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# Effect of Maternal Age on Offspring Social Behaviour in *Drosophila Melanogaster*

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**Effect of maternal age on offspring social behaviour in *Drosophila*  
*melanogaster***

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## **Abstract**

Aging can be defined as the natural and progressive decline in physiological functioning leading to increased risk for disease and death. Although the effects of age are well characterised, much less work has been done to study whether these detrimental changes can be transmitted to offspring. Advanced parental age has been correlated with higher incidence of neuropsychiatric disorders such as autism in children. As average maternal age increases in North America, it is becoming increasingly relevant to study the effects of maternal and paternal age on offspring social behaviour. We hypothesize that advanced maternal age in *Drosophila melanogaster* will affect offspring social behaviour as measured in the social space assay. This assay measures the distance to the nearest neighbour (cm) after stable social group formation as an index of social behaviour in the fruit fly. To test the effect of maternal age, we aged Canton-S females to 7, 30, and 50 days corresponding to 100%, 90%, and 50% population survival respectively, and mated them with 7-day old young Canton-S males. Their eggs were collected and allowed to develop into first generation (G1) offspring between the ages of 7–9-days old, which were then separated by sex and tested in the social space assay. We found that female G1 from 50-day old mothers were significantly less social ( $p < 0.0001$ ,  $n = 7$ ) compared to controls from 7-day old mothers ( $n = 18$ ). Males demonstrated a similar trend of increased social space when mothers were 50-days old. In conclusion, there is a sex-specific effect of maternal age on offspring social behaviour in *D. melanogaster*.

**Keywords:** maternal age effect, social behaviour, aging, drosophila, parental age

## **Introduction**

Aging can be defined as the natural and progressive decline in physiological functioning leading to increased risk for disease and death (Grotewiel *et al.*, 2005; López-Otín *et al.*, 2013). Examples of age-related deteriorations in function include reduced fertility, less efficient replacement of old tissue due to cellular senescence, and stem cell exhaustion (Kirkendall & Garrett, 1998; López-Otín *et al.*, 2013). Overall, the elderly constitute the fastest growing age demographic in Canada, and are expected to become the largest proportion of the population within the next few decades (Statistics Canada, 2011). Accordingly, there has been increased research exploring the mechanisms and effects of age on individuals.

There are several proposed mechanisms for aging. Increased genome instability due to an accumulation of mutations throughout an organism's lifespan may contribute to increased cell cycle arrest, otherwise known as cellular senescence (López-Otín *et al.*, 2013). These mutations may be caused by exogenous sources of DNA damage, such as radiation or chemical exposure. DNA integrity may also be challenged by endogenous threats such as reactive oxygen species generated by mitochondria, or imperfect DNA replication that causes errors in DNA sequence (Turrens, 2003; Hoeijmakers, 2015). Epigenetic modifications have also been shown to occur throughout the lifespan; changes to DNA methylation or chromatin organisation can contribute to altered gene regulation and affect cell and tissue function (Fraga & Esteller, 2007; Talens *et al.*, 2012). In summary, aging is a multi-factorial process caused by the combined effects of various genetic and molecular changes occurring over time.

Although the effects of aging are well-characterised, it is less clear whether these effects can be trans-generational. In other words, can parents conceiving at older ages transmit some of these detrimental age-associated changes onto their children? In humans, there are several studies that show a correlation between parental age and risk for neuropsychiatric disorders (Risch *et al.*, 1987; Eichenlaub-Ritter, 1996). For example, both maternal and paternal age are risk factors for autism spectrum disorder, and increased paternal age is associated with risk for schizophrenia development (Malaspina *et al.*, 2001; Croen *et al.*, 2007; Sandin *et al.*, 2012). Furthermore, advanced maternal age is associated with increased risk for chromosomal abnormalities like Down's syndrome, while paternal age has been implicated as a major cause for *de novo* genome mutations through constant DNA replication in spermatogenesis (Risch *et al.*, 1987; Eichenlaub-Ritter, 1996; Kong *et al.*, 2012). Animal studies have also shown a parental age effect; for example, in mouse studies, male offspring from middle-aged mothers had significantly lower body weight and smaller gonads compared to male offspring produced by younger mothers (Wang & vom Saal, 2000). In fruit flies, both maternal and grand-maternal age impact offspring reproductive fitness and progeny viability (Hercus & Hoffmann, 2000). Overall, there seems to be an effect of parental age on both the physical and behavioural characteristics of offspring; however, the behavioural effects have not been well-studied.

