MATE CHOICE AND THE EVOLUTION OF MENOPAUSE

EXPERIMENTAL INVESTIGATION OF THE MATE CHOICE THEORY OF MENOPAUSE WITH *DROSOPHILA MELANOGASTER*

By

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LAYMAN ABSTRACT

All animals are expected to remain fertile until they die. Menopause is an enigma and an unsolved problem in evolutionary biology. Numerous theories have been proposed to explain menopause, but there is no clear understanding of how this fertility reducing trait evolved in the human population. It has been proposed that biased mate choice i.e., preference for younger females can lead to accumulation of fertility-reducing mutations and the evolution of menopause. In this study, mate choice theory was tested using altered (biased) mating schemes with *Drosophila melanogaster* and the results support the mate choice theory of menopause.

ABSTRACT

Menopause, the complete cessation of menstrual cycles, apparently is a detrimental trait, yet all women experience it. Numerous theories have been proposed to explain the origin of menopause, but none has been satisfactory. In 2013, Morton *et al*. proposed a mate choice hypothesis to explain menopause and, using a computational model, showed how a bias in mating (*i.e*., older men preferring younger women) could have allowed such an otherwise detrimental trait to evolve neutrally through accumulation of female fertility-reducing mutations. To investigate whether biased mating could affect fecundity and fertility in a real system, two experimental populations were established using *Drosophila melanogaster*. Older males were mated with younger females and *vice versa*. Information was obtained, including data on fecundity, fertility, ovariole and matured egg chambers, and lifespan, for experimental, age-restricted-mating and control populations. A negative effect on the fecundity and fertility of the younger-mating sex was observed in restricted-mating compared to control populations. Age-restricted mating had no effect on longevity. Menopause could evolve according to the mate choice hypothesis.

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I dedicate this thesis to

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In Gratitude of the help and support I received, and for their unconditional love and dedicated partnership for success in my life.

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LIST OF ABBREVIATIONS

POF – Pre-Ovarian Failure

oM – Older Males mated with Younger Females

oF – Older Females mated with Younger Males

mM – Males of oM Experimental Population

fM – Females of oM Experimental Population

mF – Males of oF Experimental Population

fF – Females of oF Experimental Population

GENERAL INTRODUCTION

Menopause is a complete cessation of the menstrual cycle observed in human females. It is defined as a loss of fertility before death, following 12 or more months of the transitional phase in the menstrual cycle, termed "perimenopause" (Dennerstein,1996). Some of the symptoms of menopause include hot flashes, vaginal dryness, and difficulty in sleeping. Menopause is associated with osteoporosis and cardiovascular diseases (Freeman et al., 2007).

According to Darwinian evolutionary theory, selection should act against the early termination of reproduction (Hamilton,1966). Natural selection should favor continued reproduction with an increase in the lifespan of humans (Hill & Hurtado,1991). Post-menopausal life is restricted to humans, captive chimpanzees, killer whales (Packer et al.,1998; Reznick et al.,2005) and short pilot-finned whales (Marsh et al.,1986), but other primates do continue to reproduce until death (Johnstone and Cant 2010, Thompson et al., 2007).

Cessation of reproduction in human females is observed around 50 years of age (Johnstone and Cant, 2019) as shown in **Figure 1**. In female killer whales, reproduction cessation is observed around 30 to 40 years of age, and they continue to live for decades (Croft et al., 2007).

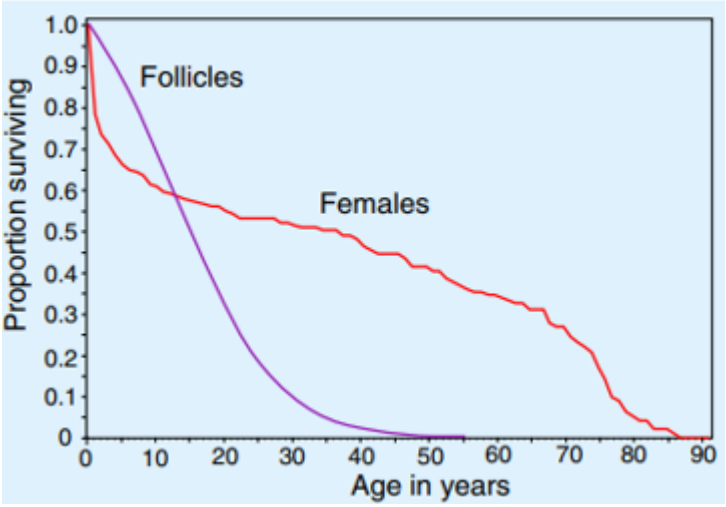

Figure 1. Reproduction and biological senescence curves (modified diagram from Johnstone and Cant 2019). Redline indicates survivorship of Hadza women and Purple line indicates the predicted ovarian follicles remaining as a function of age.

Menopause Theories

There are many theories of menopause. Physiological theories explain menopause as the natural depletion of the number of follicles in the ovaries with increasing age (Armstrong, 2001). Ovaries contain immature follicles, some of which mature each month. Follicle-stimulating hormone (FSH) and luteinizing hormone (LH) production from the pituitary gland cause one follicle every month to mature, thereby facilitating ovulation. Though there are millions of follicles, only ~400 follicles mature to release oocytes in an entire lifespan

(Vaskivuo et al., 2001). However, significant degeneration of follicles is due to programmed atresia (i.e. loss of follicles that do not ovulate; Armstrong,2001), as shown in **Figure 2**. The hormonal changes that lead to ovarian follicular atresia are poorly understood (Vaskivuo et al., 2001).

A decline in oocyte quality or reproductive capability in women is tied to a decrease in ovarian follicular reserve. The decline in ovarian follicular reserve is non-uniform and is accelerated with age (Titus et al., 2013). This decrease in the ovarian reserve might occur possibly due to increased genetic defects in oocytes over time (Gosden et al.,1998; Titus et al.,2013). These genetic defects include meiotic DNA mutations or failure in DNA repair mechanisms that are culled through poorly understood genetic pathways (Titus et al., 2013). Studies have identified some of the genes associated with ovarian failure. They include *BMP15* and *FOXl2* (Welt et al.,2004). Pituitary abnormalities may also decrease ovulation, leading to a decline in fertility (Armstrong, 2001).

Although menopause is believed to be completely natural in a physiological sense, premature ovarian failure (POF) can result in early menopause. Women in this condition may have irregular periods but typically remain fertile. POF can be spontaneous or induced. Induced POF occurs when women undergo surgery for reasons such as uterus cancer or endometrium. Damage to the ovaries might also

result from chemotherapy and radiation. Lifestyle habits such as cigarette smoking may contribute to spontaneous POF (Ebrahimiet al., 2011). Mutations in the germline may also lead to POF but rarely (Ebrahimiet al., 2011).

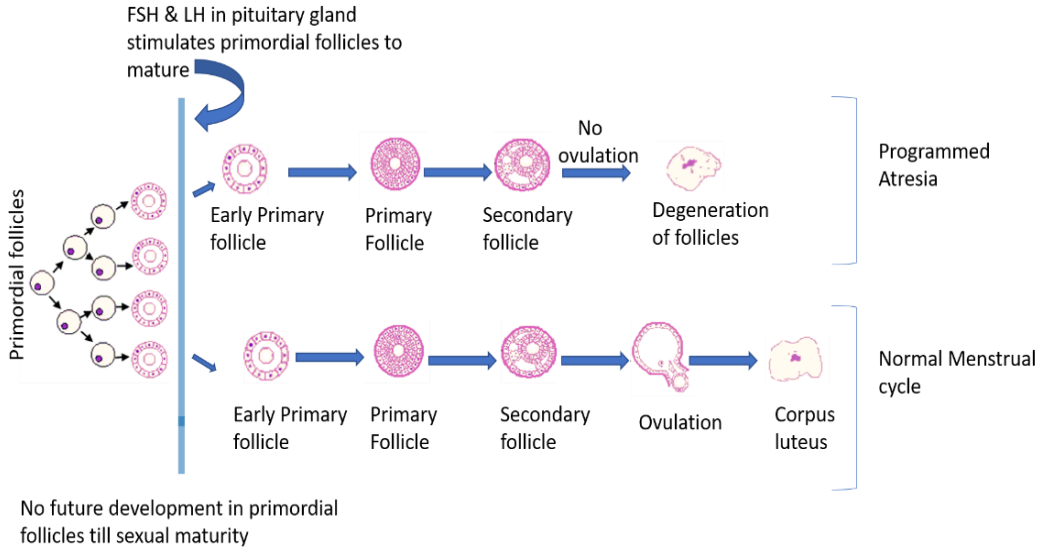

Figure 2. Physiological steps involved in [Menopause].

The evolutionary theories of menopause are based on evolutionary changes due to social-interaction change (i.e., on kin selection), for example, where older non-reproductive women help to rear their grandchildren. The grandmother hypothesis, which is based on kin selection theory, explains how grand mothering played an important role in increasing the longevity of humans, decreasing mortality and increasing fertility in females, thereby compensating for the loss of fertility through menopause (Hawkes et al., 1998). Hawkes el al.,

described that grand-mothering can strengthen selection against late-onset mortality causing mutations by increasing the sharing of genetic information of post-menopausal women through the increased reproductive fitness of their daughters. Although grand-mothering has benefits, it has its own limitation (i.e., it doesn't explain how menopause evolved in the first place regardless of whether it came before or after the life-span extension; Takahashi et al.,2017).

Another theory, based on the life-span artifact, explains how reproduction senescence arose as a result of extended life span from hunter-gatherers to modern human population (Washburn,1981; Peccei,2001). It explains menopause as an 'already built-in' trait and a by-product of the increased lifespan that is observed in humans. Various intrinsic and extrinsic factors allowed females to continue living after menopause (Peccei,2001). Intrinsic factors that have extended life-span are speculated to have included reduced telomere degradation (Atzmon et al., 2010) and extrinsic factors include barriers, like escaping from predators, agricultural and medical developments. But the life-span artifact theory was unable to explain why fertility remained intact with the increased lifespan in males but not in females (Takahashi et al.,2017).

There are several other theories of menopause that are variations on the theme of kin selection, such as the mother hypothesis, patriarch hypothesis, absent father hypothesis, reproduction cost hypothesis and reproduction conflict hypothesis (William, 1957; Lahdenpera et al., 2012; Rogers, 1993; Penn & Smith 2017; Peccei,2001; Thouzeu& Raymond., 2017). But the theme of kin selection alone cannot explain the evolution of menopause. Kin selection processes are also observed in other animals. For example, the bird species Florida scrub, which is famous for helping its close relatives (via altruistic behavior) during the breeding season, should also favor a trait like menopause, which is not the case (Woolfenden,1975).

Kin selection-based theories try to explain why a trait like menopause would have evolved, but they do not explain the population genetics of menopause (i.e., how infertility mutations were selected and became fixed in populations; Morton et al., 2013).

In 2013, Morton et al. postulated a population genetic theory (Mate Choice Theory) and based on computational simulations, explained how non-random mating (men preference of mating with younger women) could have led to

accumulation of deleterious mutations affecting female fertility over a period of time, thereby leading to the origin of menopause in populations.

The author's idea was to test whether age-dependent, sex-dependent fertility-reducing mutations could accumulate in females (or males) beyond the age of allowed reproduction but before death. That is, if reproduction is denied to older females (or males), could fertility-reducing mutations accumulate because they do not affect fitness. These mutations are age-dependent and sex-dependent in expression. Thus, individuals could live beyond their ability to reproduce as a result of mutation.

In their simulation experiment, keeping the population size constant, mutations were included for a late age of onset that affected mortality as well as fertility without any pre-existing diminishment in female fertility. When the authors used an age-indifferent preference for mating by the male [AP model], they observed that there was no age-dependent mortality or sex-specific infertility-causing mutations in the population. The fertility of females remained high, allowing them to extend their lifespan as fertile men do by selection against age-dependent, mortality and infertility-causing mutations.

When the authors used age-preference by males (i.e., older men preferring younger females for mating) [YP model], they observed female-specific accumulation of mutant alleles in the population and menopause occurred early, indicating that mating strategies can affect the fertility of females in the population. The male menopause never appeared as male-specific infertility-causing mutations did not accumulate.

An important aspect of the mate choice theory, besides explaining the origin and evolution of menopause, is that it shows how a change in the mating system can allow detrimental mutations to evolve neutrally. This opens the possibility that other female traits, whether independent from or associated with menopause, may also have evolved through mate choice theory. If the theory is correct, it should produce symmetrical results and should predict the evolution of male menopause in populations practicing preferential mating between females and younger males. Takahashi et al., 2017 modified mate choice theory and combined three theories: grandmother, lifespan artifact, and mate choice to better explain the origin of menopause.

To supplement mate choice theory, we have used *Drosophila melanogaster* as a model organism to understand the role of mate choice in the evolution of

menopause. The lifespan of *Drosophila melanogaster* is approximately 30 to 60 days at room temperature (25°C). Female *D. melanogaster* remains virgin for 24 hours post eclosion and male *D. melanogaster* remain virgin 12 hours (Ashburner 1973).

A mating scheme has been developed using *D. melanogaster*. Two simulated experimental populations were created: older males mated with younger females (oM) and older females mated with younger males (oF). In turn, each experimental population has three replicates.

Reproduction age is restricted to three days in younger counterparts of experimental populations, so any old age expressing mutations are not allowed to be expressed and thus are not selected out. We hypothesized that if the idea of mate choice is correct, these mutations should segregate in the populations (**Figure 3**).

Fecundity and Fertility Experiment:

Previous experiments in the Singh lab involved measuring fecundity and fertility within the selected and control populations. Fecundity is measured as eggs laid each day by a female fly (Barnes et al., 2003). Fertility is the measurement of offspring produced. My project involved a test of fecundity and fertility, where

experimental young females (Old male/young female) were mated with control fertile aged males and compared with the mating of control (control aged fertile males mated with control young fertile females).

Lifespan Experiment:

Longevity experiment was conducted using experimental (oF and oM) and control flies. Understanding the lifespan of flies was important because menopause is defined as senescence of fertility before biological senescence (Kirkwood & Shanley, 2010). Survival curves were made, expected lifespan for control and experimental flies was calculated.

Ovary Morphology Experiment:

Ovariole number impacts the production of eggs and offspring in *D. melanogaster* (Kelpsatel et al., 2013). Staining techniques were used to count ovariole numbers and measure stage fourteen egg chambers in experimental (oF and oM) and control flies. The reduction of ovariole and matured egg chambers (associated with fertility) in old male/young female (oM) compared to old female/young male (oF) and control flies may be predicted as early termination of reproduction in old male/young female (oM) experimental population.

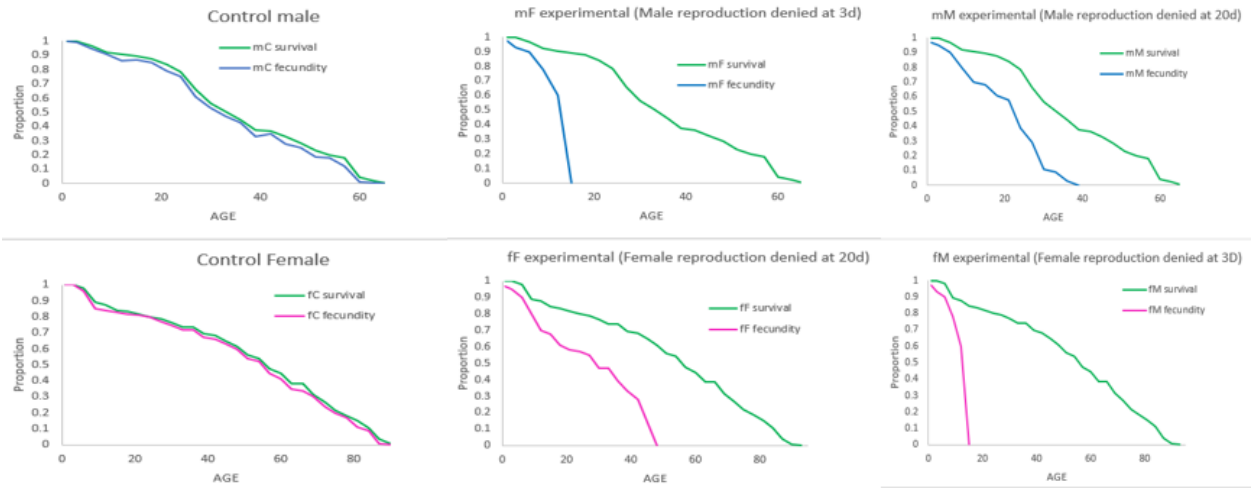

Figure 3. Hypothetical Sample Diagram of Survival and Fecundity curves for control and simulated age-restricted mating populations in *D. melanogaster*. The survival curve is shown in a green curve. Male fecundity curve is shown in blue and Female fecundity curve is shown in pink.

Objectives of the study:

In general, the theories of menopause explained very little about the origin and persistence of menopause in the human population. Morton et al. (2013) tried to explain the origin and evolution of menopause due to mate choice observed in the human population that led to an accumulation of fertility-reducing mutations in females. These fertility-reducing mutations are observed under very specific conditions. The objective of this research was to apply these conditions and test mate choice theory by investigating the effect of age-restricted mating on fecundity, fertility, and lifespan of *Drosophila melanogaster*. The purpose was to investigate if menopause can evolve because of the accumulation of sterility-causing mutations in older females as a result of being deprived of reproduction. Since the theory applies equally to both sexes, we also explored if menopause can evolve in the situation where females are allowed to have a preference for younger males in our simulated mating population.

CHAPTER 1

Test of Fecundity and Fertility in the Experimental Young Females mated with Control Aged Males

1.1 BACKGROUND

Mate choice is an important feature and is observed in various organisms including humans. Mate choice is defined as male/female morphology or behavior that biases mating towards certain opposite sexes, which has direct or indirect effects on their reproductive success (Anderson 1994; Gavrilets et al., 2001; Friberg and Arnqvist, 2003). In humans, it is believed historically that the older men prefer younger women for mating over older women (Tuljapurkar et al., 2007) or younger women outcompete older women (Mulder 2009 & Bus et al., 1994).

In 2013, Morton et al. proposed that due to mate choice, older women deprived of reproduction would accumulate fertility-reducing mutations, ultimately leading to menopause. To test this idea, two age-restricted mating populations were created in the lab, where older males were mated with younger females and vice versa (**Appendix Figure 1**).

After performing twenty rounds of age-restricted mating on three replicates of two experimental populations, a number of eggs and offspring production was

observed in the age-restricted mating populations and wild type flies. It has been showed by a previous research student from the Singh lab, that the number of eggs and offspring production in age-restricted mating population was less compared to wild type females (Mitali Chaudary 2018).

The previous results from Singh lab supported the mate choice theory. The age-restricted mating negatively affected the number of eggs and viable offspring production. The pairs chosen to mate for these experiments were within the experimental populations and control (**Appendix Figure 2**). To supplement the previous research done in the lab, experimental females (oM experimental population) were mated with control fertile males. We were interested to see if the experimental female's fecundity is negatively affected even when mated with fertile wild type males. To be clear, we were interested to understand the effect of age-restricted mating in the fruit fly and the origin of sex-specific fertility-reducing mutations in *D. melanogaster.* We were testing the phenomenon of fertility loss, but not the physiological symptoms of it.

Female *D. melanogaster* become sexually mature after 24-hours of post-eclosion (Ashburner 1973). With the single insemination, a female fruit fly can lay eggs up to 11 days (Ashburner, 1973). Larvae hatch 22 hours after the eggs are laid on food medium. After four days from this point, they undergo metamorphosis and

larvae pupate. After seven to eight days, the pupae eclose into adults. Usually, fruit flies live up to 30 to 60 days under laboratory conditions and lay eggs until they die (Ashburner, 1973). Pair fecundity in the fruit fly is always difficult to measure since many factors effect mating such as age, body size, temperature, nutrition, etc. (Alexei et al., 2007; Stella et al., 2013; Churchill et al., 2019). All the factors were taken into consideration in the experimental design.

