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Testing Whether Male Age or High Nutrition Causes the Cessation of Reproductive Aging in Female *Drosophila melanogaster* Populations

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ABSTRACT

Fecundity seems to stop declining and plateaus at low levels very late in *Drosophila melanogaster* populations. Here we test whether this apparent cessation of reproductive aging by a population, herein referred to as fecundity plateaus, is robust under various environmental influences: namely, male age and nutrition. The effect of male age on late age fecundity patterns was tested by supplying older females with young males before average population fecundity declined to plateau levels. The second possible environmental influence we tested was nutrition and whether late-life fecundity plateaus arise from a decline in the calories available for reproduction. This hypothesis was tested by comparing average daily female fecundity with both low- and high-lifetime nutrition. Both hypotheses were tested by measuring mid- and late-life fecundity for each cohort under the various environmental influences, and statistically testing whether fecundity stops declining and plateaus at late ages. These experiments demonstrate that mid- and late-life population fecundity patterns are significantly affected by the age of males and nutrition level. However, male age and nutrition level did not affect the existence of late-life fecundity plateaus, which demonstrates the robustness of our earlier findings. These results do not address any issue pertaining to the possible role, if any, of lifelong inter-individual heterogeneity in *Drosophila* fecundity.

INTRODUCTION

MORTALITY RATES decelerate and "plateau" late in life in *Drosophila*, medflies, wasps, yeast, nematodes, and humans.[1–13] There is no widely accepted explanation for these plateaus, although several theories have been proposed.[9,14–20]

Similar to the plateauing of late-life mortality-rates, the decline in average population fecundity has been shown to decelerate and plateau at late ages in several large cohorts of *Drosophila melanogaster*.[21] This observation was based on age-specific population fecundity data obtained from large starting cohorts and not on individual female fecundity patterns. Therefore, it should be understood that the plateauing in late-life fecundity that we observed is a characteristic of the population, analogous to mortality-rate plateaus, and not necessarily characteristic of individual female fecundity patterns.

It is conceivable these late-life fecundity plateaus were generated by heterogeneity in in-

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dividual female fecundity, with the high egg-layers dying before the onset of the plateau. However, we have data from over 2,800 individual females, not presented here, that suggest otherwise.

In this study, we test two plausible explanations for late-life fecundity plateaus using *D. melanogaster*. These hypotheses are that (1) male sexual inadequacy attenuates female fecundity in such a way that a spurious plateau is created, or that (2) a physiological decline in female reproductive ability related to high nutrition attenuates female fecundity in a manner that is inconsistent and spurious. Although these possible explanations are not exhaustive, eliminating them as causes is essential in determining whether late-life fecundity plateaus are a robust population phenomenon. In addition, this study is the first of its kind to look at the effect of male age and nutrition on late-age fecundity patterns.

We chose to test male age and nutrition, because male and female cohorts were handled in parallel and provided high nutrition in the initial study that established the existence of late-life population fecundity plateaus.[21] This experimental design resulted in a supply of older mates for older females, raising the prospect that older males may have limited female fecundity at later ages due to diminished sexual function associated with aging. In addition, sustained high nutrition might have resulted in a physiological decline in female reproductive ability, also limiting late age fecundity.

Various components of male sexual function in *Drosophila* decline with age, including overall mating success.[22–27] Thus, it is easy to imagine that the decline in average population fecundity with female age, and even the plateau itself, may be a result of an age-related decline in the sexual performance of males.

If older males cause late-life fecundity plateaus, then supplying younger mates to older females should either delay the onset of the fecundity plateau, until the new mates become old, or obliterate the plateau altogether, because few females would survive to late enough ages for the plateau to be observable. Therefore, the supply of young males to females in mid-life should cause the population fecundity plateau to disappear. On the other hand, if older males do not cause fecundity plateaus, then we should still observe a population fecundity plateau when old females are in the presence of young males. This plateau might occur earlier or later, because of the physiological effects of supplying older females with younger mates.

