertical chamber. When the flies are first added to the chamber they will try to escape, then they will begin to explore the chamber, and finally, they will decide where to settle in a stable group formation. We then take an image of this settled group and process the picture with the free software ImageJ (National Institutes of

Health, Bethesda, Maryland, United States) to measure the precise distances between neighbouring *Drosophila*, and use the statistical program GraphPad Prism (Prism version 7.00 for Mac, GraphPad Software, La Jolla California USA, www.graphpad.com) to determine significant differences (Schneider et al., 2012). Many other measures could also be performed, such as the orientation of flies within the arena or measuring changes through time (Hahn et al., 2013; Schneider and Levine, 2014). Although these methods have the capability to measure the group formation over time for every individual interaction, I chose a rapid method of identification that required less computer programming and analysis and is less expensive. These other methods may be used in the future if a change with age is found.

The data obtained follow a non-Gaussian distribution and are thus non-parametric. The data were therefore represented using either histograms (Burg et al., 2013; Simon et al., 2012), social space index (Burg et al., 2013; Simon et al., 2012) or box and whiskers (Kaur et al., 2015; McNeil et al., 2015; Wise et al., 2015). One limitation to using histograms is that they require the experimenter to arbitrarily define the bin size and is therefore open to user bias. Another previously used representation is the social space index, although it has been known to exclude large portion of the data and is therefore not an accurate representation of the results. Finally, although the box and whiskers representation is an accurate representation of the distribution of the social space of the individuals, it is ineffective at delivering the desired take home message of statistically significant differences. It is ineffective because overlapping adjacent boxes skew the visual interpretation and obscure the fact that even though there is an overlap in distribution, the medians and/or the internal variances can be significantly different.

Therefore, an alternate mode of accurate representation that is more visually effective will aid in the efficient delivery of the take home message of the data. Indeed, the box and whisker representation includes the flies that are far away from the group but have no actual impact on the median, which is what is actually tested with the statistical tests. But these far away flies skew the distribution strongly in a non-reproducible manner such that although the medians are highly reproducible, the means of the social space assay are not. Here, I thus show a method of transforming the original data points from a non-

parametric distribution to a parametric distribution by removing the statistical outliers, as described previously (Motulsky and Brown, 2006). This is also beneficial as statistical tests for parametric data are often more powerful (Conover and Iman, 2010). I will use a one-way ANOVA or student t-test to test for statistical significance between the means, instead of the whole distribution, as was previously compared when the data were non-parametric. One final way I represent the data is using violin plots, which are a form of box plot that also shows the probability density around a certain value, resulting in a structure that could resemble a violin.

I also show three ways data can be gathered from the social space images using in-house or published (McNeil, 2015) specially designed ImageJ macros: distance of each fly to its closest neighbour, distance between each fly and all other flies in the group, and the number of flies within each body length radius around each fly to interpret clustering patterns. Each of these measures gives different information about individual and group settling behaviour. The questions to address here are: does each measure follow the same trend? And if so, which measure is most effective at demonstrating the social spacing between flies?

2.2 Methods

2.2.1 Aging *Drosophila melanogaster* For The Social Space Assay

The laboratory control strain Canton-S *Drosophila melanogaster* was reared in mixed sex in bottles over Jazz Mix media (brown sugar, corn meal, yeast, agar, benzoic acid, methyl paraben and propionic acid; Thermo Fisher Scientific, Waltham, MA, USA). All flies were maintained at 25°C, 50% humidity with a 12:12 light: dark cycle. New bottles were made bi-weekly when the parents were less than seven days old. Every Monday, existing flies in bottles were removed to prevent new emerging flies cohabitating with their parents. Every Wednesday, flies were collected from the bottles (40 flies/ vial, seven vials/week) under cold anesthesia (Fernandez et al., 2014). Flies were transferred to new media in vials every two-to-three days and were maintained for up to seven weeks. Here I

used seven- and 30-day-old *Drosophila melanogaster* to demonstrate data analysis of the social space assay.

An unnamed mutant provided by a collaborator was used to demonstrate how different strains of flies might have different trends in social spacing between the nearest neighbours and between all flies in a group. Because this mutant was used in another yet unpublished study, it is not named here or described further. The data were collected by Sam Jolley and were the subject of an Honour's Thesis.

2.2.2 Social Space Assay And ImageJ Analysis

The social space assay was performed and analyzed using the free image processing software ImageJ and the accompanying macros as previously described (McNeil et al., 2015). In short, after flies were acclimated to the assay room (25°C, 50% humidity) for two hours, male and female flies were added via aspiration to the 2-dimensional vertical chamber composed of a series of glass and acrylic pieces that form a hollow triangle (15 flies per chamber, separated by sex, n=9 replicates). Flies explore the chamber before settling along the glass, which usually occurs around 20 minutes following addition to the chamber (Simon et al., 2012). Images of the chamber were then taken at 30 minutes and were analyzed using ImageJ. Each fly's distance to its closest neighbour, distance to every other fly in the chamber (denoted distance to all flies) and the number of flies within one-to-15 body lengths (average of 0.23 cm per male body length or 0.27 cm per female body length, denoted XY radius) were then gathered using specifically designed macros (denoted 1, 2, and 3, respectively) and all values were kept in a Microsoft Excel spreadsheet.

2.2.3 Analysis Of The Data From The Social Space Assay Using GraphPad Prism 7

The distances to the nearest neighbour by group were compiled into columns in Prism7 and represented as box and whiskers to observe the raw distribution. A Kruskall-Wallis non-parametric statistical test (three groups or more are compared) or Kolmogorov-

Smirnov (two groups are compared) was performed by ranking the values and evaluating the means of these ranks for significance. This was also performed for the distance to all flies and the number of flies per body length away from each fly.

In order to transform the data from non-parametric to parametric data, outliers were removed using robust regression and outlier removal (ROUT) analysis on the original data by fitting the data to a model with a robust method in which outliers do not impact the fitting to the model (Motulsky and Brown, 2006). The outliers were identified through the false discovery rate (FDR) where the value used, Q, is set to its lowest rate (Q=0.1%) and only data points that were very far from the rest of the data (as predicted by the model) were removed as definitive outliers. The resulting data were then put into a new spreadsheet in Prism 7 and analyzed using either a one-way analysis of variance (ANOVA, $p< 0.05$) with a Holm-Sidak post test to correct for multiple comparisons or a student t-test as the data can now assume Gaussian distribution. These data were represented using a line indicating the mean and bars showing the standard error to the mean.

2.2.4 Representation Of Data Using Violin Plots

Violin plots were generated using the raw data gathered from the nearest neighbour macro (macro 1). The website I used to generate this visualization can be found at http://boxplot.tyerslab.com as this method was not available in GraphPad Prism 7 (accessed June 2016). This method of representation shows the probability density of data at different values.

2.3 Results

In order to determine which mode of quantification of social spacing would best represent my data, I investigated several ways of measuring the social group. Figure 2.1 shows a schematic diagram of transforming the original image taken during the social space assay into a black and white image using the software ImageJ. This program can

measure the distance to every fly and its nearest neighbour to give information about pairs, each fly's distance in relation to every other fly in the group to give information about the whole group and finally the average number of flies located in each body length radius away from that fly to provide information about group formation. Each representation is shown as a box and whiskers representation with the horizontal line in the box representing the median and a "+" representing the mean. The box represents 50% of the data points, the whiskers each represent 15% of the data points, and beyond the whiskers (above and below) each represent 10% of the data points. Young flies tend to be two-to-three body lengths away from their nearest neighbour and that is what is shown here (Simon et al., 2012). In the case of this aging study, all three measures indicate that each 30-day-old fly is more distal to its nearest neighbour, every other fly in the chamber, or there are fewer flies within each body length radius away from that fly (XY radius). When performing non-parametric tests such as the Kolmogorov-Smirnov test, that assess the cumulative ranking of the values for significance, the two groups were significantly different in each of the three measures, although the significant difference in the XY radius only arose after four body length radii away from each fly.

Figure 2.2 shows an unnamed mutant and its control following the social space assay. Both the distance to the nearest neighbour and the distance to all flies are shown. Notably, the mutant's median and mean distance to the nearest neighbour is more proximal than control ($p < 0.01$), where the median and mean of the mutant in the distance to all flies is more distal ($p < 0.0001$), exemplifying a scenario where these measures do not show the same trend.

Figure 2.3 shows an additional mode of data visualization using the violin plot. The violin plots are used to represent the distances to the nearest neighbour at different ages to see if the values cluster around a specific distance. This representation is different from the box plot, as the box and whiskers will show the distribution with the size of the box but not where these values cluster. The seven-day-old males appear to have a more narrowed distribution indicative of the distribution within the social space chamber whereas the 30-day-old males have a wider and taller distribution indicative of a more

spread out pattern within the chamber, although this does not capture more information than was already provided by the box and whiskers in this case.

While the box and whiskers representation has been used in the past, Figure 2.4 shows how the raw data represented as box and whiskers can be transformed and shown as a mean and standard error to the mean by conservatively removing the definitive outliers (Q=0.1%, robust regression and outlier removal analysis). In this case, the mean distance to the closest neighbour of seven-day-old male flies before outlier removal was 0.85 ± 0.04 cm and 30-day-old flies had an average distance of 1.062 ± 0.05 cm with significant difference between the two groups (Kolmogorov- Smirnov non-parametric t-test p< 0.0001). Additionally, the median for seven-day-old flies was 0.61 cm and for 30 days was 1.06 cm. Following outlier removal, the seven-day-old males had an average distance of 0.70 ± 0.03 cm with a median of 0.54 cm and 30-day-old males remained on average 1.06 ± 0.05 cm and median of 1.06 cm as no outliers were found. The median for seven-day-old males change during this transformation as it is much closer to the mean value. I performed this same analysis on the distance of each fly to every other fly in the chamber and the number of flies within each body length radius away from that fly using sex-specific body lengths (average of 0.23 cm per male body length or 0.27 cm per female body length) and found no outliers in either data set among young and old flies. This may be because of the large data set for both groups (15 measurements per fly per chamber with nine chambers total) and thus all measurements were considered part of the normal variation.
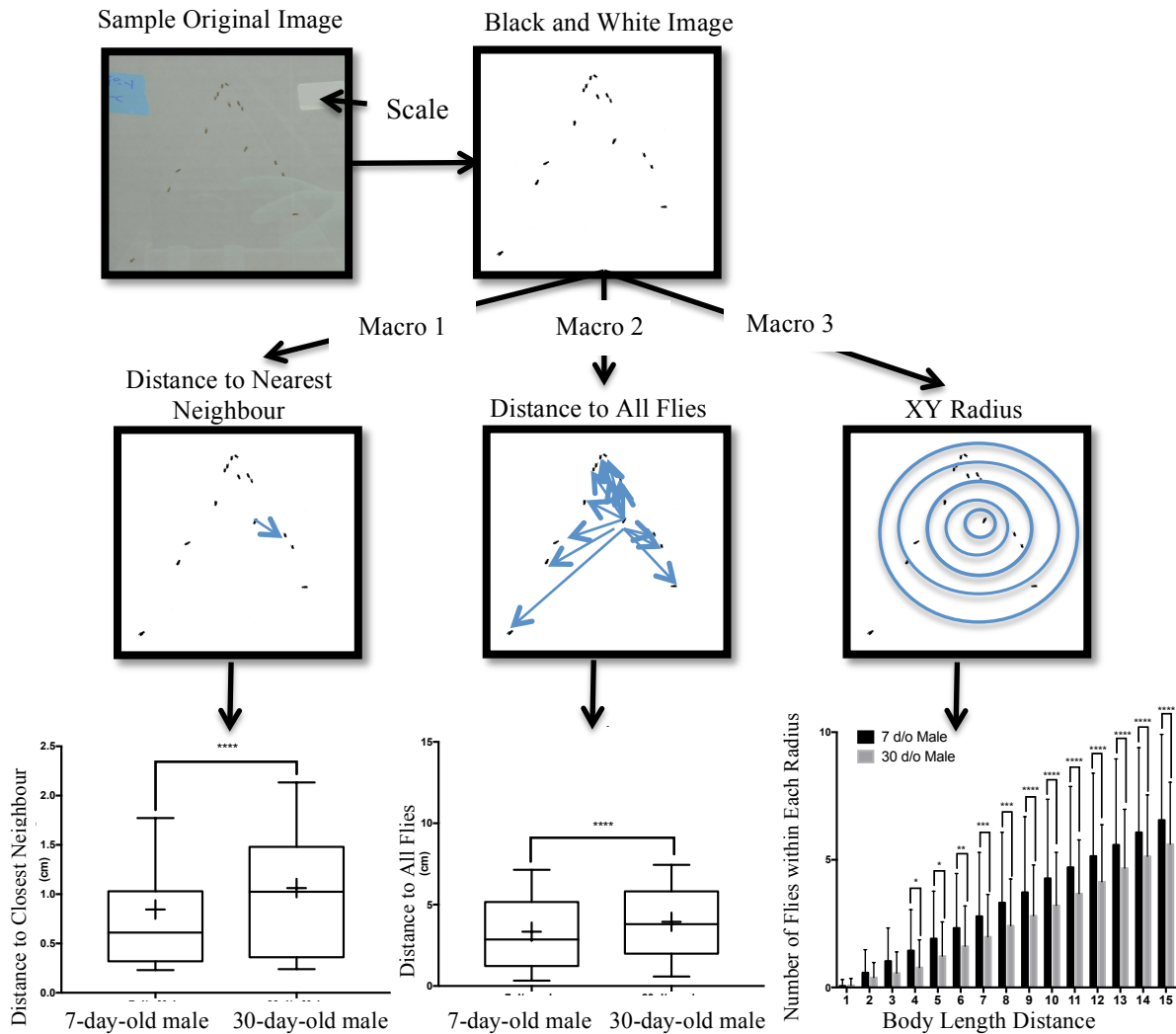
Figure 2.1 Schematic diagram of obtaining distance to nearest neighbour, distance of each fly to every other fly in the chamber (all flies) and the number of flies occupying each body length radius around each fly (XY radius) from the original social space image. The original image is converted to 8-bit to transform each fly into a black figure on a white background. The scale sticker indicates a constant width of 1.9 cm on each image to be used during analysis for consistency among images. Using previously designed macros, distances to the nearest neighbour (NN), all flies (AF) and how many flies are within body length radii (XY radius) are given as an output. These measurements are then put into GraphPad Prism 7 for representation and analysis, as shown at the bottom of the diagram in a box and whiskers representation for the NN (left) and AF (center) and mean with standard error to the mean (XY radius - right). The box represents 50% of the distribution and the whiskers include 10-90% or the data points while the mean is represented within the box as a horizontal line and the mean as a + sign. In the case of aging flies, older flies (30-day-old males) are significantly further apart from their nearest neighbour (****p<

0.0001) and from all flies in the group (****p< 0.0001). As for how many flies lie within each body length radius (0.23mm per male body size), the differences between seven-day-old and 30-day-old group size are only significant after four body length radii away, as older flies are further away from the group than younger flies (*p< 0.05, **p< 0.01, ***p< 0.001, ****p< 0.0001). Kruskall-Wallis non-parametric test with a Dunn's *post hoc* test.
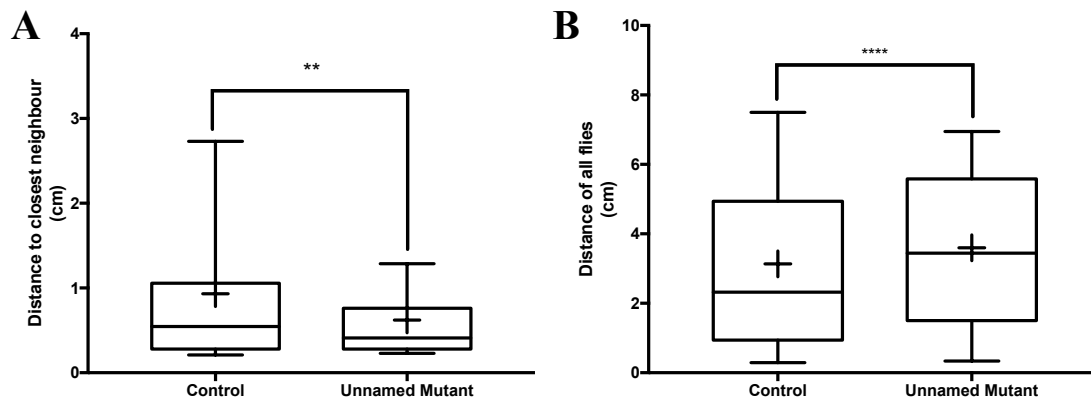
Figure 2.2 Distance to nearest neighbour and distance to all flies for a control and an unnamed mutant shown with box and whiskers (+ indicates mean, horizontal line is the median). (A) The unnamed mutant is closer to its nearest neighbour in relation to the control and therefore have pairs of flies that are closer together (**p< 0.01; Kruskal Wallis). (B) The unnamed mutant has a further distribution of flies when measuring the distance of each fly to every other fly in the chamber as compared to control (****p< 0.0001). Kruskall-Wallis non-parametric test with a Dunn's *post hoc* test.
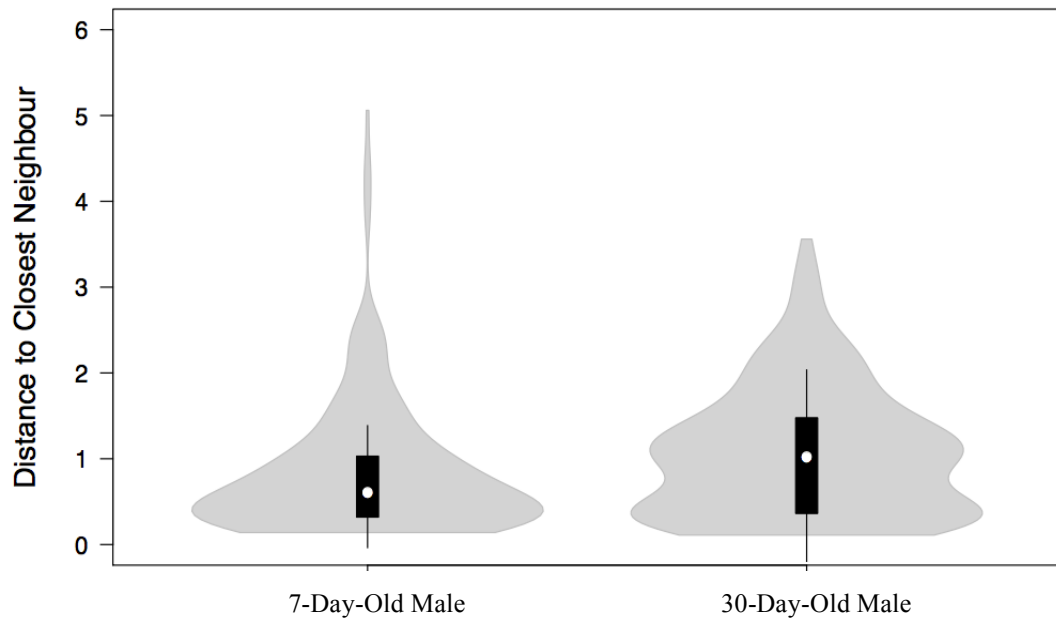
Figure 2.3 The Violin plot representation shows the probable density distribution around each data point for the distance to the closest neighbour in seven and 30-day-old males. The black box is a box and whiskers representation with the white dot as the mean value. The shape surrounding the box is the clustering of values around a specific distance. In both the seven-day-old and 30-day-old males shown, there is clustering around the average of the box but a second cluster is seen in the 30-day-old males. However, this method does not yield more clarity regarding the distribution of the values for the distance to nearest neighbour than the box and whiskers.
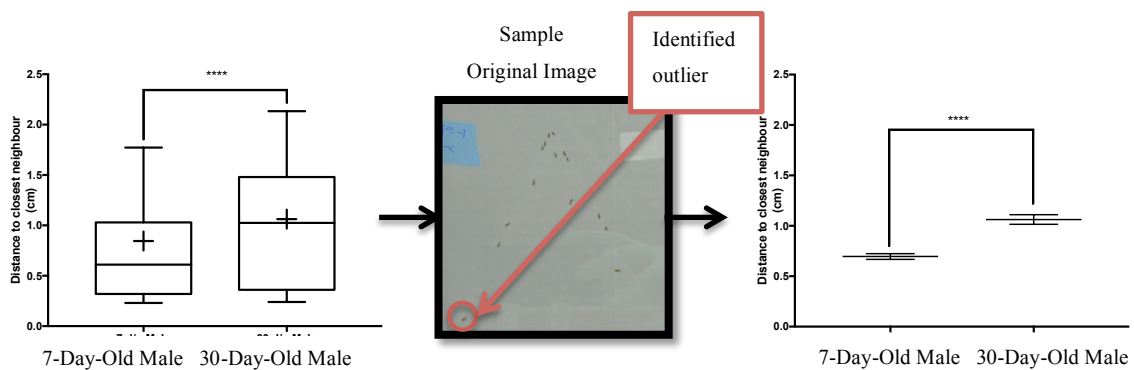
Figure 2.4 Schematic diagram of the transformation from raw data with ROUT analysis to identify outliers. ROUT analysis is applied to the raw data to remove only definitive outliers. The data now follow a normal distribution and can be represented using the mean and standard error to the mean in the distance to the nearest neighbour. The median of seven day old flies was 0.61 cm and for 30 days was 1.06 cm (Kruskall-Wallis non-parametric test with a Dunn's *post hoc* test). Following outlier removal, the seven-

day-old males had an average distance of 0.70±0.03cm with a median of 0.54 cm and 30-day-old males remained on average 1.06±0.05 cm and median of 1.06 cm as no outliers were found (one-way ANOVA with a Holm-Sidak post test). As no outliers were found in the data set of the distance to all flies or XY radius, the ROUT analysis was not included subsequently in this thesis.