By the time of this experiment, twenty-four rounds of age-restricted mating were performed on the three replicates of the experimental population. Fecundity and fertility of three replicates of oM experimental population and control were observed and measured. A number of eggs laid every day is taken into consideration as a measurement of fecundity. Fertility is the number of viable offspring produced to the number of eggs laid by the female.

Our results showed that experimental young females of oM experimental population (old male/young female) fecundity and fertility were less compared to the control females. The results supported the previous work done in the Singh lab.

Menopause is the single most major factor affecting the health of women. The results of this research will not only shed light why women get infertile and what

can be done about it but also it will shed light on the connection between fertility genes, hormones, and health.

1.2 Methods

Standard control jars were cleared the day before collection of virgin flies i.e. when pupae were ready to eclose into adults. Virgin control males were collected (30 in total) and were kept separately in vials (food-filled) for twenty days. Flies were changed into fresh food-filled vials every seven days.

Experimental oM has three replicates named 1.M, 2.M and 3.M. Jars of three replicates were cleared when the collected virgin males of control population were 17 days old. 10 virgin females were collected (one in each vial) from each replicate of oM (30 females in total) and aged for three days. The 20-Day aged control male was allowed to mate with a 3-Day young oM female for three days. Male was discarded after 3 days of the mating window; the female was transferred into fresh food-filled vial and was allowed to oviposit. The number of eggs laid was counted every day in the twenty-day observation period by transferring the female into fresh vail every twenty-four hours. The ability to lay

eggs and produce offspring by the experimental oM population replicates were recorded and was compared with the standard control population.

To compare the test of fecundity of the experimental population, a standard control was created, where 20-day old control male was mated 3 days old virgin female in the same manner as done in the experiment. Fecundity and fertility were measured within the control population to serve as a control for comparison. The vials of every third day in twenty-day observation were kept aside and a number of offspring produced were observed and recorded for each population of oM and control population females.

1.3 RESULTS

Fecundity and Fertility of experimental and control females were measured for twenty days. Graphs were made with GraphPad PRISM 6.0 (version 6.01) software.

Using R windows software (version 1.1.46), analysis of variance was done to examine the difference between the two populations.

1.3.1 Measurement of Fecundity in oM and Control females

Fecundity is measured in terms of oviposition (i.e. number of eggs laid by a female every day after the male has been discarded). The mean number of eggs laid by control females is larger than the mean number of eggs produced by oM experimental females (**Figure 1.1**). A linear decrease ina number of eggs laid is observed both in control and oM females. Whereas, within the replicates of the experimental population, there was no difference observed. All the three replicates of oM females (1.M, 2.M, and 3.M) shown a general trend in the decrease in egg-laying capability (**Figure 1.2**). Interestingly, two of the 1.M replicate females laid no eggs from day 18. The highest number of eggs laid is observed before day 5 in both control and oM females.

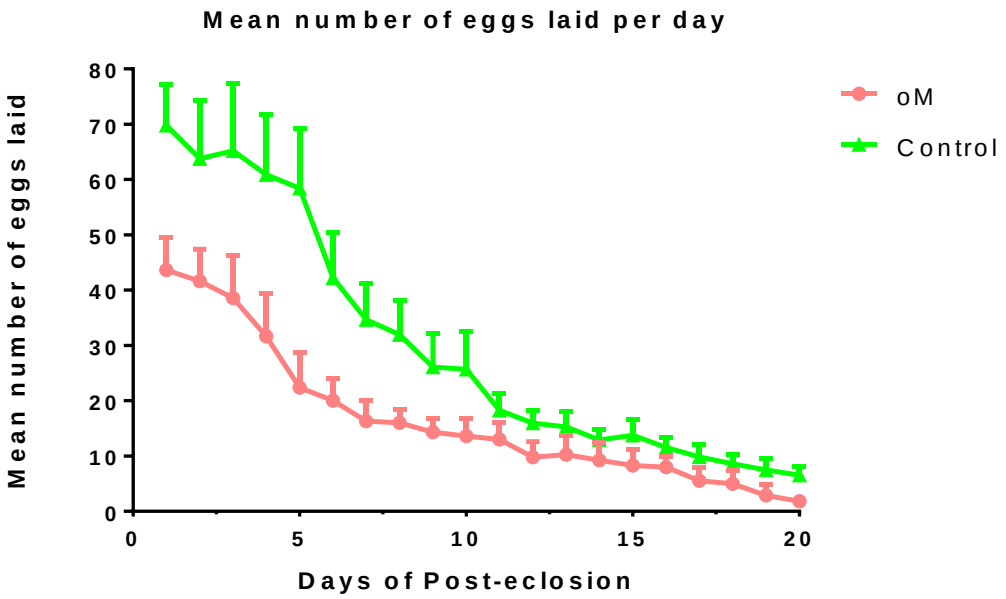

Figure 1.1: Mean number of eggs laid by control and oM (older males/younger females) population over a time. Bars in the figure represent SEM (Standard error of the mean).

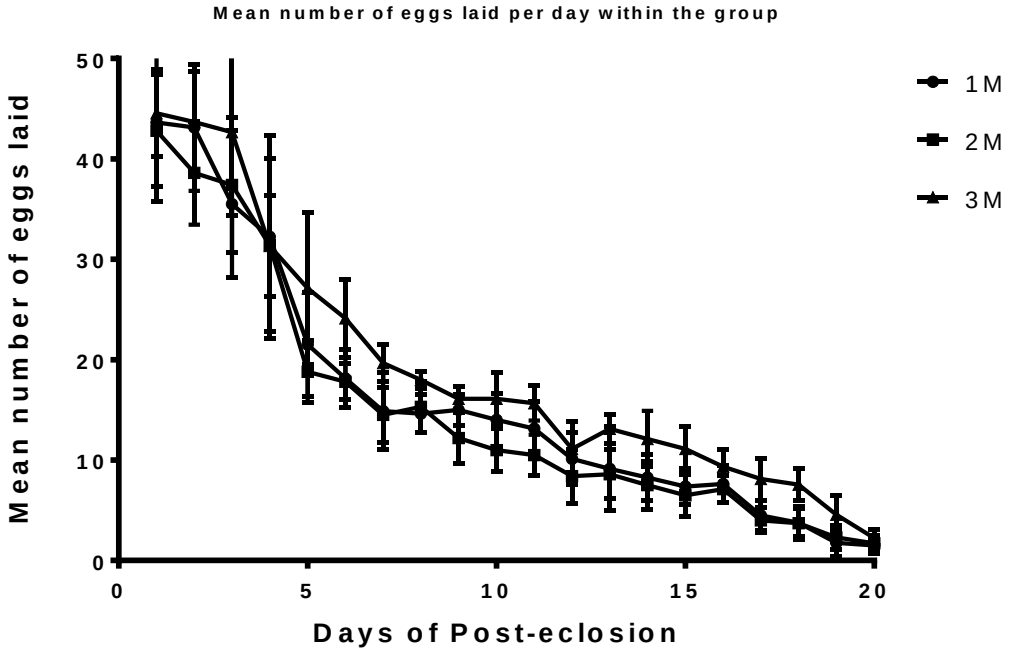

Figure 1.2. Mean number of eggs laid by three oM population replicates (1.M, 2.M, and 3.M). Bars in the figure represent SEM (Standard error of the mean).

1.3.2 Measurement of offspring in oM and Control females

The mean number of offspring produced by the control females is significantly higher than the average number of offspring produced by oM females. Most of the oM females produced no offspring after day 15 post-mating period. Controversially, control females produced offspring even after day 15 post-mating period (**Figure 1.3**).

Within the old male/young female population, there was no difference in the offspring produced (**Figure 1.4**). Almost three populations reached zero after day 15 post-mating period.

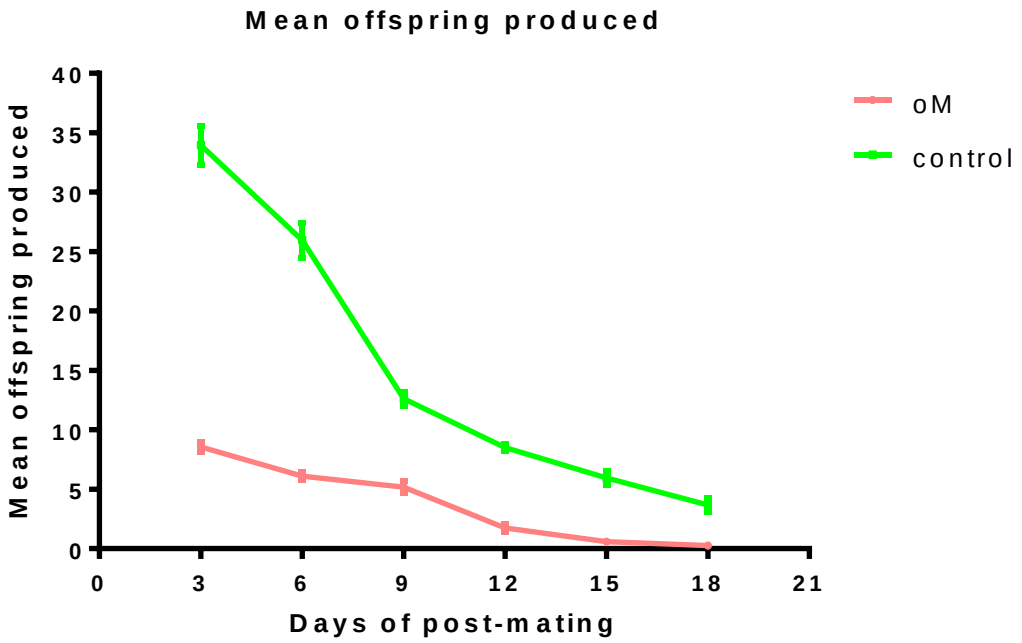

Figure 1.3. Mean number of offspring produced by control and oM population females observed every third day. The bars in the figure represent standard error of the mean.

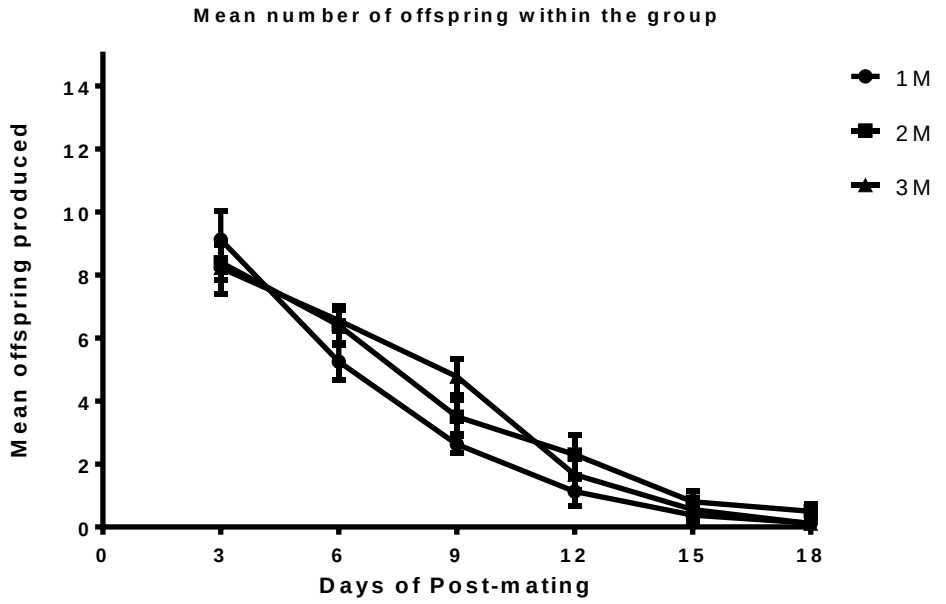

Figure 1.4. Mean number of offspring produced by old male/young females (1.M, 2.M, and 3.M). The bars in the figure represent SEM (standard error of the mean).

1.3.3 Fertility over time

Fertility in the study was measured as a number of viable offspring produced from the eggs laid. The percentage of eggs eclosed into adults is observed greater in control females than the oM experimental females (**Figure 1.5**).

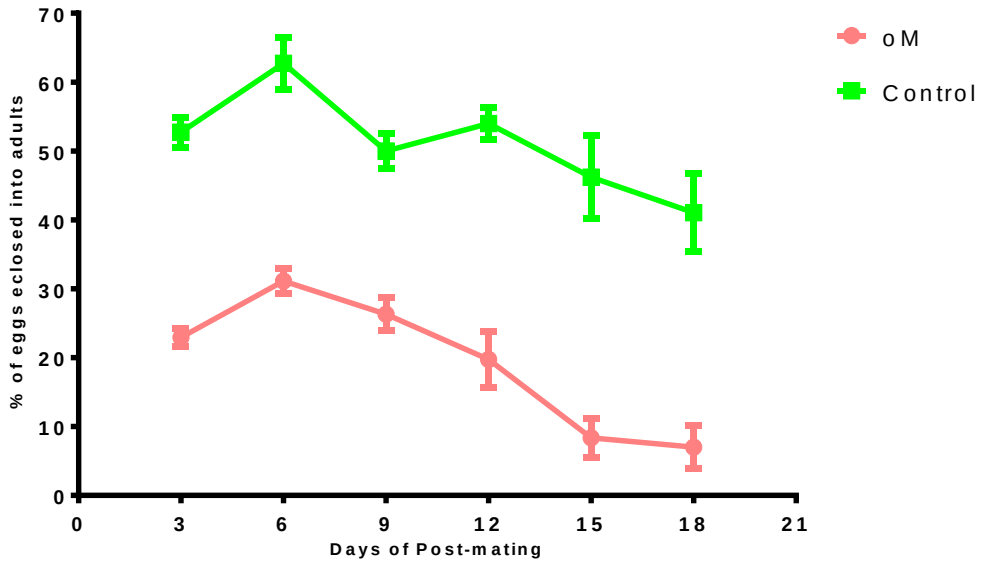

Figure 1.5. Percentage of eggs eclosed into viable offspring over time in old male/young female and control females. Bars in the graph represent SEM (standard error of the mean).

1.3.4 Percentage of females remained fertile over time

Almost all females in the control population remained fertile, except that, the percentage dropped to 88% on day 18 of post-mating. Whereas, in oM females, initially all females were fertile till day 9 post-mating period and it dropped sharply further from day 12 post-mating.

Only 22% females of oM were fertile at the end of the observation (i.e. on day 18) post-mating, and in control population 88% females were fertile, indicating a great difference between the fertile females between control and oM females (**Figure 1.6**).

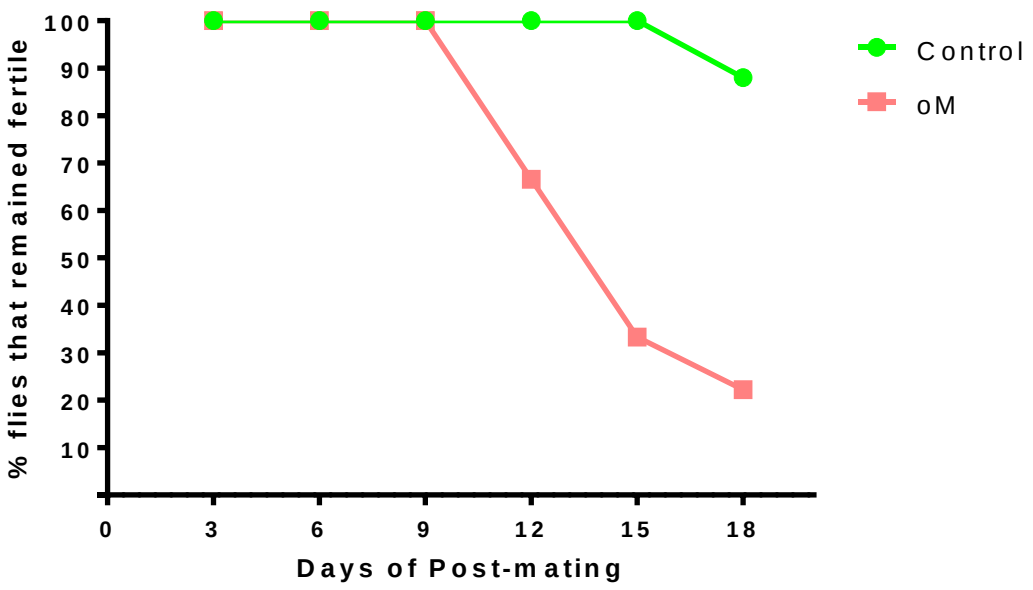

Figure 1.6. Percentage of the females remained fertile over time in control and old male/young female population replicates observed every third-day post-mating.

1.4 Statistical analysis

1.4.1 Total Eggs laid

One measure of the female's fecundity is the total number of eggs laid, obtained from the number of eggs per day summed from day 1 to 20 (female age 3 to 23 days post-eclosion). A total number of eggs laid for each female is obtained for oM and control populations. There are total 10 observations for 1.M, 2.M, 3.M and 20 observations for control.

Boxplot for the data was done to see the distribution and variability in the values (**Figure 1.7**). The boxplot of the data shows four outliers i.e. four females died before twenty-day observation and were removed.

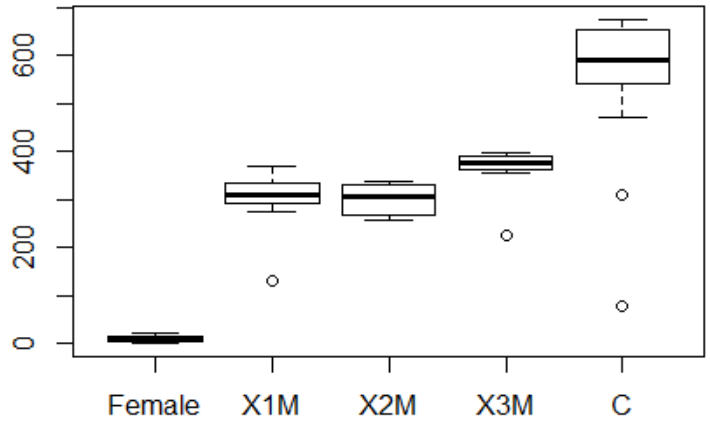

Figure 1.7. Boxplot of total eggs laid by females of oM experimental replicates and control females. Bars in the figure represent distribution of eggs laid by the females. Y axis the name of the population and X axis the number of eggs.

The outliers were removed, and the total egg data of females were again obtained from the boxplot graphical point (**Figure 1.8**).

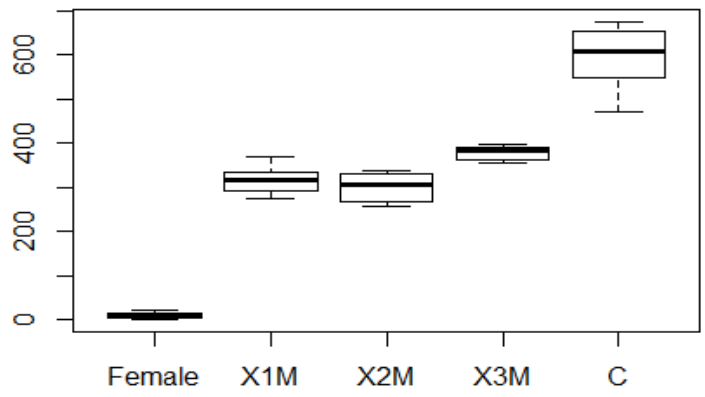

Figure 1.8. Boxplot of total eggs laid by females of oM experimental replicates and control females, after excluding outliers. Bars in the figure represent distribution of eggs laid by the females. Y axis the name of the population and X axis the number of eggs.