There are several potential mechanisms by which the effects of age may be transmitted from parents to offspring through germline cells. In older human females, less effective cellular machinery or damaged proteins involved in meiosis may contribute to increased rates of chromosomal nondisjunction in offspring (Dailey *et al.*, 1996).

Maternal germline cells are also the source of mitochondria for offspring in humans; therefore, the effects of mitochondrial dysfunction with increased age may be passed down by mothers conceiving at older ages (Cummins, 2002; Shokolenko *et al.*, 2014). In males constantly undergoing spermatogenesis, intrinsic errors in DNA replication are the major source for *de novo* mutations in gametes (Risch *et al.*, 1987; Kong *et al.*, 2012). This accumulation of mutations in germline cells may be a mechanism for the paternal age effect. Furthermore, both maternal and paternal genomes undergo epigenetic changes that could be passed down to offspring and impact gene regulation. Specifically, epigenetic changes occur throughout life in a fairly predictable manner in a variety of organisms; epigenetics has been proposed as a “time-keeping mechanism” for age-related changes in cellular function (Fraga & Esteller, 2007; Calvanese *et al.*, 2009). Because epigenetic changes might be transmissible onto offspring, this is also a potential mechanism for altered gene expression in offspring from older parents.

Simple animal models such as *Drosophila melanogaster* are powerful tools for studying effects of age because of their short generation time, abundant offspring, and genetic homology with humans (Grotewiel *et al.*, 2005; Hales *et al.*, 2015). Furthermore, flies demonstrate well-characterised simple and complex social behaviours that are robust measures for normal and aberrant social behaviour. There are several experimental paradigms established to measure various aspects of fly behaviour, such as learning and memory, aggression, and courtship assays (Grotewiel *et al.*, 2005). One such measure for behaviour is the social space assay; this experimental paradigm measures the mean distance between individuals within a stable social group as a metric for social behaviour (Simon *et al.*, 2012; McNeil *et al.*, 2015). Specifically, proper social space is the balance

of attractive and repulsive cues between flies, and is a foundational precursor to more complex behaviours (Simon *et al.*, 2012). Overall, social space is demonstrated to be a consistent and robust metric for social group formation and interaction between flies.

This project aims to isolate for *Drosophila* maternal age effect on behaviour, as a parallel study in our lab is exploring potential paternal age effect. Furthermore, we have previously established a parental age effect on offspring social behaviour when both parents are old (Figure 2, Appendix). In female fruit flies, oogenesis occurs similarly to spermatogenesis; in contrast with human oogenesis, female fly eggs are constantly replicating and maturing at different stages throughout the reproductive tract similarly to sperm (Bratu, 2015). Furthermore, female fruit flies pack mitochondria, histones, proteins, and various coding and non-coding mRNAs into large eggs prior to fertilization (Bratu, 2015). Thus, maternal age effects in the fruit fly would likely be transmitted through potential mechanisms such as *de novo* mutations or through changes in maternal germline egg cell packaging.

To study the effect of maternal age on offspring social behaviour, we will test the offspring of old and young mothers mated with young fathers in the social space assay. However, controlling for sperm age produces a unique challenge. After mating a single time, female flies can store sperm within their reproductive tract spermathecae for up to two weeks to facilitate continuous fertilisation (Lefevre & Jonsson, 1962). During this time, she may reject other males (Lefevre & Jonsson, 1962; Manning, 1967). Furthermore, female flies cannot be kept virgin until they reach desired mating age, because older virgin females demonstrate lowered sexual receptivity compared to older non-virgin females (Manning, 1967). As such, before we age the females, we will mate



them once to genetically modified males whose sperm have been tagged with green fluorescence protein (GFP). This will be done to confirm that old sperm have been ejected from the female's reproductive tract prior to re-mating at older ages. A parallel fertility and fecundity curve of females mated a single time will also show when stored sperm have been ejected from the female reproductive tract.