2.4 Discussion

The social space assay is a behavioural assay that measures the distance between flies in a group. This information can vary based on mating status of the flies and previous social experience (Simon et al., 2012), certain mutant backgrounds (Wise et al., 2015), and finally age, as I will explore in later chapters. There are several ways of gathering and analyzing data from the social space assay. Each measure provides different information about the group's spatial formation in the chamber. The distance to closest neighbour gives information regarding the spacing of pairs within the chamber, but not in relation to the group. To gain insight into the formation of the group, the number of how many flies occupy each body length radius away from a single fly is measured, which is information that is built upon the closest neighbour. The final measure is the distance to all flies, which is a collection of measurements of how far one fly is from every other fly in the group, which informs the formation of the entire group.

When evaluating *Drosophila* group behaviour in the social space assay, all three measures of the social space assay must be evaluated for each condition. Although the overall group spacing (distance to all flies) may not be variable among treatment groups, one must keep in mind that these are averages of each fly to every other fly in the chamber and the mean may be masking some of the group properties. For example, flies may be gathered in smaller groups dispersed throughout the chamber, and a measure that would capture this may be the distance to nearest neighbour, where the nearest neighbour would be closer than the average of all flies. Similarly, the number of flies within each body length radius would reveal this pattern of smaller groups that would be lost in the distance to all flies measurement. Therefore, all three measurements should be performed for each experiment and then evaluated to see if the measures show a similar or different

trend. In the context of this thesis, analyzing the social spacing of *Drosophila* at different ages does not yield different trends among the three measures. This means that only one measure may be chosen as a proxy for the others as the pairing, small groups, and overall group information follows the same trend. However, in the case of the unnamed mutant, the distance to closest neighbour and distance to all flies did not yield the same trend. Here it is possible that the overall distribution of the population was dispersed throughout the chamber but was arranged in pairs or other small groups such that the distance to the nearest neighbour was more proximal but the overall social space in the chamber was more distal than control. Therefore, each measure must be evaluated to identify potential differences in pair and group formation.

There are also several modes of representing these data. The first mode of representation is box and whiskers, as the data gathered from the social space assay is non-parametric. While this method represents the entire distribution, it is not as visually informative and can lead to some confusion as the statistics are performed on the entire cumulative distribution and may lead to significance, although the boxes may overlap, giving the appearance that their distributions are similar. However, we can eliminate this biased visual and generate parametric data by removing outliers of the entire distribution and representing the information as the mean with standard error to the mean. Finally, violin plots look at the shape of the distribution and where individuals choose to cluster. The violin plots showed the distribution of values clustered around the mean and therefore did not give more clarity or different information than the box and whisker do, and are thus not more visually different than the mean and standard error to the mean in this case.

The outliers that are removed in the social space assay data are often "primer" flies that veer away from the group and look for new food sources to then direct the rest of the group to this source (Tinette et al., 2004). These primer flies are not reproducible at the same distance away from the group and may be constantly moving as the group is stable. While the reasons why these flies leave and how they behave may be of interest, as they may be entirely asocial, they are not of interest to the present study. These flies may be skewing the distances between flies and within the group and are therefore removed as statistical outliers. When the outliers are removed, the data are parametric where an

analysis of variance (ANOVA) or student t-test can be calculated to determine significant differences between groups. Visually this graph effectively produces a visual representation that demonstrates the statistical difference between the groups. Therefore, the mean and standard error to the mean will be used for all future studies.

In conclusion, each of the three measures of distances between flies in a group should be used to understand the pair and group dynamics of the social model, *Drosophila*. Additionally, several modes of representation may be employed to visualize the data in a meaningful way. Within the contexts of this project, each of the three measures led to a similar conclusion and for simplicity, only the distance to the nearest neighbour will be used for future analysis. Additionally, only the mean with standard error to the mean error bars will be used to represent these data, as this is the most effective way to demonstrate these results.

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Chapter 3 – Thirty and 50-Day-Old *Drosophila melanogaster* And Their Progeny Are Less Social Than Seven Day Old Flies

3   Abstract

I explored whether social behaviours, specifically social spacing and social avoidance, change with aging using the social space and social avoidance assays. I also tested the transmissibility of these age effects by testing the progeny of aged *Drosophila* with both assays. I then investigated the effects of having one aged parent on the social behaviour of the progeny. I chose to study the effects of the progeny of aged fathers. In parallel, a student in our lab (Shirley Long, Honour's Thesis 2015-2016) studied aged mothers and found that maternal age did not impact the social space of the male or female progeny.

3.1   Introduction

3.1.1 Behavioural Changes To Individuals With Age

Specific behaviours are variable over time. Some behaviours have been shown to decline over time, such as locomotion, phototaxis, and geotaxis (over three proposed phases of aging, see Figure 3.1). Conversely, some behaviours have been shown to be more stable with aging, such as free fall flight, emission of *Drosophila* stress odorant (dSO) in response to a stressor, and avoidance of electric shock (Cook-Wiens and Grotewiel, 2002; Simon et al., 2006). In an analysis of *Drosophila* behaviours from life to death, walking, resting, feeding and flying behaviours each declined with age and were correlated with time-of-death (Carey et al., 2006). Similarly, a decline in negative geotaxis at four weeks of age has been seen in both males and females in addition to increased activity at night and increased courtship behaviour at night in males (Ratliff et al., 2015). However, fewer studies have reported the changes to group behaviours, such as the social spacing between individuals within a group, with aging. Therefore, I am investigating how group social behaviours change with aging. My first aim for this chapter is to characterize the social behaviour of aged individuals, particularly in terms of their social spacing and

ability to avoid vials that were previously occupied with stressed flies. My second aim is to determine if aging of the parents causes a change to the behaviour of the first generation via heritable material. If so, my third aim will be to determine for how many generations this effect persists. And finally, my last aim is to determine which parent is responsible for a change to the social behaviour of the progeny.
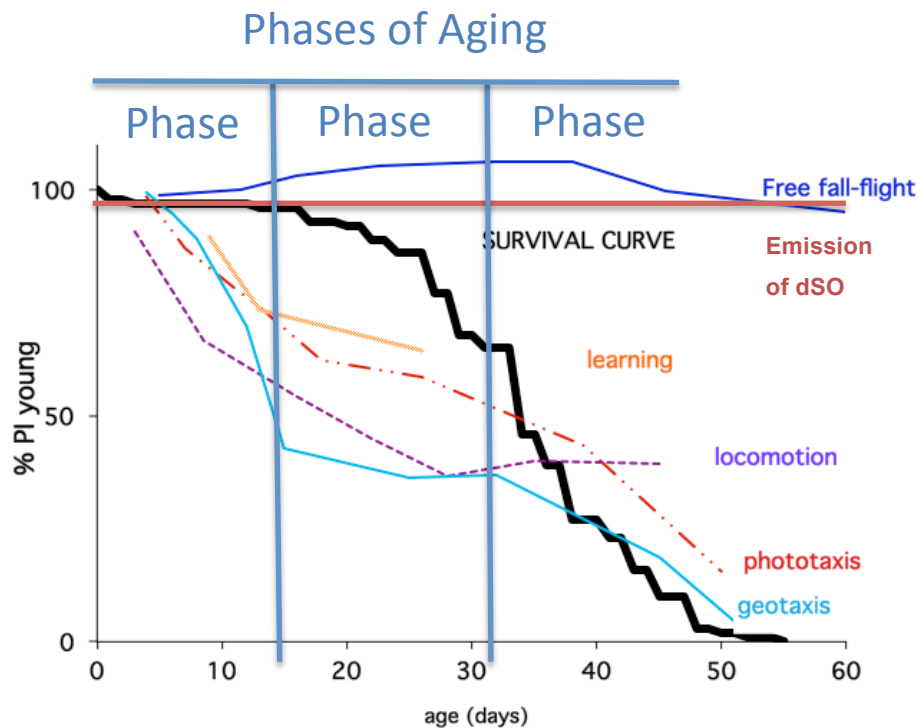
Figure 3.1 Some of the behaviours that have been previously characterized as changing with age in *Drosophila melanogaster*. The performance of each behaviour begins at 100% when individuals are young (performance index on the y-axis, denoted %PI) and declines with aging at different rates. In the first phase of aging, survival is relatively stable but after two-to-three weeks, behaviours such as locomotion, phototaxis and geotaxis begin to decline in performance. In the second phase of aging, *Drosophila* begins to die and there is a plateau in the behaviour. In the third and fourth week of life where phototaxis, geotaxis, and learning appear to display stable behaviour while locomotion is also still declining. In the third phase of aging, the decline in locomotion has plateaued but phototaxis and geotaxis decline until death. Both free-fall flight and emission of the *Drosophila* stress odorant (dSO) are stable throughout life (Simon et al., 2006; Yost et al., unpublished lab data). This figure is used with permission granted from the authors (personal communication Dr. Anne Simon, University of Western Ontario).

3.1.2 Changes To The Progeny Of Aged Parents

Advanced parental age has been linked to decreases in progeny viability and behaviour (Nystrand and Dowling, 2014). It is suggested that this tradeoff occurs so parents can allocate more energy to producing high quality offspring at the expense of their own survival (Partridge and Gems, 2006). Likewise, it has been shown that rapid reproduction in early life reduces longevity in *Drosophila*, as resources have been invested into reproduction at the cost of longevity later in life (De Loof, 2011). Tradeoffs among longevity and fertility have been shown in relation to smaller testis size that produced fewer sperm, but these sperm were shown to also produce highly viable offspring in older male *Drosophila melanogaster* (Decanini et al., 2013). Studies have even shown that female *D. melanogaster* prefer to mate with older males as they are thought to have sperm of higher quality with genes that have allowed them to live longer (Rezaei and Krishna, 2015). Although both parents can contribute damage to the next generation, the type of damage introduced by each parent is often different. It is suggested that, in humans, issues arise in very young fathers due to fertilization with immature spermatids, whereas older sperm in fathers over 45 years have accumulated *de novo* mutations (Weiser et al., 2008). Alternatively, older mothers contribute increased trinucleotide repeats to the progeny (Weiser et al., 2008). Interestingly, parental age has been shown to affect the progeny in a sex-specific manner where mothers have been shown to have shorter lived *D. melanogaster* daughters whereas, to a lesser extent, fathers had shorter lived sons (Priest et al., 2002).

In this chapter, I address how parental age can affect the social spacing of the next generation as advanced parental age has been shown to cause negative and irreversible effects on the progeny of these aged parents. Some traits that are affected include offspring viability, offspring longevity and changes to typical social and non-social behaviour in *Drosophila* (Hercus and Hoffmann, 2000), humans (D'Onofrio et al., 2014; Malaspina et al., 2001), and mice (Janecka et al., 2015). Many physiological traits change with age and affect the next generation, although this effect of aged parents on behaviour in the progeny has been less explored. I focus on both the joint effects of aged parents and the effect of having just an aged father. This is because recent studies have linked

behavioural disorders such as the Autism Spectrum Disorders (ASD) and Schizophrenia to older fathers (D'Onofrio et al., 2014). This is of particular interest to this project as ASD and Schizophrenia are types of neuropsychiatric disorders that include some form of social deficit. Thus, studying the effects of aging and social behaviour in *Drosophila* may give some insight into how these disorders may manifest in humans from having an aged parent.

3.1.3 Oogenesis In *D. melanogaster* Changes With Age

To determine how parental age can have an effect on the next generation, it is important to first understand how gametes are produced in both males and females and where damage can be introduced that will affect the progeny in terms of behaviour. Female gamete formation, or oogenesis, is complete in 14 stages and an overview is given in Figure 3.2 (Becalska and Gavis, 2009; Miller et al., 2014). The age of the female mostly has an adverse affect on the early stages, known as the previtellogenic phase, where the germ stem cells (GSCs) have developed into the oocyte and accompanying nurse cells. One way female age can affect oogenesis is via GSC exhaustion, which may account for some of the reduction in fecundity (progeny viability) with aging (Zhao et al., 2008). For example, in *Drosophila serrata*, increased maternal age resulted in decreased fecundity, which was compounded when both mother and grandmother were aged, while fathers consistently remained young (Hercus and Hoffmann, 2000). Similarly, in *Drosophila melanogaster*, aged mothers have been shown to lay eggs with lower egg-to-adult viability, lower larval-to-adult viability, and reduced egg-hatching success (Kern et al., 2001). Additionally, changes in the production and sensing of hormones with aging may cause changes to egg production. For example superoxide dismutase, an enzyme that resists free radicals, has been linked to GSC proliferation and longevity and reduction in this enzyme may accelerate GSC depletion (Pan et al., 2007). Therefore, damage can be introduced to the female gametes during oogenesis that will subsequently affect the next generation.
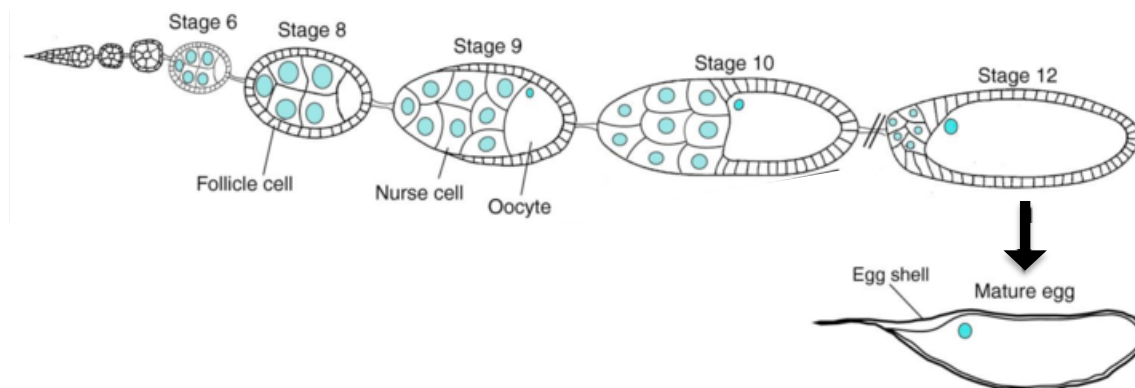
Figure 3.2 Oogenesis of *Drosophila* is divided into 14 stages. Throughout the female *Drosophila* lifespan, eggs are constantly made from a limited pool of germ stem cells (GSC) and somatic stem cells (SSC) within the germarium. The GSCs will become cytoblasts that will further divide into nurse cells and an oocyte and the SSCs divide to become the egg chamber. The following stages of oogenesis are divided into the early stages, known as the previtellogenic phase, and the later phase called the vitellogenic phase (adapted from Becalska and Gavis, 2009). This figure is used with permission granted from the authors (personal communication Dr. Elizabeth R. Gavis, Princeton University).

3.1.4 Spermatogenesis In *D. melanogaster* Changes With Age

The process of spermatogenesis is divided into three stages including the proliferative, meiotic, and spermiogenesis phases (Marchetti and Wyrobek, 2005; Figure 3.3). Spermatogenesis in *Drosophila* is maintained by GSCs in the tip of testes around somatic cells called the apical hub. These hub cells are an important part of the stem cell niche in *Drosophila* and one study saw the level of a secreted ligand called unpaired (Upd; important for GSC self renewal) decreased with age (Boyle et al., 2007). Measuring sperm viability, motility, and identifying testes morphology have all been used to test the quality of sperm (Sloter et al., 2004). One study found that when comparing older (30- and 50-day-old) male flies to young (1-to-2 day old) male flies, the testes appeared much thinner under phase contrast microscopy with fewer differentiated germ cells, indicative of decreased spermatogenesis (Boyle et al., 2007).

Over time, mature spermatozoa can become damaged due to thermodynamic changes in DNA leading to deamination, depurination, and the formation of thymine dimers (Siva-Jothy, 2000). Additionally, sperm have very little cytoplasm containing proteins to facilitate the repair of these types of damage (Siva-Jothy, 2000). The sperm genome is also highly compacted in many organisms, including humans and *Drosophila*, compared to the genome of other cells (Belloc et al., 2009). This is due to the exchange in histones for more basic proteins called protamines, which further compact the genome and make repair more challenging (Belloc et al., 2009). Also, metabolic activity over time within the sperm can result in oxidative stress of DNA which can lead to a reduction in fertility, as shown in humans (Ahmadi and Ng, 1999). Interestingly, in *Drosophila*, advanced paternal age has not been shown to significantly affect overall mortality and only slightly affected egg-to-adult viability of the progeny after fathers were five weeks of age (Price and Hansen, 1998). However, damage to the sperm genome with aging can cause changes to the progeny, particularly in terms of their social behaviour. For example, in mice, the progeny of old fathers were less social with other individuals and displayed less exploratory behaviour when alone (Smith et al., 2009). A similar study even showed this effect when just the grandfathers of young mice tested were aged and the parental generation remained young (Sampino et al., 2014). Therefore, I will be investigating the effect of parental and paternal aging on social behaviours, including the social spacing between individuals and the avoidance of stressed individuals.
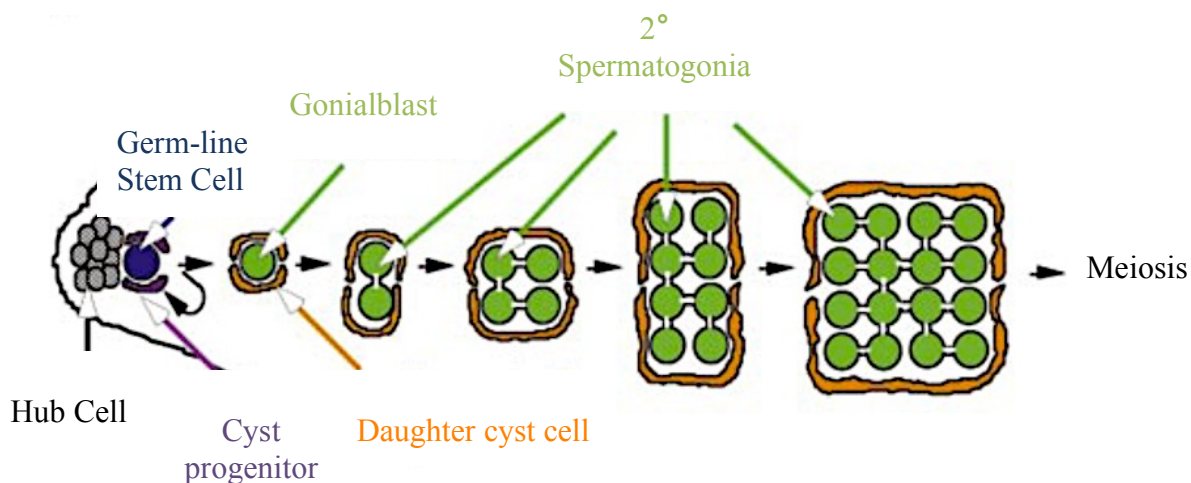
Figure 3.3 Spermatogenesis in *Drosophila* is comprised of three main stages. The first phase is the proliferative phase where stem cells undergo division in spermatogonia to become meiotic spermatocytes, The second phase is the meiotic phase where recombination generations haploid spermatids. The final stage is the spermiogenesis phase where mature spermatozoa are created by morphological and biological changes (Marchetti and Wyrobek, 2005). Spermatogenesis in *Drosophila* is maintained by germline stem cells (GSC) in the tip of testes around somatic cells called the apical hub (Tran et al., 2000). This figure is adapted from a review on spermatogenesis with permission granted by the author (personal correspondence Dr. Steve DiNardo, University of Pennsylvania).

3.1.5 Significance And Hypothesis

Many of the studies that have been done on advanced parental age and social behaviour in human has been in the context of disorders such as ASD. ASDs are a heterogeneous group of disorders in which a social deficit is an important criterion for diagnosis, in addition to communication deficits and repetitive or restrictive behaviour (Holt and Monaco, 2011). Importantly, one example of the social deficit experienced by individuals with ASD is difficulty regulating personal space (Gessaroli et al., 2013). Additionally, fathers over the age of 45 have been linked to children with neuropsychiatric disorders such as ASD and Schizophrenia, which are diseases that include a change in stereotypical social behaviours (D'Onofrio et al., 2014). Interestingly, one study found that individuals born to both very young fathers (under 20 years old) and older fathers (over 45 years old)

had poorer social function than those born to fathers in between these ages (Weiser et al., 2008). Similar changes in children were correlated with very young mothers and mothers over the age of 35 years and particularly affected male children (Myrskylä and Fenelon, 2012). Individuals with psychopathy, which is another neuropsychiatric disorder, actually prefer to have closer interpersonal space, although no information regarding parental age and psychopathy has been determined (Vieira and Marsh, 2014).

