Men in menopause? Experimental verification of the mate choice theory of menopause with *Drosophila melanogaster*

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Abstract

Menopause is an unusual fertility reducing trait and it goes against natural selection. Group selection-based theories, such as the Grandmother hypothesis can, in principle, explain the evolution of menopause, but they do not explain how a fertility reducing mutation became fixed in populations. A population genetics theory of menopause, Mate Choice Theory, explains the evolution of menopause resulting from asymmetric mating involving older males and younger females leading to accumulation and neutral evolution of infertility causing mutations in older females. In this study we tested the mate choice theory using *Drosophila melanogaster.* Long term laboratory experiments involving asymmetric mating between young females and old males and vise-versa, were conducted over 40 generations and measurement of fitness parameters affecting female survival, fertility, fecundity, and male survival, fertility and testis length and width, were measured. The results showed, as expected, that younger females mated with older males produced infertile females with fewer ovarioles, fewer matured eggs, and fewer offspring; and younger males mated with older females produced sterile males with thinner testis compared to the control. The outcome of the experiment was symmetrical in that it works irrespective of sex, making both sexes, males and females, menopausal depending on which sex is deprived of reproduction in older age. These results show that change in mates' behavior, such as preferential mating, can have profound impact on women's health and could provide a basis for understanding reproductive senescence with age in both aging females and males.

Key Words: Mate choice, menopause, fertility, mutations, testis, ovary, female reproduction, women's health

Introduction

Human menopause is defined as a complete cessation of the menstrual cycle, and therefore a loss of fertility, usually characterized as such one year after a woman's final menses [1]. This phase is often called 'post-reproductive life', signifying reproductive senescence before biological senescence [2]. According to Darwinian evolutionary theory, selection should operate against the early termination of reproduction and against survival beyond reproductive senescence [3]. This post-reproductive life trait is almost uniquely observed in humans [4], otherwise barely observed in captive chimpanzees [5,6], killer whales [7] and short-pilot fined whales [8], while other mammals continue to reproduce until death [9]. This pattern of early reproductive cessation and prolonged post-menopausal survival is extremely inconceivable [10]. In human females, cessation of reproduction occurs around 50 years of age, characterized by years of diminishing fertility beforehand [11]. Human life histories are remarkable for the support in reproduction and upbringing of children by older, less fertile women, thus making a decline of reproduction in older women an evolutionary puzzle [12].

The solutions for this evolutionary puzzle can be grouped into two perspectives: a medical perspective that focuses on complications of health in post-menopausal women [13] and an evolutionary perspective that focuses on the origin and evolution of menopause in humans [12]. Under the medical perspective, cessation of reproduction (i.e. loss of fertility) following the menopausal phase results from a continuous and lifetime loss of follicles from the ovaries, starting from a maximum at birth [13]. This follicular loss occurs through ovulation, as each follicle carries an immature ovum that is developed in response to the hormonal signalling in the phase of the first

13 days of the menstrual cycle and is released on the 14th day of menstruation [13]. Programmed atresia, the degeneration of follicles that do not ovulate, is believed to be a major cause of follicular loss, with ovulation accounting for a small part [14]. Consequently, a decrease in oocytes with age might also occur due to genetic defects in oocytes over time [15]. These defects may include unrepaired meiotic DNA damage or breakdown in DNA repair mechanisms that are culled through poorly defined genetic pathways [16,17].

The evolutionary perspective of origin and evolution of menopause were sub-categorized in two approaches [18], non-adaptive and adaptive, which are summarized in Table 1. Under the non-adaptive approach, menopause is an epiphenomenon, an indirect result of selection due to antagonistic pleiotropy that favored early reproduction in females' fertile lives, or is simply a by-product of increase in lifespan, known as the "lifespan artifact hypothesis" [18,19]. The increase in lifespan is believed to be based on intrinsic factors, such as reduced telomere degradation, or extrinsic factors, such as human intelligence, social and cultural transmission of information, advances in medical and agricultural technologies etc. [18, 20-23]. The non-adaptive models suggest that an increase in human longevity could have allowed the menopause trait to appear and be expressed in older ages of females [18,24], but the theory was unable to explain why fertility remained intact in males with the increased lifespan but not in females [12]. The explanations in the second, adaptive, approach tried to contextualize the above shortfall of why fertility in females did not increase with lifespan.

Under the second approach, menopause is explained as an adaptive trait, the direct result of favourable selection of post-reproductive life that increased maternal investment in existing offspring or gaining inclusive fitness by older women through grandparenting, known as the "grandmother hypothesis" [25]. The grandmother hypothesis explains how grandmothering 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 [25]. This hypothesis suggested that grandmothering 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 sons or daughters. Although grandmothering has benefits, this theory has its own limitation, i.e. it could not explain how menopause evolved in the first place, regardless of whether it came before or after the lifespan extension [26].

A population-genetic model, the mate choice theory

Recently a non-kin-selection-based and non-adaptive, population-genetic theory postulated that a change in mating behaviour—e.g. preference of men mating with younger women—could have led a menopause-like phenotypic trait to evolve in women [12]. Such a mating preference towards younger women would relax selection on older women within the populations. In this kind of reproduction dynamic, the accumulation of female-specific, late-onset fertility-reducing mutations gets rendered neutral and fix in the population, leading to cessation of reproduction and, ultimately, menopause to evolve [12]. Authors used computational simulation and showed that in populations with the mating system involving older men preferring younger females for mating, accumulation of female-specific mutant alleles took place and menopause occurred early, indicating that mating strategies could affect the fertility of females in the population [12]. Although this theory is propitious, applicable to both men and women, and applicable to all sexually reproducing organisms, it has not yet been assessed in living organisms.

To experimentally verify the mate choice theory and provide support to the findings of the computational model [12], we used *D. melanogaster* as a model organism to understand the role of preferential mating in the evolution of menopause. Our aim was to investigate the effects of

age-restricted mating in *D. melanogaster* on the origin of sex-specific fertility-reducing mutations like those predicted in human menopause. Our interest was, of course, not in menopause-related physiological symptoms in flies but in the phenomenon of fertility loss. We examined the effect of age-restriction mating on reproductive ability in *both younger females and younger males when paired with older mates*. The rationale was that deprivation of reproduction in older females (or males) would accumulate sex-specific infertility mutations and result in sterility in older females (or males). If the mate choice theory of menopause is true, we hypothesized that the fertility-reducing mutations should segregate in the populations and express in the later stages of life when the "young mates" get old.

Here we present the results from the age-restricted mating experiment that has been running for 40 generations (and continuing). The expression of reproductive ability in experimental flies was measured and compared to wildtype flies in terms of fecundity and fertility, ovariole number and testis length and width during the lifespan of the flies. We report that our experiment produced results as predicted by the computer simulation study of the mate choice theory [12], making infertility appear in female flies where young females had been mated with older males. We further report that in lines using age-restricted mating of young males with older females, males became sterile or "menopausal", which is in line with the prediction of the mate choice model, showing that the evolution of female menopause is the result of asymmetric age-restricted mating and has nothing to do with the sex of the mates per se. In both experiments, the younger sex developed menopause; the older sex remained fertile.

Table 1: Hypotheses of menopause and sources, modified from Morton et al. (2013)

Hypothesis	Description
Follicle-depletion hypothesis	Woman has fixed number of follicles and menopause ensues when the follicles are depleted [27,28].
Lifespan artifact hypothesis	Menopause is a built-in process observed when human longevity is increased [19,29].
Grandmother hypothesis	Menopause allows grandmothers to contribute to the survival of their grandchildren, thus increasing their inclusive fitness [25].
Reproduction-cost hypothesis	Investment in reproduction is higher in women than in men, thus leading to physiological deterioration and susceptibility to becoming infertile [30].
Mother hypothesis	(An adaptive version of reproduction-cost hypothesis) Aging mother increases the survival probability of children; menopause also prevents fertilization of nonviable ova [31].
Patriarch hypothesis	The origin of menopause allowed men to mate with younger women, thus increasing the longevity of both women and men, as well as the status of men in society [32].
Senescence hypothesis	Menopause is the natural effect of aging. Reproductive aging proceeds faster than somatic aging [33].
Absent father hypothesis	Reduced paternal investment and increased maternal age were factors of menopause. This is a complement to the grandmother hypothesis [34].
Evolutionary trade-off	Menopause is a trade-off between a female's future production and enhanced survival of the offspring [35].
Reproduction-conflict	Menopause is the evolutionary outcome of resource-based competition between generations (i.e. between grandmother & her daughter-in-law, who are immigrants to the family); on the basis genetic relatedness, fitness can be optimized if immigrants reproduce with the help of grandmothers [21,29].
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 [12].
Inclusive mate choice / triumvirate hypothesis approach	Explains menopause including mate choice theory, lifespan extension and grandmother-assisting to rear their grandchildren [26].