Fecundity is known to be highly responsive to nutrition. Egg laying, utilization of sperm, mating frequency, and vitellogenesis all increase with increasing nutrition in *Drosophila*.[28–31] Therefore, it is conceivable that lifelong high nutrition increased early reproduction, resulting in late-age reproductive exhaustion in Rauser et al's study.[21]

If high nutrition causes population fecundity to plateau at late ages due to physiological shortfalls resulting from high reproductive output at earlier ages, then supplying flies with low nutrition should eliminate the plateau altogether. On the other hand, if nutrition does not affect the existence of fecundity plateaus, then we should observe a plateau in the population fecundity of females given low nutrition.

This study tests the robustness of our earlier finding that average population fecundity plateaus at late ages by manipulating various environmental components. In addition, this is the first study of the effects of male age and nutrition on average population fecundity at late ages. We are testing both the effects of male age and nutrition on late-age population fecundity, and specifically whether fecundity plateaus continue to arise as these extrinsic environmental components are experimentally varied.

MATERIALS AND METHODS

Experimental populations

We used replicated laboratory-selected populations of *D. melanogaster* derived from the South Amherst, Massachusetts, IVES population (IV),[32] collected from the wild in 1975. The IV population served as the ancestral population of the five replicate O populations (having subscripts 1–5) in 1980, which were cultured using females of increasingly greater ages until females had to attain 70 days of age from

egg.[33] In 1989, five corresponding CO populations were derived from the five O populations and have latterly been cultured using females 28 days of age.[34] These populations have been maintained at effective population sizes of at least 1000 individuals and cultured as described above for at least 100 generations at the time of the assays. Replicate populations CO_1, CO_2, and CO_3 were employed to test both hypotheses.

General culture and assay methods

All flies used in the fecundity assays were raised as larvae in 5 mL of standard banana-molasses food at densities of 60–80 eggs per 8-dram vial for two generations. During this controlled density rearing, the CO_i populations were reared using a 2-week generation time and kept in incubators at 25°C and constant illumination.

During each assay, adults were kept in 5-mL food vials containing charcoal colored medium, so that eggs could easily be counted using a dissecting scope. Each vial contained varying amounts of yeast, depending on the treatment. At the beginning of each assay, four females and four males, age 12 days from egg (all reported ages are days from egg), were placed in each vial and transferred to fresh vials daily so that eggs could be counted. Egg counting began at age 29 days from 50 randomly selected vials from each treatment within each replicate population until the number of vials fell below 50 at which time eggs from all remaining vials were counted. We did not count eggs at earlier ages because we were only concerned with the effects of male age and nutrition on mid- and late-life fecundity patterns, as the effects on earlier patterns are well established. Flies from different vials were combined daily as mortality occurred to forestall any age-dependent density effects.[35–40]

All assays started with 3,200 females per replicate population, and as many males. Half of each population was assigned one treatment and the other half served as the control. The treatment of each control population was the same for all assays and both experiments. Males and females were the same age throughout each assay and the amount of yeast within each vial was 5 mg, or a high yeast concentration, similar to the protocol used in our assays that first demonstrated the existence of late-life population fecundity plateaus.[21] Each fecundity assay continued until all flies were dead.

Male age assays

All procedures were as described above until age 40 days, when males in half of the vials were removed and replaced with young (age 14 days from egg) males to test for the effect of male age on late-life plateaus in fecundity. The introduction of young males at age 40 days occurred during the decline in mid-life female fecundity and sometime before the onset of the late-life fecundity plateau.