Based on this, I hypothesize that social space will change with age and this effect will be passed onto the next generation. I first determine what is considered "aged" for *Drosophila* by generating a survival curve and expect that at 25°C *Drosophila* will live until around three months according to previous studies (Maynard-Smith, 1958). I then characterize *Drosophila* at 100%, 90% and 50% survival using the social space assay and test the progeny of flies at these ages as well and test subsequent young generations to see how long the effect, if any, will last. I also characterize the avoidance of a form of stress in both aged individuals and their progeny. And finally, I characterize the social space of the progeny of old fathers and perform morphological measures of the sperm in males of different ages.

3.2 Methods

3.2.1 Fly Handling

All flies were maintained at 25°C, 50% humidity with a 12:12 light: dark cycle. Weekly fly collections and sexing was always done over cold anesthesia. All flies were maintained over Jazz mix media (brown sugar, corn meal, yeast, agar, benzoic acid, methyl paraben and propionic acid; Fisher Scientific).

3.2.2 Stocks Of Canton-S *Drosophila melanogaster* And Aged Flies

The laboratory control strain Canton-S *Drosophila melanogaster* was reared in mixed sex in bottles over Jazz Mix media. Fresh food bottles of *Drosophila* are made bi-weekly when the parents are less than seven days old. Every Monday, existing flies in bottles

were removed to prevent new emerging flies cohabitating with their parents. Every Wednesday, flies (one-to-three days old) were collected from the bottles (40 flies/ vial, seven vials/ week) under cold anesthesia (Fernandez et al., 2014). Aging flies were transferred to new media in vials every two-to-three days.

3.2.3 Survival Curve

Survival curves were performed as in Simon et al. 2003. Specifically, Canton-S *D. melanogaster* were collected from stock bottles under cold anesthesia at two to three days old (40 flies mixed sex flies/ vial, n=9 vials, tested in parallel over three consecutive weeks (n=3 per week for 3 weeks)) and maintained over Jazz Mix media (50% humidity, 25°C, and 12:12 light: dark cycle) until death. *D. melanogaster* were transferred to new food every two-to-three days and the number of dead flies were then counted. The resulting survival curve was generating using Microsoft Excel. This curve was then used as the basis for all subsequent studies that required flies at 100%, 90% and 50% survival.

3.2.4 Fertility And Fecundity Curves

Fertility and fecundity curves were performed as in Simon et al. 2003. Fertility is measured here as the number of eggs laid per female over the course of the *Drosophila* lifespan. Fecundity, however, is a measure of the number of progeny that arise from the eggs laid, and can also be referred to as the egg-to-adult viability. Both fertility and fecundity are average values per female per day and are represented as cumulative values over time. Virgin female Canton-S *D. melanogaster* are collected with young male (< 2 days old) *D. melanogaster* and maintained over Jazz Mix media containing several drops of blue food dye (club house®) for contrast to visualize eggs (5 males and 5 females per vial, n= 3 vials tested in parallel, repeated on 3 different weeks for a total of n= 9 vials). Flies were transferred daily into fresh vials and the number of eggs laid per day was counted daily to quantify fertility. The number of dead males and females were also quantified for later calculations of the number of eggs and progeny laid per female. Fecundity (egg-to-adult viability) was assessed by counting the resulting progeny ~11 days later, when they emerge as adults. The fecundity of the progeny of 30-day-old flies

(first generation) and the progeny of 30-day-old grandparent flies (second generation) were also measured. The resulting cumulative curves of fecundity were generated using Microsoft Excel.

3.2.5 Generating Old Flies And The Progeny Of Old Flies

The old flies were generated through maintaining Canton-S *Drosophila melanogaster* by transferring them to new food every two days (see 3.2.2). At 90% survival (30 ± 1.53 days, Figure 3.4) the progeny of old flies were saved and allowed to develop to adulthood (first generation). At one week old, the progeny of this first generation were collected and allowed to develop to adulthood and were also be used for behavioural tests (second generation). This cycle of maintaining flies and collecting the progeny of both young and aged flies continued for several generations (second, third, fourth and fifth generations of young and old flies) and were tested with the social space assay.

3.2.6 Generating The Progeny Of Old Fathers

In order to generate progeny from an old father but a young mother*, D. melanogaster* were collected from stock bottles under cold anesthesia (two-to-three days old; 40 flies/ vial, 7 vials/ week) and were aged to 30 days by transferring them to new Jazz Mix media every two-to-three days. Thirty-day-old *D. melanogaster* males were then separated from females under cold anesthesia and were set aside to be mated (5 males/vial; n= 9). Virgin female *D. melanogaster* were collected from stock bottles several hours after removing existing flies and were put into vials containing 30-day-old males (5 females/ vial; n= 9). Three days following mating, the flies were removed and the resulting eggs were allowed to emerge to adulthood. The progeny of 30-day-old fathers were then separated by sex at seven-days-old (15 flies/ vial; separated by sex; n= 9).

3.2.7 Social Space Assay

The social space assay was performed as previously described (McNeil et al., 2015; Simon et al., 2012), and in Chapter 2. The assay was always performed at the same time of day, Zeitgeber time (after the onset of light) 4 to 7 (12 pm to 3 pm) as the time of day

has been shown to affect social spacing (McNeil et al., 2015). In short, *Drosophila* was acclimated to the behavioural room (25°C, 50% humidity) for two hours prior to being added via aspiration to a two-dimensional vertical space composed of a series of glass and acrylic pieces that form a hollow triangle, known as the chamber (15 flies per chamber, separated by sex, n= 9 replicates). Flies were permitted to explore before settling along the glass (~20 minutes until settled). Images of the chambers were taken at 30 minutes when flies have settled (Simon et al., 2012). The images were analyzed using ImageJ to get the distance to the closest neighbour. These distances were analyzed with the statistical program Prism7 to assess significance using a one way-ANOVA with a Holm-Sidak test to correct for multiple comparisons in groups larger than two, or an unpaired t-test for groups of two (all measurements expressed as a mean ± standard error to the mean, see chapter 2 for details). Social space assays were performed using flies at 14, 21, 30 or 50 days of age flies and the progeny (first generation) of flies at seven, 30 and 50 days of age, as compared to seven-day-old flies.

3.2.8 Social Avoidance Assay

The social avoidance assay was performed as previously described (Fernandez et al., 2014). In short, I utilized a T-maze apparatus to provide a binary choice to groups of same sex flies (responders): whether to enter a clean vial or enter a vial that has been filled with *Drosophila* stress odorant (dSO) by vortexing (emitter) flies. The performance index was calculated as the number of responder flies that avoided the stress minus the number of flies that did not avoid the stress multiplied by 100 and divided by the total number of flies in the assay. Responder and emitter flies were collected at least 24 hours prior to the assay. As sex does not affect emission of dSO (Fernandez et al., 2014), emitter flies were seven days old in equally mixed sex per responder group. Responder flies were young (seven days old), old (30 days old) or the progeny of young or old flies (20 flies/ vial, separated by sex, n= 9 per sex and condition). All conditions within the behaviour room are described above. All results are reported as a performance index that evaluates the number of flies that enter the stress vial, the non-stress or empty vial, and those that do not make a choice (Kruskall-Wallis non-parametric test with a Dunn's *post hoc* test).

3.2.9 Microscopy Of *Drosophila melanogaster* Testes Morphology And Quantification Of Sperm Bundles

Male *Drosophila* at seven, 30, and 50 days of age were submerged in testes buffer (deionized water, 183 mM KCl, 47 mM NaCl, 10 mM Tris-HCl; n= 10 males per age group) on a glass dish under a dissection microscopy (Nikon SMZ1500). Tweezers were used to remove the testes and surrounding accessory gland tissue and whole testes were transferred to a new glass slide containing testes buffer and secured with a glass coverslip. The gross testes morphology was evaluated and the sperm bundles are counted before adding DAPI solution (0.2% mg/ml) to visualize the sperm heads (Sitaram et al., 2014). The number of bent versus the number of straight sperm heads was also quantified and a two-way ANOVA was used to compare the number of straight and bent sperm heads at different ages.

3.3 Results

3.3.1 Survival and Fecundity Of *Drosophila melanogaster*

A survival curve was generated to determine when Canton-S *Drosophila melanogaster* begin to die and can then be considered "old" for future studies (Figure 3.4). *Drosophila* at seven days is used as control from now on because there is no known senescence at this age and there is 100% survival (referred to as "young"). Thirty-day-old flies are considered "old" as this is when survival begins to decline (90% survival) and will continue to decline (50 days old, 50% survival; 72 days old, 10% survival). Although groups of flies can be aged until 10% survival, these aged flies were not used in this study due to the inherent low quantity limitation. The cumulative fecundity (egg-to-adult viability) of female *Drosophila* with age is also shown on Figure 3.4, where egg-to-adult viability is maintained until 49 ± 3.18 days (65.53 progeny per female, cumulative), at which point fecundity then plateaus until female death. Interestingly, there appears to be a lag period of fecundity under 10 days, as seen in other studies of age, before a steep increase in the egg-to-adult ratio per female between 10 and 40 days (Novoseltsev et al., 2003).
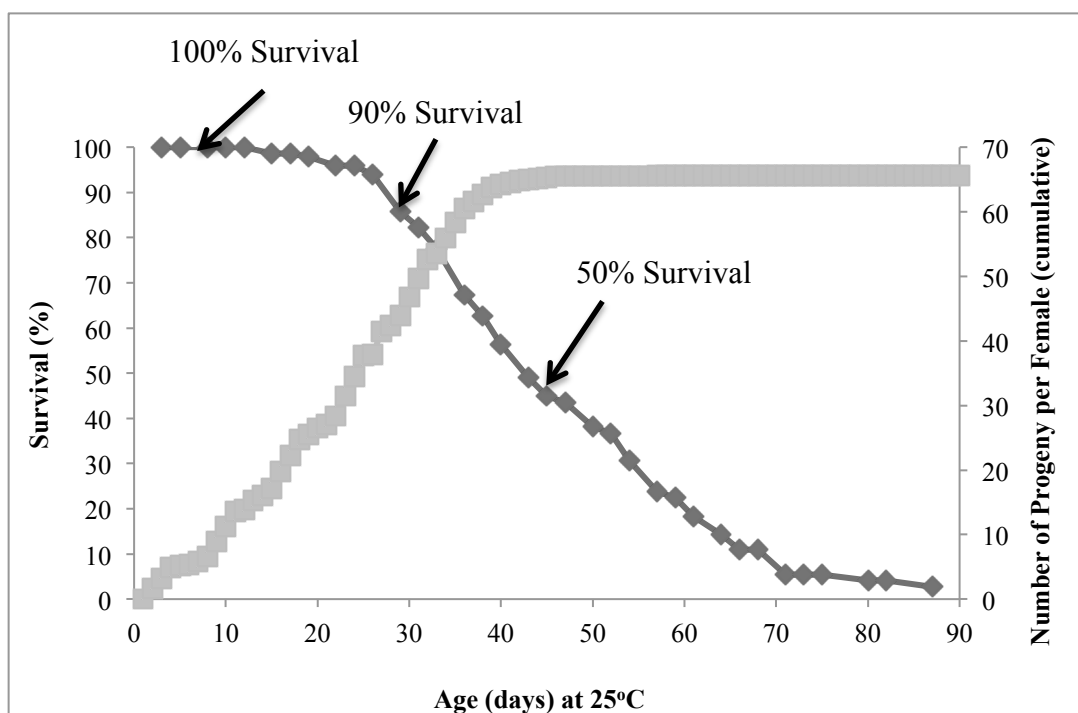
Figure 3.4 Survival and fecundity curve of Canton-S *Drosophila melanogaster* at 25°C (40 flies/ vial, n=9 vials). Seven-day-old flies have 100% survivability and are considered young, whereas 30-day-old (90% survival) and 50-day-old (50% survival) flies are both considered old (40 flies per vial, mixed sex, n= 9). Egg-to-adult viability of the progeny is stable until 49 ± 3.18 days when fecundity reduces until female death (5 male and 5 female per vial, n= 9 vials).

3.3.2 Social Space Of *Drosophila* Changes With Aging

Now that I have established a baseline for the ages to study, I chose to test young *Drosophila melanogaster* (seven-day-old, control) compared to those at 14, 21, 30 and 50 days when *Drosophila* survival declines. Young flies are around three body lengths apart from their nearest neighbour, which falls into the upper boundary of what has been previously reported for this age (Simon et al., 2012). Young *Drosophila* is more proximal to their closest neighbour at seven days of age than those at 30 and 50 days of age in both sexes (one-way ANOVA, ****$p < 0.0001$; Figure 3.5A). However, flies at 14 and 21 days of age are more proximal to their closest neighbour than those at seven days old for both sexes (****$p < 0.0001$ males, **$p < 0.01$ females). Interestingly, this time period overlaps with the steep incline in fecundity seen in Figure 3.4. All values for the distances between neighbouring flies at different ages can be found in Appendix A.

3.3.3 Changes To Social Space Are Passed On To The Next Generation Only

I then studied the progeny of the aging flies. The seven-day-old progeny of parents that are either seven, 14, 21, 30 or 50 days old are tested with the social space assay (Figure 3.5 B). All values for the distances to the nearest neighbour can be found in Appendix B. The progeny of 30-day-old parents and 50-day-old parents are more distal to their closest neighbour than the progeny of seven-day-old parents (***$p < 0.001$ males, *$p < 0.05$ and ****$p < 0.0001$, respectively in females; one-way ANOVA). In males, the progeny of 14-day-old parents and 21-day-old parents are no more or less distal to the nearest neighbour than controls or the progeny of 30-day-old parents. However, the female progeny of 14-day-old parents are more proximal to the nearest neighbour than the progeny of seven-day-old parents (*$p < 0.05$), whereas the progeny of 21-day-old parents are no more or less proximal. Interestingly, their progeny also displays the pattern observed in the parents, which is consistent with an inherited factor or mechanism.

Finally, the effect of having aged parents is somewhat ameliorated by the second generation as the social space between the progeny of old grandparents and the progeny of young grandparents is not significantly different (Figure 3.5 C). This is also true for the next three generations where the intervening generations remain young and only the parental generation is aged, although some variation is observed (Figure 3.5 D-F; Appendix B).
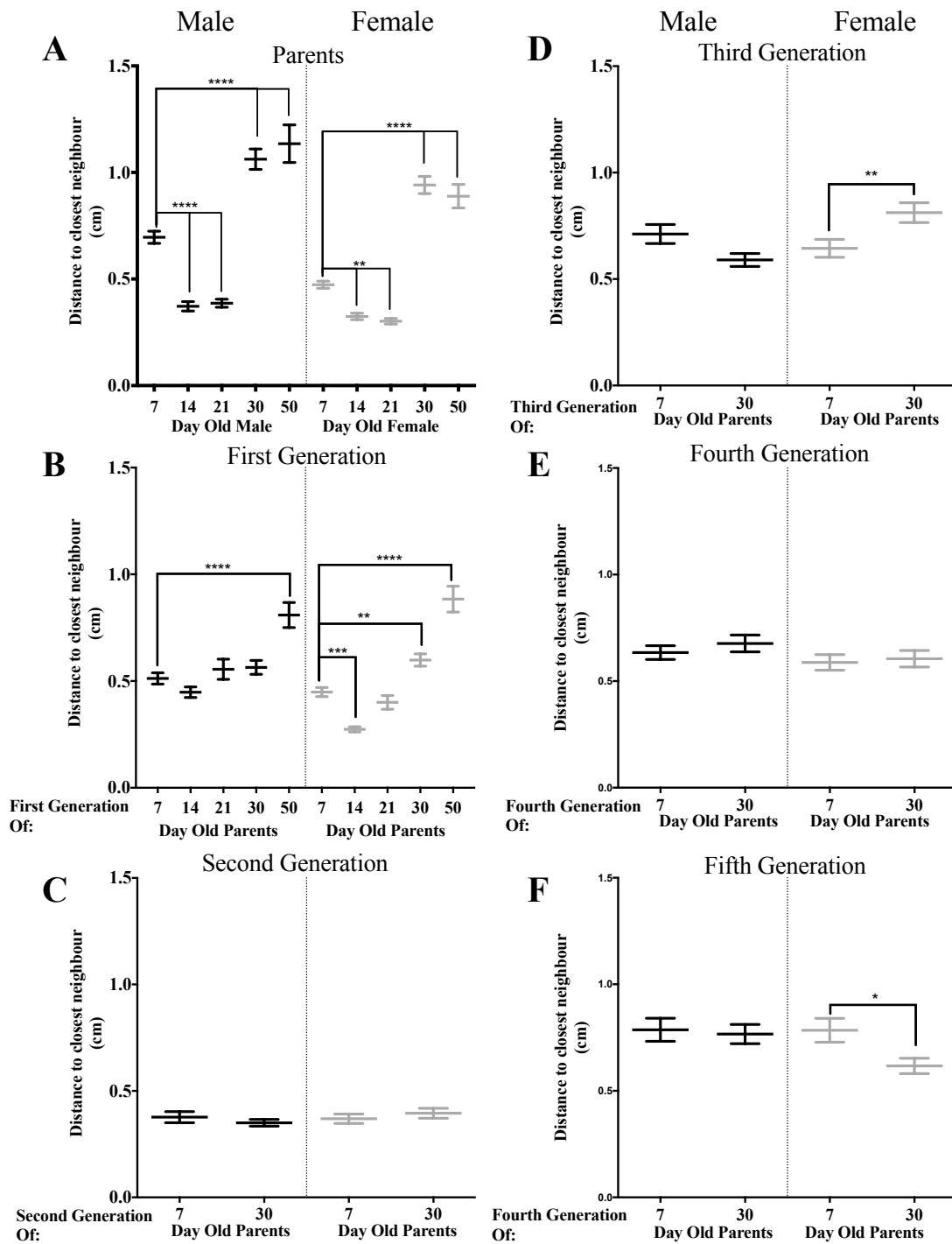
Figure 3.5 Aged *D. melanogaster* are more distal to their closest neighbour and this effect is transmitted to the next generation but then stops in the following generations. (A) to (F): each graph represents the distance of each fly to its nearest neighbour in the social space assay. The sex of the animal is indicated above each column. Social spacing is shown as the mean and standard error to the mean of the

closest distance between neighbouring flies (One-way ANOVA followed by a Holm-Sidak *post hoc* test in each sex separately - Each set of asterisks represents statistical significance: *p< 0.05, **p< 0.01, ***p< 0.001, and ****p< 0.0001.). (A) Effect of age: Social space of males and females as compared to seven days old, 14, and 21 days old are closer to their closest neighbour and older flies at 30 and 50 days old are further (B) Effect of having parents aged beyond seven days old: Social spacing in the progeny of parents aged to seven, 14, 30, or 50 days old while the progeny are tested at seven days old. The progeny of 30- and 50-day-old *D. melanogaster* are more distal to their closest neighbour in both males and females than the progeny of seven-day-old flies. The male progeny of 14 and 21-day-old parents are no more or less social than the progeny of seven-day-old. However, the female progeny of 14-day-old flies only is significantly closer to their closest neighbour than control. (C,E,F) The second, fourth and fifth generations of seven and 30-day-old flies do not differ in social space in both sexes. (D) The third generation of seven and 30-day-old flies do not differ in social space in males but the third generation of 30-day-old flies in females are further apart from the third generation of seven-day-old flies.

