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WITHIN-POPULATION VARIATION IN CYTOPLASMIC GENES AFFECTS FEMALE LIFE SPAN AND AGING IN *DROSOPHILA MELANOGASTER*

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Abstract.—It has been suggested that mitochondrial DNA (mtDNA) may play an important role in aging. Yet, few empirical studies have tested this hypothesis, partly because the degree of sequence polymorphism in mtDNA is assumed to be low. However, low sequence variation may not necessarily translate into low phenotypic variation. Here, we report an experiment that tests whether there is within-population variation in cytoplasmic genes for female longevity and senescence. To achieve this, we randomly selected 25 ''mitochondrial founders'' from a single, panmictic population of *Drosophila melanogaster* and used these founders to generate distinct ''mt'' lines in which we controlled for the nuclear background by successive backcrossing. Potential confounding effects of cytoplasmically transmitted bacteria were eliminated by tetracycline treatment. The mt lines were then assayed for differences in longevity, Gompertz intercept (frailty), and demographic rate of change in mortality with age (rate-of-senescence) in females. We found significant cytoplasmic effects on all three variables. This provides evidence that genetic variation in cytoplasmic genes, presumably mtDNA, contributes to variation in female mortality and aging.

Key words.—Aging, cytoplasmic, *Drosophila melanogaster*, female, Gompertz, life span, mitochondria, mtDNA, senescence.

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Genetic variation in the mitochondrial genome has traditionally been considered selectively neutral. Consequently, sequence divergence in mitochondrial DNA (mtDNA) has been used extensively as a tool to infer evolutionary histories, with the assumption that mtDNA evolves solely by accumulating neutral mutations (see Gemmel et al. 2004; Ballard and Rand 2005). However, this traditional view of the mitochondrial genome is currently changing. In fact, non-neutral evolution of mtDNA has now been implied in several recent studies demonstrating that specific cytoplasmic gene products exhibit reduced or altered function when coexpressed in nuclear genomes derived from different populations (reviewed in Rand 2001; Rand et al. 2004; Ballard and Rand 2005). Thus, mtDNA is likely to play a role in adaptive evolution. However, our knowledge of its influence on phenotypic variation is still very incomplete (for recent reviews see Rand et al. 2004; Ballard and Whitlock 2004; Gemmel et al. 2004; Ballard and Rand 2005).

Mitochondrial DNA has also been suggested to play an important role in the evolution of aging (Harman 1956; Tanaka et al. 1998; de Benedicts et al. 2000; Niemi et al. 2005; reviewed in Ballard and Whitlock 2004). Products of mtDNA clearly have a profound role in energy metabolism. Because variation in metabolic rate may be associated with variation in life span (e.g., Sacher 1977), genetic variation in mtDNA could conceivably affect both metabolism and aging (Beckman and Ames 1998; Speakman 2005). Currently, empirical evidence for an association between metabolic rate and aging is limited (Brand 2000; Speakman et al. 2004). Khazaeli et al. (2005) found no relationship between metabolic rate and survival in *Drosophila melanogaster* lines artificially selected for increased life span. However, the results of studies directly testing effects of mtDNA mutations on aging have provided strong support for the mitochondrial aging hypothesis. First, Trifunovic et al. (2004) created an ''mtDNA mutator'' mice phenotype that exhibited an increase in the level of somatic mtDNA mutations. This manipulation resulted in reduced life span, as well as in an earlier onset of several aging-related phenotypes, including reduced body mass, reduced fertility, and hair loss. Kujoth et al. (2005) employed a similar technique to reveal a link between accumulation of mtDNA mutations and aging. These two studies provide evidence for the effect of mtDNA mutations on aging and support the possibility that variation in mitochondrial genes affects the aging process. In line with the results of experiments that employed mtDNA mutators, James and Ballard (2003) recently found evidence that interpopulation variation in mtDNA haplotypes was related to variation in life span in *D. simulans*.