Analysis of variance was performed to examine the relation between egg production of control and oM experimental population females. ANOVA results revealed that there is a statistical difference observed between two populations ($p < 0.0001$).

1.4.2 Total offspring produced

Total offspring produced by each female in the observation period was noted in MS Excel. There were total 27 females for oM population (i.e. 8 females for 1.M,

10 for 2.M and 9 for 3.M) and 17 females for the control population after removing the outliers (females that died before the observation period are excluded from the analysis).

Boxplot for the data was performed using R software to see the distribution of the offspring data (**Figure 1.9**).

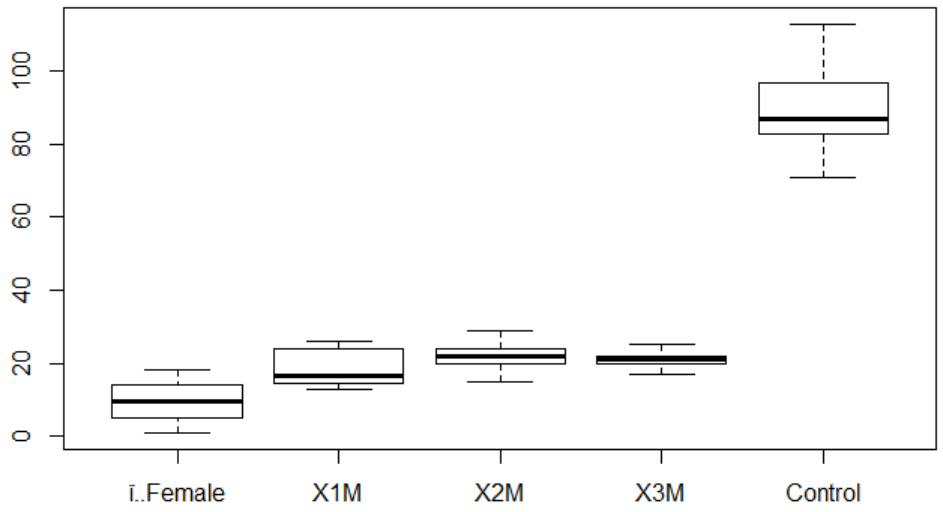

Figure 1.9. Boxplot of total offspring produced by females of oM experimental replicates and control females. Bars in the figure represent distribution of offspring produced by the females. Y axis the name of the population and X axis the number of offspring. Female column is the default produced in R software.

Analysis of variance was performed to examine the relationship between offspring production of control and oM experimental females. ANOVA results

revealed that there is a statistical difference observed between two populations ($p < 0.0001$).

Emmeans is the package in R that provides marginal means of the populations and calculates the difference between the populations. Analysis has been done to check the difference between the populations of oM and between the oM and control populations (**Table 1.1**).

It is observed that within the experimental populations (1.M, 2.M, and 3.M), there was no difference and between the experimental populations and control population, there was a significant difference between the offspring production.

Table 1.1. Differences between the populations obtained by Emmean pairs (Package of R software).

Pairs	p-value
1M-2M	0.8411
1M-3M	0.851
1M-C	0.001***
2M-3M	1.000
2M-C	0.001***
3M-C	0.001***

1.5 DISCUSSION

After performing twenty-four generations of age-restricted mating, it was found from the study that the young female's fecundity and fertility of old male/young female (oM experimental poulation) were negatively affected compared to control females. With the increase in age, the production of eggs and offspring decreased in both oM and control females, oM population females affected more rapid than the control females. Within the experimental oM population, the females had shown a similar trend of decline in fecundity and fertility and shown no statistical difference between replicates. The percentage of females that remained fertile was significantly higher in control females (88%) than the oM experimental females (22%) at the end of the observation. One important factor to consider here is that the experimental young female's fecundity and fertility were measured when mated with the control old fertile male, not from the within oM experimental male population.

Another important factor to consider in this study is the age of flies when are mated. The control wildtype 20-D old male mated with a 3-D old female from the same line, allowed us to examine the effect of age-restriction mating in young experimental (oM) females when mated with control 20-D old males. The control wild type flies exhibited similar production of eggs compared to the literature

and therefore considered to be a good representation of wildtype flies reproductive capability (David et al., 1975). From the studies, it is observed that the production of offspring lowers quickly than the actual reproductive capability (David et al., 1975). The same was observed in our study. The production of offspring declined more rapidly with age than the number of eggs laid as a function of age (**Figure 1.1 and Figure 1.3**). Almost three replicates females of oM experimental population produced no offspring after 15 days of post-mating (**Figure 1.3**).

Overall, it is assumed that the age-restricted mating had negatively affected the experimental female flies. The young females from the experimental population had a significant decrease in the production of eggs and offspring over time, meaning that there may be some underlying changes occurred in young females due to the age-restricted mating with older males. The results from this chapter supported the previous work done in the Singh lab (**Appendix Figure 2**).

The study also supported the computational work done on mate choice by Morton et al., 2013 on how the selection favored early loss in the fertility of a woman due to the historical preference of older male towards a younger female. It is possible in humans, that the older women, deprived of reproduction may have accumulated the fertility-reducing mutations, ultimately led to menopause.

These mutations may be responsible for the hormonal changes that are observed at the time of pre-menopause and menopause.

To strengthen our results, it is recommended to do the sperm viability test, i.e. to dissect the female seminal receptacle to examine how much sperm is stored. This would provide clear results and eliminate any confounding factors on pair-fecundity. Our experiment was based on the assumption that the female can lay eggs up to eleven days with single in-semination (Ashburner, 1973). However, in our experiments, we have noticed the egg production even after 11 days. So, it is unclear about at which point, the female ends laying eggs. Also, it will be interesting to see the effects of age-restricted mating after every five rounds of selection. This would provide an insight into the changes that are occurring over time and also be interesting to see the complete sterility in fruit flies. Lastly, it is important to know about where the changes are occurring at a genetic level. The genetic level changes can be examined by doing the sequencing experiments. By this, it will be more obvious to see the difference between the age-restricted and age-unrestricted populations.

Menopause is the ultimate outcome of every woman's life. Understanding the genetic cause of the origin of menopause will not only shed the light on how infertility arose in human females, but this will also provide great insight on the

inter-connection between genes affecting hormones and other physiological related changes seen in humans. This research can also be useful in understanding the origin of menopause in other organisms like captive chimpanzees, killer-whales and short-fined pilot whales.

1.6 Appendix

Table 1: Preparation of Standard cornmeal-molasses-agar medium

Cornmeal	78 g
Agar	7.8 g
Yeast	15.6 g
Cold water	200 ml
Boiling water	1000 ml
Molasses	55 ml
Propionic acid	6 ml
Tegosept	24 ml

Combine cornmeal, agar, and yeast into a glass beaker. Add the boiling and cold water simultaneously then place onto a hot plate with a magnetic stirrer. Wait for mixture to boil, then remove beaker from hot plate and add the molasses while stirring. Wait for mixture to cool to 60^{o}celsius, then add in the propionic acid and tegosept and pour into 250 ml jars and 35 ml vials.

EXPERIMENTAL SET UP USING *DROSOPHILA MELANOGASTER*

In an effort to test the computational simulation model of Morton et al. (2013), we carried out experiments with *Drosophila melanogaster* to study the effects of restricted reproduction in fruit flies over time.

MATERIALS AND METHODS

Drosophila melanogaster

A diversified base population was created by previous researchers in Singh lab in the year 2016 by mixing ten different *Drosophila melanogaster* populations that were ordered from *Drosophila* Stock Centre, California.

Drosophila stock culture

The *Drosophila* population was maintained in 250 mL glass jars on approximately 25-30 mL standard cornmeal-molasses-agar medium (appendix table 1). The population was maintained at 22° C under a12-hour dark and light cycle.

Random Mating Scheme

We maintained the fruit fly culture by changing the populations into fresh food-filled glass jars every two weeks. The populations in the jars were randomly mated. This is considered as the standard or base control populations.

Non-random mating scheme

A mutation accumulation experiment is currently underway by mating older males to younger females and *vice versa*. In the older male, younger female non-random mating scheme denoted as oM, as the control population was growing, on day 9, we cleared the jars when the pupae were ready to eclose, as shown below. Females remain virgin 8-10 hours after eclosion and males remain virgin for 12 hours after eclosion. Virgin flies are easily detectable due to their larger body size than the older flies. Virgin flies do not have dark pigmentation as older flies and most importantly, they have a dark greenish spot, called meconium (the last meal before pupating) on their abdomen (Ashburner, 1979). On day 10, we collected 100 virgin males, placing 10 in each food-filled vial. We aged the males for 20 days, changing them into new vials every 7 days to prevent food contamination. Meanwhile, on day 26, we cleared the control population jars again, to be able to collect 100 virgin females on day 27. We then aged the females for 3 days. On day 30, we mated the 20-day old virgin males with 3-day old virgin females in 3 jars to avoid the risk of losing complete line, the progeny in which went on to be the next generation. Given a 3-day mating window, once it was visible that there were enough eggs laid on the food, the jars were cleared to start the new population, with the last day of mating becoming day 0 for the next

generation. The populations were maintained this way for 23 generations. We created 3 independent replicates of oM that were denoted as 1.M, 2.M, and 3.M. Vice versa, this procedure was also used to create older female, younger male non-random mating scheme denoted as oF, with 3 independent populations labelled as 1.F, 2.F, and 3.F.

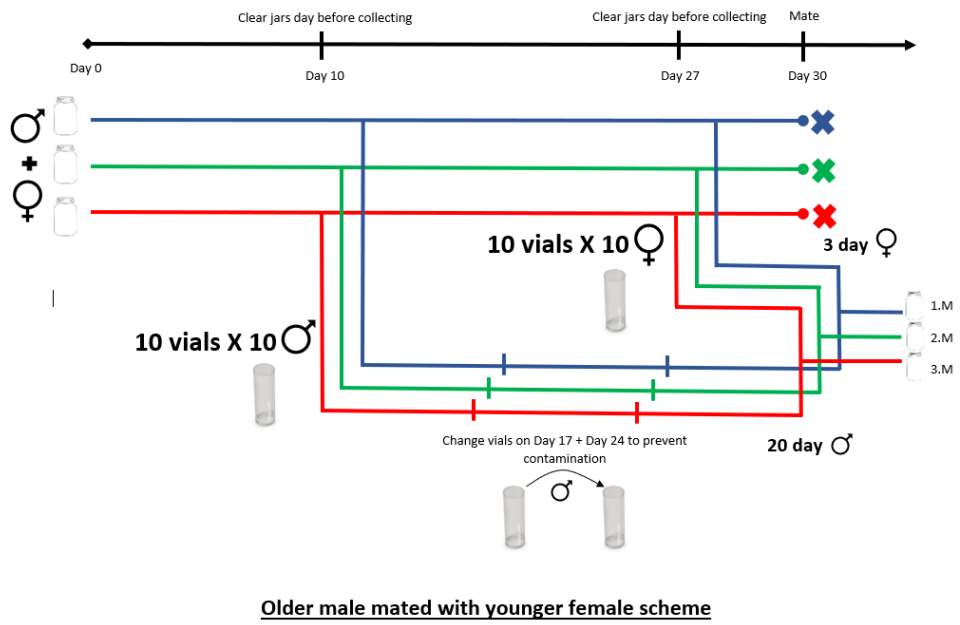

Figure 1: Simulated age-restricted mating in *Drosophila melanogaster*

The non-random mating scheme experiment was started in the year 2016. For 1.M, 1.F and 3.F populations, the generation zero was started in September 2016 and for 2.M, 3.M and 2.F populations, the generation zero was staring in May, June and July months of 2016.

It takes approximately thirty days for one round of non-random simulation (see Figure). At present, in June 2019, for old male/young female, 1.M is in 30th generation of simulation. 2.M is in 33rd and 3M is in 34th generation of simulation. For old female/young male, 1F is in 31st, 2F in 32nd, 3F is in 29th of non-random simulation generation.

Previous work:

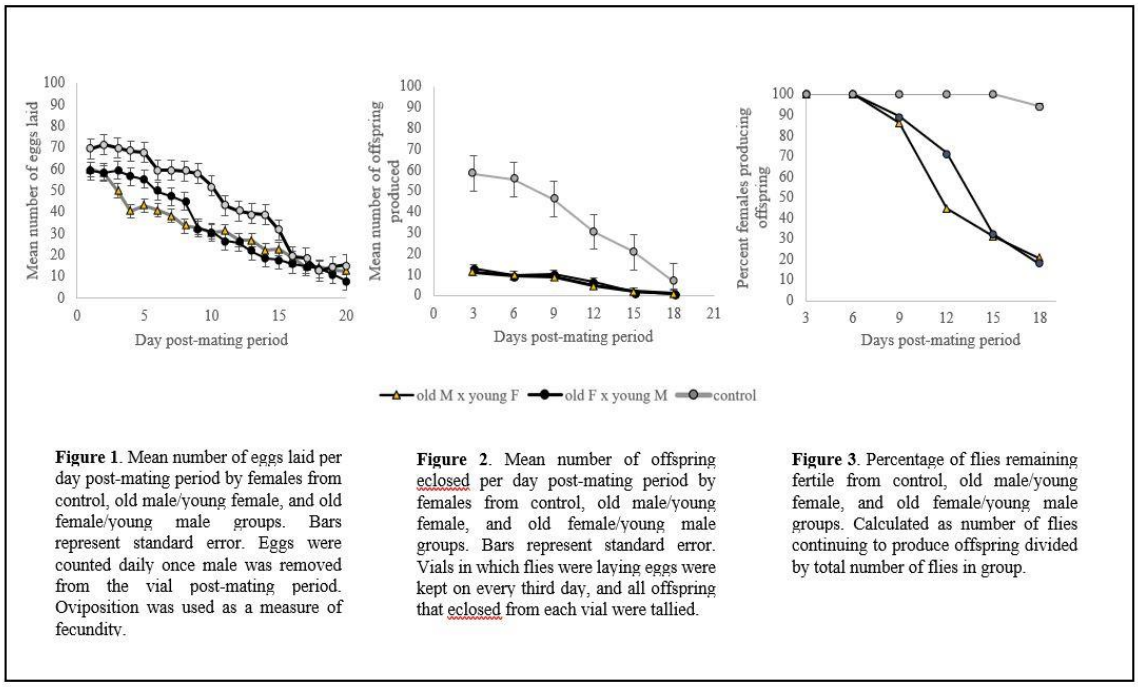

Figure 1. Mean number of eggs laid per day post-mating period by females from control, old male/young female, and old female/young male groups. Bars represent standard error. Eggs were counted daily once male was removed from the vial post-mating period. Oviposition was used as a measure of fecundity.

Figure 2. Mean number of offspring eclosed per day post-mating period by females from control, old male/young female, and old female/young male groups. Bars represent standard error. Vials in which flies were laying eggs were kept on every third day, and all offspring that eclosed from each vial were tallied.

Figure 3. Percentage of flies remaining fertile from control, old male/young female, and old female/young male groups. Calculated as number of flies continuing to produce offspring divided by total number of flies in group.

Figure 2: Previous work done in Singh lab by Mitali Chaudary, 2018.

Raw Data:

Table 2. Egg laying raw data for 1.M

Days_of_egg_laying	1M_1	1M_2	1M_3	1M_5	1M_6	1M_7	1M_8	1M_9
1	52	53	49	33	48	42	36	36
2	54	41	43	36	51	38	39	43
3	41	39	29	46	42	31	27	29
4	17	43	17	38	36	37	30	40
5	21	19	18	31	21	14	22	26
6	19	13	18	23	16	18	19	19
7	15	11	13	23	16	11	14	16
8	15	13	14	19	13	15	14	14
9	17	14	15	17	14	16	13	14
10	14	16	15	16	16	11	9	15
11	10	17	14	16	11	11	11	15
12	9	6	11	13	11	7	11	13
13	0	9	9	14	10	9	9	13
14	9	5	9	11	6	6	9	11
15	7	5	8	10	8	5	7	9
16	7	8	9	10	8	5	9	5
17	5	3	3	6	3	4	5	7
18	5	2	2	4	3	6	4	4
19	2	0	2	0	1	3	2	4
20	1	0	2	2	1	2	2	2

Table 3. Egg laying raw data for 2.M

2M_1	2M_2	2M_3	2M_4	2M_5	2M_6	2M_7	2M_8	2M_9	2M_10
42	37	43	39	39	38	48	39	52	51
40	29	36	37	37	34	43	40	43	47
46	35	39	36	37	26	29	39	48	39
37	33	36	43	44	19	23	27	23	29
22	17	14	20	21	18	14	20	19	23
17	16	17	18	20	19	14	19	19	19
12	13	11	18	17	12	13	19	15	15
11	13	14	17	17	18	13	16	19	15
10	9	9	16	15	11	12	15	13	12
9	9	7	13	11	11	12	14	12	12
10	10	6	12	10	11	11	14	10	11
7	6	5	12	7	9	9	13	6	10
7	6	5	11	7	9	9	13	9	10
5	5	4	11	8	7	7	11	8	9
5	4	3	9	8	7	5	9	8	7
6	8	8	8	5	5	7	9	8	7
4	2	2	5	5	4	3	5	5	5
3	2	2	5	3	7	2	4	4	5
3	1	1	3	2	1	2	4	2	4
1	1	1	3	2	1	1	3	2	2

Table 4. Egg laying raw data for 3.M

3M_1	3M_2	3M_3	3M_4	3M_5	3M_6	3M_8	3M_9	3M_10
51	49	45	48	41	38	42	46	41
48	38	41	41	48	35	47	49	46
62	47	38	38	47	39	38	36	39
26	30	36	35	39	23	29	32	32
23	29	38	27	29	21	21	39	17
21	29	26	23	21	19	21	29	28
21	21	21	22	19	19	19	16	19
19	17	18	17	19	17	18	18	19
17	15	15	14	17	16	18	17	16
13	13	16	13	19	19	17	19	16
15	15	18	14	19	14	16	15	15
10	11	13	9	13	12	16	7	9
13	11	13	11	13	15	15	13	14
18	9	11	9	11	14	13	13	11
11	9	9	9	12	14	15	11	10
9	6	10	8	9	11	12	9	10
7	6	9	7	5	9	9	12	9
9	4	7	9	7	8	8	7	9
4	2	3	5	4	8	7	3	5
2	2	3	2	1	3	3	1	3

CHAPTER 2

Measurement of Lifespan in Age-restricted Mating Populations of *Drosophila melanogaster*

2.1 Introduction

Aging is a natural process of physiological deterioration that reduces fitness and increases the risk of diseases. Aging is greatly influenced by various factors such as reproduction, temperature, nutrient intake, and environmental stress, etc. although the inter-relationship among these variables has been poorly demonstrated (Hughes, 2010).

Existing studies on longevity demonstrate differences in survival probabilities within or between species (Carlson and Hetcher, 2003). Evolutionary theories suggest that selection acting against mortality-causing mutations will become weaker with age (Hamilton, 1966). The mortality rate is highly age-dependent, for instance, young individuals showing lower mortality rates compared to older individuals (Fried et al., 2001; Reznick et al., 2006).

A group of organisms is said to have a mortality risk when individuals typically experience a decline in survival even though they live under similar conditions

(Luckinbill and Claire, 1984). This mortality risk varies among the species (Carlson and Hetcher, 2003). Humans are believed to have much longer lifespans among all the primates (Finch, 2010). The extension of human longevity has happened only after the industrial revolution. The longer lifespans may be due to the advancements in medicine, food, and the environment (Finch, 2009). Even under high mortality risk experienced by hunter-foragers, the life expectancy of humans at birth is double the wild chimpanzees (Finch, 2010).