Results

The results presented here include fecundity, fertility, longevity, ovariole number and testis length and width. We also measured lifespan because menopause is defined as the senescence of fertility before biological senescence [2]. The results uniformly point to significant negative effects on the fecundity and overall fertility, including testis size and ovariole number of the younger mate, of a population preferentially experiencing a non-random, age-restricted mating strategy.

The results are based on three sets of populations: a random, mated control population; an age-restricted experimental population involving young females mated with old males (fM); and another age-restricted experimental population involving old females mated with young males (Fm). The control and the experimental populations consisted of three replicates each.

Measurements of fecundity and fertility (at generation 20)

Fecundity and fertility measurements were repeated twice but slightly displaced in time—at generation 20 and 24. There was an overall decline in fertility in both control and experimental flies with age. However, a complete loss of fertility in young females mated with older males (fM) was observed much before the end of female survival. In contrast, the control females reproduced throughout the lifetime period. Fecundity was measured in terms of oviposition—i.e. number of eggs laid by a mated female each day for 20 days post-mating (males were discarded after 3 days of mating). Control females produced, on average, the largest number of eggs per day as compared to the experimental populations until day 16, after which mean egg production matched that of the experimental populations (Fig 1A). All three populations demonstrated a general linear decline in oviposition over their lifetimes.

The average number of offspring produced per day by control females was significantly greater as compared to the cumulative averages of both experimental populations, though the disparity decreased with the increasing age of the experimental females (Fig 1B). Control flies exhibited a linear decrease in the number of offspring eclosed per day. The experimental populations also demonstrated a linear trend in decline in offspring number eclosing over time and were not significantly different from the control in mean offspring produced per day. Egg fertility (hatchability) was measured as the viable offspring produced from the eggs laid. The proportion of eggs laid per day that eventually eclosed into viable adults was greatest in the control population throughout the experiment, but it generally decreased over time. The percentage of eggs eclosed into adults was comparably similar in the two experimental populations (Fig 1C).

Measurement of female fertility over lifetime. Almost all females continued to produce offspring throughout the control experiment, as the percentage of females producing offspring dropped to only 94.4% by day 18 (Fig 1D). In the experimental group, conversely, the percentage of females producing offspring over time demonstrated a sigmoidal curve. In the young female–old male pairings (fM), the curve exhibited an inflection point at around 10 days post–mating period. By day 18 of the post–mating period, only 6 of 29 females (20.69%) were producing offspring. The old female–young male pairs (Fm) produced a curve with an inflection point at around 14 days post–mating period. By day 18, only 5 of 28 females (17.86%) were producing offspring.

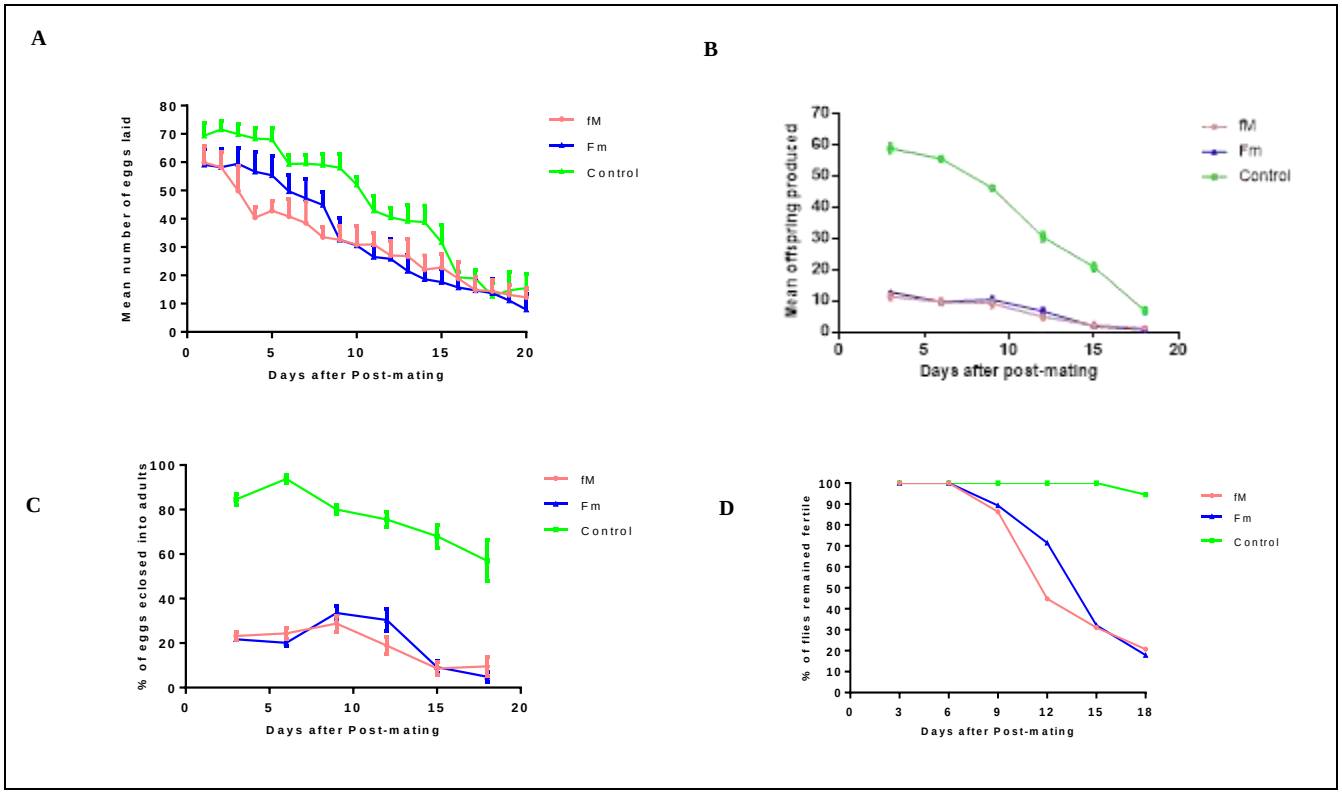

Fig 1. Fecundity and Fertility curves for control and experimental (fM and Fm) populations

(A) Mean number of eggs laid per day by females from control and experimental (fM, Fm) populations. Eggs were counted daily after male was removed from the vial. Oviposition was used as a measure of fecundity. **(**B) Mean number of offspring eclosed per day by females from control and experimental (fM, Fm) populations. Egg hatchability was measured using eggs laid every third day and all offspring that eclosed from each vial were counted. (C) Percentage of eggs eclosed by females from control and experimental (fM, Fm) populations. It was calculated as the mean number of eggs laid in total on a given day divided by the number of viable offspring produced on average on that day. (D) Percentage of flies remaining fertile from control and experimental (fM, Fm) populations. It was calculated as the number of flies continuing to produce offspring divided

by the total number of flies in the population. Bars in all figures represent standard error of the mean.

Measurements of fecundity and fertility (at generation 24)

In this experiment, the observation of fecundity and fertility was limited to the control and one experimental population only: comparison of young experimental females (fM) mated with older control males (cM) and a control. The idea was to test the fecundity and fertility of young females (fM) when mated with fertile older males (cM). The results presented here show a similar pattern to that observed in the 20th generation of selection. The mean number of eggs laid by control females is larger than the mean number of eggs produced by young females mated with older control males throughout the observation period (Fig 2A). A linear decrease in eggs laid with age is observed in both control and experimental females.