A necessary criterion for mitochondria to contribute to variation in aging within populations is that sequence polymorphisms exist at this level. Some early theoretical work suggested that within-population variation in mtDNA for fitness will be low, due to fast fixation/purging of adaptive/deleterious mutations (Takahata 1984; Clark 1984). Later work has shown that mtDNA polymorphisms may nevertheless be maintained by interactions with nuclear genes. Although some models suggest that fairly restricted conditions (sex-specific viability selection and/or frequency-dependent selection) are required to maintain mtDNA polymorphism through such mitonuclear fitness interactions (Clark 1984; Gregorius and Ross 1984), work by Rand et al. (2001) has shown that mitonuclear fitness interactions should be more potent in maintaining mtDNA polymorphism if the nuclear genes involved are located on the X chromosome.

Here, we present the results of an explicit test for within-population cytoplasmic variation in senescence and life span, across 25 randomly sampled cytoplasmic lines from a single outbred population of *D. melanogaster*. We find cytoplasmic genetic variation for longevity as well as age-specific mor-

tality rates (frailty and the rate-of-senescence). Our results provide support for the hypothesis that mtDNA may directly affect variation in aging.

Materials and Methods

Stock Flies

Flies used in this experiment came from LH_M, a large outbred laboratory population (>1700 flies/generation) of *D. melanogaster*. This population is cultured on a discrete 14-day generation cycle (for a detailed description of this population and its culturing protocol see Chippindale et al. 2001). Flies and larvae were reared in 10-dram vials containing 10 ml cornmeal-molasses-killed-yeast medium. Adult flies were also fed live yeast. Both larval and adult numbers were regulated to moderate densities with 150–200 larvae per juvenile-competition vial and 16 pairs of adults per adult-competition vial. Flies were reared at 25°C, on a 12:12h light: dark cycle. At the start of these experiments, the population had adapted to this laboratory environment for more than 300 generations.

Construction of ''mt'' Lines

Cytoplasms were sampled from the laboratory population by randomly collecting 25 mated females. Each female was essentially a mitochondrial founder, used to found a separate ''mt'' line that was fixed for her mito-/cytotype. To identify genetic variation among these mt lines, it was necessary to disassociate each sampled cytoplasm from the nuclear genetic background with which it was originally associated. Taking advantage of the strict maternal transmission of cytoplasmic DNA in *D. melanogaster*, this was achieved by backcrossing the female offspring of each line with males that were randomly sampled from the outbred stock population for 27 generations, successively increasing population size. For the first 20 generations of backcrossing, eight daughters were collected from each line and mated individually to eight males from the stock population. From generation 21 to 25, numbers used for backcrosses were increased to a mean of 70 daughters (range: 42–100) per line mass-mated to 50 stock males. At generation 26, we created five vials per line with 30 daughters and 20 stock males per each vial. In generation 28, 160 daughters per line were then backcrossed to 160 stock males. From that point and onward all lines were closed and maintained as separate populations of approximately 320 individuals per line. This provided us with 25 mt lines, each with a specific cytotype expressed in many different nuclear genetic backgrounds representing the LH_M population. According to diagnostic polymerase chain reaction (PCR) conducted at generation 15, all lines were free from *Wolbachia* infection. Nonetheless, at generation 26, all lines were treated with tetracycline hydrochloride to ensure they were uninfected with cytoplasmically transmitted bacteria such as *Wolbachia*. The survival assay was conducted four generations after the lines were closed (i.e., six generations after tetracycline treatment).