In summary, age-related changes in physiology have been well described in aging adults. However, less work has been done to characterise whether these detrimental changes can be transmitted to offspring by old parents. Although research has been done in *Drosophila* to study the maternal age effect on progeny reproductive fitness, no studies look at parental age-associated behavioural changes. Hence, our aim was to characterize the effect of advanced maternal age on progeny social behaviour using *D. melanogaster* in the social space behavioural assay. We hypothesized that advanced maternal age in *D. melanogaster* will affect offspring social behaviour in the social space assay.

## **Materials and Methods**

**Fly stocks:** We investigated the effect of advanced maternal age on offspring social behaviour using wild-type laboratory Canton-S *D. melanogaster*. Genetically modified male flies whose sperm protamines were labelled with green fluorescence protein (GFP) were also used; they were prepared according to the transformation protocol as described by Manier *et al.*, 2006. All flies were maintained in large mixed sex groups within standard bottles containing “Jazz mix” fly food from Fischer Scientific (water, brown sugar, agar, yeast, corn meal, benzoic acid and methyl paraben). Environmental

conditions were kept constant in an incubator at 25°C, 50% humidity, and a 12 hour light:dark cycle.

**Aging mothers, mating, and collecting progeny:** We collected virgin females from Canton-S laboratory stocks under cold anaesthesia using ice, and mated them for three days at 25°C to males with GFP-tagged sperm. This was done to allow for sperm imaging within the female reproductive tract, and to prevent the lowered sexual receptivity seen in older females that have never mated. After the initial three-day mating, we removed the males. All females were aged in groups of 10-20 in standard vials with “Jazz mix” fly food, and transferred every other day. At 30 or 50 days, we re-mated females to 5–7-day old Canton-S males for three days. These time points were chosen because they correlate to 90% and 50% population survival respectively according to our lab’s survival curves; these time points approximate middle and advanced age in the fruit fly (Figure 1, Appendix). Control parents were young 5–7-day old Canton-S females mated once with 5–7-day old Canton-S males. Post-mating, we collected eggs and allowed them to develop into first generation adults (G1) between the ages of 5–7-days old, and tested them in the behavioural assay.

**Behavioural testing:** Progeny behaviour was assessed using the social space assay. This assay assesses social behaviour by allowing us to measure the average distance to the nearest neighbour once the flies have settled into stable social groups within pre-constructed glass arenas (Simon *et al.*, 2012; McNeil *et al.*, 2015). We collected G1 adults between the ages of 5–7-days old under cold anaesthesia and sorted them by sex into groups of 15 flies 1–2 days prior to testing. On testing day, flies were transferred into fresh vials and allowed to acclimatise to the testing room (25°C, 50% humidity) for two

hours. Subsequently, we placed the flies into social space assay chambers between 4–7 hours after lights-on, and allowed them to settle into stable social groups. Pictures were taken at 20, 30, and 40 minutes after entry into the chamber. Using ImageJ software and GraphPad Prism 6, pictures were analysed to determine the distance to the nearest neighbour as a measure for social space distribution.

**Statistics:** Outliers were removed using ROUT analysis ( $Q = 1\%$ ). A one-way ANOVA and Holm-Sidak post-hoc test were used to compare the mean distances to the nearest neighbour between treatment groups.