The mean number of offspring produced and the proportion of eggs eclosed into adults by control females is significantly higher than the average number of offspring and viable adults produced by the young experimental females. Most of the young experimental females produced no offspring after day 15 of the post–mating period. Conversely, control females continued to produce offspring after day 15 of the post–mating period (Fig 2B).

Measurement of female fertility over lifetime. Almost all females in the control population remained fertile, and the percentage dropped to 88% on day 18 post-mating. In the experimental females, all females were fertile till day 9, and then it dropped sharply after day 12. Only 22% of females of experimental population were fertile at the end of the observation, i.e. on day 18, compared to 88% in the control (Fig 2C). Fertility decreased in older experimental young females.

Analysis of variance was performed to examine the relation between egg production and offspring production of control and experimental populations. ANOVA results showed a statistical difference between control and experimental populations ($p < 0.0001$).

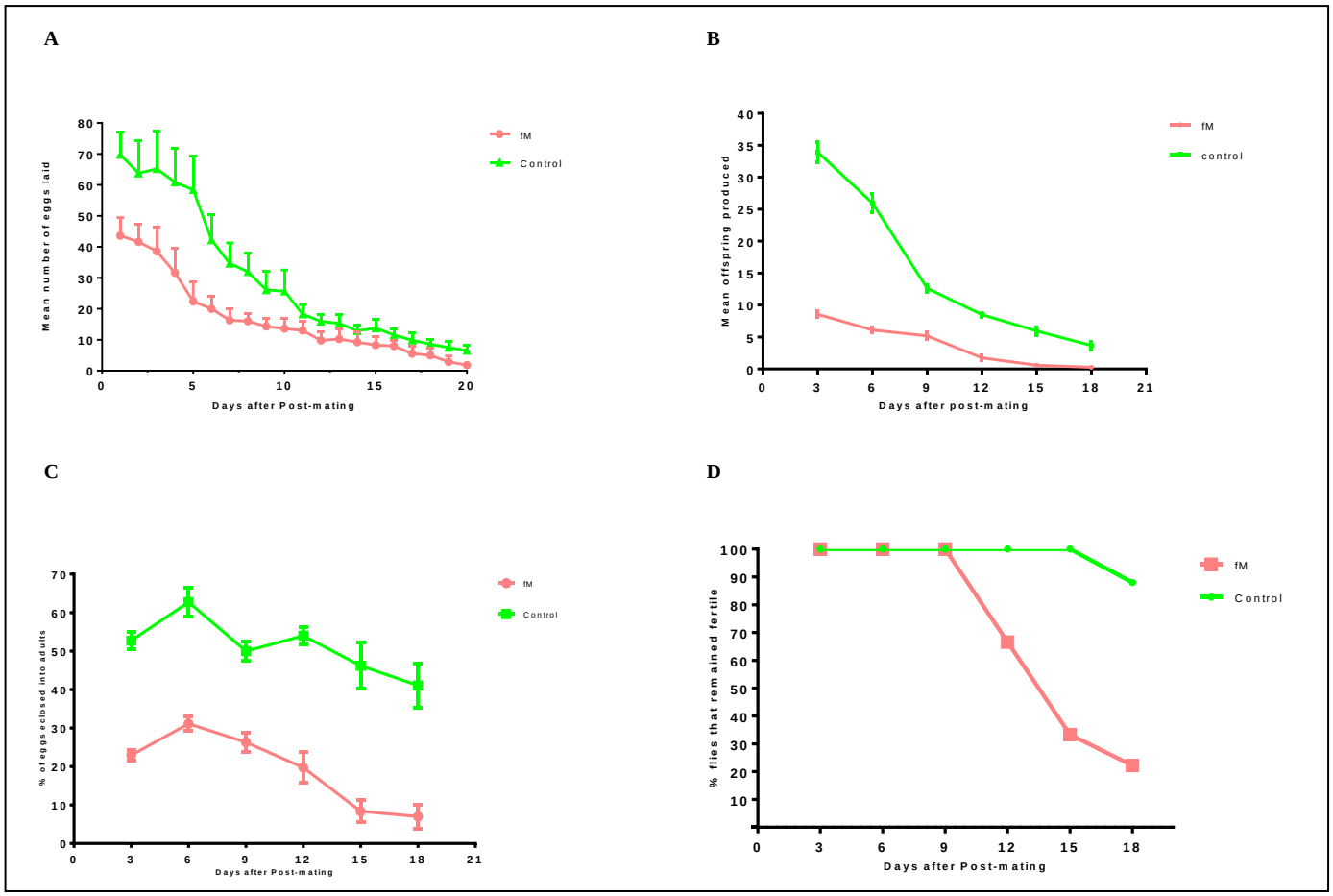

Fig 2. Fecundity and Fertility curves for control and experimental (fM) populations

(A) Mean number of eggs laid per day by females from control and experimental female (fM) populations. Eggs were counted daily once male was removed from the vial. Oviposition was used as a measure of fecundity. (B) Mean number of offspring eclosed per day by females from control and experimental (fM) populations. Egg hatchability was measured using eggs laid every third day, and all offspring that eclosed from each vial were counted. (C) Percentage of eggs eclosed by females from control and experimental (fM) populations. It was calculated as the mean number of eggs laid in total on a given day divided by the number of viable offspring produced on average on that day. (D) Percentage of female flies remaining

fertile from control and experimental (fM) populations. It was calculated as the number of flies continuing to produce offspring divided by the total number of flies in population. Bars in all figures represent standard error of the mean.

Survivorship and lifespan

The outcomes of the lifespan experiment included survivorship, expected life span, and log-rank analysis of the survival curves. Survival curves—the fraction of flies surviving at each interval—were created with the Kaplan-Meier survival method. Kmsurv is an R-package that produces life-table information based on the survivorship function and outputs the average survival at each interval. These average survival curves were then plotted using matplot in R software. The life expectancy of each population was calculated using R code and the outcome is included in the Fig 3 legend. Life expectancy is calculated as the average number of days in a life remaining from the beginning of the interval to the end. The R code for life expectancy is available online in the support documentation (**TEXT**). Log analysis is the test used to analyze survival curves based on the observed and expected number of deaths [36]. Further, having observed the number of recorded deaths, we calculated the expected number of deaths at each interval based on log rank analysis. A Chi-square test was done to test the significance of the differences between the populations.

The average expected lifespans for females and males were 53 and 33 days, respectively. Overall, in all populations, virgin females were observed to live longer than virgin males. Three populations in total, one from male populations (2f) and two from female populations (1f and 2m), were identified as outliers and removed from analysis to reduce bias.

All survival curves for the male population, including the control and experimental populations, showed a similar decrease in lifespan over time, although the Chi-square test, χ^2 (5, N = 50) = 1.118, p-value = 0.409, showed that the difference between the control and the two

experimental male populations was not significant. Similar results were found for the female populations. Two of the populations that were identified as outliers were removed from the Chi-square test, χ^2 (4, N = 50) = 11.1, p-value = 0.34, which showed that the difference between the control and the two experimental female populations was not significant.