While human longevity has extended, the phenotype of menopause has been detected along with it. According to the lifespan artifact theory, menopause is a 'built-in' trait that was observed only after the human longevity was extended (Washburn, 1981; Peccei, 2001). But lifespan artifact alone could not explain the origin of menopause.

With the increase in age, there will be a decline in fertility of all the organisms (Kirkwood et al., 1991). Understanding the lifespan is important because complete reproductive senescence in human females is observed before the biological senescence (Kirkwood & Shanley, 2010). But the mechanism of the links between reproduction and survival is not well understood (Prowse and Partridge, 1997).

A previous study showed that age preference mating observed in humans (i.e. either older men preferring younger women or younger women competing older women for mating) could produce age-dependent infertility at older ages in women, leading to menopause (Morton et al., 2013). But we do not know if age-dependent infertility causing mutations have any role in the mortality of humans at older ages.

To understand this better, we have conducted an experiment on *Drosophila melanogaster* with age-restricted and age-unrestricted mating populations to see if there is any effect of age restriction mating on longevity in fruit flies.

The fruit fly species *Drosophila melanogaster* is an attractive model organism to observe age-specific mortality and survival. In this study, the mortality risk and the death rates of *D. melanogaster* virgin females and males were measured (in cohorts) between specific intervals of age for control and two treatment populations (old females = oF and old male = oM, defined subsequently). The treatment populations comprise simulated (age-restricted) mating populations, where older males were mated with younger females (oM experimental population) and older females were mated with younger males (oF experimental population).

Based on the results of computational simulations conducted previously by Morton et al. (2013), where fertility causing mutations were introduced in the population, we hypothesized that there would be no difference between the life spans of control and experimental populations (oM and oF). This null hypothesis was used to test for a difference between life spans of control and treatment populations.

2.2 Methods and Materials

2.2.1 Materials:

250mL food-filled jars, CO_2 bed, paintbrush, light stand, magnifying lens

2.2.2 Experiment design:

A total of 900 virgin flies (450 male and 450 female) with 300 (150 male and 150 female) in each population (Control; oM Experimental; oF Experimental) were used in this study. For the control, 50 male and 50 female flies were collected separately from a standard population in three replicates. For oM and oF experimental populations, 50 male and 50 female flies were collected separately from three replicates of oM (1.M, 2.M, and 3.M.) and oF (1.F, 2.F, and 3.F.) experimental populations respectively. All flies were collected in 250 mL fresh food-filled jars.

The flies used in the control and experimental were all virgins and never had been exposed to mating. Observations were recorded on every third day to count the number of flies that were alive. Also, for uniformity, the number of dead flies in the food jars were counted under light microscopy [CO_2 gas was used to

anesthetize the flies and flies were counted on the CO_2 bed with the help of paintbrush and magnifying lens not more than 2-3 minutes to make sure that the flies are not much exposed to CO_2]. Few flies that were stuck in the food jars were checked under the light microscope to see if there is any movement in their legs. If there was any movement, those flies were considered alive and were carefully transferred to the jars using the brush. A movement in legs and attraction towards bright light was considered as a step in observing the alive flies in the jars. Flies were transferred into the fresh food-filled jars every third day. A strict 12-h dark and light cycle was followed throughout the experiment and all flies were kept at room temperature, 22^oC.

R software for windows (Version 1.1.456) was used to produce the survival curves. Log-rank analysis and Chi-square test, also referred to as the χ^2 test was performed in R software to examine the association between the populations.

2.3 RESULTS

The outcomes of the experiment included survivorship, expected life span, and log-rank analysis of the survival curves.

2.3.1 Survivorship

Survival curves and analysis were performed with the Kaplan-Meier survival method. Kmsurv is an R-package available that produces life-table information based on survivorship function s (t) and outputs - the average survival summaries. It creates a cohort life tables based on the column of days and number of alive/dead flies (Campos-Filho and Franco, 1988). Kmsurv produced average survival summaries and also a number of other variables as listed in **Table 2.1**. Survival curves were created from the surv (Fraction of alive flies at each interval) produced from the Kmsurv R package.

Table 2.1. Number of variables obtained from raw data using Kmsurv function in R software.

Nsub	number in the original cohort surviving at each interval
Nlost	input of no. of individuals lost to the cohort during the interval
Nrisk	no. of individuals at risk (for the event = death) during the interval
Nevent	no. of events i.e. deaths during the interval
Surv	survivorship function [p(x)] for x = beginning of interval
Pdf	death rate during interval
Hazard	age-specific mortality risk

2.3.2 Expected life span

The survival curve p(x) is the fraction of flies surviving to x days. Expected life span is computed from the survivorship (surv produced from Kmsurv package of R).

The code that we created (appendix code) calculates the average number (as a fraction of the initial cohort) alive in an interval. At the beginning of each interval, the total number of days remaining to the cohort is calculated from the average number alive by summing over the average alive to the end of the life. The life exp is calculated as an average number of days of life remaining that is expected from the beginning of the interval.

2.3.3 Log-rank analysis:

Log-rank analysis is used to analyze the survival curves (Bland and Altman, 2004). The analysis is based on the events [i.e. number of flies' dead at each interval]. In each interval, the observed number of deaths and number expected are calculated. Number of expected deaths is calculated based on an assumption [i.e. if there is no difference between the populations] (Bland and Altman, 2004). From the number of variables Kmsurv produced: risk, nlost, nevent and nlost were used to calculate the expected values. Observed values are obtained by summing the xnevents**.**

An Example of Calculation: If there are two populations (X and Y): Number of

Expected eventcalculation = xnrisk* (xnevent+ynevent)/ (xnrisk+ynrisk+xnlost+ynlost)

After observed and expected values were calculated, Chi-square test was done in R software to see if there exists any difference between the populations.

2.3.1 Survivorship

2.3.1a Survivorship of control males and females

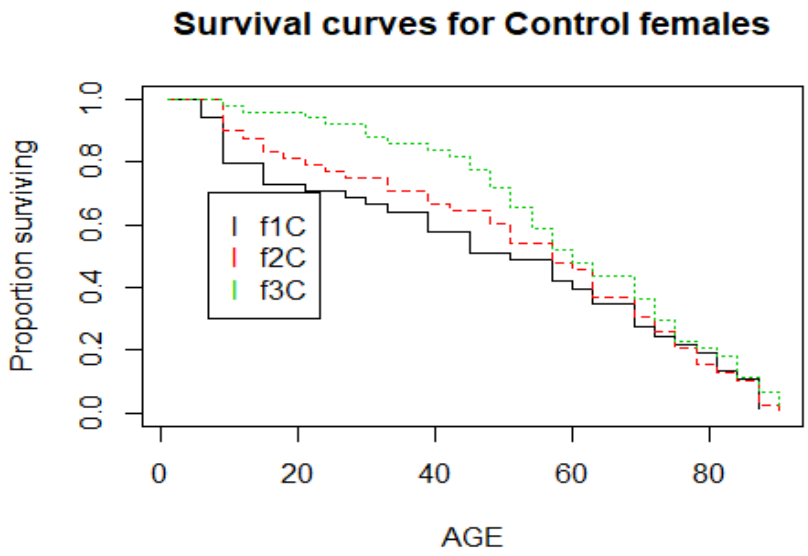

Figure 2.1. Survival curves for control females. Y axis is the proportion of surviving at each interval. X axis the age of the females. f1C, f2C, f3C are the control female replicates (150 females collected in 3 jars, 50 in each). Log-rank survival curve analyses were conducted to obtain the observed and expected nevents (number of deaths at each interval).

All the three replicates of control female population shown a similar trend of decline over a time (**Figure 2.1**). The average lifespan for the three replicates was around 52 days. Chi-square test were performed to examine the relation between three female replicates. The expected and observed values were similar for all the three replicates (**Table 2.2**), and the difference was not significant, χ^2 (2, N = 50) = 1.066, p-value = 0.586.

Table 2.2. Observed and expected survivorship (in days) for females in control replicates.

Replicate	Observed	Expected
f1C	42	35.13
f2C	43	40.96
f3C	44	50.38

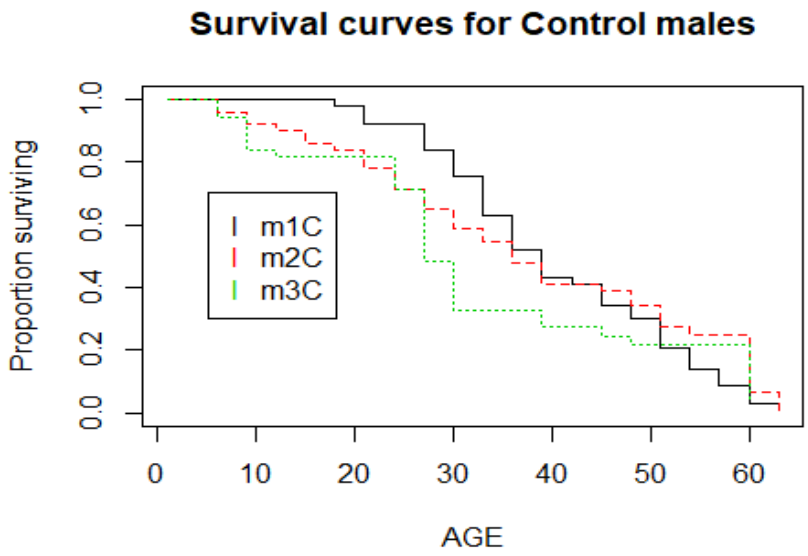

Figure 2.2. Survival curves for control males. Y axis is the proportion of surviving at each interval. X axis the age of the females. m1C, m2C, m3C are the control male replicates (150 males collected in 3 jars, 50 in each).

Survival curves were made for three replicates of control male population (**Figure 2.2**), the survival curves followed similar trends over a time Log-rank survival curve analyses were conducted to obtain the observed and expected values. The observed and calculated values were not different in three control male replicates (**Table 2.3**). A Chi-square test was done once expected values were obtained from log-rank analysis. The relation between the survival curves over a time were not significantly different, χ^2 (2, N = 50) = 0.4728, p-value = 0.789.

Table 2.3. Observed and Expected survivorship (in days) for males in control replicates.

Replicate	Observed	Expected
m1C	44	46.41
m2C	44	37.69
m3C	40	39.00

Also, no differences were detected among replicates for males and females, they were pooled and survival curves for the pooled populations were created (**Figure 2.3**).

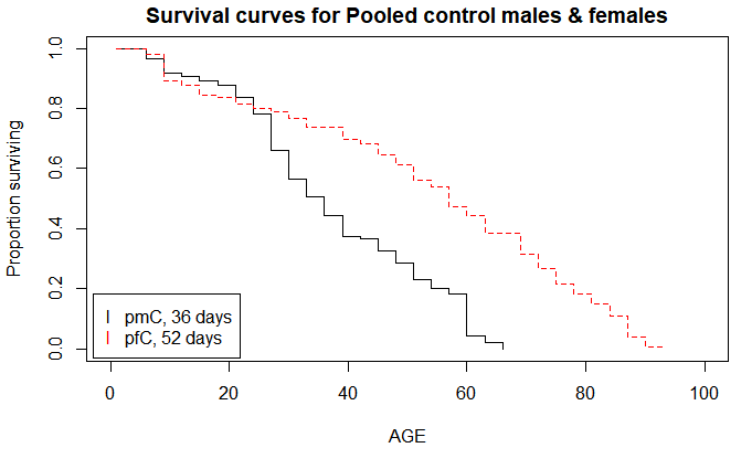

Figure 2.3. Survival curves for pooled control males and females with expected life spans. Y axis is the proportion of surviving at each interval. X axis the age of the females.

Life-expectancies of pooled control males and pooled control females were calculated. The average life-expectancy of control females is 52 days whereas for control males, it is 36 days, showing a great difference between the genders of the *Drosophila melanogaster*.

Log-rank analysis was done to obtain the observed and expected values (**Table 2.4**). Chi-square test was done to examine the relation between control males and females, and is observed that the significant difference is observed, χ^2 (1, N = 150) = 32.4728, p-value = 0.001.

Table 2.4. Observed and Expected survivorship (in days) for pooled control males and pooled control females.

Population	Observed	Expected
mC	128	80.27
fC	132	131.51

2.3.1b Survivorship of Experimental population (oF)

Survival curves were made using mat plot in R software (**Figure 2.4**). Log-rank analysis was done to obtain the observed and expected values from three replicates of oF (**Table 2.5**).

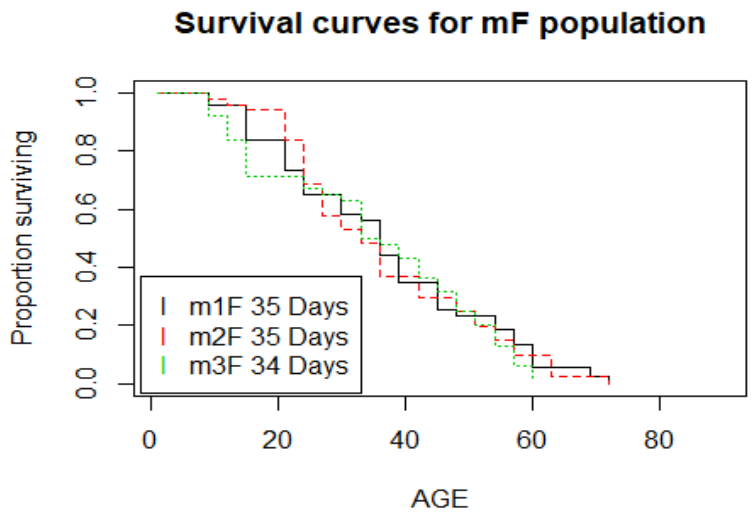

Figure 2.4. Survival curves for males of oF population with expected life spans. Y axis is the proportion of surviving at each interval. X axis the age of the females. m1F, m2F and m3F are the males from three replicates of oF population (1.F, 2.F and 3.F)

Table 2.5. Observed and Expected values (in days) for oF population males (m1F, m2F and m3F)

Replicate	Observed	Expected
m1F	44	44.95
m2F	44	43.80
m3F	43	39.82

The expected life spans are included in the figure legend (**Figure 2.4**). We observed that the average life expectancy of the males of oF populations is approximately 35 days (**Figure 2.4 and Table 2.5**). A chi-square test was performed to examine the relationship between the survival curves of oF population males. The relation between three population males were not significant, χ^2 (2, N = 50) = 0.11, p-value = 0.9465.

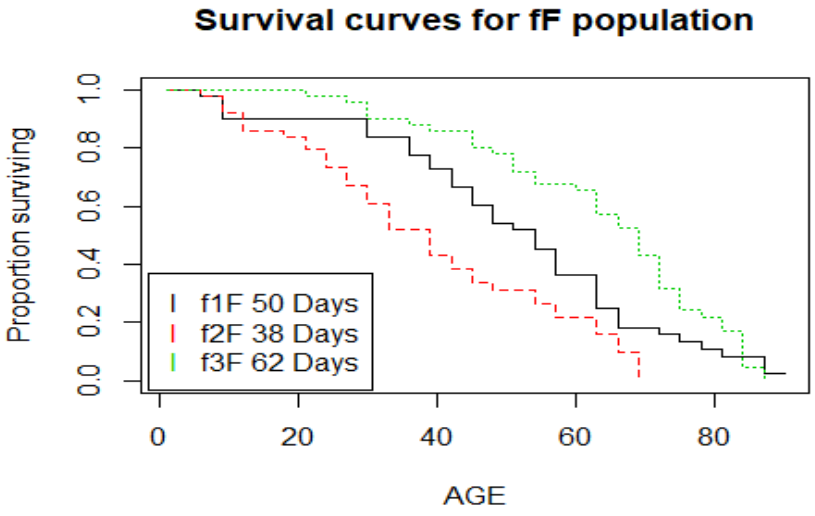

Figure 2.5. Survival curves for females of oF population with expected life spans. Y axis is the proportion of surviving at each interval. X axis the age of the females. f1F, f2F and f3F are the female replicates of oF experimental population.

Once the survival curves were obtained in R software (**Figure 2.5**), life-expectancies were calculated. We observed that the life expectancies for f1F and f3F were similar (50 and 62 days) and f2F life expectancy was approximately 38 days. Log-rank analysis was done to obtain the observed and expected values (**Table 2.6**). Chi-square test was performed to examine the difference between three female replicates of oF experimental population, the relation between three is significant, χ^2 (2, N = 50) = 10.288, p-value = 0.005.

The f2F replicate expected value and the survival curve was significantly different from two replicate populations (f1F and f3F), so the f2F was treated as an outlier and the log-rank analysis and Chi-square test were performed without the f2F population to examine the relation between f1F and f3F, the relation between two replicate populations is not significant, χ^2 (1, N = 50) = 1.58, p-value = 0.173.

Table 2.6. Observed and Expected values (in days) for oF experimental females

Replicate	Observed	Expected
f1F	45	44.00
f2F	43	22.15
f3F	45	64.94

2.3.1c Survivorship of experimental population (oM)

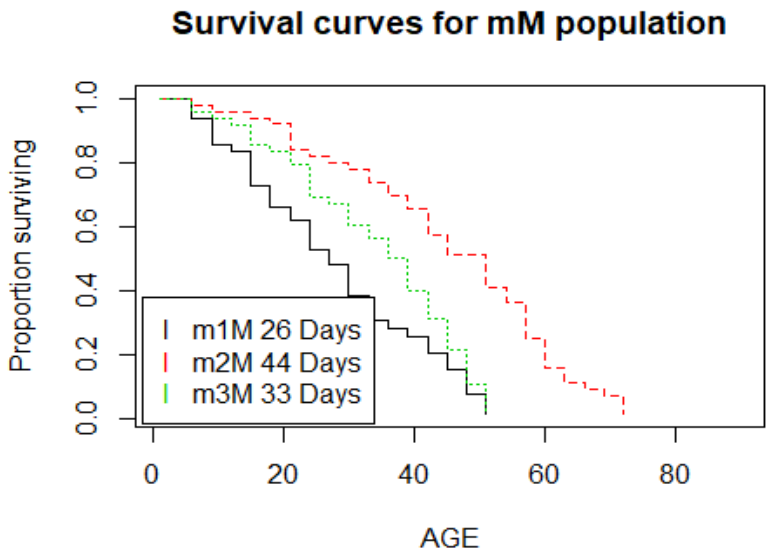

Figure 2.6. Survival curves for males of oM population with expected life spans. Y axis is the proportion of surviving at each interval. X axis the age of the males. m1M, m2M and m3M are the males of three replicates of oM experimental. The expected lifespans were calculated and included in the figure legend.

Survival curves were created using mat-plot in R software (**Figure 2.6**). Log-rank analysis was done to obtain the observed and expected values from the three replicates of oM experimental population (**Table 2.7**). Chi-square test was performed to check relation between the three replicate populations. The results indicate that the relation between three replicates is significant, χ^2 (2, N = 50) = 11.00, p-value = 0.004.

Table 2.7. Observed and Expected values (in days) for oF population males (m1F, m2F and m3F)

Replicate	Observed	Expected
m1M	42	23.99
m2M	46	70.25
m3M	44	34.81

Based on the survival curves and expected values that were obtained, it was observed that m2M replicate of oM experimental population is different from the

two replicates (m1M and m3M). The value for m2M was treated as outlier. Without m2M, when the log-rank analysis and Chi-square test was done to examine the relation between m1M and m3M, the relation between two replicates was not significant, χ^2 (1, N = 50) = 1.11, p-value = 0.29.