Nutrition assays

All procedures were as described above except that half of each population was fed a low yeast concentration (0.2 mg per vial) while the other half of the population was fed a high yeast concentration (5.0 mg per vial) throughout each assay, following the procedures described in Chippindale et al.[28]

Modeling population fecundity at late ages

To assess the effects of males and nutrition on the existence of late-life population fecundity plateaus in *Drosophila*, we utilized a simple model of this process. The model we fit to mid- and late-life fecundity data was a three-parameter, two-stage linear model, having a second stage slope of zero, analogous to the two-stage models fit to our mortality[11,12] and fecundity data.[21] The first stage of this model is only meant to represent the mid-life decline in population fecundity just prior to the plateau, and so the start of this stage may vary according to experimental treatment. We chose this model *a priori* and used it to experimentally test the assumption that population fecundity plateaus at late ages by statistically testing the fit of our data to the model. Under the two-stage model the fecundity at aged t-days is

$$\begin{cases} \phi_1 + \phi_2 t & \text{if } t \le \phi_3 \\ \phi_1 + \phi_2 \phi_3 & \text{if } t > \phi_3 \end{cases} \quad (1)$$

This nonlinear regression model was fit to the fecundity data using nonlinear least-squares in

the R-project for statistical computing ⟨www.R-project.org⟩. We wrote a self-starting R-function for the two-stage linear model that provided initial estimates for the parameter values as well as the predicted fecundity from equation (1).

Statistical tests

Each experiment was conducted with three replicate CO populations. Although these populations are maintained under identical selection conditions, they are genetically independent. Fecundity in each CO population was measured under two environmental conditions in each experiment: old and young males and high and low nutrition. Assays separated in time will vary due to uncontrolled aspects of the environment, which can be considered block effects. In these experiments, the random effects due to different populations is completely confounded with the random block effects. For our purposes it is not important to disentangle these two sources of random variation.

In our statistical model, we let i indicate one of the three populations, and j indicate one observation within a population. The smallest unit of observation is the number of eggs produced by four females in one vial. Individual female fecundity was not measured because we are testing whether plateaus exist at late ages under various environmental conditions, which is a population, rather than an individual, phenotype. However, we present the results from individual vials as the average fecundity per female. Each population has a total of n_i observations that are split between the two experimental treatments. We assume that observations 1 up to m_i are either the old male or high nutrition treatments, depending on which experiment we are considering. Observations m_i+1 up to n_i are then the young male or low nutrition treatments. The number of eggs per female in population-i, observation-j, is y_{ij}. The basic nonlinear model is given by

$$y_{ij} = f(\phi_i,\nu_{ij}) + \varepsilon_{ij}$$

where ϕ_i is the vector of parameters, ν_{ij}, is the covariate vector, and ε_{ij} is the within population variation. The covariate vector contains the age of the females in the jth observation, t_{ij}, and the experimental treatment, δ_{ij}, where δ_{ij} is zero if $j \leq m_i$, and 1 otherwise.

For the two-stage linear model, the functional relationship is

$$f(\phi_i,\nu_{ij}) = \begin{cases} \phi_{1i} + \phi_{2i}t_{ij} & \text{if } t_{ij} \leq \phi_{3i} \\ \phi_{1i} + \phi_{2i}\phi_{3i} & \text{if } t_{ij} > \phi_{3i} \end{cases}$$

We assume that both fixed and random effects may affect the values of the model parameters. The magnitude of the fixed effects, either male age or nutrition, can be examined to determine if they have a significant effect on age-specific fecundity. The parameters are also assumed to vary randomly between the population/blocks as described previously. These assumptions translate into the system of equations:

$$\begin{aligned} \phi_{1i} &= \beta_1 + \gamma_1\delta_{ij} + b_{1i} \\ \phi_{2i} &= \beta_2 + \gamma_2\delta_{ij} + b_{2i} \\ \phi_{3i} &= \beta_3 + \gamma_3\delta_{ij} + b_{3i} \end{aligned} \qquad \text{(2a–c)}$$

where the γ_k ($k = 1 - 3$) are the fixed effects due to either male age or nutrition, and the b_{ki} are the random population/block effects. An important statistical test will be to determine if the γ_k are significantly different from zero. If so, this will indicate that the experimental treatment has a statistically significant effect on the regression model parameter.