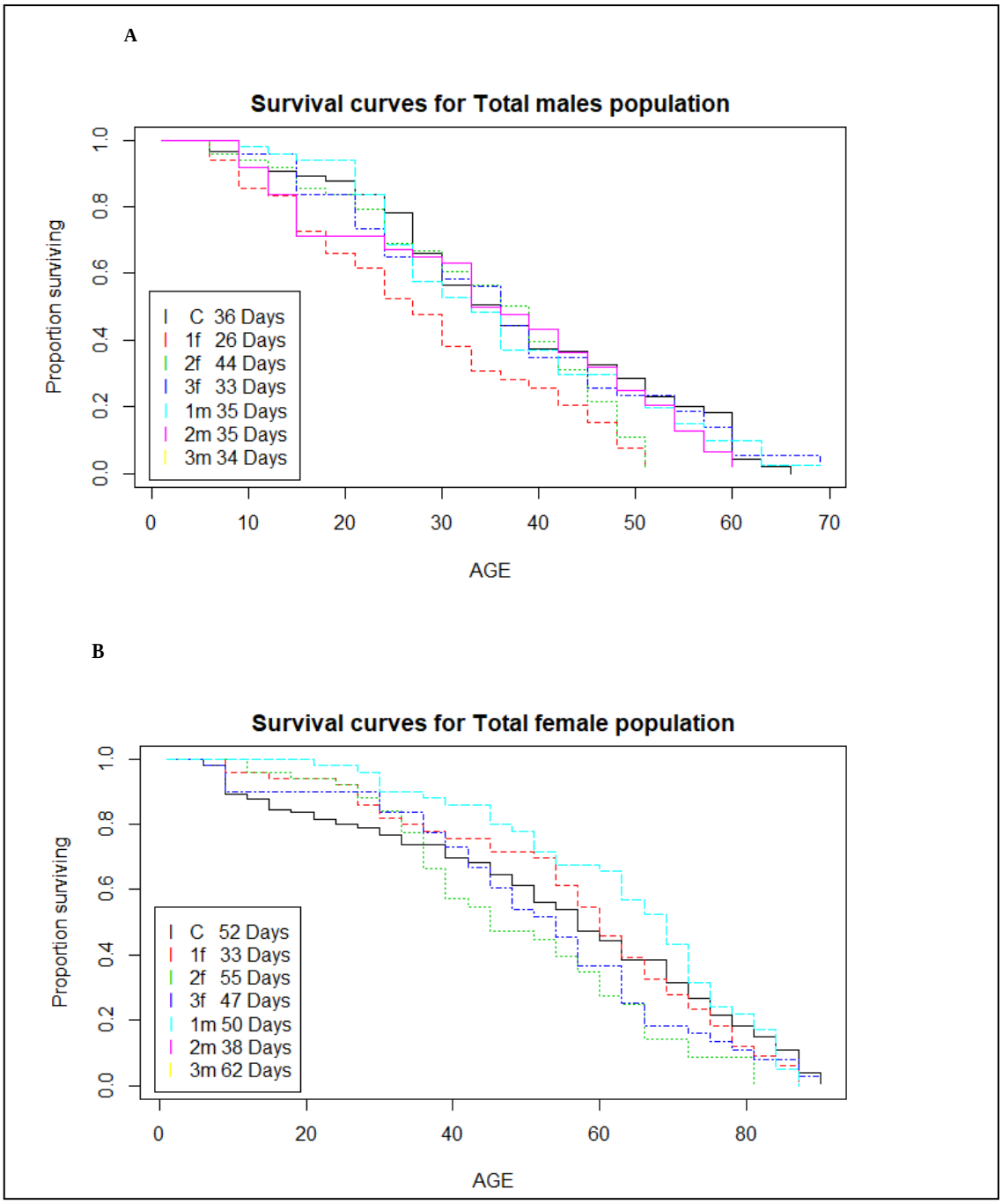

Fig 3. Survival curves for the total number of males and females—pooled control and two experimental populations.

Expected lifespans are included in figure legend. The y-axis is the proportion of those surviving at each interval; the x-axis is the age of the fly. The three repetitions of the fM population are simply named as 1f, 2f and 3f and the three reps of Fm population as 1m, 2m and 3m. Control males were named as C to enhance accuracy in data entry and analysis.

Egg chamber and ovariole count

The mean number of stage-14 egg chambers was observed every third day post-eclosion for control and experimental populations (fM and Fm) and is shown in Fig 4A. The number of egg chambers was quite variable, but it generally decreased over time, reaching a low of 4 on day 30. The experimental population involving younger males followed a pattern similar to the control population. It was also quite variable and decreased from 10 on day 3 to 3 on day 30. The experimental population involving younger females produced the fewest number of stage-14 egg chambers throughout the observation period. The mean number of egg chambers decreased from 6 on day 3 to nearly 0 on day 30. One of the young female populations (Rep 1f) produced no matured egg chambers on day 30. The one-way ANOVA produced 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. young male, control vs. young female and young female vs. young male populations. There was no significant difference in the stage-14 egg chambers for the control (M = 8.34, SEM = 1.01) and the experimental population of old females mated with young males (Fm) (M = 6.92, SEM = 1.01); t (18) = 0.95, p = 0.35. However, a significant difference was observed between the matured egg chambers for the control (M = 8.34, SEM = 1.01) and the experimental population of young females mated with older males (fM) (M = 3.58, SEM = 0.57; t

(18) = 4.09, p = 0.0007). Also, a difference was observed between young female (fM) (M = 3.58, SEM = 0.57) and old female (Fm) experimental populations (M = 6.92, SEM = 1.01; t (18) = 2.71, p = 0.014).

The number of ovarioles per female presented here is the average number of ovarioles in the left and right ovary. The mean number of ovarioles for the control was higher than that for the two experimental populations. Ovariole count started at 14 for the control, while it was similar for the young female (fM) and young male (Fm) experimental populations (i.e. 13.5) on day 3. Notably, there was a sharp decrease in the ovariole count for the young male (Fm) population on day 21. We noticed a gradual increase, followed by a decrease of the mean number of ovarioles for the young female (fM) population. The highest mean ovariole counts recorded for control populations and old female (Fm) experimental populations were on day 6 and day 18, respectively, whereas for the young female (fM) population, it was on day 6. The one-way ANOVA that was performed returned a p-value = 0.0001, rejecting the null hypothesis of no difference among populations. Like egg chamber count, two-tailed *t*-tests were performed on two-way comparisons: control vs. young male, control vs. young female and young female vs. young male populations. There was a statistically significant difference in the ovariole count for control and young male (Fm) experimental population; t (9) = 2.53, p = 0.032. There was also a significant difference observed in the ovariole count for the control and the young female (fM) experimental population; t (9) = 7.43, p < 0.0001; and a difference was observed between the two experimental populations (fM and Fm); t (9) = 2.54, p = 0.031.