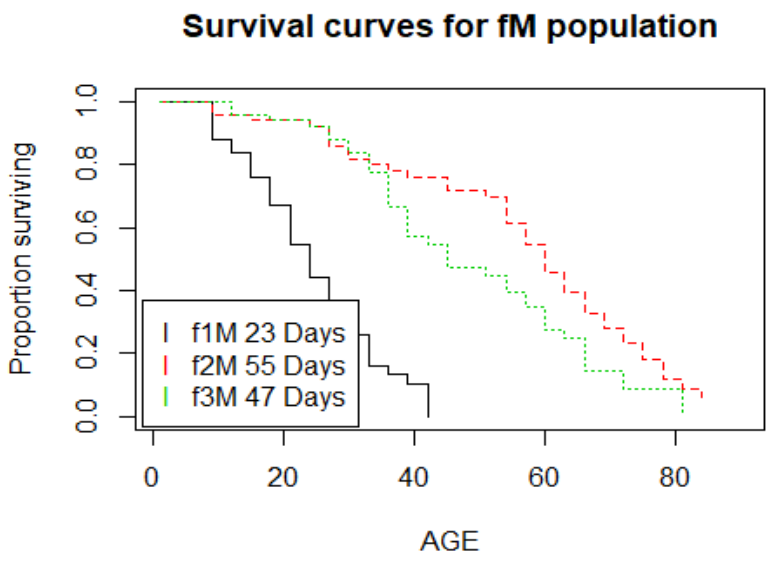

Figure 2.7. Survival curves for females of oM experimental population with expected life spans. Y axis is the proportion of surviving at each interval. X axis the age of the females. f1M, f2M and f3M are the females of three replicates of oM experimental (1.M, 2.M and 3.M respectively). Life-span expectancies of three replicates in oM experimental were calculated and added in the figure legend.

We observed that the f1M replicate expected life span is 23 days, whereas life expectancy of f2M and f3M is 55 and 47 days respectively. The survival curve of f1M is greatly different from the f2M and f3M replicates. The f1M females died

sooner than other two replicate females. Log-rank analysis was done to obtain the expected values from the oM experimental populations (**Table 2.8**). When Chi-square test was performed, the relation between the three replicates was significant, χ^2 (2, N = 50) = 19.76, p-value < 0.005.

As f1M females expected value and life expectancy is different from the f2M and f3M, the value was treated as an outlier. Without f1M, when the Chi-square test was done from the observed and expected values of f2M and f3M, the relation between both was not significantly different, χ^2 (1, N = 50) = 1.744, p-value = 0.186.

Table 2.8. Observed and Expected values for oM population females (f1M, f2M and f3M)

Replicate	Observed	Expected
f1M	44	14.47
f2M	44	66.56
f3FM	42	46.91

2.3.1d Pooled male's survivorship and Longevity

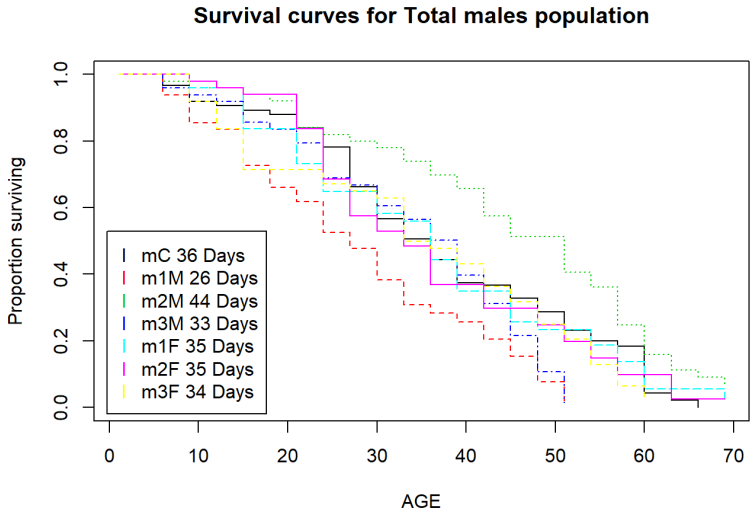

Figure 2.8. Survival curves for Total males of pooled control and two experimental populations with expected life spans. Y axis is the proportion of surviving at each interval. X axis the age of the fly.

The survival curves for total males from control and two experimental populations were created using matplot function in R software (**Figure 2.8**). The life-expectancies were calculated for all the males and included in the figure legend. Log-rank analysis was done to obtain observed and expected values

(**Table 2.9**). Chi-square test was done to see if there is any difference between the survival curves of total males from control and two experimental populations, the relation between the male population of control, oM and oF experimental is not significant, χ^2 (6, N = 50) = 11.59, p-value = 0.07. But, when done the within population analysis, we have already observed that m2M value of oM population was different from m1M and m3M. Hence the m2M value was treated as an outlier among the males from different populations. The log-rank analysis was done to obtain the expected values from different populations without m2M.

When Chi-square test was performed without m2M, the relation between the males of control, oM (except m2M) and oF was not significant, χ^2 (5, N = 50) = 1.118, p-value = 0.409. However, the result outcome of the experiment was not affected whether we included m2M or not.

Table 2.9. Total male observed and expected values from control and two experimental populations

Population	Observed	Expected
mC	128	130.52
m1M	42	23.4
m2M	46	70.08
m3M	44	34.8
m1F	44	43.11
m2F	44	41.5
m3F	43	38.57

2.3.1e Pooled female's survivorship and Longevity

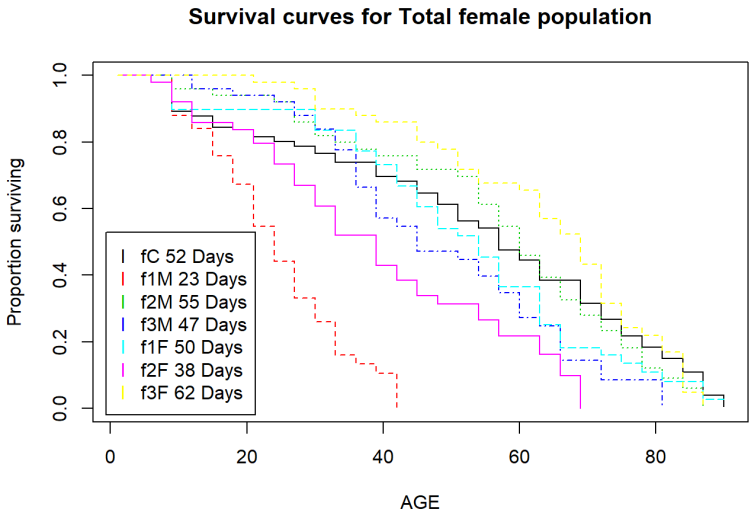

Figure 2.9. Survival curves for Total females of pooled control and two experimental populations with expected life spans. Y axis is the proportion of surviving at each interval. X axis the age of the fly.

The Survival curves for total females from all the populations (control and two experimental) were created (**Figure 2.9**). Life-expectancies were calculated and added in the figure legend. Log-rank analysis was done to obtain the observed and expected values (**Table 2.10**). Chi-square test was done once the expected values were calculated for each population.

Table 2.10. Total male observed and expected values from control and two experimental populations

Population	Observed	Expected
fC	132	157.07
f1M	44	11.04
f2M	44	51.52
f3M	42	34.65
f1F	45	45.45
f2F	43	24.62
f3F	45	64.61

Chi-square relation obtained from the females of control, oM and oF experimental indicate that there is a Signiant difference observed between the females, χ^2 (6, N = 50) = 31.66, p-value < 0.005. But, when analysis was performed the within experimental replicates, we observed that f1M of oM population and f2F of oF population were different from the rest of the experimental replicates and were considered as outlier values. One replicate from each experimental

population (f1M from oM and f2F from oF) were removed as they were very different from other survival curves. Including those two female replicates (f1M and f2F) were greatly affecting the results.

The log-rank analysis was done without f1M and f2F females. With the obtained expected and observed values from log-rank analysis (**Table 2.11**), Chi-square test was performed to examine the relation between females of control and experimental populations except f1M and f2F, the relation between the females were not significantly different, χ^2 (4, N = 50) = 11.1, p-value = 0.34.

Table 2.11. Total female observed and expected values from control and two experimental populations without f1M and f2F

Population	Observed	Expected
fC	132	145.52
f2M	44	47.11
f3M	42	30.42
f1F	45	41.31
f3F	45	59.85

2.4 DISCUSSION

Two major conclusions were made from the above conducted experiment. Firstly, the results of the study indicate that there is no significant difference in lifespan between the control and each experimental population (oM and oF). Secondly, there was a significant difference observed between males and females of all the populations (i.e. female flies lived longer than did males).

Overall in all the populations, it was observed that females lived longer than the males. The average expected lifespan for the females was around 53 days whereas for the males, it was 33 days (**Figure 2.8 and Figure 2.9**). Our results showing difference between the males and females supports research conducted previously by Maynard Smith (1958), who found that the virgin females lived longer than did virgin males in *Drosophila subobscura.* However, when Marynard Smith (1958) conducted the experiment with non-virgin males, those mated males lived longer than the mated females.

In this experiment, three outlier populations were identified (m2M, f1M and f2F). These three replicates did not follow similar trends in survival curves like other flies. The p-value differed whether these replicates were included, especially the f1M and f2F replicates in the total female analysis (**Figure 2.8**). The f1M, f2F

females died quicker than the rest of the females in the experiment. This can be explained scientifically, as there are several factors that affects the longevity of *Drosophila melanogaster* such as parental genetic variability, larval overcrowding, adult stress, choice of diet, physical anomalies in food (example: bubbles, bacteria etc.), body size etc. (Linford et al., 2013). The early deaths in f1M and f2F might have happened due to food spoilage or other unknown factors that includes some unknown mutation reducing the lifespan in the population.

The first conclusion (i.e. there is no difference between the lifespans of control and experimental populations) support the idea of Morton et al., 2013. They created a computational simulation for mating matrix; one was age indifferent mating preference (AP) and the other was age different mating preference, where older male mating preference towards younger women (YP) was imposed. When the AP model was used, selection operated against sex-specific sterility-causing mutations and mortality-causing mutations. But when YP model was used, female specific sterility-causing mutations accumulated, became neutral and fixed in the population. However, the mortality causing mutation did not accumulate in both the sexes and followed the same trend as in AP model, because male mating was retained at old age.

Identical results were observed in *D. melanogaster.* Previous studies done on fecundity and fertility in our lab show that there was decline in the fecundity and fertility of young female when mated with older males (Mitali Chaudary, 2018). But in longevity experiment we performed, it is clear that, though there was reduction in fecundity and fertility observed (in egg laying experiment) in experimental populations, the longevity of the age-different and age-indifferent flies were same. That means, the age-restricted mating strategy did not have any affect on the longevity of the *D. melanogaster.*

Our research concludes that there is no effect of age-restricted mating on the longevity of flies. This may be also true in humans, meaning sterility causing mutations (due to mate choice) does not have affect on mortality observed in humans.

This research is limited to the virgin flies. It is known that reproduction has effect on longevity. Further analysis of the current research is suggested with mated flies to see if there is any effect of age-restricted mating on longevity of flies. Future research may strengthen the idea of infertility and mortality-causing mutations. Also, repeating the experiment for three replicates m2M, f1M, f2F would be recommended for the future experiments in order to be balanced.

2.5 Appendix

Table 1. Raw Data for Total females:

Time	fC.lost	fC.Deaths	f1M.lost	f1M.Death	f2M.lost	f2M.Death	f3M.lost	f3M.Death	f1F.lost	f1F.Death	f2F.lost	f2F.Death	f3F.lost	f3F.Deaths
1	0	0	0	0	0	0	0	0	0	0	0	0	0	0
3	0	3	0	0	0	0	0	0	0	1	0	1	0	0
6	5	13	0	6	0	2	0	0	2	4	0	3	0	0
9	0	2	0	2	0	0	0	2	0	0	2	3	0	0
12	1	5	2	4	0	1	0	0	0	0	0	0	0	0
15	0	1	0	4	0	0	0	1	0	0	0	1	0	0
18	0	3	0	6	0	0	0	0	0	0	0	2	0	1
21	0	2	0	5	0	1	0	1	0	0	0	3	0	0
24	0	2	2	5	0	3	0	2	0	0	0	3	0	1
27	0	3	0	3	1	2	1	2	0	3	0	3	0	3
30	0	4	1	4	0	1	3	3	0	0	2	4	0	0
33	1	0	0	1	0	1	0	5	0	3	0	0	0	1
36	0	6	1	1	0	1	3	4	0	2	0	4	0	1
39	0	2	0	3	0	0	0	1	0	3	0	2	0	0
42	0	5	0	0	0	2	0	3	1	3	1	2	1	3
45	0	5	0	0	0	0	0	0	0	3	0	1	0	1
48	2	7	0	0	0	1	0	1	0	1	0	0	0	3
51	0	3	0	0	2	4	0	2	0	3	0	2	0	2
54	2	9	0	0	0	3	0	2	1	4	0	2	0	0
57	0	4	0	0	0	4	0	3	0	0	0	0	0	1
60	1	8	0	0	0	3	0	1	0	5	2	2	3	4
63	0	0	0	0	1	3	1	4	0	3	0	2	0	2
66	2	9	0	0	0	2	0	0	0	0	0	3	0	4
69	2	6	0	0	0	2	0	2	0	1	0	0	1	5
72	0	6	0	0	2	2	0	0	1	1	0	0	0	3
75	0	4	0	0	0	2	0	0	0	1	0	0	0	1
78	0	4	0	0	0	1	0	3	0	1	0	0	0	2
81	0	5	0	0	0	1	0	0	0	0	0	0	0	5
84	1	8	0	0	0	2	0	0	0	2	0	0	0	2
87	1	3	0	0	0	0	0	0	0	0	0	0	0	0
90	0	0	0	0	0	0	0	0	0	1	0	0	0	0

Table 2. Raw Data for Total males:

Time	mC.lost	mC.Death	m1M.lost	m1M.Deat	m2M.lost	m2M.Deat	m3M.lost	m3M.Deat	m1F.lost	m1F.Deat	m2F.lost	m2F.Deat	m3F.lost	m3F.Death
1	0	0	0	0	0	0	0	0	0	0	0	0	0	0
3	0	5	2	3	0	1	1	2	0	0	0	0	0	0
6	2	7	0	4	0	1	0	1	0	2	0	1	1	4
9	0	2	0	1	0	0	0	1	0	0	0	1	0	4
12	0	2	2	5	0	1	1	3	2	6	0	1	1	6
15	0	2	0	3	0	1	0	1	0	0	0	0	0	0
18	3	6	0	2	0	4	0	2	0	5	3	5	0	0
21	0	8	2	4	0	1	0	5	0	4	0	7	0	2
24	6	17	0	2	0	1	0	1	0	0	2	5	0	1
27	1	13	0	4	0	1	0	3	3	3	0	2	0	1
30	1	8	1	3	1	2	0	2	0	1	0	2	2	6
33	2	8	0	1	0	2	0	3	0	5	0	5	0	1
36	0	9	0	1	0	2	0	5	0	4	0	0	0	2
39	0	1	0	2	0	4	1	4	0	0	1	3	0	3
42	1	5	0	2	0	3	2	4	0	4	0	0	0	2
45	0	5	0	3	0	0	0	4	0	1	0	2	0	3
48	0	7	1	2	2	5	1	3	0	0	0	2	0	2
51	0	4	0	0	0	2	0	0	0	2	0	2	2	3
54	1	2	0	0	0	5	0	0	1	2	0	2	0	2
57	5	15	0	0	0	4	0	0	0	3	0	0	1	1
60	0	1	0	0	0	2	0	0	0	0	0	3	0	0
63	0	1	0	0	0	1	0	0	0	0	0	0	0	0
66	0	0	0	0	0	1	0	0	0	1	0	0	0	0
69	0	0	0	0	1	2	0	0	0	1	0	1	0	0

Sample: Code for calculating life expectancies for 1.F

```
```{r}
n <- nrow(lt)
ave_alive<- c(rep(0,n))
for (i in 1:n ) {
ave_alive[i] <- (lt$surv[i] + lt$surv[i+1])/2.0
}
ave_alive[n] <- ave_alive[n-1] / 2.0
lt$ave_alive<- ave_alive
fly_days<- c(rep(0,n))
for (i in 1:n ) {
  dint = times[i+1] - times[i]
  sum <- 0.0
  for (j in i:n ) {
    if (!is.na(ave_alive[j]) ) {
      sum <- sum + ave_alive[j]
    }
  }
fly_days[i] <- sum * dint
}
lt$fly_days<- fly_days
str(lt)
# print(lt$fly_days)
life_exp<- c(rep(0,n))
for (i in 1:n ) {
life_exp[i] <- lt$fly_days[i] * lt$surv[i]
}
lt$life_exp<- life_exp
str(lt)
# print(lt$life_exp)
write.csv(lt, "Longevity_1M_F_longevity.csv")
```
```

CHAPTER 3

Measurement of Ovariole and Egg Chamber Counts in Age-restricted Mating Populations of *Drosophila melanogaster*

3.1 Introduction

In their mate choice theory of menopause Morton et al. (2013) proposed that older females deprived of reproduction would accumulate sterility mutations leading to the evolution of menopause. In the model experimental system, *Drosophila melanogaster*, was set up to test the mate choice theory, we were interested to know what kind of sterility mutations, such as mutations affecting ovariole number, egg chambers, number of eggs, and egg fertility, would arise and accumulate.

Ovariole numbers impact the production of eggs in *D. melanogaster*. It is a quantitative trait that affects the reproductive success of a fruit fly (Bouletreau-Merle et al., 1982; Kelpsatel et al., 2013).

Ovarioles are tubular, finger-like projections in the ovaries. Each ovariole has an anterior germarium, where somatic and germ cells are connected and organized and a posterior vitellarium (Koch et al., 1966). The germarium contains primordial germ cells (PGCs) that produce a cluster of cells, one of which will

become an oocyte and the rest become nurse cells (Koch et al., 1966). The vitellarium consists of series of interconnected egg chambers. Egg chambers leave the germarium and continue developing as they move to the posterior in the ovariole (see **Figure 3.1**). Each egg chamber consists of an oocyte and fifteen nurse cells surrounded by a layer of follicle cells (Koch, Smith and King,1967).

The development of egg chambers is divided into different consecutive stages, starting from stage one to stage fourteen (Cummings and King, 1969). Stage fourteen is the mature primary oocyte that is ready for fertilization (Robinson et al., 1994). The stage fourteen egg chamber is not produced until the female is 48 hours old in *D. melanogaster* (Robinson and Cooley, 2002). In *Drosophila melanogaster*, development of a mature egg involves the coordination of thousands of somatic and sixteen germline cells (Robinson and Cooley, 2002). Typically, only the stage fourteen egg chamber has yolk, and will be ready for fertilization, so the number of stage fourteen eggs in the *D. melanogaster* has direct impact on the production of offspring (Carlson et al., 1998).

Variation in ovariole count in *Drosophila* has a significant genetic component (Wayne et al., 2001; Bergland et el., 2008). Some of the candidate genes for ovariole number have been identified. They include ***babl*** and ***InR*** **genes**

(Sarikaya et al., 2012; Sarikaya and Extrayour 2015). But the extent of genes that contribute to genetic variability in ovariole number is unknown (Sarikaya et al., 2012).

There are other factors that affects the ovariole number in *Drosophila*, including stress, nutrition, selection, and body size (Lefranc and Bundgaard,2004). Lefrane and Bundgaard (2004) found that there is a direct effect of body size on the ovariole number. Affect of early and late fecundity on ovariole number has been studied in *D. melanogaster* (Rose, 2003; Carlson et al., 1998), however, research on the effect of age-restricted mating on the development of mature oocytes in *D. melanogaster* is scarce.