3.3.4 Social Avoidance Of Aged Flies And The Progeny Of Aged Flies

I then assessed how the aging process affected another social behaviour: avoidance of the marking left by stressed flies, or dSO. Seven-day-old males and females have a higher performance index, and are therefore more able to avoid the stressor, than flies at 14, 21, and 30 days of age (Figure 3.6). Due to a lack of flies at 50 days old required for this assay (20 flies per replicate versus 15 for social space that was also carried out over an extended period of time), I was unable to test flies at this age with social avoidance but was able to obtain enough progeny to test them with the social avoidance assay. Because the 14- and 21-day-old flies appeared to have a lower performance index similar to those at 30 days old, I chose to test the progeny of 30 days old and not the progeny of 14 or 21 days old. The progeny of 30-day-old flies and 50-day-old flies have a lower performance index than the progeny of young flies, but not significantly differently. All values for performance index at different ages and the progeny of different ages can be found in Appendix C.
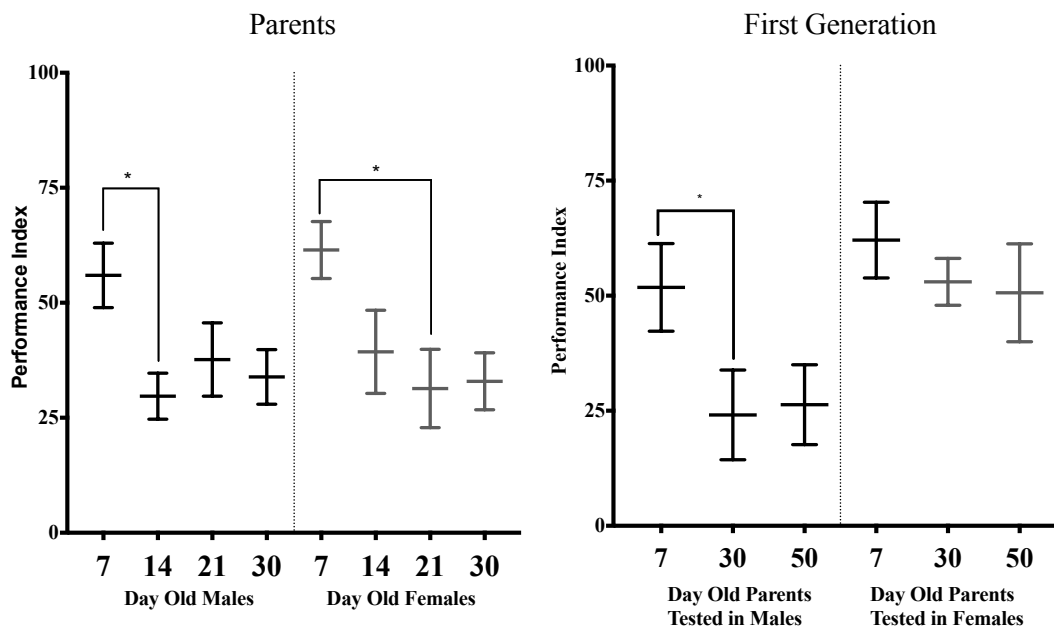
Figure 3.6 Aged flies and the progeny of aged flies have a lower performance in the social avoidance assay than young flies. (A-B) both graphs represent the performance index of *Drosophila* at different ages or the progeny of parents at different ages using the social avoidance assay to test the ability of these flies to avoid vials with previously stressed flies. A lower performance index indicates a fly's lack of ability to avoid this stress. The sex of the animal is indicated below each column. Social avoidance is shown as the mean and standard error to the mean of performance (Kruskal-Wallis non-parametric test followed by a Holm-Sidak *post hoc* test in each sex separately, the asterisks represent statistical significance with *p< 0.05). (A) At 14 days, males are significantly lower on the performance index of avoiding stress than control seven-day-old males and 21- and 30-day-old males follow the same trend, although not significantly different. Similarly, aged females have lower performance, where only females at 21 days old are significantly lower in performance. (B) The young male progeny of 30 days old flies are significantly lower on the performance index of avoiding stress than control seven-day-old males and the male progeny of 50-day-old parents follow the same trend, although not significantly. Females follow a similar trend of decreased performance although not significantly.

3.3.5 The Progeny Of Aged Parents Live Longer And Have Reduced Fecundity

To identify how aging of parents would affect life history traits of the progeny and grand-progeny, a survival and fecundity curve of the first and second generation of aged parents is generated. I also extracted the number of eggs laid and progeny that developed from those eggs at 100%, 90%, and 50% survival for each generation.

The seven-day-old progeny of 30-day-old *Drosophila* are able to live up to 160 days in the lab (Figure 3.7A), exceeding the lifespan of the progeny of seven-day-old *Drosophila* who are able to live until 90 days. The second generation of 30-day-old flies do not live longer than the first generation or control. Additionally, differences in survival among the three groups (control, first generation of aged parents and second generation of aged parents) are significant after each group reaches 50% survival, as shown in Figure 3.7A. Control flies reached 50% survival at 52 ± 7.51 days, whereas the first generation reached this survival at 66.5 ± 0.50 days and the second generation at 33 ± 8.00 days (Figure 3.7B). The maximum survival also significantly different among the three groups, as the control lived to a maximum of 91.3 ± 2.60 days, the first generation of old parents lived to 148 ± 9.00 days and the second generation of old parents was 64 ± 1.00 days (Figure 3.7B). Fertility per female (Figure 3.7C) and fecundity per female (Figure 3.7D) each show no difference in the egg laying and progeny viability at the different age points for each group. The differences among fertility (number of eggs laid, cumulative) and fecundity (number of progeny developed, cumulative) rates were not different at 100% and 90% survival. However, the fertility and fecundity at 50% survival of the control (fertility: 102.08 ± 11.82, fecundity 56.92 ± 5.09) were significantly different from the first generation (fertility: 161.92 ± 25.90, fecundity: 96.76 ± 14.48) and second generation (161.62 ± 21.77, fecundity: 102.73 ± 14.25) of old parents. Additionally, maximum fertility and maximum fecundity was greatest for the second generation of old parents (fertility: 202.02 ± 31.67, fecundity: 105.81 ± 13.68), followed by the first generation of old parents (fertility: 164.45 ± 27.25, fecundity: 101.82 ± 16.14), in comparison to control (fertility: 112.51 ± 8.67, fecundity: 57.52 ± 4.91).

A Survival and Fecundity of *Drosophila* (control)
Survival and Fecundity 1st generation old parents
Survival and Fecundity 2nd generation old parents

B
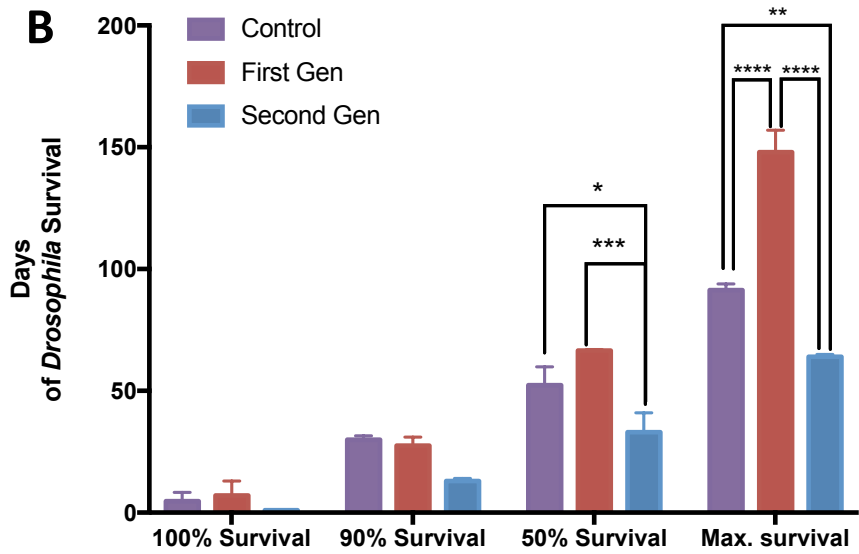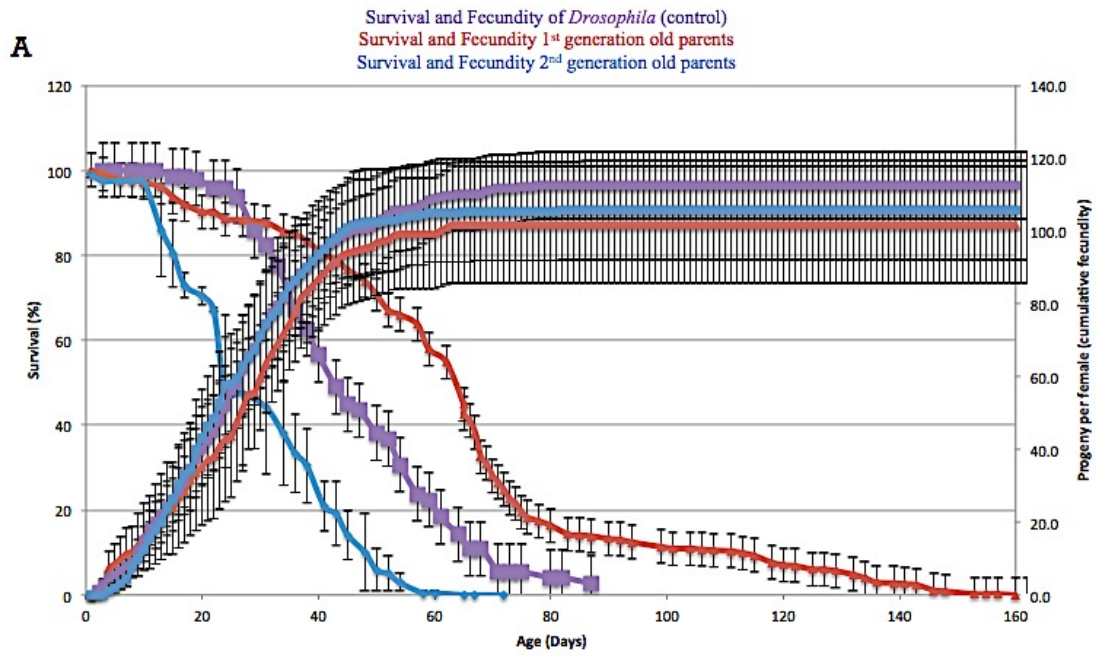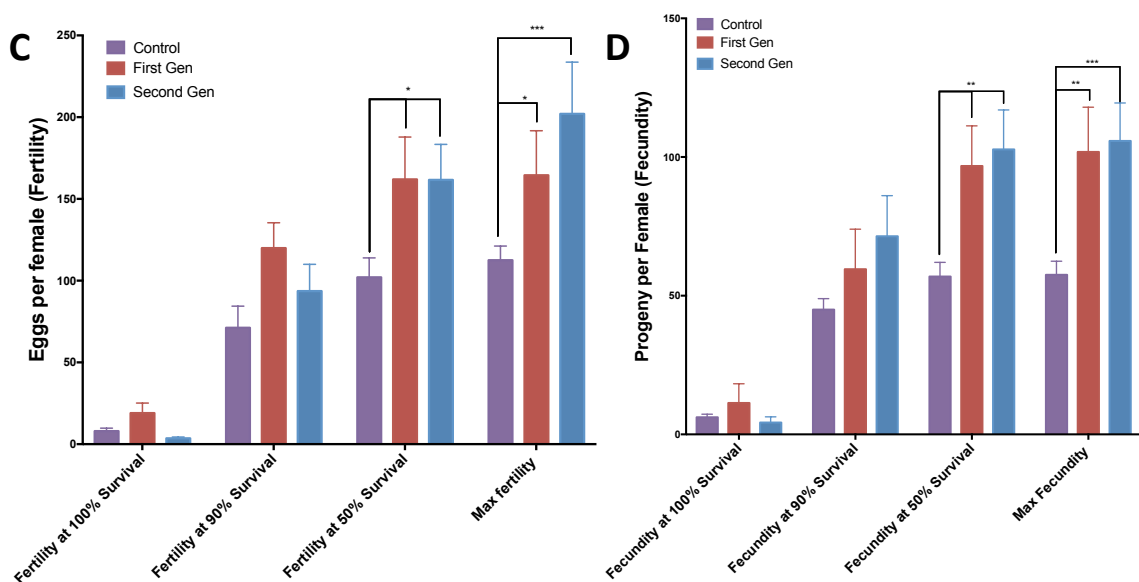
Figure 3.7 The progeny of 30-day-old parents (red) has increased longevity and reduced fecundity, which is not fully recovered in the second generation. (A) The first generation of old parents have increased survival until 160 days. The progeny of young individuals (control, purple) has similar longevity to the progeny of old grandparents (blue). The second generation of old parents (old grandparents) survives until 72 days. Cumulative fecundity among for each generation is also shown on the secondary axis. (B) Differences among survival only arise when comparing 50% survival in the three generations. At 50%, the control generation (parental, mean survival 52.3 ± 7.51 days) and the first generation (66.5 ± 0.50 days) are not statistically different. However, the second generation (33 ± 8.00 days) is significantly different from both control (*p< 0.05) and the first generation (***p< 0.001). The maximum (max) survival of the control (91.3 ± 2.60 days) was significantly different from both the first generation (148 ± 9.00 days; ***p< 0.001) and the second generation (64 ± 1.00 days; ****p< 0.0001; two-way ANOVA with a Holm-Sidak *post hoc* test). (C) The fertility among the different generations was not significantly different at 100% and 90% survival, but was different at 50% survival and in terms of maximum number of eggs laid per female. Control flies laid fewer eggs per female (102.08 ± 11.82 eggs) by 50% survival, than the first generation of old flies (161.92 ± 25.90; *p< 0.05) and the second generation (161.62 ± 21.77; *p< 0.05). Control flies also had reduced maximum fertility per female (112.51± 8.67) relative to the first generation (164.45 ± 27.25; *p< 0.05) and the second generation (202.02 ± 31.67; ***p< 0.001; two-way ANOVA with a Holm-Sidak *post hoc* test). (D) The fecundity among the different generations was not significantly different at 100% and 90% survival, but control flies had fewer adult progeny per female (56.92 ± 5.09) by 50% survival than the first generation (96.76 ± 14.48; **p< 0.01) or the second generation (102.73 ± 14.25; **p<0.01). Control flies also had fewer progeny overall per female (57.52 ± 4.91) as compared to the first generation (101.82 ± 16.14; **p< 0.01) and the second generation (105.81 ± 13.68; ***p< 0.001; two-way ANOVA with a Holm-Sidak *post hoc* test).

3.3.6 30-Day-Old Fathers Have Seven-Day-Old Progeny That Are Further Apart And Have Altered Sperm Morphology

To see the impact of having one aged parent on the progeny, I mated old male flies (30 days) with virgin females and tested their progeny in social space. I found that the male progeny of 30-day-old males are more distal to their closest neighbour than the male progeny of seven-day-old males (**p< 0.01;Figure 3.8; Appendix D). However, the female progeny of 30-day-old males is no more distal to their closest neighbour than the female progeny of seven-day-old males.

I then used both light microscopy and fluorescent microscopy to see if there were any visual differences in the number of sperm bundles, testes morphology and sperm head morphology of young and old males that may give an indication into how the fathers are affecting the progeny (Figure 3.9A). On average, 7-day-old males yielded 18.6 ± 1.50 sperm bundles per testis, whereas 30-day-old males had 14 ± 1.18 sperm bundles per testis and 50-day-old males had 10.4 ± 1.02 sperm bundles (Figure 3.9B). Due to the reduction in visual sperm heads in 50-day-old males, only 30-day-old males sperm are shown but the sperm bundles present within the testes were still visible. When sperm were treated with a dye that intercalated between the bases of DNA, DAPI, the overall morphology of the sperm heads was visible. As seen in Figure 3.9A, the sperm heads of young males appear straight whereas the heads of older males appears bent. In each testis of seven-day-old males there were on average 39 ± 2.48 straight sperm heads and no bent sperm heads, whereas in 30-day-old males there were an average of 15 ± 4.95 straight sperm heads to 12.5 ± 5.39 bent sperm heads and 50-day-old males had an average of 19.5 ± 8.65 straight sperm heads and 8.5 ± 2.99 bent sperm heads (Figure 3.9C).
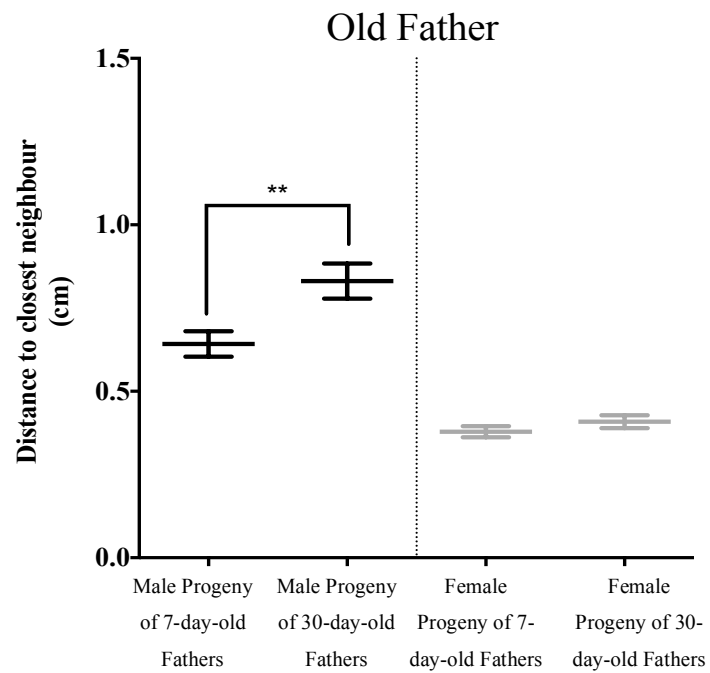
Old Father

Figure 3.8 The male progeny of aged fathers are less social. The male progeny of 30-day-old fathers are more distal to their closest neighbour than the progeny of young fathers (unpaired t-test; **p< 0.01). Females are not statistically different if their fathers were young or old.
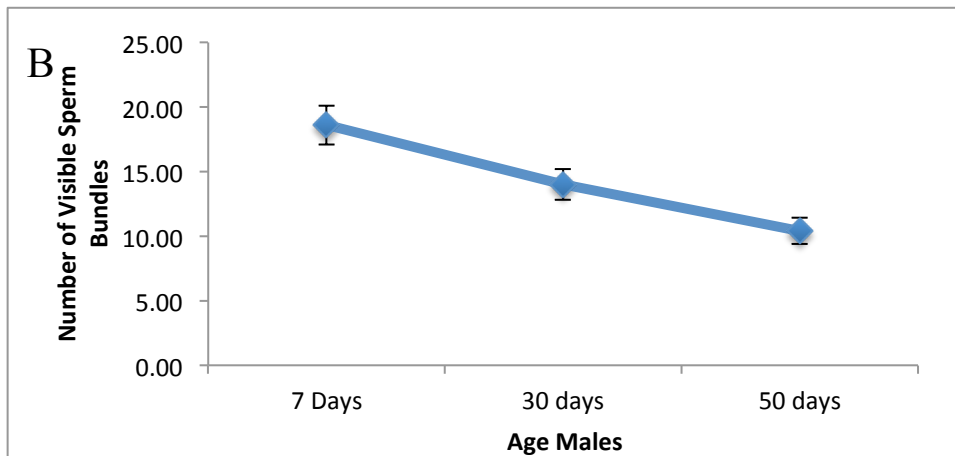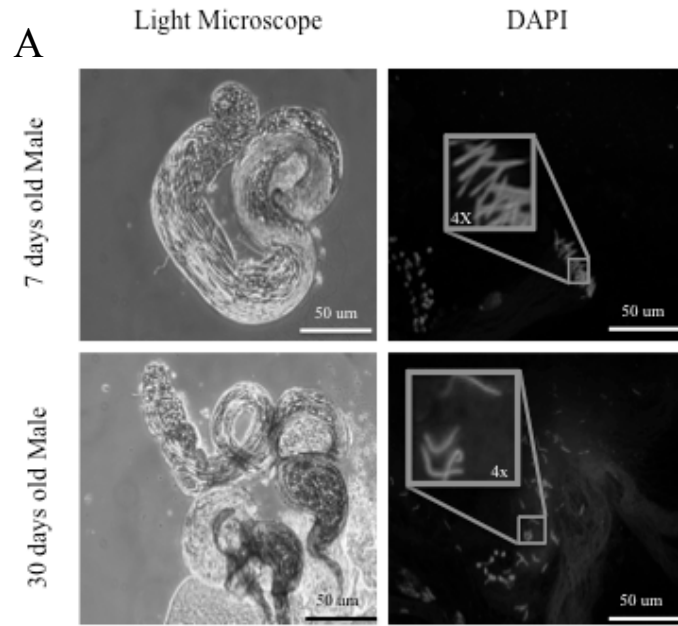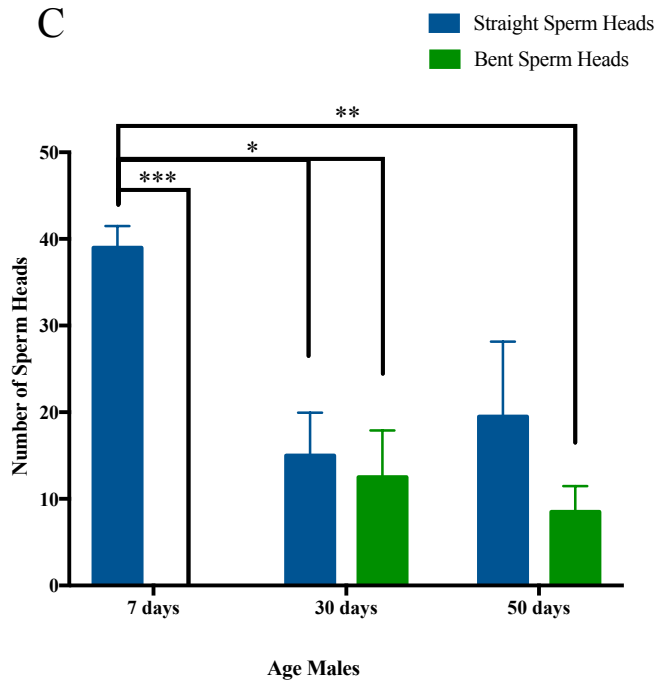
Figure 3.9 Older males have altered testes morphology and a bent sperm head shape. (A) The images on the left show the sperm and testes of seven-day-old males (top) and 30-day-old males (bottom) under light microscopy. Testes in older males appear thinner and darker with fewer sperm bundles than those in younger males. Images on the right show the same males under fluorescent microscopy where sperm heads are visualized with DAPI. The sperm heads of older males appear bent or misshaped as compared to the straight heads of seven-day-old sperm. **(B)** With age, the average number of visual sperm bundles present in the testes declines with age (18.6 ± 1.50 bundles in seven-day-old flies, 14 ± 1.18 bundles in 30-day-old males, and 10.4 ± 1.02 bundles in 50-day-old males; n= 5, mean and standard error to the mean). **(C)** With age, the number of straight sperm heads decreases with age and there are bent sperm heads at older ages (39 ± 2.48 straight: 0 bent at seven days old, 15 ± 4.95 straight: 12.5 ± 5.39 bent at 30-days-old, and 19.5 ± 8.65 straight: 8.5 ± 2.99 bent at 50-days-old). Seven-day-old males have significantly more straight sperm heads than 30-day-old males ($*p< 0.05$), 50-day-old bent sperm heads ($**p< 0.01$), and than seven-day-old bent sperm heads ($***p< 0.001$). However, the differences between straight and bent sperm heads were not significantly different among 30- or 50-day-old males (two-way ANOVA).