The average number of eggs laid decreases substantially with age in these populations. Therefore, we should model within population variance as a function of mean fecundity. The general formulation is

$$Var(\varepsilon_{ij}) \cong \sigma^2 g^2(\hat{u}_{ij},\nu_{ij},\delta)$$

where $\hat{u}_{ij} = E(y_{ij}|\mathbf{b}_i)$. In this analysis, we used $g(.) = |y_{ij}|^\delta$, where δ is estimated from the data. The $\mathbf{b}_i$ were assumed to be distributed as

$$\mathbf{b}_i \sim N\left(\mathbf{0}, \begin{bmatrix} \Psi_{11} & 0 & 0 \\ 0 & \Psi_{22} & 0 \\ 0 & 0 & \Psi_{33} \end{bmatrix}\right)$$

The maximum likelihood techniques used to estimate the model parameters and test their significance are reviewed in Pinheiro and Bates,[41] and implemented with the non-linear mixed effects package in R (version 1.8).

Since the plateau height is a nonlinear function of the underlying parameters of model (1), it is not readily tested with the methods described

above. However, we want to test whether the height of the fecundity plateau is significantly different from zero for each of the four treatments, so a model, identical to equations (2a–c), with no γ parameter, was estimated from the three replicate populations for each treatment. The parameter estimates were used to determine the height of the late-life fecundity plateau for a given treatment by the following equation:

$$\hat{\beta}_4 = \hat{\beta}_1 + \hat{\beta}_2\hat{\beta}_3 \quad (3)$$

The variance of $\hat{\beta}_4$ was estimated from the delta method as follows:[42]

$$Var(\hat{\beta}_4) = Var(\hat{\beta}_1) + \hat{\beta}_3^2 Var(\hat{\beta}_2) + \hat{\beta}_2^2 Var(\hat{\beta}_3) + 2\hat{\beta}_3 Cov(\hat{\beta}_1\hat{\beta}_2) + 2\hat{\beta}_2 Cov(\hat{\beta}_1\hat{\beta}_3) + 2\hat{\beta}_2\hat{\beta}_3 Cov(\hat{\beta}_2\hat{\beta}_3) \quad (4)$$

Asymptotic 95% confidence intervals on the plateau height, $\hat{\beta}_4$, were estimated as, $\hat{\beta}_4 \pm 1.96\sqrt{Var(\hat{\beta}_4)}$. The variances and covariances in equation (4) were estimated from the maximum likelihood procedure used in R.

RESULTS

Older males do not cause average population fecundity plateaus

Supplying females with young males before average population fecundity declined to plateau levels did not eliminate late-life plateauing in fecundity (Fig. 1). The height of the late-life fecundity plateau, along with the 95% confidence intervals, was determined for both treatments (Table 1), and the number of eggs per female per day after the break day, was significantly greater than zero (Fig. 2). This result demonstrates that fecundity plateaus at late ages regardless of male age.