To test the mate choice theory, two simulated age-restricted mating populations (older males mated with younger females, and older females mated with younger males) were created in the laboratory and the experiments were run for over 30 generations. To see if there was any effect of age-restricted mating on ovariole number and mature oocytes, we performed ovariole and egg chamber counts in the age-restricted mating populations of *D. melanogaster*.

We hypothesized that, among the experimental populations, the younger females mated with older males (oM) will have fewer ovarioles and stage fourteen egg chambers due to the accumulation of age-specific sterility-causing mutations. The

research carried out here has direct bearing on the importance of mutation accumulation in age-restricted mating populations and how it reduces fertility.

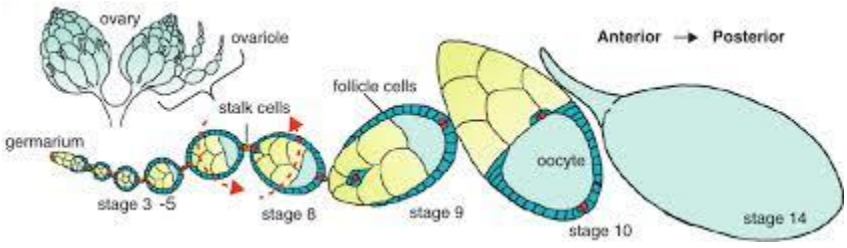

Figure 3.1. Picture representations of ovary, ovariole and stage fourteen egg chamber of *D. melanogaster.* Adopted from Automatic analysis of gene expressions in *Drosophila* microscopy images by Jiˇríˇ Borovec, 2017.

3.2 Materials and Methods

3.2.1 Establishment of stock population

A diversified base population was created by the lab researchers in the year 2016 by mixing ten different populations of wild *D. melanogaster* from *Drosophila* stock, California.

3.2.2 Fly maintenance

The flies were reared at room temperature (22°C) on corn-agar-molasses-yeast medium in 250 mL food jars under 12 -hr light/dark cycle.

3.2.3 Simulated non-random population

Two experimental populations were created: One with older males (20-day old) mated with younger (3 days old) females (oM experimental population); and another with older females (20-day old) mated with younger (3 days old) males (oF experimental population). Each experimental population consisted of three replicates named as: oM (1.M,2.M & 3.M), and oF (1.F,2. F& 3.F). Thirty generations of non-random mating have been carried out by the time of this writing in order to examine the effects on ovariole and mature egg chambers count.

3.2.4 Experimental design

Virgin females from experimental and control populations were collected post-ecloson in vials (with food) separately. Every third day, three females from each experimental and two from control populations were sacrificed on a concave slide to observe stage fourteen egg chambers and the ovariole number using dissecting stereomicroscope. Egg chambers with developed dorsal appendages

were considered as stage fourteen (mature) egg chambers (**Figure 3.2**). Flies were changed into fresh vials every sixth day. Each female was dissected into a drop of PBS (Phosphate Buffer Solution) using a dissecting stereomicroscope. With the help of fine needles, ovarioles and eggs were separated from each other in the ovary. To make it easier for identifying and counting individual ovariole, 1µL of crystal violet stain was added into the sample. The number of eggs with dorsal appendages (stage fourteen eggs) were counted and recorded from the left and right ovary. The average number from the left and right ovary was considered as a number of the stage fourteen eggs per female.

A total of 200 females were dissected (three from each replicate population of experimental and two from control every third day) to count the ovariole and stage fourteen eggs till day 30. All observations were recorded in Microsoft Excel. One-way ANOVA and Two-tailed t-tests were performed to compare the ovariole number and stage fourteen egg chambers between the experimental and control populations and within the experimental populations using PRISM 6.0 software.

3.3 Results

The mean number of stage fourteen egg chambers and ovariole number were counted every third day till day 30. Crystal violet stain was used to detect individual ovarioles. The mean number of stage fourteen egg chambers and ovariole number for experimental and control were calculated (**appendix Table 2**) and compared graphically and with one-way ANOVA.

3.3.1 Total stage fourteen egg count and statistical analysis

The mean number of stage fourteen egg chambers for control populations are shown in **Fig 3.3**. The number of egg chambers oscillated a lot, but it generally decreased over time reaching a low of four on day 30.

The experimental population (1.F, 2.F & 3.F), older females followed a pattern similar to the control populations. It was also quite variable and decreased from ten on day 3 to three on day 30.

The experimental population involving younger females produced the fewest number of stage fourteen egg chambers. The mean number of egg chambers decreased from six on day 3 to nearly zero on day 30. (**Figure 3.3**). One of the populations (Rep 1.M) produced no matured egg chamber on day 30.

The one-way ANOVA gave a p-value = 0.0034, rejecting the null hypothesis of no difference among populations. Two-tailed t-tests were performed on two-way comparisons: control vs. oF, control vs. oM, and oM vs. oF populations (**Table 3.1**).

Table 3.1. Two tailed T-test performed p-values for control/oF. Control/oM and oM/oF populations

Control and oF population	P-value = 0.351
Control and oM population	P-value = 0.0007 **
oM and oF population	P-value = 0.014*

There was no significant difference in the stage fourteen egg chambers for control (M = 8.34, SEM = 1.01) and experimental population oF (M = 6.92, SEM = 1.01); t (18) = 0.95, p = 0.35. A significant difference was observed between the matured egg chambers for control (M = 8.34, SEM = 1.01) and experimental population oM (M = 3.58, SEM = 0.57; t (18) = 4.09, p = 0.0007). Also, a difference was observed between oM (M = 3.58, SEM = 0.57) and oF experimental populations (M = 6.92, SEM = 1.01; t (18) = 2.71, p = 0.014).

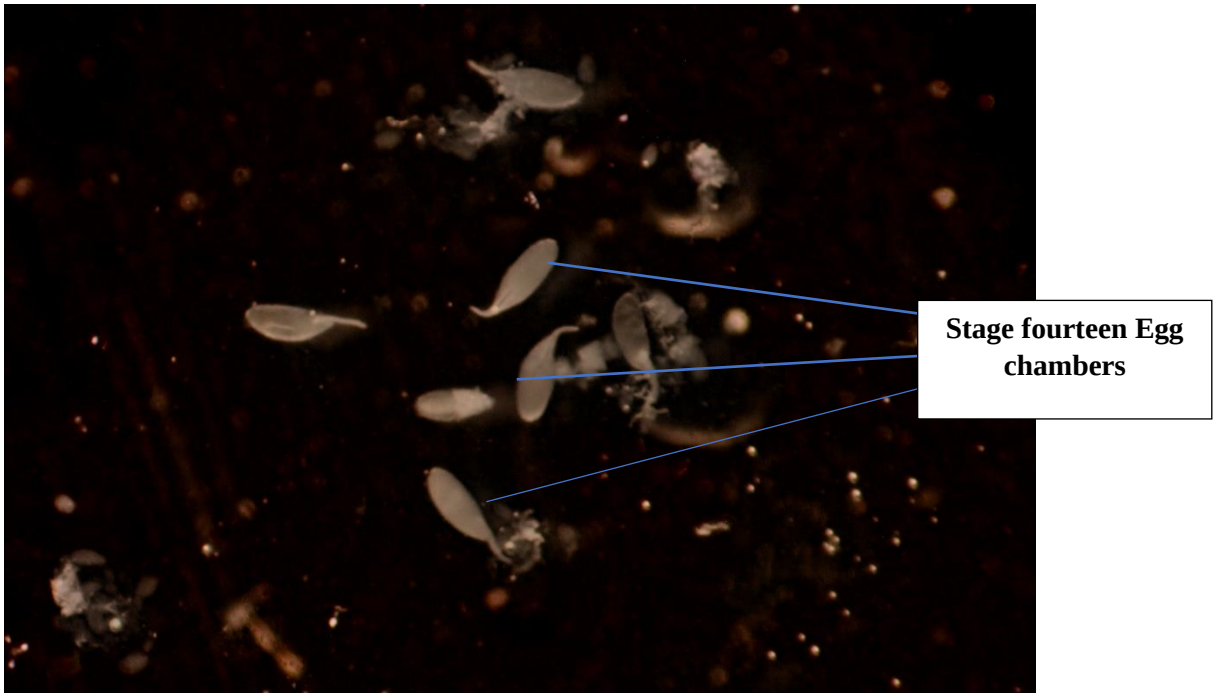

Figure 3.2. Image of stage fourteen egg chamber with dorsal appendages captured under the dissecting stereo microscope at low magnification

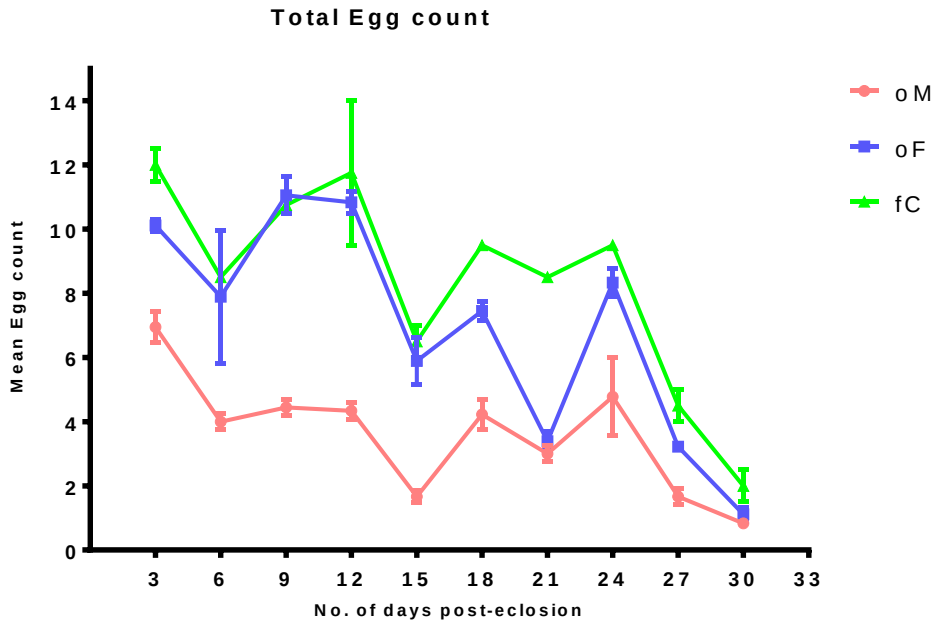

Figure 3.3. Plot of stage fourteen egg chambers and number of days post-eclosion in females. Mean number of stage fourteen egg chambers observed every third day post-eclosion for control, old female/young male, old male/young female. Bars in the graph represent standard error of mean.

3.3.2 Stage fourteen egg count & statistical analysis within the populations

3.3.2a oF population Egg count:

Within the old female/young male population (oF experimental), a one-way ANOVA was performed to compare the three populations. There was no statistical difference between the means of three populations ($F(2,27) = 0.182$, $p = 0.83$) in oF experimental.

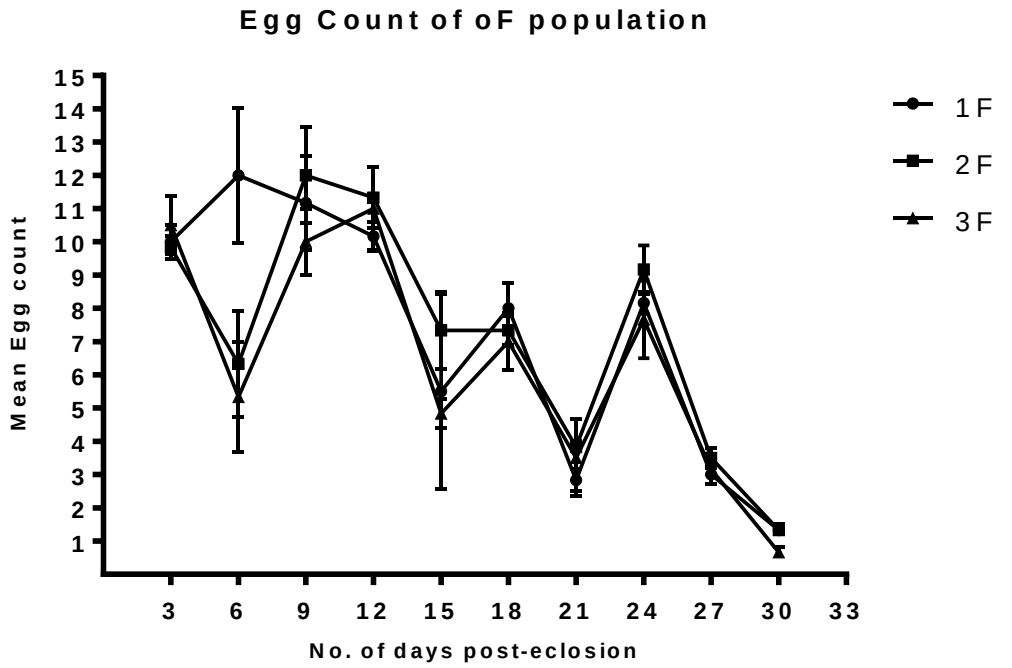

Figure 3.4. Mean (+/- SE) egg chambers as a function of days post-eclosion. Mean number of stage fourteen egg chambers for three replicates of old female/young male. Bars represent standard error of mean.

3.3.2b oM population Egg count:

Within the old male/young female population (oM), a one-way ANOVA was performed to compare the three replicates. There was no statistical difference between the means of three populations ($F(2,27) = 0.1796$, $p = 0.836$) in oM experimental.

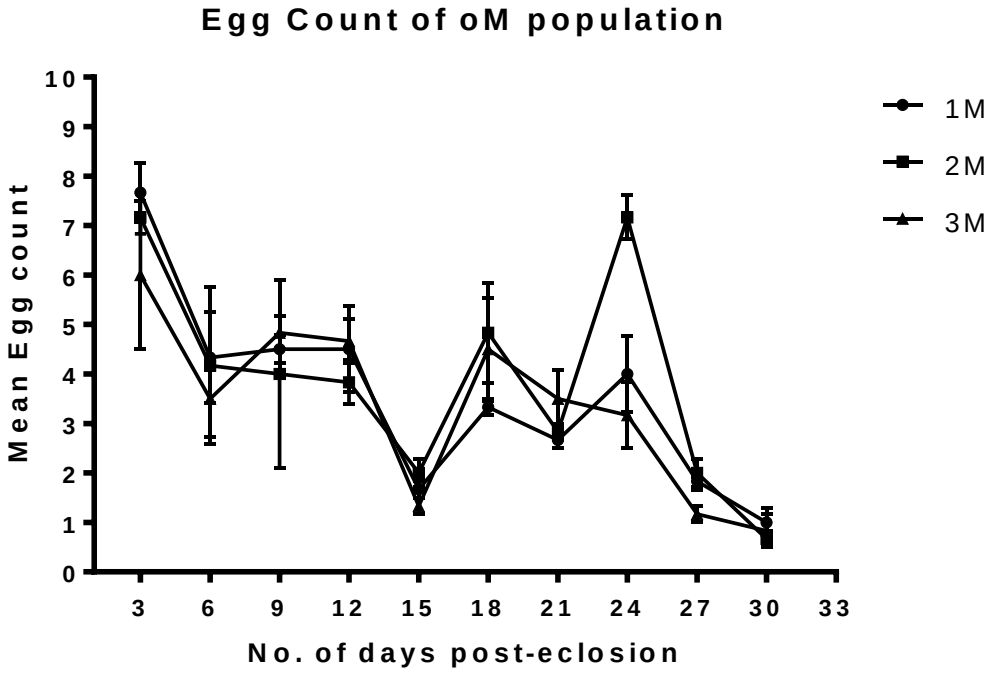

Figure 3.5. Mean (+/- SE) egg chambers as a function of days post-eclosion
Mean number of stage fourteen egg chambers observed every third day post-eclosion for 3 replicates of old female/young male. Bars represent standard error of mean.

3.3.3 Total ovariole count & statistical analysis

The number of ovariole per female is considered as the average number of ovariole count recorded in left and right ovary. The mean number of three replicates of oM (1.M, 2.M & 3.M), three replicates of oF (1.F, 2.F & 3.F) and control were calculated (**appendix Table 4**) and plotted in **Figure 3.8.** The mean number of ovarioles for control were higher than the two experimental

populations. Ovariole count started at fourteen for the control, whereas it was similar for the oM and oF population (i.e. 13.5) on day 3. Notably, there was a sharp decrease in the ovariole count for oF population on day 21. We noticed a gradual increase and decrease of mean number of ovarioles for the oM population.

The highest mean ovariole counts recorded for control populations and oF populations on day 6 and day 18 respectively, whereas for the oM population, it was on day 6 (appendix Table 4).The one-way ANOVA was performed returned a p-value = 0.0001, rejecting the null hypothesis of no difference among populations. Two-tailed t-tests were performed on two-way comparisons: control vs. oF, control vs. oM, and oM vs. oF populations (**Table 3.2**).

Table 3.2. Two tailed T-test performed p-values for control/oF. Control/oM and oM/oF populations

Control vs. oF	P-value = 0.032*
Control vs. oM	P-value = 0.0001***
oM vs. oF	P-value = 0.0317*

There was a statistically significant difference in the ovariole count for control and oF experimental population; t (9) = 2.53, p = 0.032.There was a significant difference observed in the matured egg chambers for control and oM experimental population; t (9) = 7.43, p < 0.0001. Also, a difference was observed for oM and oF experimental populations; t (9) = 2.54, p = 0.031.