3.4 Discussion

3.4.1 Survival And Fecundity Decrease With Aging

Drosophila melanogaster survival begins to decline at 30 days and further at 50 days so these ages were used continuously throughout this study as measures of aged individuals and parents, which corresponds to what others have used in aging studies (Hu et al., 2014; Maynard-Smith, 1958; Simon et al., 2003, 2006). I found that fecundity, or egg-to-adult viability, a common measure of progeny sustainability, declined after 49 ± 3.18 days (similar to Simon et al. 2003). However, the progeny could still be tested and collected from the parents of 50-day-old individuals (Price and Hansen, 1998). Between one and around nine days, there was a lag in the rate of egg and progeny production that then increased after 10 days, which has been reported by other researchers (Novoseltsev et al., 2003). Perhaps this is due to the presence of seminal fluid in the female reproductive tract following the onset of mating in early life (under one week old) that has been shown to affect behaviour and up-regulate oogenesis (Wolfner, 1997).

Interestingly, the fecundity and fertility of the first generation of old parents was not reduced but longevity was increased, as compared to the progeny of young parents and the second generation of old parents. Some studies have shown that the first generation of parents that have been exposed to a stressor (aging, in this case) have increased longevity and reduced fertility and fecundity as a mode of stress resistance. It is suggested that energy is diverted away from progeny production in order to extend longevity (De Loof, 2011). However, this tradeoff was not seen as the progeny production of the first generation of old flies was increased relative to control and was maintained in the second generation of aged parents. Therefore, it is possible that the aging stressor was not a powerful enough force to induce this strong trade-off with longevity and fecundity, although behaviour was affected, as discussed below. Interestingly, the second generation of aged parents had reduced longevity relative to control but maintained fertility and fecundity.

3.4.2 Aged Flies And The Progeny Of Aged Flies Are Less Social

Group formation is proposed to be necessary for more complex behaviours to take place, such as courtship and feeding (Dankert et al., 2009; Hahn et al., 2013; Schneider and Levine, 2014). I found here that the social spacing between pairs of flies in a group changes with aging and can be passed on to the next generation, in an atypical manner for behaviour. Changes through age of social spacing did not follow any of the patterns described in Fig. 3.1. Instead of a progressive change, I found that there was a stepwise pattern.

I found that at 14 and 21 days of age, both males and females were more proximal and therefore more social with respect to the nearest neighbour as compared to those at seven days old. This may be correlated with increased fertility and fecundity at this age as individuals have a high fecundity rate at this age. It is possible that females would have an increase in seminal fluid buildup, as discussed above, which could lead to changes in female behaviour and thus more proximal social space at this age. Additionally, other chemical compounds transferred during copulation could be affecting social spacing. For example, cis-vaccenyl acetate (cVA), which has been implicated in proper social spacing and aggregation (often around food), is also transferred during copulation from males to females (Bartelt et al., 1985). Thus when *Drosophila* females are most fertile, they are most receptive to copulation and will have a buildup of these chemicals possibly resulting in more proximal social space (Lof et al., 2008). Similarly, when individuals are highly fertile, they may be more social as to look for sexual partners, which may explain the increased social space in individuals at 14 and 21 days of age. Interestingly, at older ages (four and seven weeks of age), individuals become more distal. An evolutionary explanation for this phenomenon may be the antagonistic pleiotropy theory, which explains how pleiotropic genes that are beneficial in early life and promote reproduction will become detrimental in later life. This may also be the pattern that is seen with social spacing, as individuals are more proximal at younger ages when they are highly fertile and then become more distal at older ages when fecundity declines.

At older ages, however, both males and females were more distal, or less social. There are several possibilities that may explain this result. Firstly, there may be an advantage to being more distal, or less social when older, as it has been suggested in humans who tend to have smaller social networks as they age so they can focus on more meaningful relationships (Charles and Carstensen, 2010). Perhaps this could be occurring in flies as they are only interacting with the same sex in the social space assay who may not be providing them with any specific benefit and thus there is no pull factor to bring them closer together. Secondly, there may be no reproductive or other incentive to be more social when older so individuals may be indifferent to being closely grouped. And finally, if sensory perception such as olfaction is important for proper social spacing, decline in the sensory modalities with age may be causing a change in the social behaviour of aged flies. The age at which flies become more (two and three weeks) or less (four and seven weeks) social falls into the second and third phase of aging, respectively, where other behaviours are in decline but have plateaued in the second phase before completely declining in the third, such as locomotion and geotaxis (Simon et al., 2006). It is possible that the change in these behaviours has an effect on social behaviours as well. Additionally, at two and three weeks, the social spacing is closer which corresponds with the plateau of some behaviour.

The effect of both more proximal social spacing at two and three week old parents and more distal spacing at four and seven week old parents was transmitted to the next generation. Because the progeny were more social with two and three week old parents and less social when their parents were older, this cannot be explained by decay in sensory modalities or an accumulation of genetic mutations that are passed on, as I would expect a gradual increase in their social spacing or a progressively less social phenotype. This also cannot be a learned behaviour from the parents as the different generations were never in contact.

Several ideas may explain this phenomenon. Firstly, random mutations have a higher chance of affecting longer genes. Many long genes are often involved in the structure and function of the synapse and thus random mutations that are likely to affect longer genes will affect synapses and thus social behaviour, including social spacing (King et al.,

2013). Perhaps fewer mutations, that accumulate in two or three weeks, result in an inhibition of repulsive cues in the environment causing the progeny to be more social, whereas more mutations that have accumulated by 30 or 50 days old in the parent result in greater repulsive cues causing them to be less social. Secondly, the damage that is introduced to the gametes is due to factors external to the genome, like certain types of RNA. Mutations in part of the RNA silencing system known as Piwi RNA have previously been shown to cause defects in oogenesis and reduce the number of GSCs (Malone and Hannon, 2009). One member of the piwi RNA (piRNA) group found in *Drosophila*, known as Aubergine (Aub) has also been shown to interfere with gametogenesis, the development of the embryo and has even been linked to accumulations of double stranded breaks in the DNA of germ cells (Harris and Macdonald, 2001; Klattenhoff et al., 2007). These piRNA are also involved in silencing transposons and defects would lead to increased transposition, which could affect genes of the next generation and possibly interfere with their ability to perceive environmental cues (Malone and Hannon, 2009).

The effect of having aged parents was not visible following the first generation, as the second through fifth generation of old parents were as social as those with young parents. This may be due to increased heterogeneity within the population, which could ameliorate the damaging effects of the aged parents, and thus changes in social behaviour were not observed. However, there were differences in third and fifth generation of old parents in females were the third generation females were less social and the fifth generation females were more social. This may be due to inherent genetic variation within the population that may also be affecting the seven-day-old controls resulting in a significant difference between the third and fifth generations of old and young parents. Perhaps these generations should be characterized again based on other behaviours to see if there is a difference.

3.4.3 Aged Flies And The Progeny Of Aged Flies Are Less Able To Avoid the *Drosophila* Stress Odorant

Social avoidance is the avoidance of a stressor, where in this context the stressor is a vial that previously contained stressed flies that emit the *Drosophila* stress odorant (dSO). In a more typical manner for behaviour with aging, both males and females were less efficient at avoiding this stressor. This may be due to a loss of sensory perception with time, like the ability to detect odors, which has been previously shown to deteriorate with aging and the effect can even be passed on to the next generation (Burns and Mery, 2010). Another explanation as to why the aged flies were less able to avoid the stressor could be due to decline in the function of the nervous system with aging (Paul et al., 2007). However this effect cannot explain why the same trend was seen in the next generation as they were tested when they were young. This suggests that the change in performance with parental age can be due to mutation accumulation within the parental gametes that are transmitted to the next generation. Because this behaviour does not follow the pattern observed with social space, as the avoidance of stressors did not improve in individuals that were 14 and 21 days old and their young progeny, I can conclude that these behaviours are affected by aging in different ways. However, it must also be considered that this is a lab artifact as these flies being housed in lab for a very long time and its possible that have learned to be indifferent to other flies, whether stressed or not, in the vial, although this still does not account for the inheritance of the phenotype.

3.4.4 Older Fathers Affect The Sons But Not The Daughters In Social Space

One notable finding in this chapter is that older fathers transmit damage to the sons but not the daughters as the sons were less social with their nearest neighbours. This is interesting because these aged fathers contribute very little cytoplasm to the zygote and thus, the material that is most likely passed on to the progeny that is damaged is genomic DNA. This may be due to reactive oxygen species that have been shown to be particularly detrimental to sperm, as ROS can attack the double bonds of unsaturated

fatty acids leading to a loss in cellular membrane integrity (Aitken and Krausz, 2001). Additionally, the sex difference observed when the sons but not the daughters are affected is interesting because these females are somehow able to resist this damage. And finally, aged fathers are sufficient to cause this change in social behaviour whereas 30-day-old mothers are not sufficient to cause a change in the sons or daughters (as demonstrated by others in the lab; see Appendix E).

One piece of evidence that leads to the conclusion that DNA is damaged with male age, and that this may be contributing to changes in social behaviour, is that the sperm heads, as visualized with fluorescent microscopy, are bent in older males. This may be due to the improper exchange of histones for protamines when the genome is compacted. As seen in mice, improper timing of the expression of protamine 1 or improper rationing of protamines 1 and 2 lead to male infertility (Aoki, 2005). And interestingly, removal of the genes for protamines lead to 20% of sperm heads having a bent shape in *Drosophila*, while remaining fertile (Rathke et al., 2010). Improper compaction of the genome can leave areas of the DNA exposed to stressors and damage such as reactive oxygen species, which will then affect the next generation. Alternatively, others suggest that improper packaging of the genome during compaction may be a way for repair mechanisms to interact with the DNA and repair damage that was previously not fixed (Belloc et al., 2009). This mechanism may be beneficial in younger males that have intact repair machinery but older males have less efficient DNA repair methods and thus improper compaction of the genome causes vulnerability that can lead to more inherited damage. Additionally, incomplete exchange of histones for protamines, as seen in both humans and mice, will result in the transmission of post-translational modifications on histones, an epigenetic mechanism, affecting the progeny (Johnson et al., 2011).

The sons of old fathers were found to be less social, while the daughters remained as social as those with young fathers. This is particularly striking, as the only difference between the groups is the sex of the fly, as they are siblings. There are several possibilities as to why the phenomenon occurred. Firstly, compensation from an additional X chromosome in the females may be ameliorating the effects of an aged X chromosome from the father that is not masked in males. Additionally, females have been

shown to be more resistant to stressors like starvation, so aging of the parent may be one of these stressors that females are able to suppress or not be affected by while males lack this ability (Matzkin et al., 2009). Alternatively, the presence of a Y chromosome may be perpetuating the damage provided by the father causing the sons to be less social. The Y chromosome will remain mostly intact from one generation to the next, as there is no cross over with an additional Y chromosome. Therefore mutations gathered here can accumulate throughout aging and will not be diluted and transferred to the next generation. It has even been reported that the neo-Y chromosome of *Drosophila miranda* has eroded over generations since there is no strong defense to resist damage (Kaiser and Bachtrog, 2010). Alternatively, hormonal differences between males and females may account for the sex difference observed in behaviour, as sex differences have previously been shown in relation to senescence (Bowen and Atwood, 2004). However, more work is needed to determine which factor, sex chromosomes or sex hormones, has a larger impact on behaviour when fathers are aged.

3.4.5 Conclusion and Significance

This chapter has illuminated the phenomenon that damage associated with parental age can be inherited and impact the next generation, including the spacing between individuals and the avoidance of stress. This effect mostly dissipates in the second generation of aged parents as survival and social spacing returns closer to parental levels. Interestingly, the fathers are more important for affecting the social behaviour of sons and not daughters. Future work on this topic includes understanding this sex difference observed in the progeny of old fathers and using neuro-genetic tools available in *Drosophila* to manipulate the sex determination pathway such that I may understand how parental aging affects the next generation based on hormonal expression in the fly. This sex difference is paralleled in human studies as males are diagnosed with the neuropsychiatric disorder, autism spectrum disorders, at a rate four times higher than females. Additionally, older fathers have been correlated with children with neuropsychiatric disorders like autism and schizophrenia in human.

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Chapter 4 - Manipulations of the Biological Aging Process Affect Social Behaviour of Parents and Progeny

4   Abstract

Previously, I showed that social spacing differs with age and that parental aging also affects the social spacing of the next generation. Here, I show which biological mechanism of aging might be responsible for the changes to social behaviours with aging and with parental aging. I used different laboratory tools to accelerate the aging process (increased rearing temperature and exposure to reactive oxygen species generator, methyl viologen) or decelerate the aging process (caloric restriction). I then tested both the parents exposed to these conditions and their progeny in social spacing. I found that accelerating physiological aspects of aging results in an aged phenotype of social spacing (more distal) earlier in individuals and can be passed on to the progeny. Alternatively, when parents are exposed to conditions that decelerate aging, I found that older parents and their progeny behave like younger parents (more proximal) or progeny with younger parents in social spacing.

4.1 Introduction

There are many interconnected mechanisms of aging that lead to an aged phenotype and may affect conserved neural circuits in the developing progeny. In the context of my study, there are many unanswered questions, such as: which mechanism of aging is responsible for causing a change in social behaviour? Additionally, do these mechanisms that can affect aging also result in a change to the next generation? Finally, is it age-related changes (biological aging) or learned social experience, such as total time spent together (chronological aging), required to result in a change in social space? In order to begin addressing these questions, I adjusted environmental conditions in order to accelerate or decelerate the biological aging process. I then used the social space assay to assess if accelerating or decelerating the aging process will have an effect on aging individuals and if this change is transmitted to the next generation.

Chronological age is length of time an organism has lived and the length is determined by heredity (Iliadi et al., 2012). In contrast, both genes and the environment influence biological aging. Throughout life, metabolism can change and can be influenced by biological aging factors and lead to an aged phenotype. One way that metabolism can affect the individual is by a gradual accumulation of metabolic by-products that can damage pathways and proteins (Conti, 2008). As *Drosophila* does not internally regulate its body temperature (Conti, 2008), one way to manipulate its metabolism is to adjust the temperature, as higher temperatures (29°C as opposed to 25°C) can speed up metabolism, and lower temperatures (18°C) can slow down metabolism. Increased metabolism at higher temperatures has been shown to affect protein and enzyme stability and may explain why individuals at higher temperatures experience reduced longevity (Conti, 2008; Halle et al., 2015). Alternatively, lower temperatures have been shown to extend longevity. For example, during colder months, insects such as *Drosophila* and grasshoppers have been shown to down-regulate metabolism and pause reproduction in order to preserve energy to survive until the warmer months and in the process, extend longevity (Flatt and Schmidt, 2009). As a consequence, it is also expected that increased metabolism would result in reduced longevity and fecundity. Previously, behaviours, such as motor activity, have also been affected by changes in temperature. For example, senescence of negative geotaxis in *Drosophila* was accelerated at higher temperatures and was slower at lower temperatures (Grotewiel et al., 2005). Therefore, it is not chronological aging, but factors that influence biological aging that can lead to senescence and changes in negative geotaxis (Grotewiel et al., 2005).

The oxidative stress hypothesis states that the rate at which aging occurs depends on the rate at which oxidative damage accumulates in cell components (Harman, 1956). Around 10% of the reactive oxygen species (ROS) generated in cells is created in a controlled way for processes such as cell signaling and immune function (Dröge, 2002). However, the remaining 90% of ROS arises from by-products of metabolism during production of ATP through the electron transport chain of mitochondria that accumulate over time (Balaban et al., 2005). Thus the generation of ROS is intimately linked with metabolic

processes like respiration. Moreover, the integrity of the mitochondrial membrane weakens over time and ROS can leak into other regions of the cell causing damage to macromolecules and possibly DNA (Balaban et al., 2005). Therefore, this damage could affect both the individual and, if present in the gametes, could be passed on to the progeny.

Oxidative stress can be a major cause of aging, especially behavioural senescence, because the brain is vulnerable to free radicals since it has a high metabolic rate. One study tested how oxidative stress affects learning and memory in *Drosophila* that were five, 25 and 50 days of age (Haddadi et al., 2014). They found a significant reduction in long term memory retention in older flies (Haddadi et al., 2014). They also saw older flies had higher order neurodegeneration in cell bodies, a reduced number of synapses, a reduction in antioxidant enzyme activity, and a decrease in the level of neurotransmitter enzymes in mushroom body extrinsic neurons (Haddadi et al., 2014). Oxidative stress and lifespan regulation have also previously been linked, although there is conflicting data regarding whether a reduction in antioxidant enzymes such as superoxide dismutase (SOD) has an effect on lifespan (Paul et al., 2007; Speakman and Selman, 2011).

Caloric restriction (CR) is the practice of reducing calorie intake by 30-40% while maintaining the same protein content and, in the case of *Drosophila*, yeast content (De Loof, 2011; Ostojić et al., 2009). This has proven successful in promoting longevity in models such as *Drosophila*, mouse, *S. cerevisiae* and *C. elegans* (Kenyon, 2010; Partridge et al., 2005). Although, the exact pathway in which CR operates could be via conserved pathways or may vary among organisms (Kenyon, 2010; Partridge et al., 2005). One evolutionarily conserved pathway that has been shown to be affected by CR is the Insulin/IGF pathway, as it is involved in nutrient sensing (Kenyon, 2010). Energy from food is divided amongst reproduction, maintenance of the soma, and storage in fat for later use so cells must be able to sense the amount of nutrients that are ingested such that energy can be shuttled into these pathways (Skorupa et al., 2008). One study suggested that by adjusting levels of protein and carbohydrates, energy shuttling and storage adapts so the animal consumes enough nutrients. Thus behaviour such as feeding and egg-laying will be affected in addition to changes in longevity when the levels of

protein and carbohydrates are changed. For example, in a study where flies were fed a low protein/ high calorie diet, flies adjusted their eating behaviour and suppressed reproduction to consume more protein, which resulted in obese flies that had shortened lifespans (Skorupa et al., 2008). However, when flies were fed the high protein/ low calorie diet, flies promoted reproduction, inhibited fat storage, and ate less. In this condition, longevity did not increase as energy was used in high reproduction (Skorupa et al., 2008). It is suggested that a reduction in calories will reduce excess ROS buildup to then lower metabolic demand. Therefore, with age, individuals on a CR diet should be biologically "younger" as there has been less ROS damage over time that would preserve macromolecules and pathways from damage associated with aging. As long as the calorie content is not reduced such that it is causes starvation, caloric restriction should extend longevity.

Mutants such as *methuselah, Indy, chico* and mutations in the ecdysone receptor of *Drosophila* have each been shown to alter longevity (Balaban et al., 2005; Cook-Wiens and Grotewiel, 2002; Simon et al., 2003). Although much can be learned from artificially accelerating or decelerating the aging process genetically, it is not an accurate representation of the normal aging process that is affected by the environment. Therefore, I will not be using mutant flies and will be adjusting rearing temperatures and diet in order to manipulate the aging process to adjust as few variables as possible.

My hypothesis is that adjusting the biological aging of *Drosophila melanogaster* will affect social spacing and that this effect will be passed on to the next generation that has not been exposed to these conditions. Here, I will accelerate the biological aging of *Drosophila melanogaster* via increased aging temperature and exposure to methyl viologen (paraquat, a ROS generator) and test the artificially aged individuals in social space. I will also test the progeny of these parents in social space, although the progeny have not been exposed to either higher temperatures or paraquat. I expect flies that are exposed to the stress condition to demonstrate the aged phenotype earlier than those who are not exposed and that this effect will be passed on to the next generation. I will also counter the aging process by feeding *Drosophila* a calorie-restricted diet and will test

these flies and their first generation in social space. I expect older flies exposed to calorie-restricted food to have the social spacing more similar to younger flies and that this effect would be passed on to the progeny.

4.2 Methods

Please see chapter 3 section 3.2.1- 3.2.7 for fly handling, separating by sex, and methods for the social space assay and statistical analysis. All modifications to these methods are listed below.