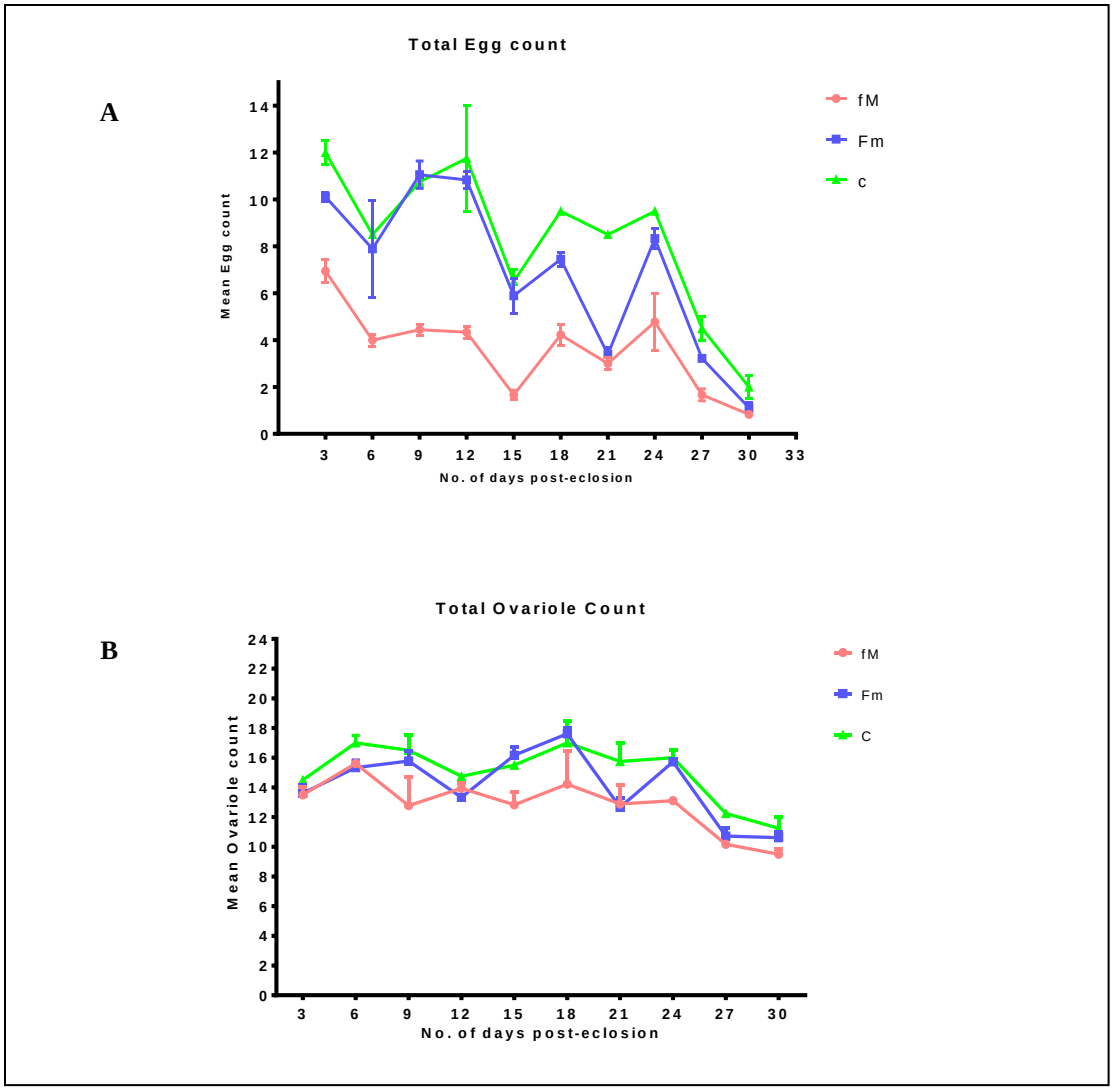

Fig 4. Plot of stage-14 egg chambers and Ovariole count for control and two experimental populations

(A) Plot of stage-14 egg chambers and the number of days post-eclosion in females. (B) Plot of mean ovariole count and number of days post-eclosion in females. Bars in the graph represent standard error of the mean.

Testis length and width

We measured the effect of age-restricted mating on younger males when mated with older females (Fm). Testis length and width were measured as a proxy of male fertility. The length of the testis showed no significant variation in the observation period (over 50 days). In contrast, the width of testes from the younger-male experimental population (Fm) was observed to be smaller than that of the control. The decline in width of the testis implied that there were some underlying changes in the germline of younger males mated with older females (Fm).

The results for the pooled mean testis length are displayed in Fig 5. "Pooled" indicates the pooling of the three replicates of the younger-male population (Fm) or of the younger-female population (fM). None of the comparisons showed significant difference in testis length. Testis length remained unchanged over the lifespan of the fly in each of the three populations. In contrast, mean testes width showed significant differences between young male (Fm) and young female (fM) populations and the control.

The two-tailed *t*-test was performed on the differences between the experimental populations and the control. Fig 6A shows significant differences in mean testis width between young female (fM) and young male (Fm) populations during the early (days 0–15) and late periods (days 35–50) of the flies' lives; Fig 6C shows the means for Fm and the control. fM and control showed no significant difference (Fig 6B).

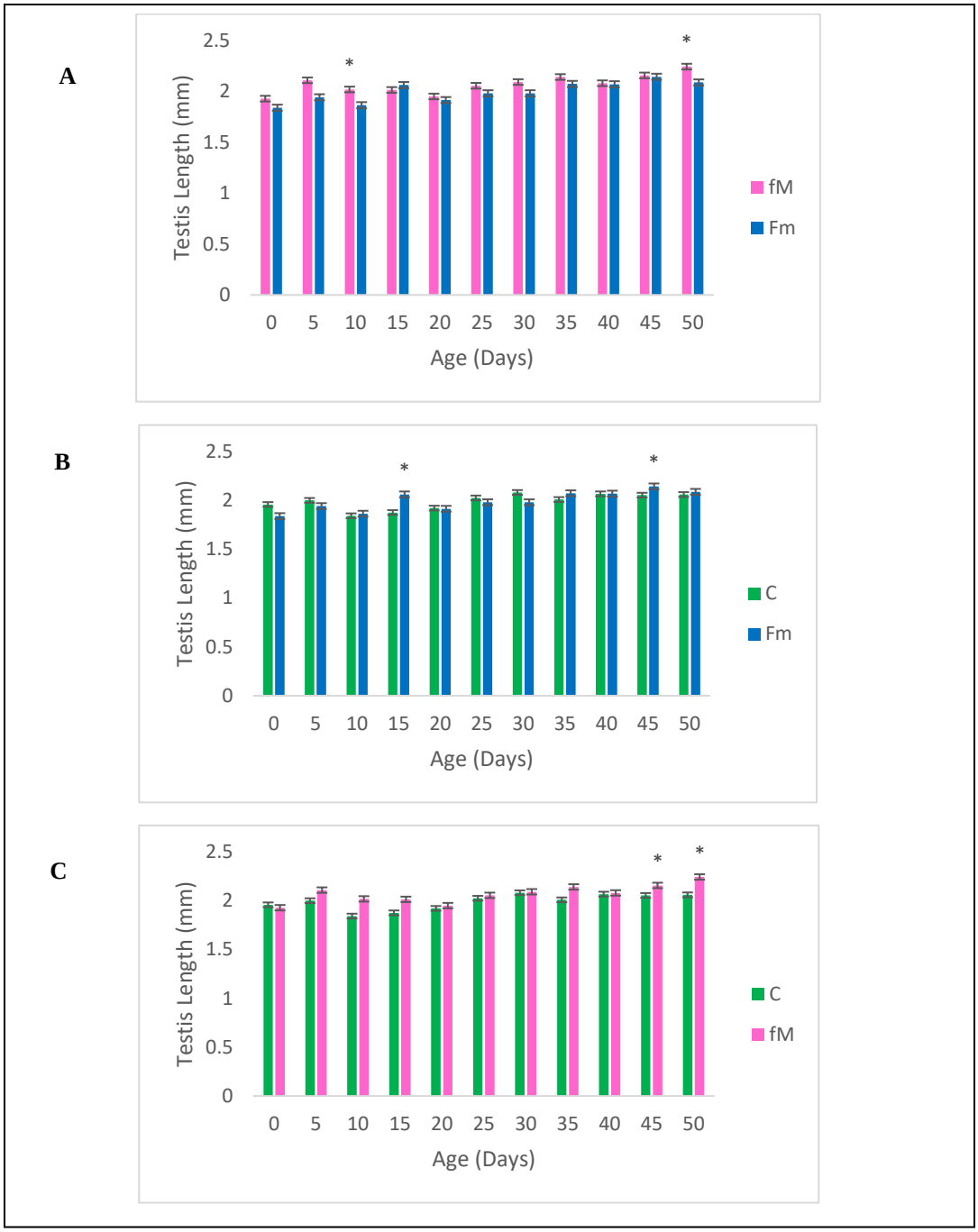

Fig 5. Pooled (over three reps) mean testis length for Fm, fM and Control (C) populations.

A single asterisk represents a p-value <0.05, double asterisks <0.02. Error bars are standard error of the mean. (A) Comparison of mean testis length of fM and Fm. (B) Comparison of mean testis length of fM and control. (C) Comparison of mean testis length of Fm and control.