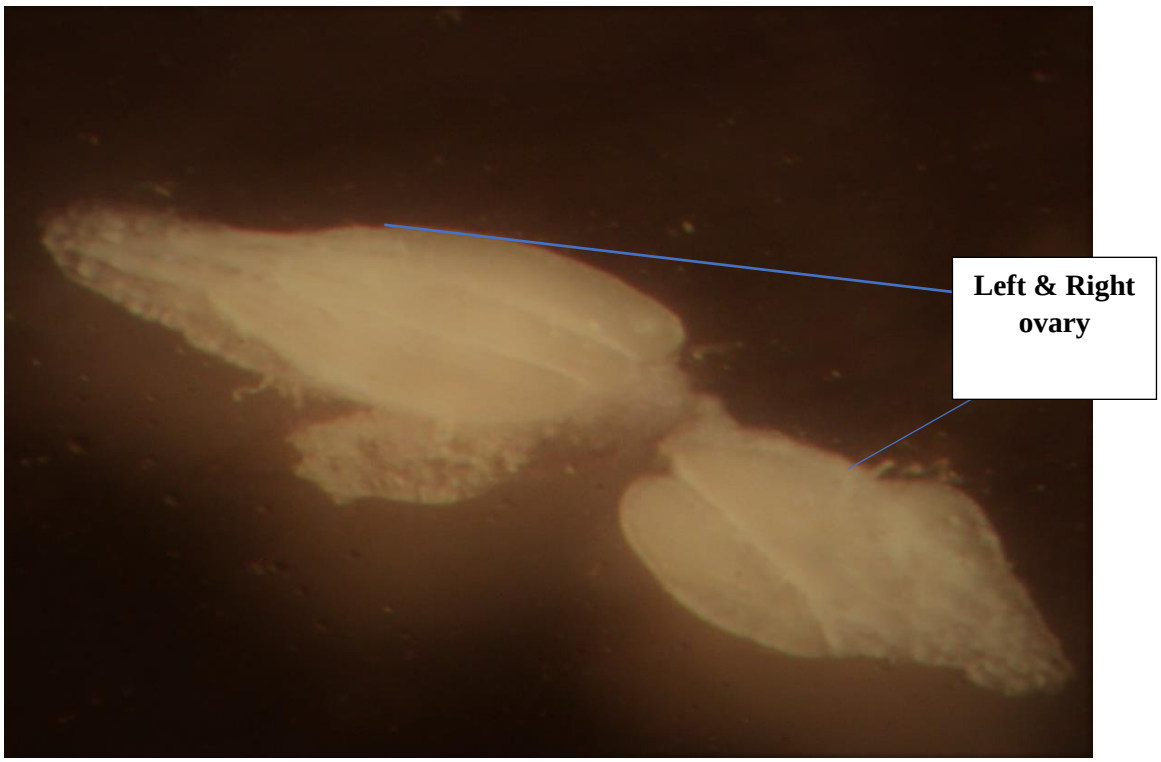

Figure 3.6. Image of left ovary and right ovary captured under the dissecting stereo microscope at low magnification

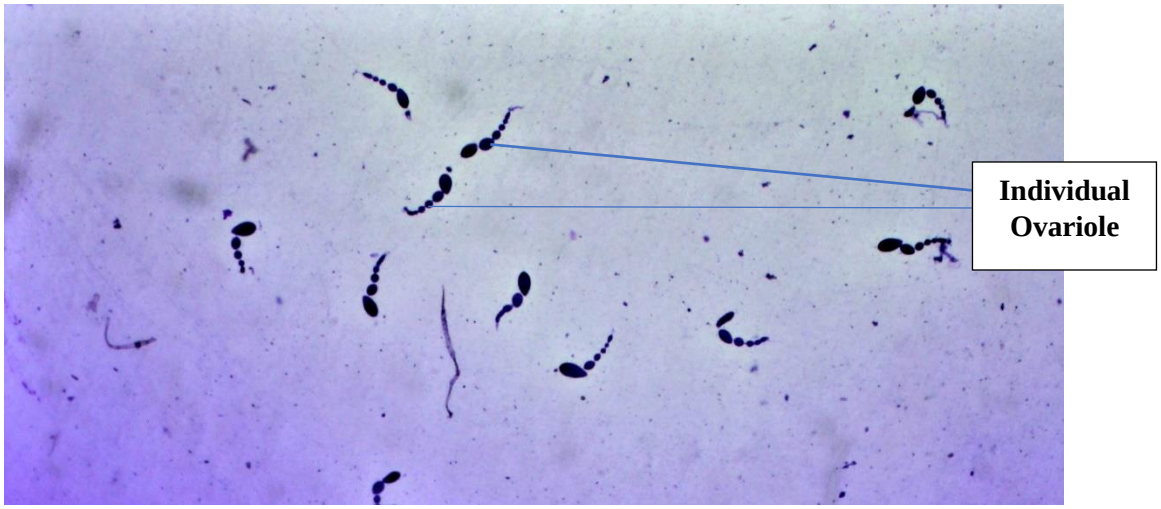

Figure 3.7. Image of ovarioles stained with crystal violet captured under the dissecting stereo microscope at high magnification

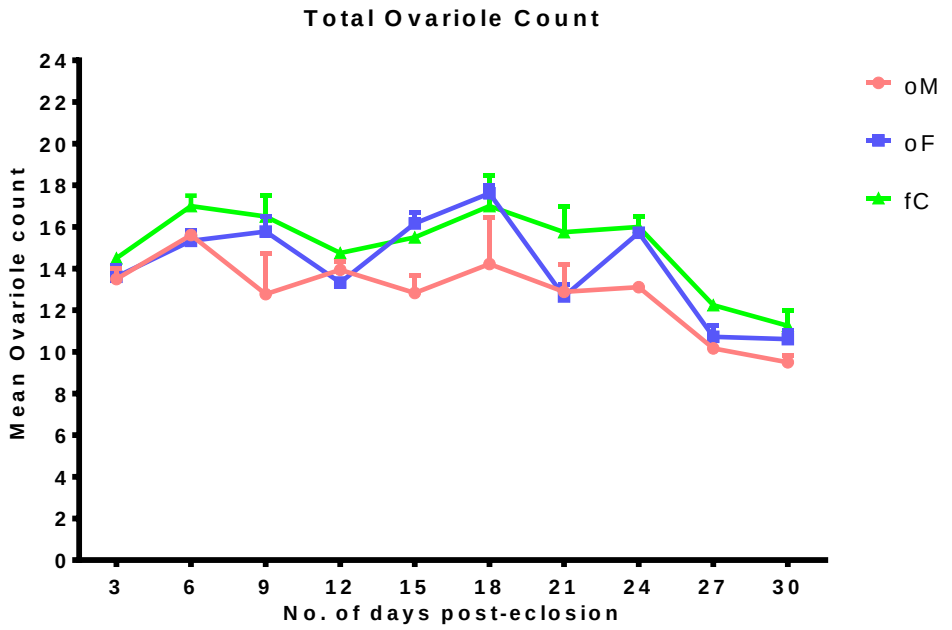

Figure 3.8. Plot of mean ovariole count and number of days post-eclosion in females. Mean number of ovarioles observed every third day post-eclosion for control, old female/young male, old male/young female. Bars in the graph represent standard error of mean.

3.3.4 Ovariole count & statistical analysis within the population

3.3.4a oF population ovariole count:

Within the old female/young male population (oF experimental), A one-way ANOVA was performed to compare the three replicates. There was no statistical difference between the means of three replicates ($p = 0.43$) in oF experimental population.

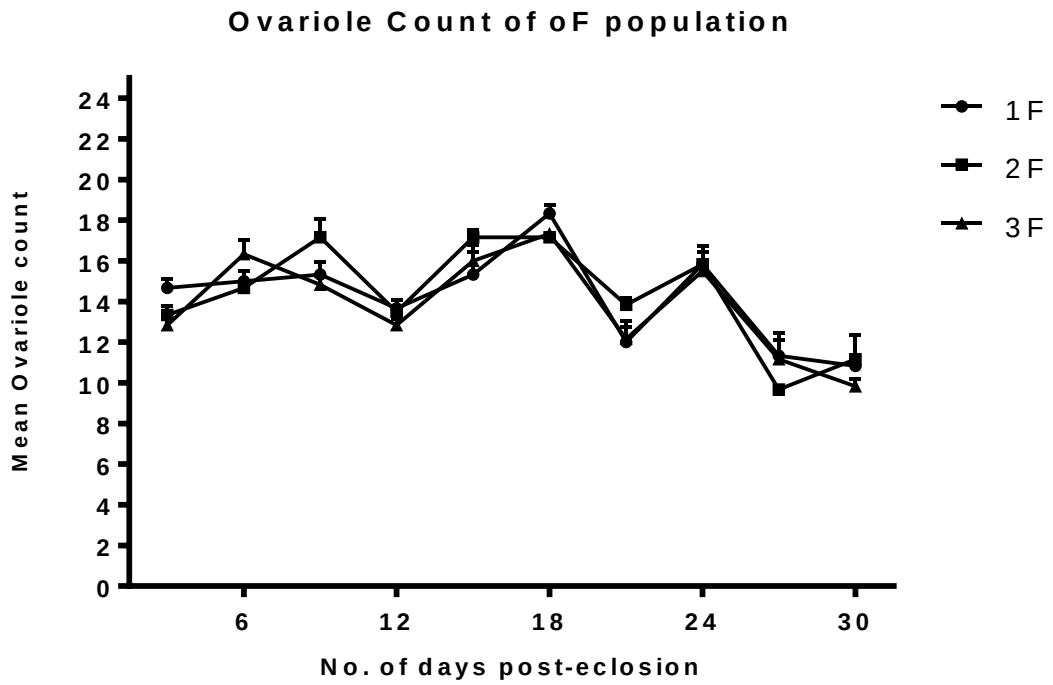

Figure 3.9. Plot of oF replicates mean ovariole count and number of days post-eclosion. Mean number of ovarioles observed every third day post-eclosion for replicates of old female/young male. Bars in the graph represent standard error of mean.

3.3.4b oM population ovariole count:

Within the old male/young female population (oM), The one-way ANOVA revealed a difference among three populations among oM experimental population ($p = 0.04$).

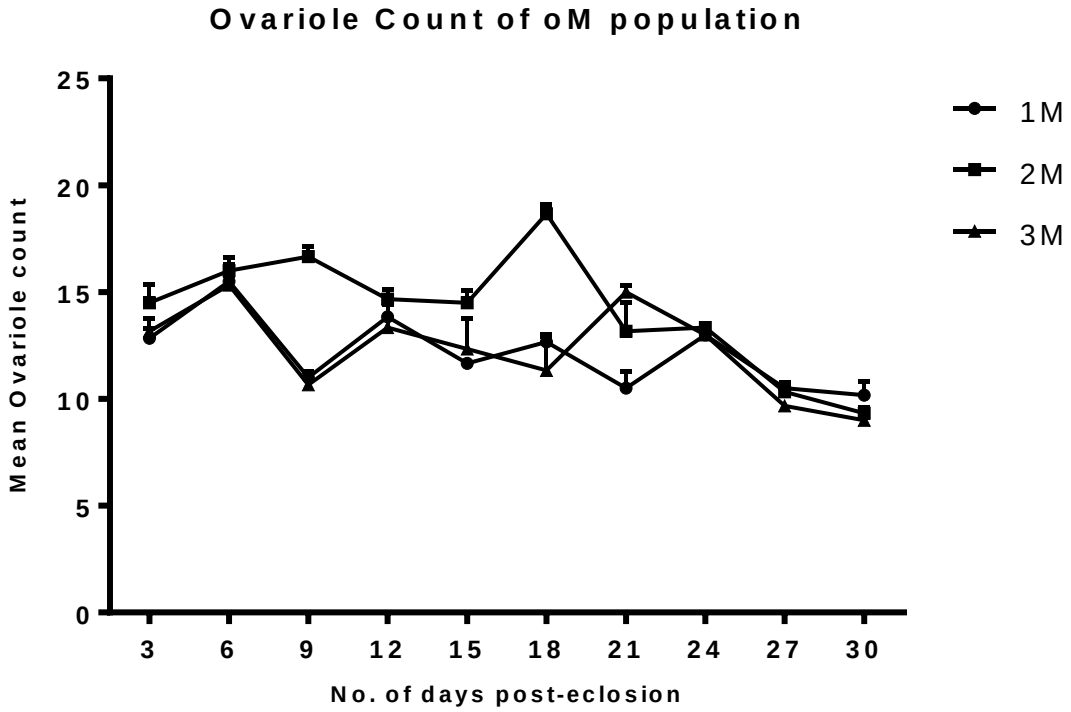

Figure 3.10. Plot of oM replicates mean ovariole count and number of days post-eclosion. Mean number of ovarioles observed every third day post-eclosion for replicates of old male/young female. Bars in the graph represent standard error of mean.

3.4 Discussion

After 30 generations of age-restricted mating scheme followed in the lab, the average number of stage fourteen eggs and ovarioles was smaller in oM experimental population when compared to oF experimental population and the control (**Figure 3.3 and 3.8**). This suggests that there may be an effect on the evolution of female's reproductive ability when mating is restricted to young females with older males (20 days old). All three replicates of experimental population showed the same pattern of decline in egg chamber count.

No difference in the number of stage fourteen eggs between oF experimental (older females mated with younger males) and control was found. Both oF replicates and control followed a similar trend. However, one-way ANOVA test revealed a statistical difference in the ovariole count of oF experimental (older females mated with younger males) compared to control (P-value 0.037). This suggests that, there may also be an effect of age-restricted mating on the oF experimental populations.

One-way Analysis of variance test revealed a difference between oF and oM experimental populations. The number of ovarioles between the oF and oM experimental were statistically different ($t(9) = 2.54$, $p = 0.031$). Also, there was a

large difference in stage fourteen egg chambers between the two experimental ($t(18) = 2.71$, $p = 0.014$). The results suggest that there may be a delay in the development of egg chambers from stage one to the matured egg chambers (stage fourteen) in the oM experimental as compared to the oF experimental. The results demonstrate that the age-restricted mating negatively affected the production and maturation of ovarioles and egg chambers in all oM experimental populations.

In *Drosophila melanogaster*, development of a mature egg involves the coordination of thousand of somatic and 16 germ line cells (Douglas N Robinson and Lynn Cooley, 1997). Our results show that mutations accumulated in the germline cells or at the coordination of somatic and germline cells in the old male/young female population that led to lesser number of matured eggs compared to old female/young male and control populations.

Germline mutations occur in gametes and will be passed on to the next generation. The evolutionary processes depend on the transmission of genetic material from one generation to the next (Scally Aylwyn, 2016), From the results observed, it suggests that germline mutations in oM experimental is higher compared to the oF experimental and the control.

Ovariole number impacts the production of mature eggs, which in turn impacts the production of healthy offspring (Kelpsatel et al., 2013). The reduction in old male/young female production of matured eggs supports the previous fecundity and fertility experiments carried out in the Singh lab (see chapter one). The reduction in matured egg chambers is may be the reason why fewer offspring were produced by old male/young females.

Our research provide support for the theory that the bias in mating systems where only younger females are allowed to mate and reproduce may lead to accumulation of deleterious mutations that reduce the fertility of females at older ages. Our research shows that the age-restricted mating, where only younger females are mated for thirty generations led to the reduction of ovariole number and maturation of egg chambers in the females.

These results are relevant to humans where preferential mating with younger females may have allowed the origin and the evolution of sterility causing mutations in the females at specific age. Such mutations could possibly act in a number of ways including those affecting hormonal levels in women. Mutations affecting number and quality of the eggs, hormonal levels, or some signalling

pathways may also be responsible for premature ovarian failure in females (Welt, Smith and Taylor 2004).

DNA sequencing of the experimental and control populations can shed light on the number and nature of sterility mutations. The results are significant to understanding human reproductive development and evolution, especially how a unique trait might, i.e. menopause, may have originated and how it changes the lifestyle and health of females associated with aging.

3.5 Appendix

1. **Table 1.** Mean number of ovariole number (average of left and right ovary ovarioles) per female in all the populations

Days	1_f1M_O	2_f1M_O	3_f1M_O	1_f2M_O	2_f2M_O	3_f2M_O	1_f3M_O	2_f3M_O	3_f3M_O	1_f1F_O	2_f1F_O	3_f1F_O	1_f2F_O	2_f2F_O	3_f2F_O	1_f3F_O	2_f3F_O	3_f3F_O	1_fC_O	2_fC_O
3	13.5	12	13	16	13	14.5	14	12	13.5	14.5	14	15.5	14	12.5	13.5	12	13.5	13	14.5	14.5
6	16	15.5	15	16	15.5	16.5	13	15.5	17.5	16	14.5	14.5	14.5	15	14.5	15	17	17	17.5	16.5
9	11	11.5	10.5	16	17.5	16.5	11	10	11	16.5	15	14.5	17.5	18.5	15.5	15	14	15.5	15.5	17.5
12	14.5	12.5	14.5	14.5	15.5	14	12	12.5	15.5	13.5	14	13.5	12.5	14.5	13.5	13.5	12.5	12.5	15	14.5
15	11.5	12	11.5	13.5	15.5	14.5	9.5	14	13.5	14	17.5	14.5	16.5	17.5	17.5	17	16.5	14.5	18	18
18	12	13	13	19.5	18	18.5	14.5	10	9.5	19	18.5	17.5	17.5	17	17	17.5	17.5	17	15.5	15.5
21	12	9.5	10	10.5	14.5	14.5	15.5	15	14.5	11	11.5	13.5	13.5	14.5	13.5	10.5	12.5	13.5	15.5	16
24	13.5	13.5	12	13.5	13.5	13	13	13.5	12.5	15.5	17	15	15.5	14.5	17.5	16	15	15.5	15.5	16.5
27	10.5	11	10	9.5	10.5	11	9.5	9.5	10	10	13.5	10.5	9.5	9.5	10	10	10.5	13	12.5	12
30	11.5	9.5	9.5	9.5	9	9.5	8	10	9	11.5	11	10	10.5	13.5	9.5	9.5	10.5	9.5	12	10.5

2. **Table 2.** Mean number of stage fourteen egg chambers (average of left and right ovary) per female in all the populations

Days	1_f1M_E	2_f1M_E	3_f1M_E	1_f2M_E	2_f2M_E	3_f2M_E	1_f3M_E	2_f3M_E	3_f3M_E	1_f1F_E	2_f1F_E	3_f1F_E	1_f2F_E	2_f2F_E	3_f2F_E	1_f3F_E	2_f3F_E	3_f3F_E	1_fC_E	2_fC_E
3	8.5	6.5	8	7.5	7.5	6.5	9	4.5	4.5	10.5	9	10.5	9.5	9.5	10.5	9	12	10.5	11.5	12.5
6	5	2.5	5.5	6	5.5	1	5	2.5	3	16	10.5	9.5	3.5	9	6.5	8.5	3	4.5	8.5	8.5
9	4	5	4.5	0.5	7	4.5	5.5	4.5	4.5	14	10	9.5	14.5	9.5	12	9	9	12	10.5	11
12	6	4.5	3	3	4.5	4	4.5	5.5	4	10	11	9.5	12.5	12	9.5	12.5	12	8.5	9.5	14
15	2	1.5	1.5	1.5	2	2.5	1.5	1	1.5	0	10	6.5	9.5	7	5.5	5	5.5	4	7	6
18	3.5	3	3.5	3	6.5	5	6.5	3	4	8.5	9	6.5	8	6.5	7.5	5.5	8.5	7	9.5	9.5
21	3	2.5	2.5	3	3	2.5	3.5	2.5	4.5	3.5	2.5	2.5	4	4	3.5	1.5	5.5	3.5	8.5	8.5
24	2.5	5	4.5	8	7	6.5	2.5	4.5	2.5	7.5	8.5	8.5	9	10.5	8	9.5	5.5	8	9.5	9.5
27	1.5	2	2	2.5	1.5	2	1	1	1.5	3.5	3	2.5	3	4	3.5	4	3	2.5	5	4
30	0.5	1.5	1	0.5	0.5	1	1.5	0.5	0.5	1	1.5	1.5	1.5	1	1.5	0.5	0.5	1	2.5	1.5

3. **Table 3.** Mean number of stage fourteen egg number observed every third day post-eclosion from females of old male/young female (oM), old female/young male (oF), control.Values describe mean ± SEM.

Days	Old M × young F (oM) (n = 90)	Old F × young M (oF) (n = 90)	Control (n = 20)
3	6.94 ± 0.49	10.11 ± 0.2	12 ± 0.5
6	4 ± 0.25	7.89 ± 2.08	8.5 ± 0
9	4.44 ± 0.24	11.06 ± 0.58	10.75 ± 0.25
12	4.33 ± 0.25	10.83 ± 0.35	11.75 ± 2.25
15	1.67 ± 0.19	5.89 ± 0.75	6.5 ± 0.5
18	4.22 ± 0.45	7.44 ± 0.29	9.5 ± 0
21	3 ± 0.25	3.39 ± 0.29	8.5 ± 0
24	4.78 ± 1.22	8.33 ± 0.44	9.5 ± 0
27	1.67 ± 0.25	3.22 ± 0.15	4.5 ± 0.5
30	0.83 ± 0.1	1.11 ± 0.22	2 ± 0.5

4. **Table 4.** Mean number of ovariole number observed every third day post-eclosion from females of old male/young female (oM), old female/young male (oF), control.Values describe mean ± SEM.