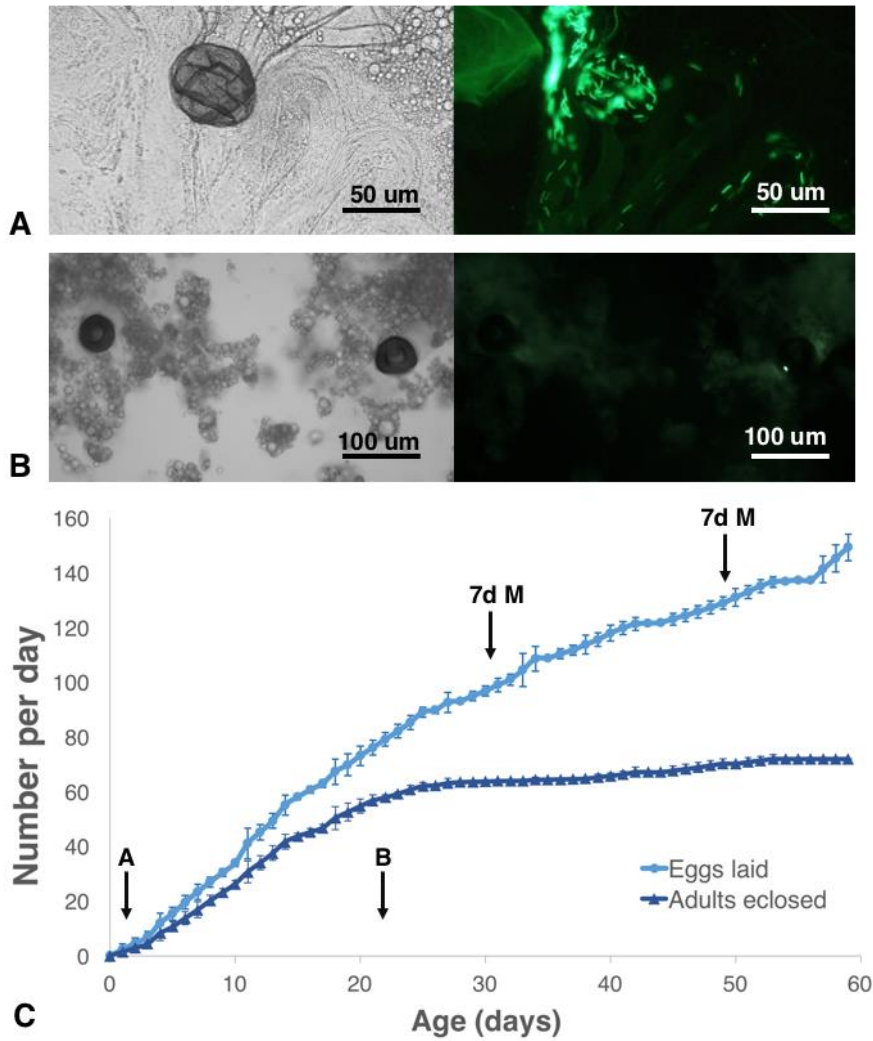
**Fertility and fecundity curve:** Virgin Canton-S females were collected in groups of five and allowed to mate with two Canton-S males for three days. After removing males, females were transferred at the same time daily and eggs per vial were counted. The number of adults that emerged from each vial was also recorded. This was done to assess fertility and fecundity across the lifetime for females that were mated once and then isolated from males. Plateaued fecundity indicates that no fertilization is occurring, and is a control for ensuring that old sperm are removed from the female reproductive tract.

**Microscopy:** A sample of Canton-S females were used for visually confirming the ejection of sperm from the reproductive tract prior to 30 and 50 days. The reproductive tracts of females mated with males expressing GFP-tagged sperm were dissected on testes buffer (water, sodium chloride, potassium phosphate, tris buffer) at 0, 1, 2, 3, and 4 weeks post-mating to visualise the female reproductive tract. The spermathecae and seminal tubules were imaged using a standard fluorescence microscope within an hour after dissection as a qualitative control to ensure sperm ejection from female flies.