4.2.1 Aging, Survival, and Fecundity Curve At 29°C

The aging *Drosophila* at 29°C as well as the survival and fecundity curves were performed as mentioned in chapter 3. Again, fecundity is defined here as the number of progeny that arise from eggs laid (fertility) and is represented as a cumulative value over the lifespan of the female fly. Following collection of Canton-S *Drosophila melanogaster* from bottles reared at 25°C, flies were placed in a 29°C incubator. Flies were transferred to new food every two-to-three days and were always placed back into the 29°C incubator. Resulting survival curves were generated using Microsoft Excel and the 100%, 90%, 50% and maximum survival were extracted and compared to 25°C using GraphPad Prism 7 and a two-way ANOVA with a Holm-Sidak test to correct for multiple comparisons.

4.2.2 Social Space Assay Of Aged Flies And The Progeny Of Aged Flies At 29°C

Drosophila aged at 29°C were tested with the social space assay at seven, 14, 21, and 30 days of age against age-matched flies at 25°C as previously described (15 flies/ chamber, separated by sex, n=9x15 flies; Simon et al., 2012). Additionally, the first generation of flies aged to seven days at 29°C were placed back at 25°C to be aged to seven days prior to testing with the social space assay against flies who have been raised and aged at 25°C (15 flies/ chamber, separated by sex, n= 9x15 flies). Flies were acclimated to the humidity chamber (24°C, 50% humidity) for two hours prior to being added via

aspiration to the two dimensional apparatus. Images of the chambers were taken at 30 minutes when flies have settled (Simon et al., 2012). The images were analyzed using ImageJ to get the distance to the closest neighbour within the chamber that were then added to the statistical program Prism 7 using a one way-ANOVA with a Holm-Sidak *post hoc* test to correct for multiple comparisons in groups larger than two, or an unpaired t-test for groups of two (all measurements expressed as a mean ± standard error to the mean, see chapter 2 for details).

4.2.3 Survival Curve Of *D. melanogaster* Exposure To Reactive Oxygen Species (Methyl Viologen/ Paraquat)

A survival curve was first generated in order to determine the 90% biological age of flies fed Methyl Viologen (Sigma Aldrich, St. Louis, Missouri, USA; hereby known as paraquat). Canton-S *D. melanogaster* were aged six days old in mixed sex prior to separation by sex and starved for six hours in an empty vial (5 males or females per vial, n= 9 per concentration of paraquat; 25°C, 50% humidity 12:12 light: dark cycle). Flies were then administered either 0 mM, 10 mM, 20 mM or 40 mM of paraquat in a solution of 5% sucrose and 1% blue dye (club house[®]) to confirm food consumption as the dye was visible in the intestines of the fly (Hosamani and Muralidhara, 2013; Lawal et al., 2010). The solution was added to Whatman[®]3 filter paper (Sigma Aldrich, 500 ul/ paper) in empty vials and new aliquots of sucrose solution were replaced every 24 hours. The number of dead flies in each vial was measured every two hours until all flies were dead, in the 10 mM, 20 mM, and 40 mM conditions. The number of dead flies in the 0 mM condition was then measured once each day until all flies were deceased.

4.2.4 Social Space Assay Of Paraquat-Exposed Flies

After 13.5 hours of either 0 mM or 20 mM paraquat exposure, both male and female flies reached the biological age of 90% survivability; therefore this time was used to measure paraquat-exposed flies in social space. Flies were starved 6 hours and were fed 20 mM of paraquat in a solution of 5% sucrose and 1% blue dye (club house[®]) for 13.5 hours before addition to the social space assay (note that a mouth aspirator was not used here, but a

funnel was used to add flies to the chamber to avoid possible paraquat exposure to the experimenter). The social space assay was then performed as previously described (separated by sex, 15 flies/ chamber, n= 9, 25°C, 50% humidity; Simon et al., 2012). The images were analyzed as mentioned in chapter 4.1.2 and chapter 2.

4.2.5 Generating The Progeny Of Males Exposed To Paraquat And The Social Space Assay

To see if ROS exposure of one parent could cause changes to the behaviour of the progeny, six-day-old males were starved and fed either 0 mM or 20 mM paraquat, as described above. After 13.5 hours, males were mated with young, non-exposed, virgin females and permitted to mate in bottles for several days before they were removed. The resulting progeny of paraquat fed fathers in these bottles were separated by sex at seven days of age (15 flies/ vial; separated by sex; n= 9) and were tested with the social space assay as previously described (Simon et al., 2012). The images were analyzed as mentioned in chapter 4.1.2 and chapter 2.

4.2.6 Social Space Assay Of The Progeny Of Paraquat-Exposed Flies

Six-day-old *D. melanogaster* were starved for six hours and flies were fed either 0mM or 20 mM paraquat for 13.5 hours as described above. Flies in mixed sex were then transferred to bottles containing Jazz Mix media (brown sugar, corn meal, yeast, agar, benzoic acid, methyl paraben and propionic acid; 50% humidity, 25°C, and 12:12 light: dark cycle) for two-to-three days before removal. The eggs in the bottles were allowed to develop to adulthood prior to testing with the social space assay as previously described (Simon et al., 2012; separated by sex, 15 flies/ chamber, n= 9). The images were analyzed as mentioned in chapter 4.1.2 and chapter 2.

4.2.7 Aging Flies, Survival, Fecundity And Social Space Over Caloric Restriction Food

All aging, survival, fecundity, and social space assays were performed as described above and in chapters 2 and 3. Upon collection of *Drosophila* from stock bottles, flies were placed over food with low yeast/low sucrose content, as this was found to be the most efficient combination of adjusting sugar and protein (yeast) to reduce the calories by 30-40% in *Drosophila* (recipe adapted from (Min et al., 2007), 50.8 kcal/ 100 ml media, Appendix G).

4.3 Results

4.3.1 Survival And Fecundity Of *Drosophila Melanogaster* At 29°C, Fed 20 mM Of Paraquat, And Fed Caloric Restriction Food

In order to see the effect of altered environmental or food conditions on *Drosophila* longevity and reproduction, survival curves were generated for all three conditions and fecundity curves was performed for individuals at 29°C and on caloric restriction (CR) food. The fecundity curve was not performed for individuals fed paraquat as this chemical kills flies within two-to-six days (depending on the concentration to which they were exposed) and thus egg laying and progeny viability were not determined.

Individuals aged at increased temperature (29°C) have both accelerated aging and reduced fecundity as compared to flies aged at 25°C (Figure 4.1A). Flies aged at higher temperatures had 100% survivability at seven days old but reached 90% survival at just 11 days followed by 50% survival at 35 days. As compared to survival values at 25°C, the 90% survival, 50% survival and maximum survival rates were significantly reduced at 29°C (respectively; Figure 4.2A). Fertility, as measured by the cumulative egg laying per female, was not different throughout life between individuals at 25°C (112.514 ± 8.67) or 29°C (77.66 ± 11.13; Figure 4.2B). However, there was a difference between the fecundity, as measured here by egg-to-adult viability, among flies at different ages at

25°C and 29°C after flies reached 90% survival (Figure 4.2C). Fecundity declined after just 18 days with a cumulative fecundity (total number of progeny per female) of 17.24 ± 3.30, which is 2.5 times earlier than flies at 25°C whose fecundity declined after 47 days (Figure 4.2B). Therefore, I was unable to use the progeny of individuals aged to 30 days at 29°C to test in social space.

To determine when *Drosophila* fed paraquat reach 90% survival, a measure of when flies begin to die, I generated a survival curve. I generated several survival curves at concentrations of paraquat in a glucose-water mixture of 0 mM, 10 mM, 20 mM and 40 mM (Appendix F). I chose 20 mM as the time to 90% survival was 13.5 hours for both males and females. This was also a convenient amount of time to prepare the flies for feeding, feed them, and test the flies in social spacing at the necessary time (Zeitgeber time 4-7 when lights are on at 8am) and because this concentration has been used to accelerate aging in other studies as it does not kill *Drosophila* too quickly but still has a measureable effect (Lawal et al., 2010; Figure 4.1B).

I also performed a survival curve on *Drosophila* fed caloric restriction (CR) food to evaluate longevity extension and determine when they reach 90% survival. However, due to methodological errors, I have not yet obtained the data. I am currently repeating this experiment and the data are currently being collected. I was able to gather data for a fecundity curve and found that egg-to-adult viability began to decline around 42 days, which is similar to those on regular food (Figure 4.1C). Both fertility and fecundity were increased in flies raised on caloric restriction following 90% survival, where the cumulative fertility was 364.77 ± 92.65 for caloric restriction and was 112.51 ± 8.67 for regular food and the cumulative fecundity was 109.6 ± 22.70 for CR and 57.52 ± 9.10 cumulative progeny per female on regular food; Figure 4.3).
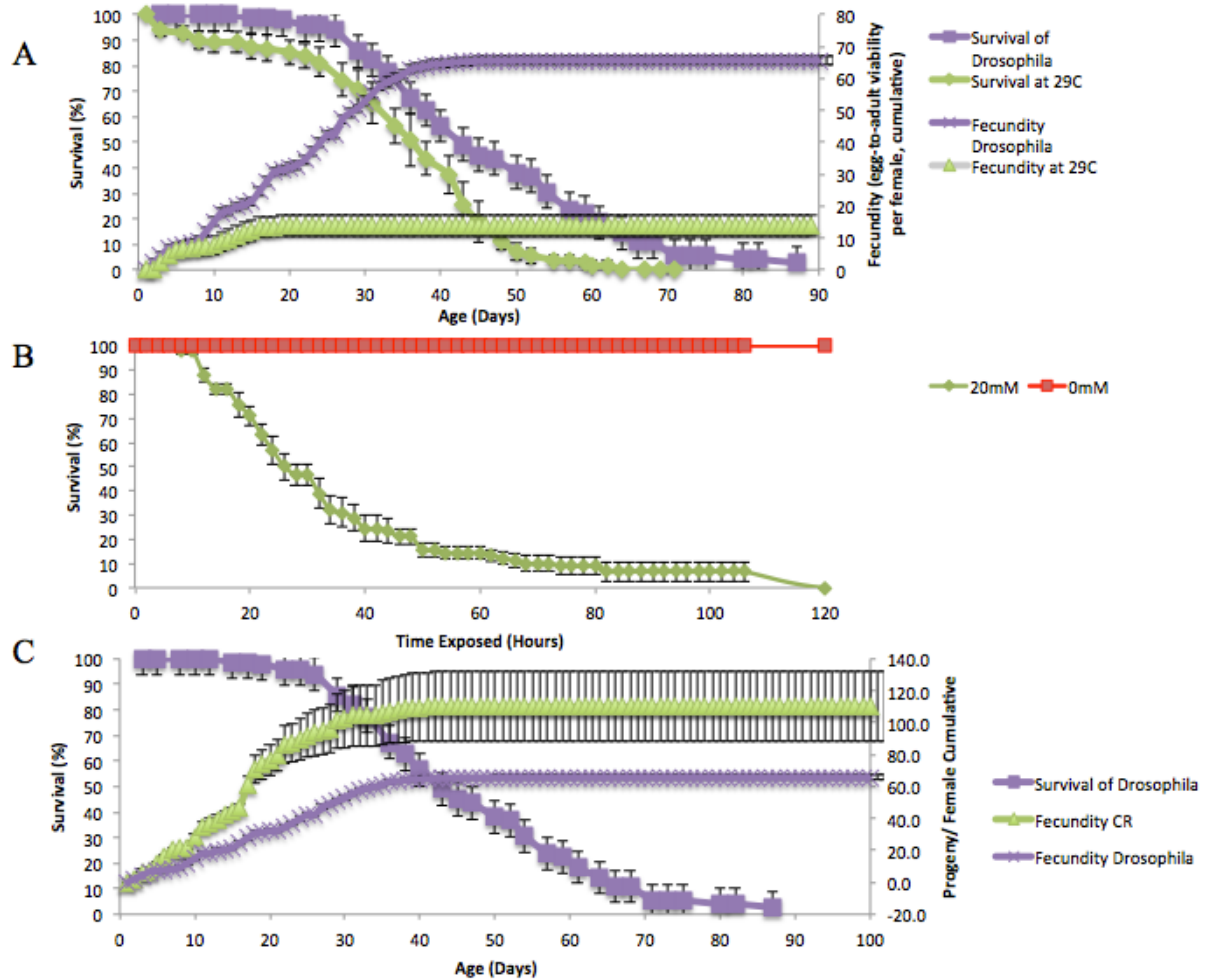
Figure 4.1 Survival and fecundity (cumulative egg-to-adult viability) in *D. melanogaster* exposed to (A) increased aging temperatures at 29°C, (B) 20 mM of methyl viologen (paraquat), or (C) calorie restricted food. (A) Survival and fecundity of *D. melanogaster* aged at 29°C (40 flies/ vial, n= 9 vials) compared to survival and cumulative fecundity of flies aged at 25°C. Seven-day old flies have 100% survivability and are considered young. Flies reach 90% survivability at 11 ± 6.77 days, and 50% survival at 35 ± 3.21 days old, which is greatly reduced compared to those raised at 25°C that reach 90% survival at 30 days at 50% survival at 50 days. Fecundity at 29°C declined after 18 days as compared to those raised at 25°C that declines after 47 days with cumulative fecundity of 13.68 ± 2.62 with reduced cumulative fecundity as compared to 25°C (65.53 ± 5.52). **(B)** Survival Curve of male and female *D. melanogaster* exposed to 20 mM paraquat over time. After 13.5 hours, flies were at 90% survival (fecundity was not tested). **(C)** Fecundity of flies fed calorie restricted food declines after 36 days but has increased cumulative fecundity (109.6 ± 22.03) compared to those on regular food.
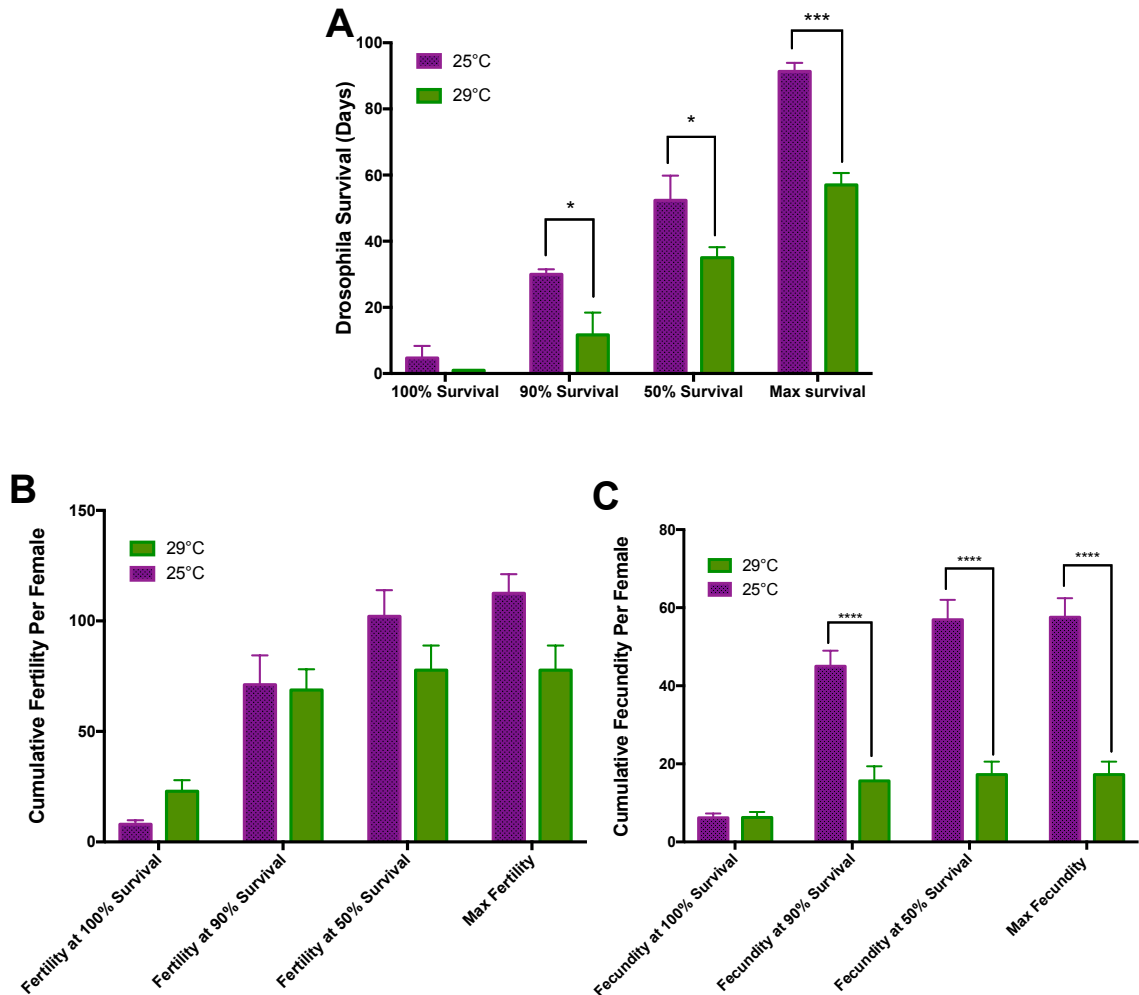
Figure 4.2 Comparison of survival and fecundity of flies at 25°C and 29°C. (A) 100%, 90%, 50% and maximum survival of *Drosophila* at 25°C and 29°C shows that individuals at 29°C die more rapidly. Two way ANOVA with a Holm-Sidak post test, with the bar graph representing the mean and standard error to the mean (*p< 0.05, ***p< 0.001). Significant differences arise at 90% survival as *Drosophila* raised at 25°C reach 90% survival at 30 ± 1.53 days, 50% survival at 50 ± 7.5 days and have max survival 91 ± 2.60 days but *Drosophila* at 29°C reach 90% survival at 11 ± 6.77 days, 50% survival 35 ± 3.21 days and maximum survival at 57±3.61 days. (B) Cumulative fertility at each age point does not differ between individuals at 25°C or at 29°C (two- way ANOVA, Holm-sidak *post hoc* test). The cumulative fertility at 25°C was 112.514 ± 8.67 and was 77.66 ± 11.13 at 29°C. (C) The fecundity of individuals at 29°C was significantly reduced compared to those at 25°C for 90% and 50% survival, in addition to cumulative fecundity (****p< 0.0001). The cumulative fecundity at 29°C was 17.24 ± 3.30 as compared to 57.52 ± 4.91 at 25°C (two-way ANOVA with a Holm-Sidak post test; ****p< 0.0001).
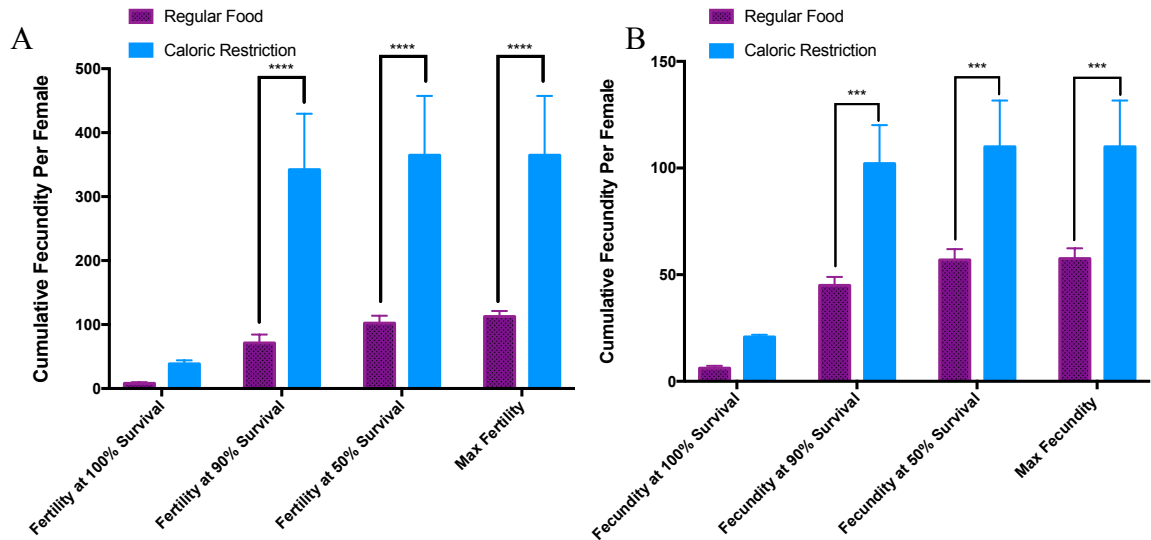
Figure 4.3 Flies on CR food have increased fertility and fecundity compared to those on regular food. (A) Flies on caloric restriction food have increased fertility as compared to those on regular food after 90% survival with a cumulative fertility of 364.77 ± 92.65 for CR and 112.51 ± 8.67 for regular food (****$p <$ 0.0001, two-way ANOVA with a Holm-Sidak post test; mean and standard error to the mean) (B) Flies on caloric restriction food have increased fecundity as compared to those on regular food after 90% survival with a cumulative fertility of 109.6 ± 22.70 for CR and 57.52 ± 9.10 for regular food (***$p <$ 0.001, two-way ANOVA with a Holm-Sidak post test; mean and standard error to the mean).