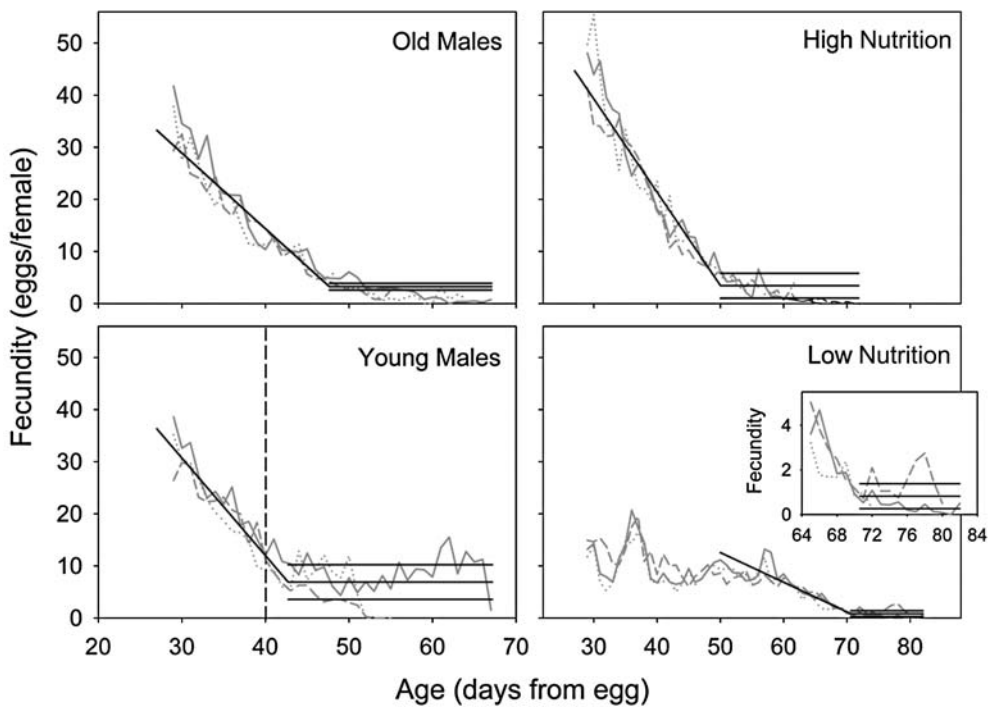

FIG. 1. Mean mid- and late-life fecundity as a function of age for each of the CO_{1-3} populations for each of four treatments. In the "Old males" treatment, males are the same age as females, and in the "Young males" treatment, old males are replaced by young males at age 40 days (indicated by the vertical dashed line). In the "High nutrition" treatment, flies were provided 5.0 mg yeast/vial, while in the "Low nutrition" treatment, flies were provided 0.2 mg yeast/vial throughout each assay. Fecundity was measured simultaneously in each CO_i population within each experiment (male age or nutrition). The two-stage linear model was estimated for the three replicate populations within each treatment, along with the 95% c.i. for the "plateau" stage of the model. For all treatments, the plateau height was significantly greater than zero. Age was measured in days from egg, and fecundity was measured as the number of eggs/female/day. The insert within the low nutrition graph depicts the fecundity pattern after age 65 days.

TABLE 1. PARAMETER ESTIMATES FROM THE TWO-STAGE LINEAR MODEL FIT TO MID- AND LATE-LIFE FECUNDITY DATA FROM EACH OF THE CO POPULATIONS FOR ALL TREATMENTS

Population	*1st-stage y-intercept* (φ_1)	*1st-stage slope* (φ_2)	*Break-day* (φ_3)	*Population*	*1st-stage y-intercept* (φ_1)	*1st-stage slope* (φ_2)	*Break-day* (φ_3)
Old males				High nutrition			
CO_1	92.16	−1.94	45.23	CO_1	106.24	−2.11	48.39
CO_2	77.23	−1.60	46.17	CO_2	113.00	−2.30	46.90
CO_3	70.41	−1.42	48.09	CO_3	91.35	−1.80	48.57
Young males				Low nutrition			
CO_1	88.61	−1.86	43.23	CO_1	46.17	−0.64	70.98
CO_2	101.21	−2.34	39.67	CO_2	39.63	−0.55	70.42
CO_3	76.79	−1.61	46.12	CO_3	29.25	−0.38	72.65

The model was fit by non-linear least squares regression.

Parameter estimates for φ_1, φ_2, and φ_3, were all significantly different than zero; $p < 0.0001$ for each of the three populations under all treatments.

Although our analysis suggests that fecundity plateaus exist regardless of the age of the mate, all of the parameter estimates from the two-stage linear model differed significantly between the two treatments (Eq. 2a–c; $p < 0.0001$; Table 2). The *y*-intercept and the slope of the first stage of the two-stage model and the breakday, or the start of the fecundity plateau, were all significantly different between the two treatments. Our results indicate that the addition of young males resulted in a more rapid onset of the fecundity plateau (Table 2). The earlier onset of this plateau may explain the increased height of the fecundity plateau (β_4) in the young males treatment (Table 2). That is, young males caused fecundity to stop declining at an earlier age than older males, resulting in a greater number of eggs per female per day during the plateau.