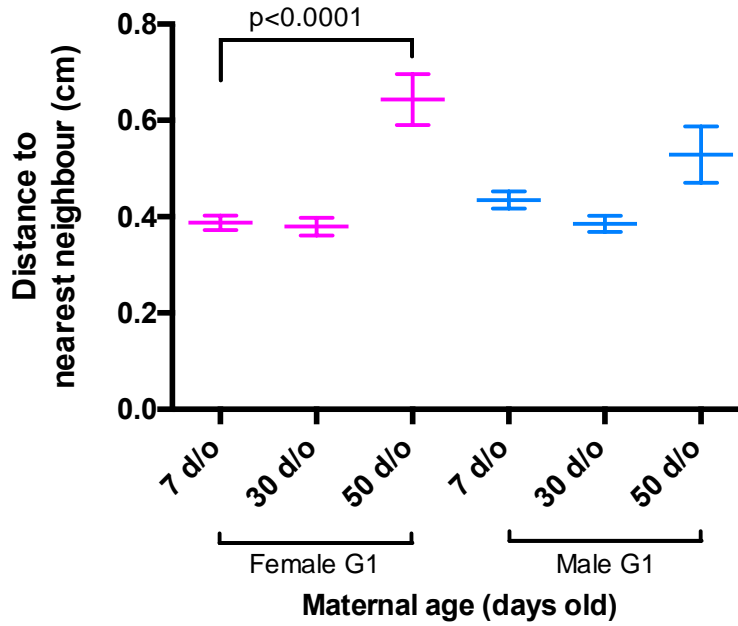
## **Results**

Sperm are present in the female fly reproductive tract after three days of mating, as visualised by standard light and fluorescence microscopy (Figure 1A). The majority of sperm are no longer visible in the female fly reproductive tract after three weeks' separation from male flies (Figure 1B). After being mated for three days and subsequently isolated from males, female flies lay eggs continuously throughout their lives as shown by a steadily increasing cumulative number of eggs per day per female (Figure 2C). The majority of these eggs yield adults until approximately 25 days, at which point the eggs stop yielding adults and the cumulative mean number of adults eclosing per day per female plateaus (Figure 2C).

In the social space assay, female offspring from 50-day old mothers were significantly further apart ( $n=7$ ,  $p<0.0001$ ) compared to female offspring from 7 or 30-day old mothers ( $n=18$ ,  $n=13$  respectively). In males, offspring from 50-day old mothers showed a trend of increased social space ( $n=8$ ,  $p=0.11$ ) compared to offspring from 7 or 30-day old mothers (Figure 2).



**Figure 1.** Representative images taken of the female fly spermatheca and reproductive tract using standard light (left) and fluorescent microscope (right) corresponding to 3d of mating (A), at which time the males are removed, or 21d post-mating (B). These time points are shown on a fertility and fecundity curve (C) plotting the cumulative mean number of eggs or adults eclosing per day per female  $\pm$  SEM (n=9). Arrows at 30 and 50 days correspond to when females are re-mated with 7 day old males.



**Figure 2.** Distance to the nearest neighbour (cm) after 30 minutes as measured in the social space assay. Flies tested were all 7–9-day old Canton-S female or male *D. melanogaster* offspring. Mothers were either 7, 30, or 50 days old (n=18, n=13, n=7 for females respectively; n=20, n=15, n=8 for males respectively) when mated to 7 day old males. \* $p < 0.0001$  as measured using one-way ANOVA and Holm-Sidak post-hoc test. Data are presented as means  $\pm$  SEM.

## **Discussion**

Female *D. melanogaster* lay eggs continuously throughout their lives after mating once for three days. Our results show that although the number of eggs laid per day stays fairly consistent over time, the number of flies able to eclose from these eggs declined and eventually plateaued around 25 days. This suggests that after approximately three to four weeks, sperm are either no longer viable within the female to fertilise eggs, or are completely removed from the female's reproductive tract. Images of the female reproductive tract under the fluorescent microscope suggest that it is the latter, as sperm can no longer be visualised within the female after three weeks. Other studies looking at sperm storage within the female fly corroborate this observation, as researchers have shown that sperm are only kept within the female for up to two weeks (Lefevre & Jonsson, 1962). Furthermore, if exposed to new males after a certain period of time, female flies preferentially re-mate to ensure the viability of the sperm for fertilization (Lefevre & Jonsson, 1962). Overall, these findings allow us to be confident that when female flies are re-mated at 30 or 50 days to young 7-day old males, females are using young sperm to fertilise their eggs.