Days	Old M × young F (oM) (n = 90)	Old F × young M (oF) (n = 90)	Control (n = 20)
3	13.5 ± 0.51	13.61 ± 0.55	14.5 ± 0
6	15.61 ± 0.2	15.33 ± 0.51	17 ± 0.5
9	12.78 ± 1.95	15.78 ± 0.71	16.5 ± 1
12	13.94 ± 0.39	13.33 ± 0.25	14.75 ± 0.25
15	12.83 ± 0.86	16.17 ± 0.54	15.5 ± 0
18	14.22 ± 2.26	17.61 ± 0.36	17 ± 1.5
21	12.89 ± 1.31	12.67 ± 0.59	15.75 ± 1.25
24	13.11 ± 0.11	15.72 ± 0.11	16 ± 0.5
27	10.17 ± 0.25	10.72 ± 0.53	12.25 ± 0.25
30	9.5 ± 0.35	10.61 ± 0.4	11.25 ± 0.74

CONCLUSION AND FUTURE DIRECTION

Using *D. melanogaster* as a model organism our aim was to show that under specific conditions of non-random mating, fertility diminishing can occur. Our aim was not to examine the physiological symptoms, as a result of menopause, but the evolution of sterility on the whole. Our research supports the theory proposed by Morton et al. in 2013. They showed that the late-onset, sterility-causing mutations can evolve neutrally under specific conditions and persist in the population. These accumulations of detrimental mutations in the population, ultimately lead to the evolution of menopause.

Our age-restriction mating strategy, followed in the lab for thirty generations, showed that the experimental populations' fecundity and fertility was negatively affected compared to the control. Age-restricted mating did not have any effect on the life span of experimental populations. The lifespan of experimental and control populations was similar, but the production of eggs, offspring and ovariole number were much less in the oM experimental populations.

My research mainly focused on the female reproduction in populations involving younger females mated with older males. Experimental investigation of population involving younger males mated with older females needs to be

carried out in order to show the effect of age-restriction mating on male reproduction when mated with older females. This can be done by microscopic examinations of the testis over a time (**Appendix A**) or by counting the number of viable sperm in experimental and control populations (**Appendix B**).

For future direction, maintaining the age-restricted mating strategy is very important. After every ten generations or so, the fecundity and fertility need to be carried out in order to test the evolution of sterility in experimental populations. Simultaneously, it would be interesting to carry out a sequencing study to see what changes have occurred at the genetical level.

Menopause is an ultimate phase of every women's life and has always been a mystery to the medical world. One biological theory proposed that menopause occurs when the number of follicles in ovary gets depleted (Van valen, 2003; Atsalis and Videan, 2009), but further research on this theory made it very complex to understand the basis of origin. Researchers all around the world tried to solve the mystery behind menopause and have offered numerous theories (**Appendix A Table 1**). Studies have identified some candidate genes that are associated with menopause (**Appendix A Table 2**).

Our research not only sheds light on understanding infertility in women's, but also the whole new idea of mate choice providing a new mechanism for the origin of sexual dimorphism in humans as well as in other species. For example, it would be interesting to know if mate choice operates in non-human species showing menopause such as killer whales and short-finned pilot whales. From a medical perspective, this line of work would provide evidence for the evolutionary aspects of human health and possible strategies for treatment. For example, human menopause is the result of mate choice, extending the mate choice window, i.e., delaying the age of mating and reproduction would be expected to delay the onset of menopause

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APPENDICES

A. Testis size as a function of age:

Testis Morphology Experiment:

It is suggested from the literature that the testis size of a fruit fly is thinned as the male ages (Monica Boyle et al., 2007). Microscopic experiment was conducted on testis morphology of experimental (oM and oF) and control flies. Any change or premature thinning in old female/young male (oFyM) is predicted as the reduction in fertility compared to old male/young female (oMyF) and control flies.

D. melanogaster male reproductive capability can be predicted by the morphology of the testis size. In this section, the supplementary work done to measure the testis size as a function of age is done.

Methodology

i. From oM experimental populations, one of the three replicates (i.e. 3M) and from oF experimental population, one of the three replicates (i.e. 3F) and control population virgin males are collected.

ii. Every fifth day, the testis of each experimental and control males was dissected in a drop of PBS solution, tissues such as accessory glands and ejaculatory duct is removed using fine forceps under the stereo dissecting microscope at higher magnification.

iii. Then the testis is transferred on to clean slide using pipette. A 22*22 coverslip is placed carefully without bursting the testis.

iv. Images with scalebar are captured at 10X using Zeiss Axioplan microscope with Nomaski optics (also called as Differential Interference contrast).

Image Analysis: Area of the testis

i. Image analysis was done using ImageJ software. The image with scale bar is loaded in ImageJ software.

ii. In Analyze, scale is set by clicking on the "set scale". With the help of straight-line tool in ImageJ, a straight line is drawn on the scale bar of (100 μm), then scale is set in analyze section. The set scale option will show the distance in pixels, then the known distance is changed to 100, also the unit of length is changed to μm. "Global option" is selected, so

that the calibrated measurements will apply to all the images (i.e. we do not have to set scale for every image).

iii. Next step is to select the "segmented line" in shape symbols section, the outline of the whole testis is drawn using segmented line shape.

iv. Then, when clicked on "Measure" in analyze section, area and length of the testis is produced in micrometer as we set our scale in μm.

v. For every image, area and length of the testis is measured and all the measurements are noted in MS Excel sheet.

vi. Using PRISM 6.0 Software, bar graph with mean and standard deviation is made.

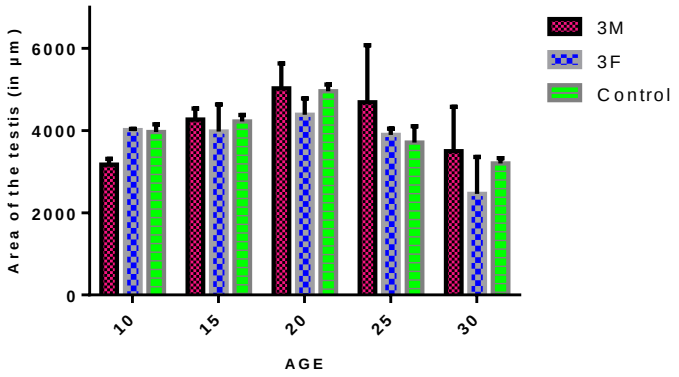

Figure 1. Testis area as a function of age. Mean area of testis (in μm) (+SD) of two experimental (oM and oF) and control

Image Analysis: Width of the testis

i. There are three regions in *D. melanogaster* testis. Apex region, Mid region and Basal region. Apex region (or apical end or apex) is defined as the area at the closed end of the testis (one tenth of the total testis). Mid region is exact middle region of the testis (one tenth of the total testis) and basal region as the area that attached to the seminal vesicle (one tenth of the total testis).

ii. After measuring area and length of the testis, width of the testis is measured using ImageJ software. By knowing the length,three regions of the testis (apical, middle and basal) is also known.

iii. A straight line is drawn at each region separately (apical, middle and basal), and "Measure" option in Analyze section is selected. It outputs the length of each region of testis.

iv. All the measurements are taken in MS Excel. The average of the three regions (apical, middle and basal) is taken as the width of the testis.

v. Using PRISM 6.0 Software, bar graph with mean and standard deviation is made.

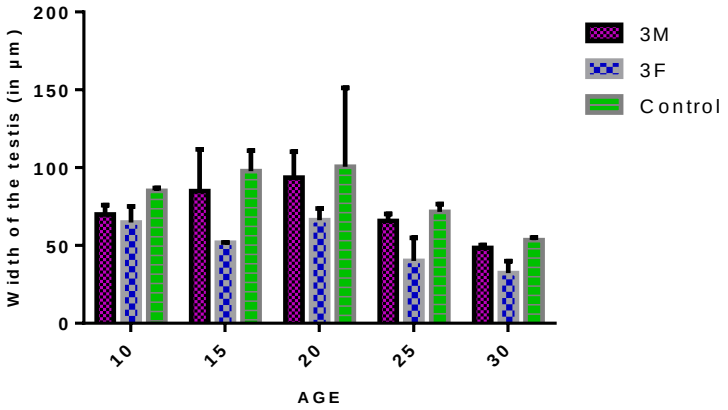

Figure 2. Testis width as a function of age. Mean width of testis (in μm) (+SD) of two experimental populations (oM and oF) and control

Table 1. Numerous hypothesis of menopause (modified from Morton et al., 2013).

Hypothesis	Description	Source
Follicle-depletion hypothesis	Women has fixed number of follicles and menopause ensues when the follicles are depleted.	S., A., & E., V. (2009). Reproductive aging in captive and wild common chimpanzees: Factors influencing the rate of follicular depletion. *American Journal of Primatology*. Van Valen, L. (2003). Ovarian excess and the evolution of menopause. *Evol. Theory*, *12*, 131-153.
Grand-mother hypothesis	Menopause allows grandmothers to contribute survival to their grandchildren, thus increasing their inclusive fitness	Hawkes, K. et al. 1998 "Grandmothering, Menopause, and the Evolution of Human Life Histories." *Proceedings of the National Academy of Sciences of the United States of America* 95.3 (1998): 1336–1339.
Lifespan artifact hypothesis	Menopause is a built-in production observed when the human longevity is increased	Caspari, R., & Lee, S.-H. (2004). Older age becomes common late in human evolution. *Proceedings of the National Academy of Sciences*. Peccei, J. S. (2001). Menopause: adaptation or epiphenomenon? *Evolutionary Anthropology: Issues, News, and Reviews*, *10*(2), 43-57.
Reproduction-cost hypothesis	Investment in reproduction is higher in women than men, thus leading to physiological deterioration and susceptible to becoming infertile	Penn, D. J., & Smith, K. R. (2007). Differential fitness costs of reproduction between the sexes
Mother hypothesis	(an adaptive version of reproduction-cost hypothesis), aging mother increases the survival probability of children; Menopause also would prevent fertilization of nonviable ova	Williams, G. C. (1957). Pleiotropy, natural selection, and the evolution of senescence. Pavard, S., Metcalf, C. J. E., &Heyer, E. (2008). Senescence of reproduction may explain adaptive menopause in humans: A test of the "mother" hypothesis. *American Journal of Physical Anthropology*.
Patriarch hypothesis	The origin of menopause allowed men to mate with younger women, thus increasing the longevity of both women and men, also the	Lahdenperä, M., Gillespie, D. O., Lummaa, V., & Russell, A. F. (2012). Severe intergenerational reproductive conflict and the evolution of menopause.

	increased status for men in society.	
Senescence hypothesis	Menopause is natural effect of aging. Reproductive aging proceeds faster than somatic aging.	Ward, E. J., Parsons, K., Holmes, E. E., Balcomb, K. C., & Ford, J. K. (2009). The role of menopause and reproductive senescence in a long-lived social mammal. *Frontiers in Zoology*.
Absent father hypothesis	Reduced paternal investment and increased maternal age were factors of menopause. This is a complement to grandmother hypothesis.	Rogers, A. R. (1993). Why menopause? *Evolutionary Ecology*.
Evolutionary trade-off	Menopause is a trade-off between female future production and enhanced survival of offspring.	Kirkwood TBL (2002) Evolution of ageing. Mech Ageing Devel 123: 737–745.
Reproduction-conflict	Menopause is the evolutionary outcome of resource-based competition between generations (i.e. between grandmother & their daughter-in-law, who are immigrants to the family); on the basis genetic relatedness, fitness can be optimised if immigrants reproduces with the help of grandmothers.	Caspari, R., & Lee, S.-H. (2004). Older age becomes common late in human evolution. *Proceedings of the National Academy of Sciences*. Kaplan, H., Hill, K., Lancaster, J., & Hurtado, A. M. (2000). A theory of human life history evolution: Diet, intelligence, and longevity. *Evolutionary Anthropology*.
Mate choice theory	A bias in mating behaviour, where only younger women are allowed to mate, produced late-onset, fertility-reducing mutations to accumulate and fix in the population, ultimately leading to menopause.	Morton, Richard A., Jonathan R. Stone, and Rama S. Singh. "Mate Choice and the Origin of Menopause." Ed. Mark M. Tanaka. *PLoS Computational Biology*9.6 (2013): e1003092. *PMC*. Web. 5 Feb. 2018.
Takahashi inclusive mate choice / triumvirate hypothesis approach	Explains menopause including mate choice theory, lifespan extension and grandmother assisting to rear their grandchildren.	Takahashi, Mike, Rama S. Singh, and John Stone. "A Theory for the Origin of Human Menopause." *Frontiers in Genetics* 7 (2016): 222. *PMC*. Web. 10 Feb. 2018

Table 2: Genes associated with Menopause

Gene name	Source
BMP15	Welt et al., 2004
FOX12	
EXO1	Stolk et al., 2009 Stolk et al.,2012
HELQ	
UIMC1	
FAM175A	
FANCI	
TLK1	
POLG	
PRIM1	
PRRC2A	
Babl	Sarikaya et al., 2012
InR	Sarikaya and Extrayour 2015
IGF1	He et al., 2010
CYP19A1	
ANKK1	He and Murabito, 2014

B. Sperm Viability test

Sperm Count Experiment:

A trial of counting viable sperm was made using LIVE/DEAD sperm viability kit that comes with two stains (SYBR-14 and PI). Protocol optimization was done with several trials that aims to count the viable sperm in experimental (oM and oF) and control flies. Any reduction in the viable sperm in old female/young male (oFyM) compared to old male/young female (oMyF) and control flies may be predicted as early senescence of fertility in old female/young male (oFyM) experimental population.

The materials and methods for sperm viability test is done as mentioned in Preethi Radhakrishnan and Kennet M. Fedorka, 2011 and 2012 articles.

LIVE/DEAD sperm viability kit from Thermo Scientific with catalogue number L-7011 was used to detect the viability of sperm in males. The kit comes with two following stains.

Propodium Iodide: Stains the damaged permeable membranes used to detect the dead sperm with red fluorescence. **SYBR-14:** Membrane permeable nucleic acid stain used to detect the live sperm with green florescence.

The dilutions of stains were made as mentioned in Preethi & Fedorka,2012 article. 1.6 μL of SYBR-14 was diluted in 10μL of DMSO & 1.6μL of Propodium Iodide was diluted in 1μL of *Drosophila* Ringers solution.

The male flies were co2 anesthetised and the seminal vesicles were dissecting in a drop of Ringer's solution on microscopic slide under Stereo dissecting microscope. Excess tissue was removed, and the seminal vesicle is carefully transferred to a new microscopic slide that contained 18μL of *Drosophila* Ringer's solution. After washing it certain times, it is then transferred on to a new microscopic slide that contained 8μL of Ringer's solution. Using fine forceps, sperm was gently punctured from the seminal vesicle. 1 μL of diluted SYBR-14 was added, mixed gently and was incubated for three min in dark moist chamber. Again, 1 μL of diluted Propodium Iodide was added, mixed gently and was incubated for one minute. This is the standardized staining protocol and concentrations of the dyes with maximum efficiency (Preethi &Fedorka, 2011).

Next, the slide is viewed under the fluorescent microscope at 200X magnification with red and green filters. Pictures were quickly taken using a microscope camera.

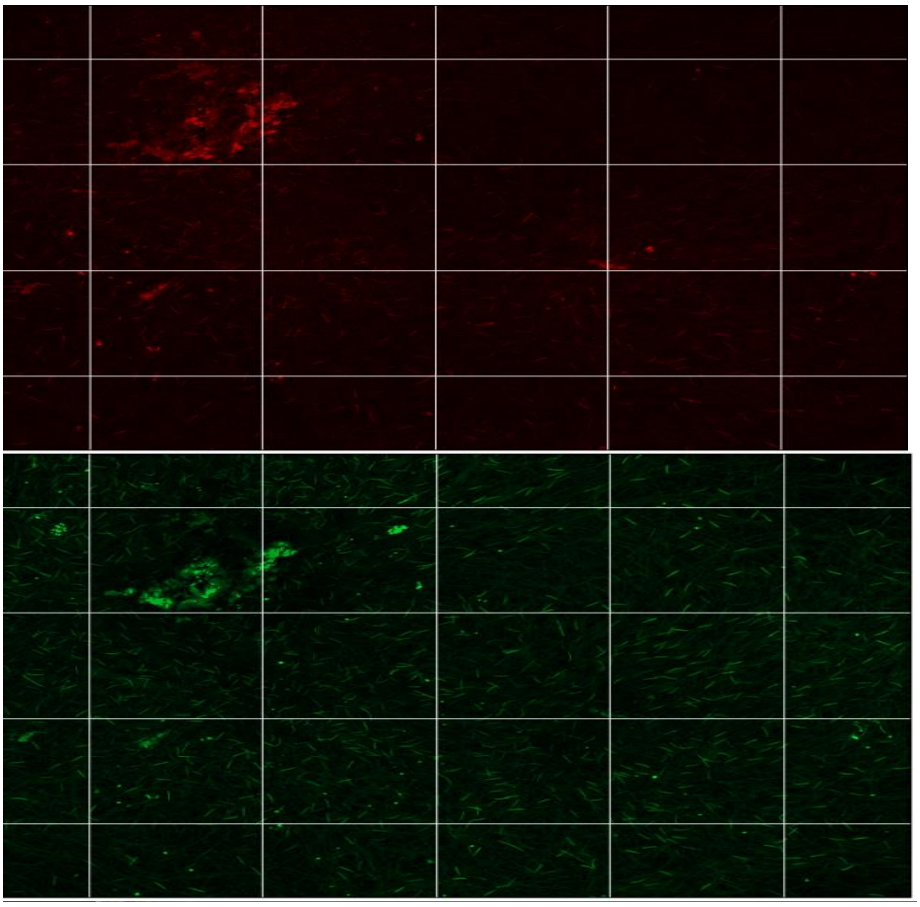

Figure 1. *D. melanogaster* sperm image taken at 20X using Fluroscent microscope. Grid lines are made using ImageJ software. Sperm with green are alive sperm and sperm with red are the dead sperm.

Sperm fluorescing both red and green are called moribund sperm (i.e. the sperm losing its cell wall integrity and is ready to die).

Using "multipoint" in ImageJ software, number of sperms are counted manually in each grid. There are 1437 sperm that were counted with green and 359 that were fluorescing red.

Alive sperm were fluorescing only green, but the dead the sperm were fluorescing both green and red (**Figure 1**). So, the number (359 sperm) is deducted from the total alive (1437) sperm. 1078 were considered as the alive sperm. In this way, the number of alive sperms in each seminal vesicle can be measured. Once the number of sperms is counted in each seminal vesicle, a graph can be made with respect to the age (**Figure 2**).

Sample Expected Outcome:

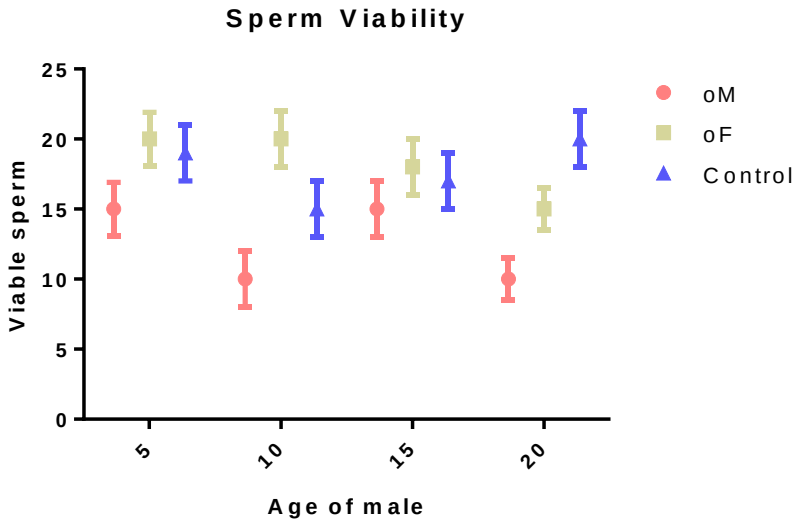

Figure 2. Plot of viable sperm in experimental and control males. X axis is the age of the males when dissected. Y axis is the sperm viability number. The bars are the standard error of means.