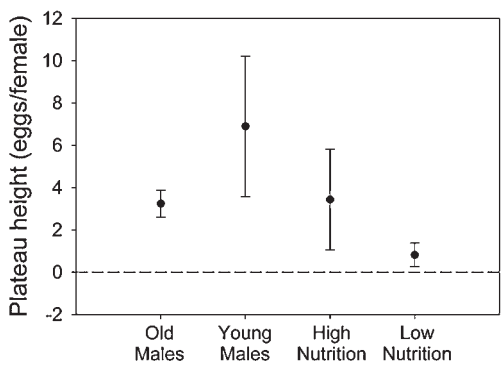

FIG. 2. Plateau height for each of the four treatments along with the 95% c.i. value. Plateau height was significantly greater than zero, regardless of male age and nutrition level. This result suggests that average population female fecundity plateaus at late ages and that the existence of these late-life plateaus are not affected by the age of mates or nutrition level. Plateau height was measured in number of eggs/female/day.

High nutrition does not cause average population fecundity plateaus

Low lifetime yeast levels (0.2 mg/vial) also did not eliminate late-life plateauing in fecundity (Fig. 1). The height of the late-life fecundity plateau was determined for both treatments, along with the 95% confidence intervals (Table 1), and was significantly greater than zero in the presence of high and low nutrition (Fig. 2). Similar to the young males experiment, fecundity plateaus existed regardless of nutrition level at some number of eggs greater than zero (Fig. 1).

Although plateaus occurred regardless of nutrition level in these cohorts, all of the parameter estimates from the two-stage linear model differed significantly between the two treatments (Eq. 2a–c; $p < 0.0001$; Table 2). Fecundity was lower under low nutrition compared to high nutrition at all ages, including ages after the onset of the plateau, and declined at a much slower rate under low nutrition compared to high nutrition (ϕ_2, Table 2). The start of the late-life fecundity plateau (ϕ_3) was much later with low nutrition compared to the high nutrition treatment and the height of the low nutrition plateau was also significantly lower compared to the high nutrition treatment

Table 2. Results from Our Experiments Testing the Effect of Male Age and Nutrition Level on Late-Life Fecundity Using a Comparison Between Females Exposed to Old or Young Males at Late Ages and High or Low Nutrition

	Treatment		*Treatment*	
	Old males	*Young males*	*High nutrition*	*Low nutrition*
Sample size (x,y-values)	3,208	2,649	4,446	1,919
1st-stage y-intercept (φ_1)	72.15	87.65[a]	90.29	40.54[a]
1st-stage slope (φ_2)	−1.44	−1.89[a]	−1.72	−0.56[a]
Break day (φ_3)	47.77	42.54[a]	50.62	70.73[a]
Plateau height (β_4) (eggs/female/day)	3.24	6.89[b]	3.43	0.82[b]

Plateau height was computed from equation (3). The x,y-values used in the regression were from the 50 vials (200 females) randomly sampled daily from each treatment having an initial population size of 1,200 vials (4,800 females). The non-linear mixed effects model was fit by maximum likelihood.

[a] $p < 0.0001$.

[b] $p < 0.05$.

(Table 2). The later onset of the plateau under low nutrition may explain the decreased height of the fecundity plateau. That is, low nutrition caused fecundity to stop declining at a later age, resulting in a lower number of eggs per female per day at plateau ages.

DISCUSSION

Younger males should have delayed the onset of late-life population fecundity plateaus or eliminated them all together if older males somehow limit fecundity at late ages and cause late-life plateaus. However, we concluded that population fecundity plateaus with both old and young male consorts. Although male age affects late-life female fecundity, it does not *cause* late-life fecundity plateaus.

The effect of young mates on the fecundity of old females is, however, interesting in itself. The earlier breakday observed in the young-males treatment suggests that young males may have stopped fecundity in late-age females from declining further, thereby forcing the average population fecundity to plateau prematurely, resulting in the observed increase in plateau height. So, to some extent, older males limit late-age fecundity, but not enough to affect the occurrence of late-life fecundity plateaus in the population. Young males are able to increase average population fecundity, but not to early-life levels.