Overall, there is an effect of maternal age on offspring social behaviour as shown in the social space assay. Previous work in our lab exploring the effect of parental age on offspring social behaviour when both parents were either 7, 30, or 50-days old showed similar results. Specifically, we show that when both parents are either 30 or 50-days old, both female and male progeny are significantly further apart compared to offspring from 7-day old parents (Figure 2, Appendix). The current study, which isolates for fly maternal age, shows no effect of maternal age on offspring social behaviour at 30 days. This

suggests that the parental age effect at 30 days is primarily mediated by the father, while the mother contributes more at advanced ages. This may in part be explained by the fecundity decline over time in female *D. melanogaster*; Millery *et al.* show that fecundity declines steadily up to 35d, at which point there is an exponential decline in fecundity until death (2014). The observed difference in behaviour between offspring from 30 and 50-day old mothers may be influenced by this dramatic decline in egg viability post 35 days, although the exact mechanisms for this decline are not yet elucidated.

There are several mechanisms by which the maternal age effect may be mediated. In the female fly, oogenesis is similar to spermatogenesis in that oocytes are continuously maturing at various stages within the ovary (Bratu, 2015). This is in contrast to human oogenesis, where a single oocyte is selected to mature per cycle. Because of this, both fly sexes may be susceptible to increased mutation accumulation in germline cells with advanced age, as the number of *de novo* mutations is correlated with greater number of DNA replication cycles (Risch *et al.*, 1987; Kong *et al.*, 2012). With increased age, there is a higher likelihood that mutations in germline cells may affect genes that play a role in social behaviour modulation.

Epigenetic changes, which are defined as heritable changes to gene regulation, may also contribute to the maternal age effect. Examples of epigenetic mechanisms include DNA methylation, histone and chromatin modifications, and microRNA (Fraga & Esteller, 2007; Calvanese *et al.*, 2009). Epigenetic changes with age are shown to occur in a variety of organisms; for example, mammals demonstrate an overall global decrease in DNA methylation with increasing age (Calvanese *et al.*, 2009). These predictable epigenetic changes are proposed to be part of an “epigenetic clock” for aging



(Calvanese *et al.*, 2009). Research in this emerging field of “aging epigenetics” aims to characterize the epigenetic profiles of “old” versus “new” cells, and study how these changes contribute to aging (Fraga & Esteller, 2007; Calvanese *et al.*, 2009). When the epigenomes of germline cells are affected by parents at advanced ages, these changes to gene regulation may be transmissible to offspring.

In *D. melanogaster*, there are specific epigenetic alterations that have been studied in detail. For example, chromatin-mediated alterations to gene transcription are heritable through both mitosis and meiosis (Cavalli & Paro, 1998; Sollars *et al.*, 2003; Chong & Whitelaw, 2004). Gene expression is also regulated through the structural reorganisation of histones; for example, histones H3K9me3 and HP1 are found to be enriched within certain areas of the fly chromosome with advanced age (Wood *et al.*, 2010; Boros, 2012). Because fly mothers pass on their histones to offspring, these changes to histone composition may in part mediate changes in gene expression within offspring (Li *et al.*, 2012). Another epigenetically transmissible element in fruit flies is the packaging of non-coding RNAs into eggs by mothers. Non-coding microRNAs are involved in post-transcriptional regulation of gene expression through RNA silencing, and are distributed throughout the egg to regulate embryo development (Leaman *et al.*, 2005). MicroRNA expression changes within flies in an age-associated manner; for example, microRNA miR-34 regulates long-term brain integrity in fruit flies and has emerged as a molecular link between aging and neurodegeneration (Liu *et al.*, 2012). As maternal microRNA profiles change with increasing age, these lasting alterations to gene expression may be transmitted to progeny through the maternal packaging of RNA into eggs. Overall, inheriting these altered histones and non-coding RNAs from mothers of

advanced age may in part mediate the behavioural changes observed from progeny of old mothers.

The effect of maternal age on offspring social behaviour is more pronounced in females, which are further apart than males. This suggests that maternal age differentially affects the two sexes. This is not entirely surprising, as male and female flies demonstrate sex-specific behaviours mediated by genes such as *doublesex* and *fruitless* (Hales *et al.*, 2015). For example, a single pheromone *cis*-vaccenyl acetate (cVA) elicits different courtship behaviours in the male and female fruit fly because of sexually dimorphic neural circuits established by the gene *fruitless* (Datta *et al.*, 2008). The sexually dimorphic nature of fruit fly neuronal development and social behaviour may explain why social space differs between males and females when mothers are old.