4.3.2 Social Space Of *Drosophila melanogaster* And Their Progeny At 29°C, Fed 20 mM Of Paraquat And Fed Caloric Restriction Food

In order to see how increased temperature affects the social spacing of aged *Drosophila*, I tested flies that were aged at 29°C for either seven, 14, 21, or 30 days. Because survival decreases quickly at 29°C, I was unable to test individuals at 50 days old. Thirty day old flies aged at 29°C are more distal to their closest neighbour than seven-day-old flies as hypothesized (one-way ANOVA, $p <$ 0.01, $p <$ 0.0001; Figure 4.4A). Fourteen and 21-day-old individuals aged at 29°C are more proximal to their closest neighbour (one-way ANOVA, $p <$ 0.0001) as compared to those aged to seven days old. However, they are still more distal than age- and sex-matched individuals at 25°C.

To see if the effects of increased temperature on parental aging can be passed on to the second generation, I tested the progeny of seven-day-old and 30-day-old flies aged at 29°C were reared and aged to seven days at 25°C using the social space assay as compared to those whose parents were aged at 25°C. I found that both male and female progeny of parents aged at 29°C were more distal than those that were both reared and aged to seven days old at 25°C, although females were not significantly different and males were (*p< 0.05; Figure 4.4B; for distance values please see Appendix H).

Male flies that were fed 20 mM of paraquat for 13.5 hours and then tested with social space were more distal to their closest neighbour than males fed 0 mM of paraquat (one-way ANOVA, p< 0.0001; Figure 4.4C; for distance values please see Appendix I). However, regardless of whether of females were fed 20 mM or 0 mM of paraquat, they had similar social space and were not significantly different. I then tested the next generation of flies whose parents had been exposed to 20 mM or 0 mM of paraquat while the individuals tested in social space were not exposed to paraquat, I found a similar result where the progeny of fed parents were more distal than those parents who were not fed paraquat (Figure 4.4D; for distance values please see Appendix I). Specifically, the male progeny fed 20 mM of paraquat were more distal to their nearest neighbour as compared to those that were fed 0 mM paraquat (p< 0.0001). Additionally, the female progeny fed 20 mM of paraquat were more distal to their nearest neighbour as compared to those that were fed 0 mM paraquat although not significantly different.

Both males and females aged 30-days or 50-days-old on caloric restriction (CR) food were not more distal to their closest neighbour as compared to those at seven days old on CR (one-way ANOVA, Figure 4.4E; for distance values please see Appendix J). Interestingly, the young progeny of parents aged either 30-days or 50-days-old on CR food were more proximal to the nearest neighbour in both males (p< 0.0001 and p< 0.05, respectively) and females (p< 0.001 and p< 0.01, respectively) as compared to the progeny of seven-day-old parents on this diet (Figure 4.4F; for distance values please see Appendix J).
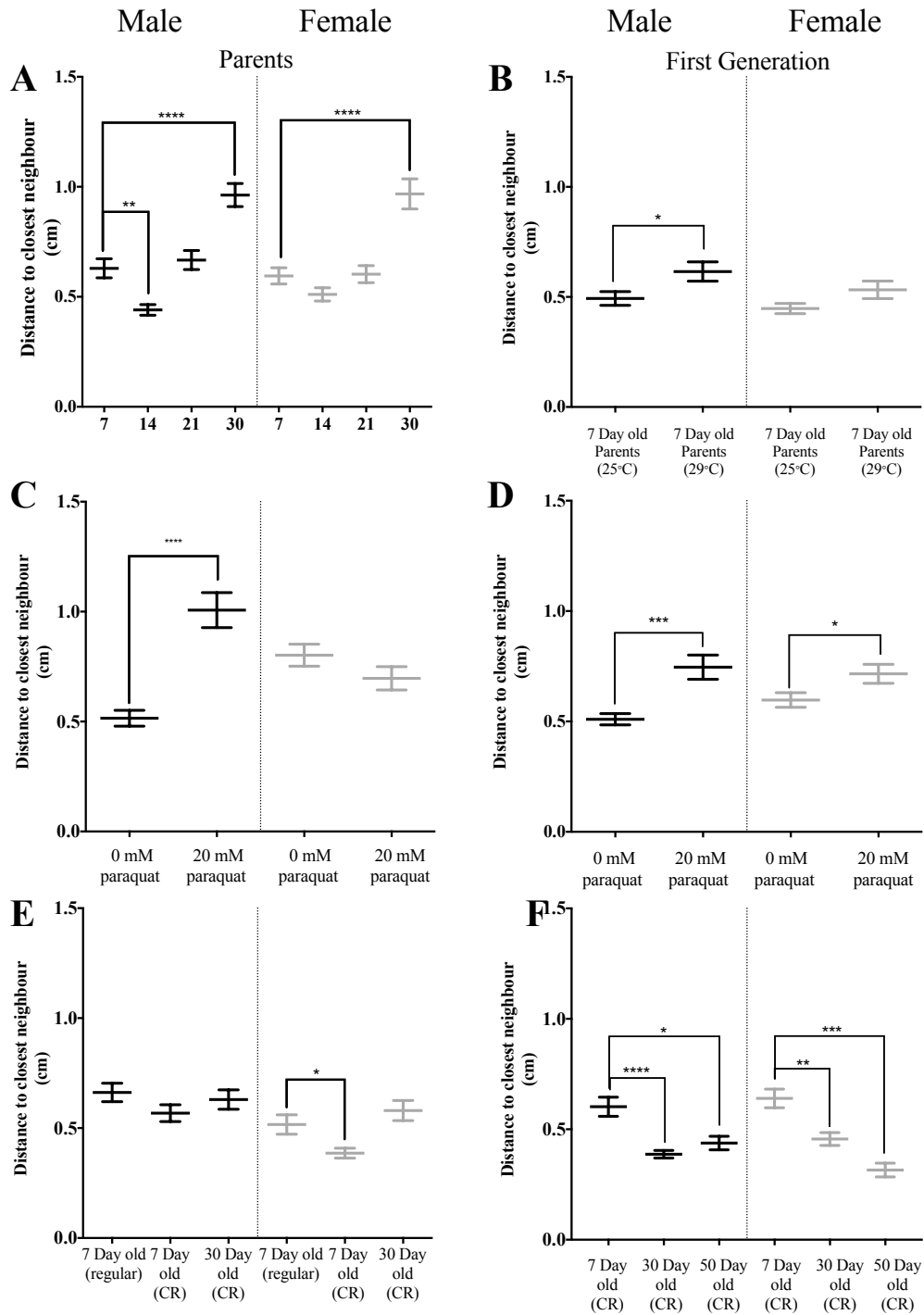
Figure 4.4 Social space of young and old *D. melanogaster* and their progeny exposed to either 29°C (A-B), paraquat (C-D) or calorie restricted food (E-F). Each set of asterisks represents statistical significance using a one-way ANOVA with a Holm-Sidak post test *p< 0.05, **p< 0.01, ***p< 0.001, and ****p< 0.0001. Each graph represents the mean and standard error to the mean. (A) *D. melanogaster* aged at 29°C are more distal to their closest neighbour. (B) The progeny of seven-day-old *D. melanogaster* at

29°C are more distal than the progeny of seven-day-old flies at 25°C. (C) Male *D. melanogaster* fed paraquat were more distal to their closest neighbour than males who were not fed but females who were fed paraquat did not differ with respect to distance. (D) The Progeny of *D. melanogaster* fed 20 mM paraquat were more distal to their closest neighbour. (E) Males on calorie-restricted food, and to some extend females, are closer to their closest neighbour at older ages and are not very different from young calorie restricted and non-calorie restricted flies. (F) The male first generation of older flies fed calorie restricted food are closer to their closest neighbour than the first generation of young flies on caloric restriction.

4.3.3 Social Space Of Fathers Fed Paraquat

Paraquat was fed to non-virgin seven-day-old males for 13.5 hours before mating with virgin females. Both the male and female progeny of males fed 20 mM of paraquat were more distal to their closest neighbour than the progeny of males who were fed 0 mM ($p < 0.01$ male, $p < 0.001$ female; Figure 4.5; for distance values please see Appendix I).
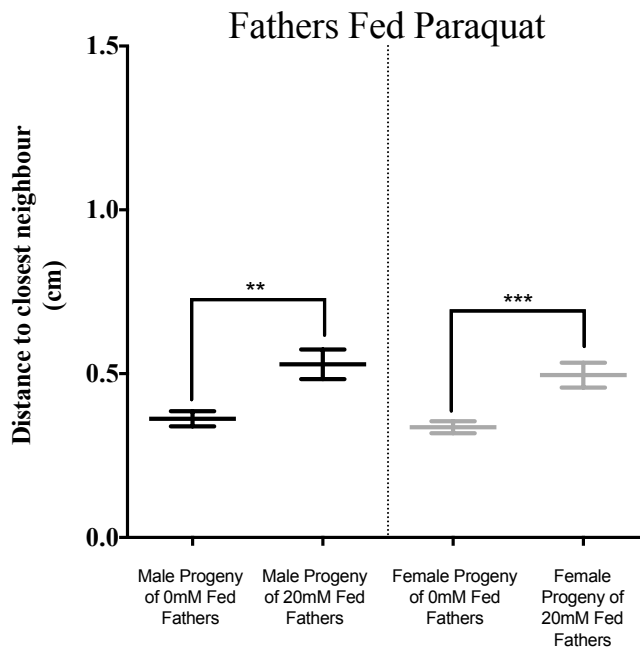
Figure 4.5 The male and female progeny of fathers fed paraquat (20 mM, 13.5 hours) are more distal to their closest neighbour compared to fathers fed 0 mM paraquat (15 flies/chamber, n= 9; One-way ANOVA; **p< 0.01, *** p< 0.001; graph represents the mean and standard error to the mean).

4.4 Discussion

4.4.1 Manipulations of the Biological Aging Process Cause Changes to Survival and Fecundity

By accelerating metabolism using increased aging temperature, I found that both survival and fecundity were reduced. When increasing the metabolism and its by-products, including ROS, I expected the longevity to be reduced, as the onslaught of chemicals generated would be detrimental to the fly. Especially as decreases in temperature have been shown to mediate the damaging effects of metabolism (Flatt and Schmidt, 2009). The reduction in fecundity may be due to a decrease in viability of eggs laid from the accelerated metabolism and a build up of by-products or damage to proteins that is impeding necessary pathways for proper development.

Due to a methodological error, I was unable to quantify the survival of *Drosophila* fed CR food but was able to measure the fertility and fecundity and found that both the number of eggs laid and the egg-to-adult viability increased. In accordance with other studies, I expected a reduction in fertility and fecundity, as individuals would be shuttling energy towards longevity and away from reproduction and thus would expect an extension in longevity (Conti, 2008). However it is possible that the increase in fertility and fecundity in early age was due to the availability of yeast in the food that is known to stimulate egg production (Skorupa et al., 2008). This effect was seen previously when *Drosophila* had greatly increased egg production but no extension in longevity when they were fed increased protein and reduced calories (Skorupa et al., 2008). The increased fecundity declines after 42 days, which is similar to what was seen on regular food. This is not what was expected as the caloric restriction theory states that progeny production would decrease to promote an extension in longevity. Therefore a study on the longevity is necessary to determine if actual caloric restriction was taking place here. A calorie reduced diet has also been shown to reduce core body temperature in homeotherms, such as mice, and thus these mechanisms (increased temperature and caloric restriction) may be opposing mechanisms in terms of accelerating or decelerating metabolism (Conti, 2008). Although, it has been suggested long ago that the mechanisms of aging are

different at different temperatures and thus individuals at lower temperatures may not have the exact opposite experience of those at higher temperatures (Maynard-Smith, 1958).

Survival at various concentrations of paraquat ranged into a scale of hours whereas the scale at 29°C was in days. Because flies must be fed paraquat in the absence of regular food, no fecundity was performed. This is because any eggs that would have been laid would have been on filter paper, which did not provide any food for the eggs. Each egg would also have to be transferred individually to a vial containing food, which can be difficult and cause physical damage. Therefore, a comparison of fecundity when flies are fed paraquat is not discussed.

4.4.2 *Drosophila* Aged At Increased Temperature And Their Progeny Are Less Social

Individuals exposed to higher temperatures for several weeks and tested with the social space assay appear to follow a similar pattern to those at physiological temperatures (Chapter 3.3.2). Flies at 25°C appear more social at 14 and 21 days old, while only male 14-day-old flies at 29°C are more social and both males and females are as social as control at 21 days. This shows that accelerating the metabolism also accelerates the aged phenotype of social space.

The highly reduced fertility at 29°C affected how we tested the next generation. Because the fecundity is greatly reduced early on (after 18 days), I chose to test the young progeny of parents that were exposed to higher temperature for just one week and the eggs laid by these parents were placed back at physiological temperature to develop. Interestingly, both males and females are less social than those whose parents were aged at physiological temperature. Therefore the effects of accelerated metabolism in the parents are enough after one week to cause a change in the social behaviour of the progeny. This phenomenon has been seen with other behaviour, as *Drosophila* parents entrained with a sensory motor task yield progeny that are able to respond the same way to a learning and

memory test (Williams, 2015). This shows an inheritance of behaviour even when the progeny are not exposed to the condition.

Some have suggested that changes in temperature do not affect longevity as simply as altering metabolism. Altering the temperature of the environment may be a selection mechanism that only promotes the survival of those that can withstand the fluctuation of temperature and thus those individuals display somewhat typical behaviour (Robert et al., 2007). As seen here, the flies that survived to the different age time points (one, two, three, and four weeks), displayed social behaviour that was similar to those at physiological temperature, although with an accelerated pattern. However, the progeny of parents exposed to increased temperature were less social than the parents exposed these temperatures. Therefore increased temperature and thus metabolism affects the gametes more than the individual. In fact, temperature fluctuation has been shown to affect sperm development (Radhakrishnan and Fedorka, 2011). From this, we can see uncoupling of changes to gametes and longevity determination. As previously suggested, energy provided to the progeny would negatively affect energy stores for the individual and thus more progeny would result in decreased longevity.

4.4.3 The Progeny Of Males Or Both Parents Exposed To Paraquat Are Less Social

When flies are fed a moderate concentration of paraquat (ROS generator) for a short period of time, males become less social while females are unaffected. In contrast, both the male and female progeny of those exposed to this stress for the same amount of time are also less social. Interestingly, when only males are fed paraquat for this same period of time and mated with virgin female, both male and female progeny are less social. Therefore, ROS affects individuals and gametes in different, but interconnected, ways. For example, it has been suggested that energy diverted to reproduction will result in a need for increased metabolism and thus higher ROS as a byproduct (Alonso-Alvarez et al., 2004). Naturally, as individuals age and continue to produce gametes, ROS is also accumulating, which can impact behaviour as ROS may affect neuronal processes. As males and females invest different amounts of energy into gamete production, the buildup

of ROS may be imbalanced and thus an individual's threshold to withstand such stress may also differ (Scharf et al., 2013). This may explain why I found that females who were fed paraquat were resistant to the stressor and displayed normal social space. Perhaps this is because more energy is required to make the large egg and therefore females may be better adept as shielding themselves from the harmful effects of ROS whereas males are more susceptible causing them to be affected and, in this case, causing a change in behaviour (Alonso-Alvarez et al., 2004). A sex difference to other stressors has also been seen in *Drosophila* as females were better adapted to resist both starvation and desiccation (Matzkin et al., 2009). However, this mechanism doesn't explain how ROS affects the progeny, where both males and females were affected the same way when parents were fed paraquat. Therefore, this alludes to different mechanisms by which ROS affects somatic and gametic tissues. Perhaps this could be an accumulation of ROS in sperm with time leading to DNA fragmentation and changes to chromatin within sperm heads (Zubkova et al., 2005). However, one other factor with the addition of ROS has caused both male and female progeny to be affected so there must be another mechanism here to explain this phenomenon.

4.4.4 Flies On Caloric Restriction Food And Their Progeny Are More Social With Age

Caloric restriction is suggested to operate by redirecting energy stores to reduce energy metabolism but increase production the synthesis of biomolecules and turnover of proteins (Weinert and Timiras, 2003). These changes in energy metabolism often include adjustments in pathways such as the Insulin/ Insulin-like Growth Factor-1 (IGF-1) pathway and target of rapamycin (TOR) pathway that has been linked to caloric restriction related increases in longevity in other animals (Kenyon, 2010). This is suggested to occur by limiting damaging by-products of metabolism that can prevent damage to macromolecules, including DNA. These damaging by-products may also account for the changes in behaviour with age and may explain why individuals fed caloric restriction (CR) food for 30 days were as social as those fed the same food for seven days or fed non-CR food. These flies were able to mitigate the effects of the by-products, which then prevented changes to social spacing behaviour. Changes in the

quantity of calories ingested has been shown to affect other behaviour such as locomotion and flight, as very high increases in caloric content were shown to impair these behaviour (Bross et al., 2005).

The effect observed in the parents fed CR food was also seen in the next generation, as the progeny of 30-day-old parents fed caloric restriction food were as social, or even more social than the progeny of parents fed CR food for one week or not at all. Similarly, alterations to parental diets have been shown to affect the behaviour of their offspring in rats. For example, when female rats were fed a CR diet during weaning (post-natal), their progeny experiences behavioural changes, where they were more sensitive to predator odor and exhibited a fear response, but also had reduced anxiety- like behaviour (Govic et al., 2014). These researchers then looked the progeny of male rats (fathers) fed a CR diet and found that progeny also had reduced anxiety-like symptoms (Govic et al., 2016). Therefore, caloric restriction of the parent causes changes to the progeny that improve certain behaviour.

One compounding variable in my experiment may be that the effects of the progeny ingesting CR food during the larval stage and first week of life as the eggs were not transplanted to non-CR food. Therefore it is possible that larvae fed CR also has an effect on behaviour that makes them more social, whereas the effects of the parental age on CR food may be less important. This has been shown previously where rats that are fed a calorie restricted diet in early life have epigenetic reprogramming of the hypothalamus-pituitary-adrenal axis that further affects behaviour (Harris and Seckl, 2011). Further testing must therefore take place to disassociate the effects of the progeny versus the parents eating CR.

4.4.5 Conclusion

Overall, I have shown that alterations to the biological aging process of *Drosophila* can cause changes to their social space. When the aging process is accelerated with either exposure to 29°C or paraquat, individuals show a pattern of accelerated aging in social space as compared to those aged at physiological temperature or in the absence of

paraquat. These effects were also passed on to the next generation. When aging is decelerated with caloric restriction, individuals and their progeny are as social or more social. This shows that chronological aging is not required for the changes to behaviour, but it is the underlying biological mechanisms that can affect behaviour. Future work will include determining if the metabolic pathway or oxidative stress pathway are more important for this change in behaviour to occur or if there are other biological aging mechanisms that may also be responsible for changes to social behaviour with age.

4.5 References

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Chapter 5 – Implications and Limitations of the Study

5.1 Implications Of The Study

I found that through aging individuals change their social spacing, as *Drosophila* is initially more social (two-to-three weeks old) and then less social (four and seven weeks old) compared to when they are one-week-old. Therefore, there is an age-specific effect on social spacing. One hypothesis to explain the age-specific effect is that cuticlar hydrocarbons, which are a known factor in promoting aggregation of flies, change with age (Bartelt et al., 1985). It is possible that at two and three weeks of age these hydrocarbons promote closeness or proximity but promote more distal interactions at older ages. Another hypothesis would be that the change in social spacing is correlated with the changes in reproduction and tighter or smaller groups may be forming at peak reproductive times. For example, the social honeybee (*Apis mellifera*) will change its behaviour from nest dwelling to foraging after two or three weeks of age (Amdam, 2011). At this time point, it has been found that honeybees that forage for pollen have a higher reproductive potential (measured by ovariole number) than those that collect nectar. Therefore, there is a relationship between types of foraging behaviour and reproductive potential (Amdam, 2011). This idea could be tested in *Drosophila* using reproductively low or reproductively high strains of *Drosophila melanogaster* to see if their social spacing is more distal in very successful strains or more proximal in reproductively unsuccessful.

Perhaps the low sociability observed in older flies is due to selection of longer-lived flies. At 30 days, 10% of the population has died off and the social behaviour of those flies may be different than what can survive to four weeks old. It is possible that these longer-lived flies have different social spacing from those on the verge of death. This effect may be diluted by other younger flies within the chamber or among different replicates that is mitigated in the sample of young flies. It is also possible that this the change in behaviour through aging is due to pleiotropic genes that promote closeness at younger ages but then

change at older ages causing individuals to be less social. One way to test this would be to do a "smurf" test where flies are fed dissolved sucrose and blue food dye for several days (Rera et al., 2012). When *Drosophila* is close to death (around two days before death), their intestines become leaky, causing the whole organism to turn blue. If we test these flies compared to the non-dyed flies, perhaps we will see a difference in the social spacing of flies that are "long-lived" compared to those about to die, which would indicate health rather than longevity underlying observed differences in social behaviour with aging.