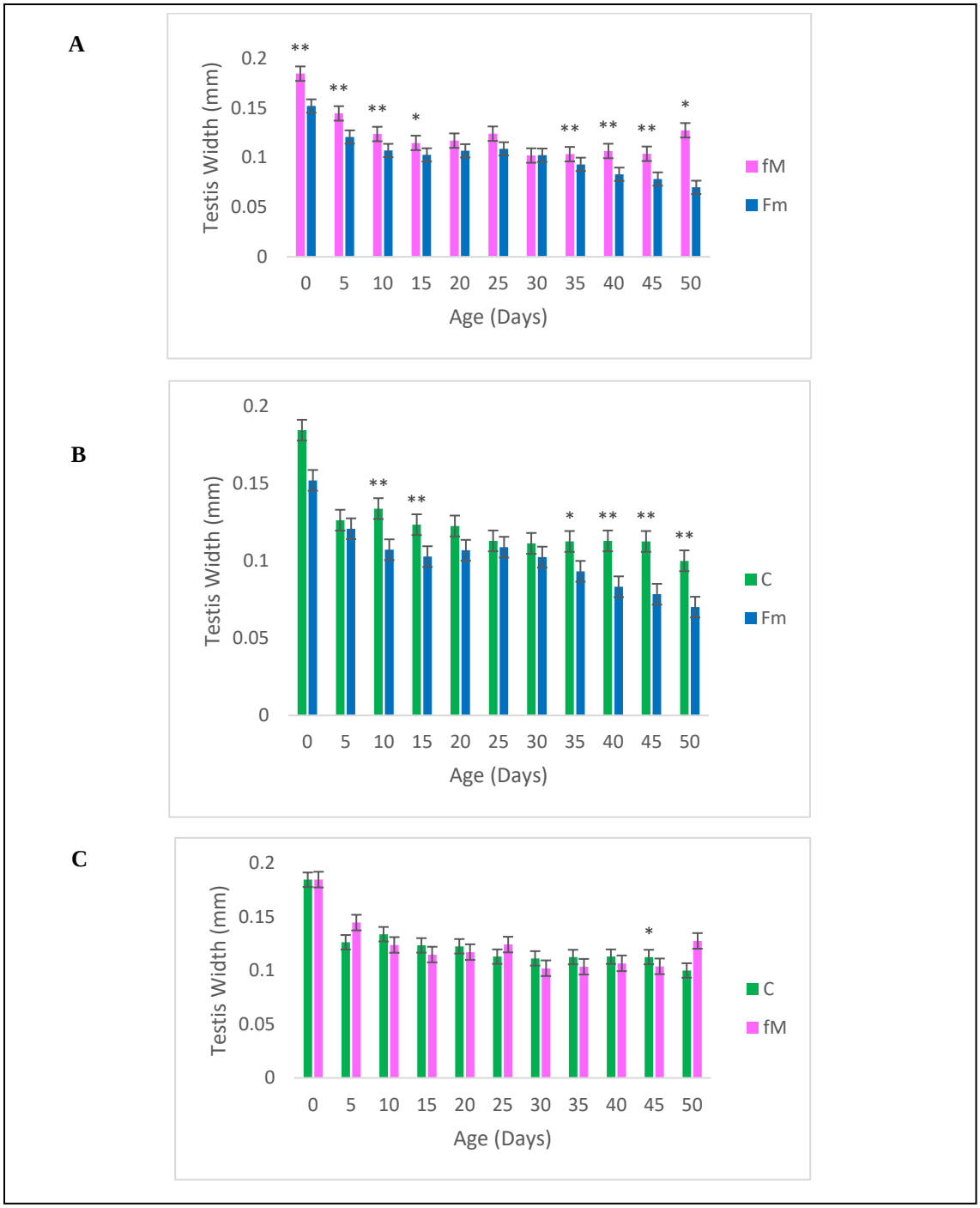

Fig 6. Pooled (over three reps) mean testis width for Fm, fM and Control (C) populations. A single asterisk represents a p-value <0.05, double asterisk <0.02. Error bars are standard error of the mean. (A) Comparison of mean testis width of fM and Fm. (B) Comparison of mean testis width Fm and Control. (C) Comparison of mean testis width of fM and control populations.

Discussion

Evolution of a trait implies the role of mutations, new or segregating, and their fixation in the population. Menopause is an unusual trait in that contrary to Darwinian expectation it leads to lower fitness of a population. Mate choice theory, while not unusual in its apparent operation, turns out to be also unusual in its effect: It allows deleterious (to female fertility) mutations to evolve as neutral alleles. Selection through one sex can affect the other. The effect can be positive, as in the case of the evolution of human longevity through selection on males [37], or it can be negative, as in the case of antagonistic evolution which leads to enhanced immunity in females [38]. Mate choice has a negative effect on female fitness, but it is indirect and there is no way for females to counteract it.

In human females, reproductive cessation occurs around 50 years of age, characterized by years of diminishing fertility beforehand [11]. In short-finned pilot whale females, cessation of reproduction occurs around 36 years of age, but they can survive up to 65 years [39]. By contrast, other mammals (e.g. elephants) breed until the end of their lifespan [40]. Mate choice theory is in line with what we know about the human mating system: Evolution from promiscuity to polygamy to monogamy, serial monogamy, harem or sexual relationship outside marriage all point to the fact that, over evolutionary history and over the globe in every society that we know of, the tendency has been for men, old or young, to prefer younger mates. Unlike the other theories of menopause, mate choice theory lends itself to genetic investigation and verification, and this is what was done in this study.

We examined the effect of age-restricted mating on fecundity and fertility in fruit flies. We found that several generations of age-restricted mating, i.e. older males mated with younger

females and vice versa, had a negative effect on the fecundity and fertility of the younger mates in their older age, as compared to the control. The effect of age-restricted mating was seen in a number of fitness traits: the number of eggs and offspring produced, count of egg chamber and ovariole number and length and width of testis. The theory worked as expected, and both the age-restricted mating strategies, with either younger males or younger females, resulted in negative impact on the flies' younger counterparts' fecundity and fertility with increasing age, implying that both males and females can experience menopause depending on the mating strategy employed.

In the following, we discuss the various aspects of fitness that were measured and show the feasibility and generality of the mate choice theory in producing menopause.

Age-asymmetric mating and the evolution of fecundity and fertility

The measurement of the fitness effects was repeated twice—a few generations apart—at generations 20 and 24. A different control was used each time to reduce any bias in the outcome. In the first measurement (gen 20), the control consisted of mating 20-day-aged males and females from the control population. In other words, in both the treatment and the control, mating involved males and females from within populations. In the second measurement (gen 24), the control consisted of mating 20-day-aged males with 3-day-old females, like the experimental population. Regardless of what control trials were used, the effect of age-restricted mating on reproductive capabilities was negative and significant in both investigations. One important aspect is that the experimental young females (fM) in generation 24 were mated with aged fertile males instead of same-line males. The idea was to test and confirm the effect of age-restriction mating in young experimental females (fM).

The second control trial, i.e. 20-day-aged males mated with 3-day-old females, is similar to the first generation of age-restricted selection carried out in experimental flies and allowed us to interpret that the observed effect of age-restricted mating on cumulative egg and offspring production in fruit flies did not just occur after one generation but occurred due to several generations of mating and mutation accumulation. Persistent age-restricted mating at each generation is important for the accumulation of sex-specific fertility-reducing mutations within the populations. This could possibly explain how, in humans, historically long-standing male preferences for younger women when choosing a partner allowed for the original cumulation of infertility in women, exhibited as the phenomenon of menopause [12].

The control wildtype flies exhibited similar production of eggs compared to the literature and were therefore considered to be a good representation of wildtype flies' reproductive capability [41]. It has been observed that production of offspring is lower than actual reproductive capability [41]. This was apparent in our experiments as well: The production of viable offspring in control and experimental populations declined more rapidly with age than the number of eggs laid as a function of age (Figs 1 and 2).

Age-asymmetric mating and the evolution of lifespan

Two major conclusions were drawn from the longevity experiment. First, the results of the study showed that there was no significant difference in lifespan between the control and experimental populations (fM and Fm), and second, there was a significant difference observed between males and females in all the populations, i.e. female flies lived longer than males. The average expected lifespan for females was around 53 days, whereas for males, it was 33 days. Our results showing a difference between the virgin males and virgin females support previous

observations showing virgin females living longer than virgin males and mated males living longer than virgin males in *Drosophila subobscura* [42].

In this study, three outlier experimental populations, identified as 2f, 1f and 2m, did not follow trends in survival curves like other flies, and including them in the data analysis differed the p-value, and so they were eliminated from the dataset to reduce the bias. The 1f, 2m females died sooner than the control. The early deaths were suspected to have happened due to food spoilage noticed during the observation period. Longevity experiments in fruit flies are often sensitive due to other factors affecting 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., as well as other unknown factors that include some unknown mutation that reduces the lifespan in the population [43]. Knowing the lifespan of fruit flies was important as it is believed that reproductive senescence in humans occurs before biological senescence [2]. In our experiments, it was clear that the senescence of reproduction took place before the biological senescence (Figs 1B, 2B and 4A).