Overall, *D. melanogaster* maternal age affects offspring social behaviour in a sex-specific manner. Although further investigation is warranted to elucidate the mechanisms for parental and maternal age effects, our research strongly suggests that parental age is emerging as an important factor in progeny social behaviour.

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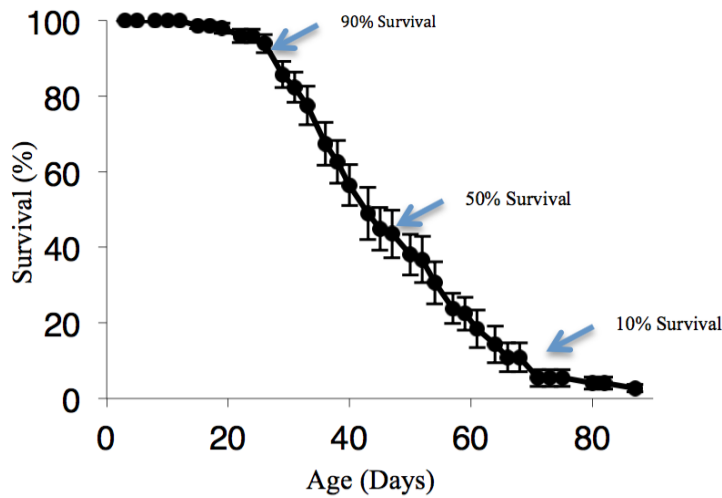
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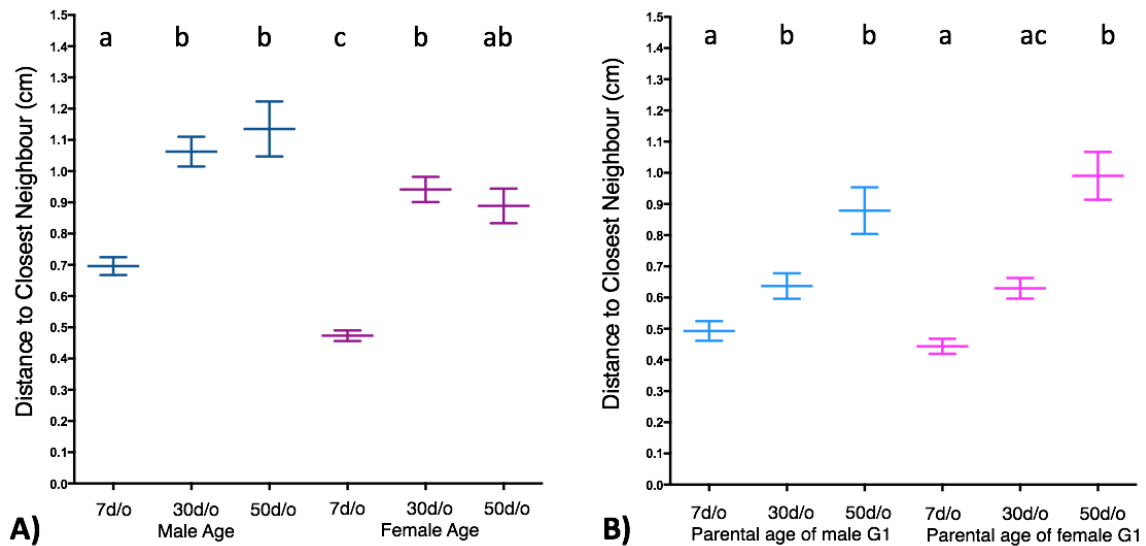
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## Appendix



**Figure 1.** Survival curve for *Drosophila melanogaster* at 25°C (N=9). Arrows indicate 90%, 50%, and 10% survival of population corresponding to 30, 50, and 70 days respectively.



**Figure 2.** Social behaviour of *Drosophila melanogaster* flies as measured in the social space assay. The assay measured distance to the nearest neighbour (cm) after 30 minutes for males and females (n=9) at various ages (A), and distance to the nearest neighbour for 7–9-day old males and females (n=9) whose parents were either 7, 30, or 50 days old (B). Data are represented as means  $\pm$  SEM and data analysis was done using one-way ANOVA and Holm-Sidak post-hoc test.