The progeny of old parents had a similar social spacing pattern to their parents; both old individuals and their young progeny were less social. It is possible that changes in the epigenome of the parent, including the presence of non-coding RNA in the gametes of aged parents was influencing the development and behaviour of the next generation. Interestingly, studies have shown evidence of imprinted RNA genes that have brain-specific expression that may be involved in neuropsychiatric disorders (Mattick and Makunin, 2006). Therefore, if non-coding RNA is introduced into the gametes and influences expression in the brain, then it could affect the development of the brain and behaviour.

One other type of material that can be inherited is the microbiome. The microbiome has previously been linked to metabolism and neuropsychiatric disorders via epigenetic mechanisms that can be transmitted trans-generationally (Heard and Martienssen, 2014). Microbes can communicate information to the host via metabolites, which has been shown to directly affect the host behaviour (Archie and Theis, 2011; Dillon et al., 2002; Forsythe et al., 2010). The microbiome of the parents may be responsible for causing changes in the neurodevelopment in the progeny of both old parents causing them to have a similar behaviour phenotype. The progeny may be exposed to this microbiome following egg laying in the vial. Because the parents are still present in the vial, any excrement from the adult flies, which likely contain bacteria, can then interact with the developing larvae and affect the fly and possibly behavior later in life. Therefore, raising parents and progeny in an axenic environment and testing them in behavioural assays may help elucidate if the microbiome is involved.

One other explanation could be changes to the epigenome. Because there is very little DNA methylation that is inherited to the progeny in *Drosophila*, it is possible that histone modifications remain in the gametes that are then passed on to the next generation (Bonasio et al., 2010). In mice and humans, it is known that 1-4% of the nucleosomes remain in the sperm with epigenetic modifications on the histones (Daxinger and Whitelaw, 2012). Additionally, this has been shown to affect the next generation as epigenetic modifications in *C. elegans* was been shown to influence lifespan of the next generation (Greer et al., 2011). Similarly, epigenetic modifications of *Drosophila* parents exposed to heat stress caused a change in eye color in the progeny (Seong et al., 2011). Therefore, because there is improper exchange of histones and protamines in the sperm with aging (Belloc et al., 2009), histone modifications can be passed on to the progeny which may explain why the effect was seen (in the sons) of old fathers but not of old mothers.

A sex effect was seen in the sons of old fathers, which implies that either the sex chromosomes or hormonal differences between males and females influence how parental changes or damage manifests in behavioural changes. This also implies that females are somewhat more resistant to damage from the father, possibly due to the presence of a second X chromosome or due to hormones.

Finally, this effect of behavioural inheritance to the next generation was only seen in the first generation of old parents. Therefore, the mechanism by which aging affects the first generation disappears in the second generation, along with the effect on survival and fecundity. Therefore more work must be done to determine if this is due to an increase in heterogeneity in the population or some other factor that is eliminating the effect seen in the first generation. The mechanism that is lessened in the second generation may be linked to repair of the metabolic pathway as caloric restriction led to more proximal social space and increased temperature led to more distal social space in the progeny. This is also of importance to other researchers as the age of the fly makes a difference to behaviour but that this difference can be reversed in future generations.

Overall, an important practical implication of this study is to stress the importance of age and reproduction. Males are often thought of as having an unlimited reproductive capacity as they could theoretically conceive at any age, although females have a very clear limit on reproduction. However, this study highlights how changes to social behaviour as a result of aging can be inherited and specifically how aged males can pass on this effect on behaviour. Thus, although males may be able to conceive at any age, there are still risks associated with fathering children at older ages.

5.2   Limitations Of The Study

One limitation of this study in terms of extrapolating the results to humans includes the fact that female gametogenesis in *Drosophila* is more similar to male gametogenesis than mammalian female gametogenesis. Therefore, female *Drosophila* was continuously making eggs like males make sperm. The limitation of this is that we may be missing some key aspect of egg damage in humans that is not captured in the fly that may be affected by age and may impact behaviours in the progeny.

Another limitation is that the methods I chose to accelerate aging (increased aging temperature and exposure to paraquat) in *Drosophila* may be too similar. Recent research has found that initially, doses of ROS will not directly affect the genome or other proteins through oxidative stress but will first interact with the electron transport and thus will have a similar effect as increased temperature on metabolism (Personal communication with Dr. Jamie Kramer, University of Western Ontario).

Finally, throughout fly maintenance I have been generating each new generation from young individuals and have thus been selecting for early senescence. This may therefore not be capturing ecologically relevant situations as flies are constantly laying eggs and new populations of flies can be generated from flies at any age, whereas I have been selecting for populations from young flies. However, this might be helping in my study of senescence, as I want to test flies that would be undergoing the aging process that would affect behaviour.

5.3   Future Work

Work in parallel to this project includes testing this phenomenon in other *Drosophila melanogaster* backgrounds as aging is a universal phenomenon and thus the aging phenotype in social spacing should be similar. The hypothesis is that aging is a universal phenomenon and should affect the social space of aged *Drosophila melanogaster* of different genetic backgrounds and their progeny similar to what is shown in this thesis with Canton-S. Preliminary results from our lab show that Oregon-R *Drosophila melanogaster* are less social with age and this effect can be passed on to the progeny, and a recently wild-caught strain (Elwood) in males (Yost, unpublished data 2016).

This thesis originally included understanding the sex difference observed in the progeny of old fathers by using a specific tool in *Drosophila* that would allow us to age the Y chromosome in the mothers and have it inherited to the sons (Hardy, 1975). The idea behind this is that if the Y chromosome is important in regulating the social spacing of the male progeny, then aged fathers who do not pass on the Y chromosome to their sons using this system will have normal social space. However, when mothers are aged and pass on this Y chromosome to their sons, their social space will be affected. However, practically, this approach did not work as males did not live past two weeks and females at four weeks were infertile. Therefore, a longevity curve must be performed on these strains to determine when these flies reach 90% survival to know when to consider them aged. Once the age at which they begin to die has been determined, I can age the old males with virgin females and test the progeny in social space. One other way to tackle this sex difference is by masculinizing female flies by using an RNAi line to the RNA transcript of important component of the sex determination pathway in *Drosophila* known as transformer (tra; Rideout et al., 2015). This will be done to see if having the hormonal expression of a male inside of a female (with an XX genome) will have a behavioural phenotype similar to a male or female and thus it may tell us if the hormonal pathway is involved in this sex difference in behaviour. The hypothesis is that masculinized females whose fathers were aged will display a change in social behaviour compared to the sons of 30-day-old fathers.

We do not know how the gut microbiome will affect social behaviours and how the microbiome will affect behaviours with age. As mentioned above, the microbiome may be passed on from parent to progeny from both mothers and fathers. As females lay eggs, the egg is exposed to both the maternal microbiome as well as the microbiome present in the environment. Similarly, males can pass on their microbiome through excrement when they are in contact with the fertilized eggs laid on the surface of the food. Therefore one approach would be to rear flies in axenic conditions and test the population through age in social space and their progeny. Additionally, the endosymbiotic α-proteobacteria *Wolbachia pipientis* has been shown to affect gene expression of *Drosophila melanogaster* (Gutzwiller et al., 2015). Therefore, to determine if these bacteria affect social spacing behavior, we can use a *Drosophila* strain devoid of these bacteria and test them with the social space assay. If *W. pipientis* does influence changes in social space behavior, then flies that do not have the bacteria will have altered social space.

And finally, as mentioned above, smurf flies (flies on the verge of death) will be tested in social space to see if there is a difference between the behaviour of longer-lived flies compared to those that are about to die. The hypothesis is that flies on the verge of death will have a different social behaviour to those that are longer lived. One prediction is that smurf flies will be less social than those that are going to live longer because they no longer depend on the social group because they are going to die soon. Whereas the flies that are not about to die still rely on or benefit from the social group and will be closer together in social space. This may then further shed some light on the mechanisms that change close to death within the brain.

5.4 References

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Appendices

Appendix A Distances to the nearest neighbour of males and females aged to seven, 14, 21, 30 and 50 days and their progeny. Individuals are 14 and 21 days old are closer together in social space than those at seven days, whereas individuals aged 30 and 50 days are further apart. This effect is passed on to the next generation. (One-way ANOVA with a Holm-Sidak post test, distances are means± standard error to the mean, ns= not significant)

Age Tested		Distance to nearest neighbour		Significance in relation to same sex control	
		Male	Female	Males	Females
Parental Age (Days)	7	0.70±0.02 cm	0.47±0.02cm	Control	Control
	14	0.37±0.02 cm	0.32±0.01cm	p< 0.0001	p< 0.01
	21	0.39± 0.02cm	0.30±0.01cm	p< 0.0001	p< 0.01
	30	1.06± 0.05cm	0.94±0.04cm	p< 0.0001	p< 0.0001
	50	1.14± 0.09cm	0.89±0.05cm	p< 0.0001	p< 0.0001
First Generation of parents at ages (Days)	7	0.40±0.02cm	0.45±0.02cm	control	control
	14	0.45±0.02cm	0.27±0.01cm	ns	p< 0.001
	21	0.56±0.05cm	0.40±0.03 cm	ns	ns
	30	0.56±0.03cm	0.60±0.03cm	ns	p< 0.01
	50	0.81± 0.06cm	0.88±0.06cm	p< 0.0001	p< 0.0001

Appendix B Distances to the nearest neighbour of the progeny of young or aged parents in the second, third, fourth and fifth generation with young generations in between. The effect of having an aged parent dissipates in the second generation, although the females of old parents in the third generation are significantly different (distances are means± standard error to the mean, ns= not significant, unpaired t-test).

Generation Tested		Distance to closest neighbour (cm)		Significantly different?	
		Male	Female	Male	Female
Second Generation (grandparents aged only)	Progeny of 7-day-old	0.38± 0.03	0.37±0.02	ns	ns
	Progeny of 30-day-old	0.35±0.02	0.4±0.02	ns	ns
Third Generation (great-grandparents aged only)	Progeny of 7-day-old	0.71±0.04	0.64±0.04	ns	ns
	Progeny of 30-day-old	0.59±0.03	0.81±0.05	ns	p< 0.05
Fourth Generation (great great grandparents aged only)	Progeny of 7-day-old	0.63±0.03	0.59±0.04	ns	ns
	Progeny of 30-day-old	0.68±0.04	0.61±0.04	ns	ns
Fifth Generation (great great great grandparents aged only)	Progeny of 7-day-old	0.79±0.05	0.78±0.06	ns	ns
	Progeny of 30-day-old	0.77±0.05	0.62±0.04	ns	ns

Appendix C Performance index of the *Drososophila melanogaster* in the social avoidance assay through aging and in the progeny of aged flies. *Drosophila*'s ability to avoid vials that previously contained stressed flies declines with age, and this effect is transmitted to the next generation (values are performance index ± standard error to the mean, ns = not significant, Kruskall Wallis non-parametric test with a Dunn's *post hoc* test).

Age Tested		Performance Index		Significance in relation to same sex control	
		Male	Female	Males	Females
Parental Age (Days)	7	55.96±7.00	61.47±6.20	Control	Control
	14	26.69±5.01	39.33±9.03	p< 0.05	ns
	21	37.65±7.97	31.36±8.51	ns	p< 0.05
	30	33.87± 5.93	32.92±6.19	ns	ns
First Generation of aged parents (Days)	7	51.80±9.53	62.08±8.22	control	control
	30	24.1±9.75	53.01±5.09	ns	ns
	50	26.32±8.68	50.64±10.64	ns	ns

Appendix D Distances to the nearest neighbour of the progeny of aged fathers in social space. The male progeny of old fathers were less social. The progeny of seven-day-old males and 30-day-old males mated with virgin females were tested in social space. Only the male progeny of 30-day-old males were further from their nearest neighbour (distances are means± standard error to the mean, ns= not significant, unpaired t-test).

Generation Tested	Distance to closest neighbour (cm)		Significance in relation to same sex control	
	Male	**Female**	**Male**	**Female**
The progeny of 7-day-old males x virgin females	0.64±0.04cm	0.41±0.02cm	Control	Control
The progeny of 30-day-old males x virgin females	0.83±0.05cm	0.38±0.02cm	p< 0.01	ns

Appendix E Social spacing of the progeny of aged mothers mated with young fathers. At 30 days, mothers do not have an effect on male or female progeny. When mothers are 50

days old, both male and female progeny are further from their nearest neighbour, where only females are significant (Shirley Long, Honour's thesis 2015-2016).

Appendix F Survival curves of *Drosophila melanogaster* fed 0 mM, 10 mM, 20 mM and 40 mM of Methyl Viologen (Paraquat).
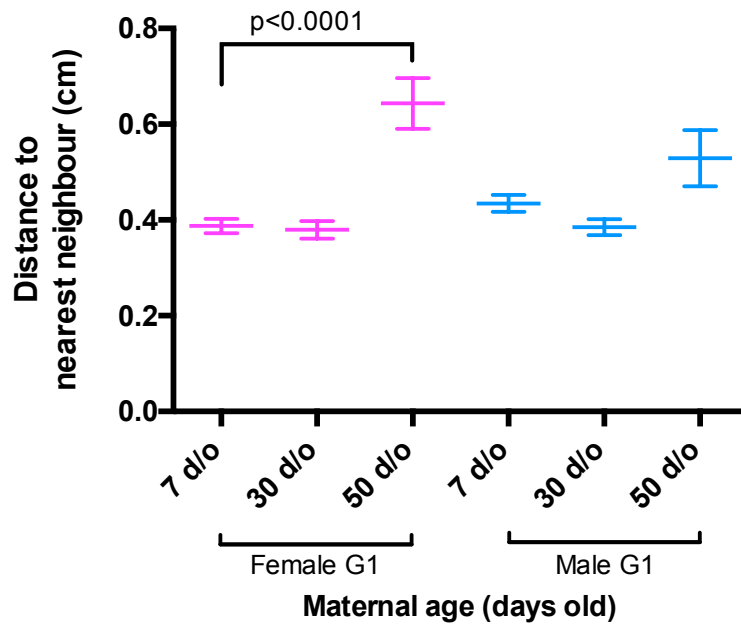
Appendix G Recipe to alter Jazz Mix media into caloric restriction food. Recipe for caloric restriction food adapted from (Min et al., 2007), components for Jazz mix media were provided by the manufacturer (personal communication). Jazz mix media is a high yeast and high sugar food source with 16% yeast and 16% sucrose content per 100ml of food. Restriction of both of these ingredients (calories) to 4% yielded individuals with enhanced longevity. The chart below shows the content of each ingredient that was provided from the manufacturer as a range of % composition. In order to calculate how to reduce the calorie content, I calculated average of the upper and lower content % in terms of grams per 100 mL of water and determined how to supplement the other ingredients.

JAZZ-Mix Ingredients	% Composition	Upper % Content (g In 100mL)	Lower % Content (g In 100mL)	Average Content (g In 100mL)	Supplemented Ingredients (g In 100mL)
Brown Sugar	60-65%	11.34	12.285	11.8125	
Yeast	7-11%	1.323	2.079	1.701	1.5
Corn Flour	12-20%	2.268	3.78	3.024	2
Agar	2.50 – 3.50%	0.4725	6.615	3.54375	2.5
Sodium Propionate	-	-	-	-	-
Benzoic Acid	-	-	-	-	-
Methyl Paraben	-	-	-	-	-

Appendix H Distances to the nearest neighbour of *Drosophila* aged at 29°C and their progeny in social space. *Drosophila* that are aged at 29°C are less social than younger flies. This pattern recapitulates what is seen at 25°C, where older individuals are further apart from their nearest neighbour. When flies are placed at 29°C for seven days and the progeny develop at 25°C until 7 days old, they are less social than those whose parents were aged to one week at 25°C (distances are means± standard error to the mean, ns= not significant, unpaired t-test or One-way ANOVA with a Holm-Sidak post test).

Age Tested		Distance to nearest neighbour		Significance in relation to same sex control	
		Male	Female	Males	Females
Parental Age (Days)	7	0.63±0.04cm	0.60±0.04cm	Control	Control
	14	0.44±0.02cm	0.51±0.03cm	p< 0.05	ns
	21	0.67±0.04cm	0.60±0.39cm	ns	ns
	30	0.96±0.05cm	0.97±0.68cm	p< 0.0001	p< 0.0001
First Generation of 7-day-old parents at 25°C		0.49±0.03cm	0.45±0.02cm	control	control
First Generation of 7-day-old parents at 29°C		0.62±0.04cm	0.53±0.04cm	p< 0.05	ns

Appendix I Distances to the nearest neighbour of *Drosophila* fed methyl viologen (paraquat) in social space. Male *Drosophila* that are fed paraquat for 13.5 hours are further apart from their nearest neighbour as compared to those that were not fed paraquat. Females are not further apart from their nearest neighbour when they are fed paraquat. Both male and female progeny of parents fed paraquat are further from their nearest neighbour. Additionally, when only fathers are fed paraquat, both male and female progeny are further from the nearest neighbour as compared to fathers that were not fed paraquat (distances are means± standard error to the mean, ns= not significant, unpaired t-test)

	Distance to nearest neighbour		Significance in relation to same sex control	
Treatment	**Male**	**Female**	**Males**	**Females**
0 mM paraquat	0.51±0.04cm	0.80±0.50cm	Control	Control
20 mM paraquat	1.01± 0.08cm	0.70±0.05cm	$p< 0.0001$	ns
Parents Fed 0 mM paraquat	0.51±0.02cm	0.60±0.03cm	Control	Control
Parents Fed 20 mM paraquat	0.75±0.05cm	0.72±0.04cm	$p< 0.0001$	$p< 0.05$
Fathers Fed 0 mM paraquat	0.36±0.02cm	0.34±0.02cm	Control	Control
Fathers Fed 20 mM paraquat	0.53±0.04cm	0.50±0.04cm	$p< 0.01$	$p< 0.001$

Appendix J Distances to the nearest neighbour of *Drosophila* fed caloric restriction food and their progeny in social space. *Drosophila* fed caloric restriction food for 30 or 50 days are as far apart from their nearest neighbour as those that are fed the same diet for only seven days. Similarly, the progeny of parents fed CR food for 30 or 50 days are also as social as those whose parents were fed for seven days (distances are means± standard error to the mean, ns= not significant, One-way ANOVA with a Holm-Sidak post test).

Age Tested		Distance to nearest neighbour		Significance in relation to same sex control	
		Male	Female	Males	Females
Individuals fed CR	7	0.66±0.42cm	0.52±0.04cm	Control	Control
	30	0.57±0.04cm	0.38±0.02cm	ns	ns
	50	0.63±0.04cm	0.58±0.05cm	ns	ns
parents fed CR	7	0.76±0.08cm	0.75±0.67cm	control	control
	30	0.56±0.06cm	0.76± 0.08cm	p< 0.0001	p< 0.001
	50	0.53±0.07cm	0.44± 0.08cm	p< 0.05	p< 0.01

Curriculum Vitae

Name: Dova Brenman

Post-secondary   Ryerson University
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                            The University of Western Ontario
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                            Honorable Mention, Poster; Fallona Family Interdisciplinary
                            Showcase December 2015
                            London, Ontario, Canada

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                            February 2016

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                            March 2016
                            Banff, Alberta, Canada

Related Work   Teaching Assistant

University of Western Ontario (Bio2290- Gray Unit, Bio 1001-
First Year Biology Lab and Literacy, Bio3597-Regulation of Gene
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Experience   The University of Western Ontario
                        2014- Present

Conferences and Oral Presentation:
Oral Presentations:

Brenman D, Long S, Kanippayoor R, and Simon AF. (**2016**). *Aged Flies And Their Progeny Are Less Social*. 7th Annual Biology Graduate Research Forum 2016. **Selected 5 min. Thesis Oral Presentation**

Brenman D, Long S and Simon AF. **(2015).** *The sons of old fathers are less social*. Fallona Family Interdisciplinary Showcase 2015, London, ON. **Selected 3 min. Thesis Oral Presentation**

Conferences:

Brenman D, Long S, Choi N, Kanippayoor R, and Simon AF **(2016).** *Aged parents have less social offspring*. The Allied Genetics Conference, Orlando, FL. **Poster**

Brenman D, Long S and Simon AF. **(2016).** *The sons of old fathers are less social*. 25th Anniversary Research Day: Neuroscience Graduate Program, London, ON. **Poster**

Brenman D, Long S and Simon AF. **(2016).** *The sons of old fathers are less social*. 8th Canadian Developmental Biology Meeting, Banff, Alberta, Canada. **Award for Best Poster Presentation**

Brenman D, Long S and Simon AF. **(2015).** *The progeny of old fathers are less social*. Fallona Family Interdisciplinary Showcase 2015, London, ON. **Honourable Mention for Best Poster Presentation**

Brenman D, Long S and Simon AF. **(2015).** *The progeny of old parents are less social*. *16th* Neurobiology Of Drosophila meeting, Cold Spring Harbor Laboratories, NY, USA. **Poster**

Brenman D, Long S and Simon AF. **(2015)**. *The progeny of old parents are less social*. 6th Annual Biology Graduate Research Forum 2015. **Poster**