Age-asymmetric mating and the evolution of egg chamber and ovariole number

To examine the effect of age-restricted mating on ovariole number and mature oocytes, we performed ovariole and egg chamber counts in the age-restricted experimental and control populations of *D. melanogaster*. Ovariole numbers impact the production of eggs in *D. melanogaster* and affect the reproductive success of fruit flies [44,45]. Each ovariole has an anterior germarium, where somatic and germ cells are connected and organized, and a posterior vitellarium [46]. The germarium contains primordial germ cells (PGCs) that produce a cluster of cells, one of which will become an oocyte and the rest of which will become nurse cells [46]. On the other hand, the vitellarium consists of a series of interconnected egg chambers, and the

development of egg chambers is divided into different consecutive stages, starting from stage 1 to stage 14 [47]. Stage 14 is the mature primary oocyte that is ready for fertilization [48]. In *D. melanogaster*, the stage-14 egg chamber is not produced until the female is 48 hours old [49]. Typically, only the stage-14 egg chamber has yolk and will be ready for fertilization, so the number of stage-14 eggs in the *D. melanogaster* has a direct impact on the production of offspring [50]. After 30 generations of age-restricted mating, we examined both stage-14 eggs and ovariole numbers in experimental and control females. The average stage-14 egg chambers and ovarioles were fewer in young females mated with older males (fM experimental) compared to young males mated with older females (Fm experimental) and control flies (Fig 4). A one-way analysis of variance showed a difference between the experimental and the control populations. The number of ovarioles in the Fm and fM populations was significantly different ($t(9) = 2.54$, $p = 0.031$). Also, there was a large difference in stage-14 egg chambers between the two experimental populations ($t(18) = 2.71$, $p = 0.014$). The results demonstrate that the age-restricted mating negatively affected the production and maturation of ovariole chambers and egg numbers in all young females mated with older males (fM populations). This suggests that there may be a delay in the development of egg chambers from stage 1 to the matured egg chambers (stage 14) in the younger female (fM) experimental population. In *Drosophila melanogaster*, the development of a mature egg involves the coordination of thousands of somatic and 16 germline cells [49]. Our results show that due to age-restricted mating strategy employed, fertility-reducing mutations could have accumulated in the germline cells or in the coordination of somatic and germline cells in the old male–young female (fM) populations that led to a lesser number of matured eggs compared to the old female–young male (Fm) and control populations.

Age-asymmetric mating and the evolution of testis length and width

The results presented in Fig 6 show a significant difference between Fm and fM as compared to but no significant difference between fM and the control. Most significantly, younger males mated with older females (Fm) were found to have lower mean testis width than fM and the control during the early (age 0 to 15 days) and late (age 35 to 50 days) stages of the flies' lives. As maturing cells move through the testis from the apical end to the seminal vesicle, there are fewer spermatocytes in the testis and more in the seminal vesicle [51]. With increasing age, the division of spermatocytes at the apical end of the testis slows down, reducing sperm production while movement of sperm towards the seminal vesicle continues. This may be responsible for the testis becoming thinner with age [51]. This observation aligns with a previous study on *D. melanogaster* that showed that the division rate of male germline stem cells (GSCs) in *D. melanogaster* slows down significantly during aging and that this slowing down correlates with a reduction in the number of sperm produced [52]. These data, combined with the measurement of testis width, show that testis width declines naturally with age due to slowed-GSC division and reduced sperm output.

According to the mate choice theory of menopause, mutations are predicted to accumulate in the younger counterpart of the mate in the age-restricted mating scheme. This was apparent in our study in testis width as significant differences with P-values of 0.05 or lower from two-tailed *t*-tests were obtained at ages 35, 40, 45 and 50 when comparing Fm to fM or the control. The control populations displayed more rapid degeneration of the testes as these populations did not undergo any restrictive mating and are thus projected to undergo normal testis degeneration. Younger males also appear to have significantly thinner testis in comparison to fM or the control early in life, at ages 0, 5, 10 and 15 days. Though the mate choice theory does not predict the

thinning of testis early in life, these observations could be the result of the selective mating scheme, as well as other mutations related to the nature of the flies. Thus, it can be attested that younger male flies have thinner testes from birth, and the testes become progressively thinner due to the accumulation of mutations described by the mate choice theory.

The testis length showed no difference between younger males (Fm), younger females (fM) and control populations (Fig 6). While the width of the testis is observed to decrease with age due to the movement of sperm out of the testis, length is a characteristic that develops early in the development of a fly and then has been found to remain static over time [53]. Length is not affected by the division and replication of sperm cells at the apical tip of the testis. The lack of a difference in the length suggests that the late-onset fertility-diminishing mutations may be affecting sperm production, rather than organ development.

Conclusion

Menopause is an unusual fertility reducing trait, and it goes against natural selection. Although group- or kin-selection-based theories such as the grandmother hypothesis can, in principle, explain the evolution of menopause, these theories are weak and have been found wanting [12, 26, 54]. To give just one example, there is a two-fold cost to grandmothering: For every child a grandmother foregoes to reproduce, she must invest or help (her son or daughter) to produce two more grandchildren above and beyond what her children would have produced without her help. The problem is not with grandmothers helping but with how grandmothers become sterile.

Mate choice theory is beautiful in its explanatory power: (1) It is a population genetics–based theory involving individual selection and does not involve founder effect, group selection, kin selection, or pleiotropic effect; (2) the theory operates by rendering deleterious mutations to evolve as neutral alleles; and (3) the theory is symmetrical in that it works irrespective of sex, making both sexes, males or females, menopausal depending on which sex is deprived of reproduction in older age.

The theory predicts that a shift in mating behaviour such that older males prefer to mate with younger females would produce menopause through accumulation of sex-specific, fertility-diminishing mutations in older females [12]. Testing this theory using an age-restricted mating scheme on *D. melanogaster* produced a number of fertility-reducing effects: younger females mated with older males produced infertile females with fewer ovarioles, fewer matured eggs and fewer offspring; and younger males mated with older females produced sterile males with thinner testis compared to the control. These results show that changes in mates' behaviour, such as preferential mating, can have a profound impact on women's health and could provide a basis for understanding reproductive senescence with age in both aging females and males. Finally, we would like to point out that menopause is neither a disease nor a "female thing"; it is the outcome of sexual lifestyle and can potentially occur in both sexes. These results raise the possibility of understanding the causes of sexual dimorphisms in disease and health.

Materials and Methods

All experiments were carried out in laboratory conditions at room temperature (22◦C) with flies reared on standard cornmeal-agar-molasses food medium.

Base population

A diverse base population was created by mixing ten different *Drosophila melanogaster* strains that were ordered from *Drosophila* Stock Centre, California.

Selection protocol

A mutation accumulation experiment was started in two experimental populations in which the mating system was altered in comparison to the control that used usual random mating. The two experimental populations were created by using age-restricted mating—by mating 3-day-old young virgin females to 20-day-old males, and 20-day-old females to 3-day-old virgin males. All populations, control and experimental, consisted of three replicates.

Drosophila melanogaster females remain virgin 8–10 hours after eclosion and males remain virgin for 12 hours after eclosion [55]. Virgin flies were easily detectable due to their larger body size relative to that of the older flies. In the population of younger females mated with older males (fM), on day 9, jars were cleared when the pupae were ready to eclose, as shown in Fig 7. On day 10, 100 virgin males were collected, 10 placed in each food-filled vial. Males were left to age for 20 days and then changed into new vials every 7 days to prevent food contamination. On day 26, the jars were cleared again, and 100 virgin females were collected on day 27. The virgin females were aged for 3 days. On day 30, the 20-day-old virgin males were mated with 3-day-old virgin females in three jars to avoid the risk of losing the line, the progeny of 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. We created three independent replicates of fM that were denoted as 1f, 2f and 3f. The same procedure was used to create the older female–younger male, denoted as Fm, with three independent replicates labelled as 1m, 2m and 3m.