Young males might have this effect because they may be able to transfer more sperm and accessory gland proteins during copulation, compared to older males. Increased female egg production in turn may cause a greater deterioration in the physiological condition of older females mated to young males. Therefore, it is expected that older females supplied young males will have some sort of immediate physiological response similar to the response we observed. That is, their egg production should immediately increase during these initial matings with young males, leading to the increased female death rate that we observed in two of the three populations.[43,44] However, this increased death rate may simply be a result of excessive harassment from the young males compared to their older contemporaries.[45]

In Rauser et al.'s study,[21] female cohorts were provided high levels of nutrition throughout each assay, raising the possibility that population fecundity declined and plateaued due to a physiological decline in reproductive output resulting from an age-related decline in female feeding and digestion. However, we determined that population fecundity plateaus still exist when females are supplied with low nutrition (Fig. 2). This result strongly suggests that nutrition level does not have an effect on the existence of late-life fecundity plateaus.

Although nutrition level did not affect the existence of the late-age population fecundity plateau, low nutrition had a significant effect

on the rate at which fecundity declined in mid-life, the timing of the start of the fecundity plateau, and the height of the plateau (Table 2). These results are consistent with dietary restriction and other nutrition studies, which have shown that low nutrition results in decreased daily and lifetime fecundity in *Drosophila*.[28,30,31,46,47] This decrease in fecundity resulting from low nutrition may be due to a slowing in ovarian maturation[31,48] and/or a decrease in the proliferation rates of germline and somatic stem cells,[49] which in turn retards egg production.

When nutrition levels were low, we observed a smaller difference between early and late reproduction, which may explain why fecundity declined at a slower rate in mid-life and plateaued at a later age and a lower height. The physiological investment in fecundity was apparently much lower with low nutrition at early ages, which allowed the prolongation of egg production before the slow decline and later plateau onset. This presumably occurs because the small investment in earlier reproduction allows for a larger investment in later reproduction, compared to high nutrition females, and an increase in lifespan.[50]

Despite the subtle effects these environmental treatments had on late-age population fecundity patterns, neither the age of males supplied to older females, nor the level of lifetime nutrition affected the occurrence of late-life fecundity plateaus. In all experimental treatments, the decline in average population fecundity slowed and plateaued at late ages at a low number of eggs per female. This population-level plateau is analogous to late-life mortality-rate plateaus, and its existence does not appear to be affected by these particular environmental manipulations. Therefore, our original discovery of late-age population fecundity plateaus is robust.

Although we are able to eliminate these possible environmental explanations as causes, there are other possible explanations for observing late-age plateaus in population fecundity. In addition, observing plateaus in average population fecundity does not preclude the idea that individual female fecundity declines to zero just prior to death, as found by Novoseltvev et al.[51] The individual females that comprise the population will die at various ages, and thus will have fecundities decline to zero at various ages. Thus, one can imagine that in an infinite population, or in very large cohorts, there will always be a number of females who will die and reproduce later than other females at very late ages. This pattern of individual deaths and fecundities at late ages will create an average population fecundity at some number of eggs greater than zero.

Our experimental tests have implicitly assumed that either old males or high nutrition may have been responsible for the appearance of late-life plateaus. However, it is possible, in principle, that both may contribute to the appearance of plateaus. How would our experimental results be affected by the dual action of old males and high nutrition? We would expect that the elimination of just one of the causes of the late-life fecundity plateaus would result in a weakening, but not complete removal, of the plateau. In the experiment in which we added young males, the plateau is in fact earlier and just as long, so there is no indication of it being diminished. In the case of low nutrition, the onset of the plateau is extended but so is the entire lifespan, thus the length of the plateau is approximately the same duration. We conclude that our results are inconsistent with either old males or high nutrition being the cause of the late life plateaus nor are the results consistent with joint action of these two factors as a cause of the plateaus.

These fecundity results are comparable to the finding that mortality rates plateau. Together, they suggest that aging is not an implacable wall of death, inexorably destroying the organism. Instead, aging is demographically a transition between two periods of relatively stable mortality and fecundity levels, which may render it more amenable to eventual control.

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