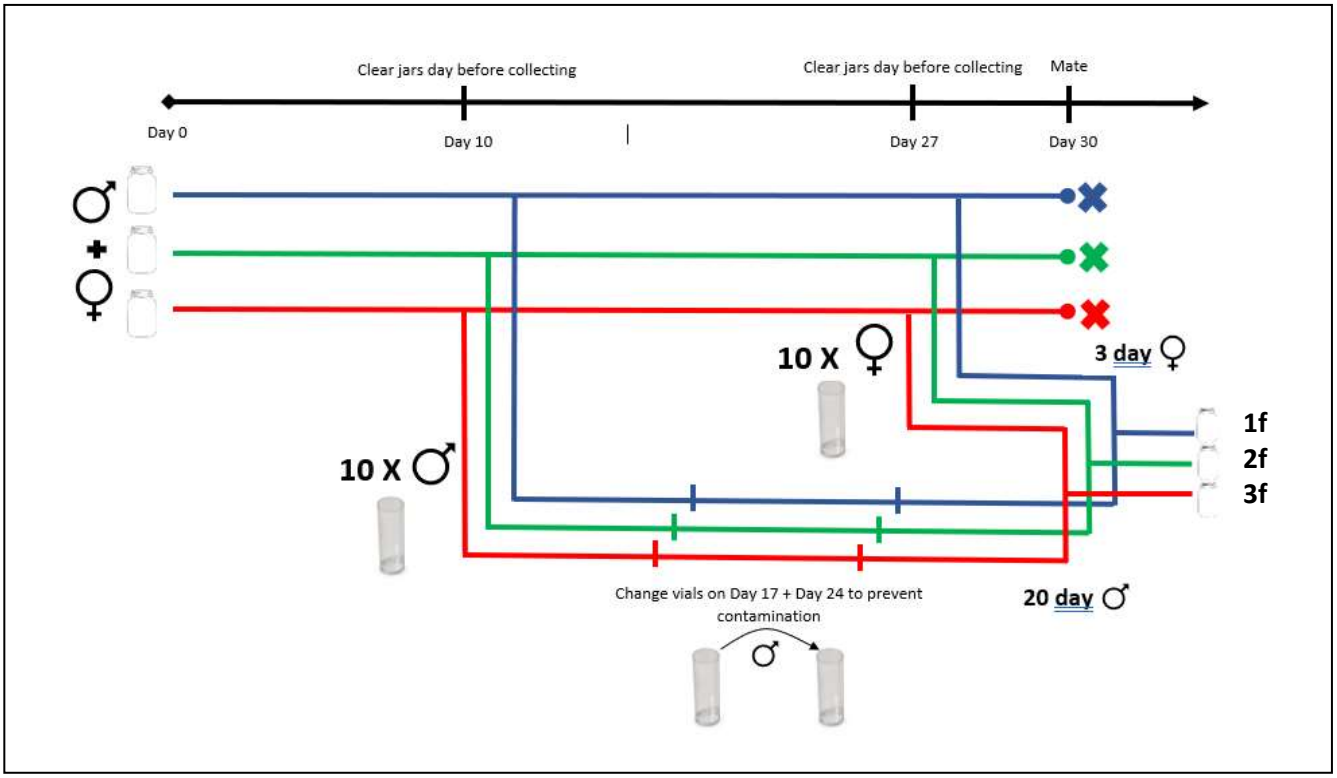

Fig 7. The age-restricted mating scheme used in the experimental populations: younger females mated with older males of *Drosophila melanogaster*.

Measurement of fecundity and fertility

To understand how the long-term selective mating protocol affected the fecundity and fertility of the fruit flies due to sex-limited aging before mating, we observed the ability of the

females to produce eggs and offspring when mated, at two different timepoints during the selection (at generations 20 and 24), using different mates.

For the 20th generation, virgin individuals were sexed immediately after eclosion using CO_2 and kept individually in food-filled vials for 20 days. On the 20th day post-eclosion, one experimental aged male fly and one young female fly from the same line were introduced into a vial and allowed to mate. Mirroring the long-term selective mating experiment conditions, the male fly was removed from the vial after three days and the female was left to oviposit. Females were transferred into a fresh vial every 24 hours to prevent larval overcrowding. The number of eggs laid after each 24-hour period was counted and used as a measure of fecundity. From vials produced every third day, the number of offspring eclosed was tallied. Fertility was calculated as the proportion of eggs produced and eclosed (hatched) into viable offspring per day. This was used as a measure of fertility. A reciprocal experiment was concurrently conducted where one experimental aged male was mated with a young female of the same line; the same procedure was applied. The three replicates of each mating scheme were tested in this manner. Two different controls were used at the two testing timepoints. For the 20th generation, wild type *D. melanogaster* from a diversified base population were sexed post-eclosion using CO_2 anesthesia. Both sexes (female and male) were kept individually in separate food-filled vials to be aged for 20 days, after which they were mated for a 3-day period. On the third day, males were removed, and females to oviposit, and fecundity and fertility were measured as described above. For the 24th generation, only one experimental population and one control population were used. At this point, the experimental young females were mated with aged control fertile males. Virgin males from the control group were aged for 20 days. On the 20th day, virgin females from the experimental young female lines were mated with 20-day-aged wildtype males. The control population was slightly

different from the 20th generation and was created by mirroring the experimental population method. Virgin males from the wildtype were sexed post-eclosion and aged for 20 days. On the 20th day, virgin females from the same line were mated with 20-day-aged males for 3 days. Once the male was discarded, fecundity and fertility were measured and tallied with the experimental populations.

All tallies were made within a 20-day observation period that began after the 3-day mating period. Flies that died within the observation period were omitted from calculations. Student's *t*-test or ANOVA was used to test for significant differences among means within and across populations ($\alpha = 0.05$).

Measurement of lifespan

A total of 900 virgin flies—450 males and 450 females with 300 (150 males and 150 females) in each population (Control; fM Experimental; Fm 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 fM and Fm experimental populations, 50 male and 50 female flies were collected separately from three replicates of fM (1f, 2f, and 3f) and Fm (1m, 2m, and 3m) experimental populations, respectively. All flies were collected in 250-mL fresh food–filled jars. The flies used in the control and experimental populations were all virgins and had never 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 was 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 were not much exposed to CO_2.) Flies were transferred into the fresh food–filled jars every

third day. A strict 12-hour dark and light cycle was followed throughout the experiment, and all flies were kept at room temperature, 22◦C.

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

Measurement of Ovariole number and stage-14 egg chambers

Virgin females from experimental and control populations were collected post-eclosion in vials (with food) separately. Every third day, three females from each experimental (Fm and Fm) replicate and two from the control populations were sacrificed on a concave slide to observe stage-14 egg chambers and the number of ovarioles using a dissecting stereomicroscope. Egg chambers with developed dorsal appendages considered as stage-14 (mature) egg chambers. 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 to identify and count individual ovarioles, 1 μL of crystal violet stain was added to the sample. The number of eggs with dorsal appendages (stage-14 eggs) was counted and recorded from the left and right ovary. The average of the left and right ovaries was used as the stage-14 egg count per female.

A total of 200 females were sacrificed to count the ovarioles and stage-14 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-14 egg chambers between the experimental and control populations and within the experimental populations using PRISM 6.0 software.

Measurement of Testis Size Using an Ocular Micrometer

Testis size from the control and two experimental populations was measured using an ocular micrometer under a compound light microscope to obtain measurements for both testis length and width. The ocular micrometer was calibrated using a stage micrometer to obtain the measurements in millimetres. Using a dissection microscope under 2x magnification, testes were dissected from *D. melanogaster* in phosphate buffered solution (PBS) on a concave slide by using forceps to pull the testes from the lower body. Individual testes from each population were isolated and placed on glass slides. Each testis was stretched using fine forceps to straighten the coiling, viewed under a compound light microscope with 10x magnification and measured by aligning the end of the testis with the ocular micrometer. The width of the testis was taken at the midpoint, by dividing the length by 2, and measuring the width at that calculated length. The mean testis length and width per fly were calculated.

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Competing Interest

Authors declare no competing interest.

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