Daughters from the mt lines were not collected immediately upon hatching, during the introgression process. Many females were therefore already mated (some to their brothers) before being introduced to random LH_M males. In effect, this slowed down the replacement rate of the nuclear background originally associated with each mitochondrial founder. However, remating rates are high in this population and range from 55 to 98% (W. R. Rice, pers. comm.). Sperm displacement is also high. The average value reported for *D. melanogaster* is 93% (Simmons 2001) and 85% for the LH_M population (William Rice, pers. comm.). Sperm displacement was also measured in this population in another experiment that was conducted at the same time as this one, and was found to be 81% (U. Friberg, D. K. Dowling, unpubl. data). To calculate the percent of the original nuclear genetic background replaced after the 27 generations of backcrossing, we conservatively assumed that all females first mated to a brother, remated to a random LH_M male with 55% probability and, if so, had 80% of their sperm replaced. This reduces the replacement rate to 22% per generation rather than 50%. Assuming all mitochondrial founders were mated to nonrelatives in the first generation, this predicts that 99.92% of the original nuclear background was replaced during our backcrosses.

Experimental Procedure

We collected 280 virgin females from each line over a period of 30 h. These females were stored in 10 vials with 28 females in each for two to three days. At this point, 42 two- to three-day-old, random LH_M males were added to each vial for exactly 2 h. During this period and under this sex ratio, nearly all females in this population will mate once (Rice 1996). All matings (approximately 7000) were conducted within one single day. The mated females were then transferred to fresh vials (10-dram vials containsing 10 ml of cornmeal-molasses-killed-yeast medium and ~3 mg of live yeast). For each line there were five vials with 50 females in each. Each mating vial thus contained three surplus females ($[28 - 3] \times 2 = 50$) that were used to cover unforeseen losses due to injury, escape, or death, if needed during this preliminary stage. Surplus females were discarded before transfer to the fresh, experimental vials. Every two days, the females were transferred to a fresh vial and the number of dead females was recorded. The food medium is slightly adhesive, so that dead flies stuck to the medium and were usually not transferred to the fresh vial. Healthy flies do not adhere to the surface. Careful efforts were made to ensure that flies that were near death and had become stuck to the food medium were nevertheless transferred to the fresh vial. Dead flies that were transferred to a fresh vial were carefully noted so that they were not counted twice. One vial was accidentally discarded during the experiment, and several flies escaped while transferring, resulting in 124 separate vials and 6104 individual flies scored for age at death. All handling of flies was conducted under light CO_2 anesthesia up to the point where the survival assays started. No anesthesia was used during the survival assays.

Analysis

We used restricted maximum likelihood analysis (PROC MIXED procedure in SAS 9.1; SAS Institute, Cary, NC) to estimate the amount of variation in female life span attrib-

TABLE 1. Mean longevities, Gompertz intercepts and rates-of-senescence with 95% lower and upper confidence intervals for the 25 mt lines used in this study. Note that the confidence intervals are based on five replicates per mt line, each consisting of 50 flies.

Lines	Longevity (95% CI)	Gompertz intercept (95% CI)	Rate of senescence (95% CI)
1	56.51 (53.76–59.27)	1.38×10^{-5} (-1.84×10^{-5}–4.49×10^{-5})	0.181 (0.142–0.221)
2	56.08 (54.13–58.04)	3.91×10^{-5} (-3.24×10^{-5}–11.10×10^{-5})	0.167 (0.099–0.236)
3	55.11 (52.02–58.19)	11.30×10^{-5} (-6.00×10^{-5}–28.60×10^{-5})	0.131 (0.092–0.171)
4	57.47 (53.34–61.61)	3.05×10^{-5} (-1.76×10^{-5}–7.87×10^{-5})	0.156 (0.121–0.191)
5	56.15 (54.49–57.81)	5.78×10^{-5} (-4.68×10^{-5}–1.62×10^{-5})	0.148 (0.103–0.194)
6	58.61 (56.88–60.35)	2.16×10^{-5} (-2.00×10^{-5}–6.33×10^{-5})	0.163 (0.114–0.212)
7	46.37 (46.01–46.74)	18.00×10^{-5} (4.71×10^{-5}–31.30×10^{-5})	0.135 (0.111–0.159)
8	55.66 (52.77–58.55)	1.49×10^{-5} (-0.99×10^{-5}–3.97×10^{-5})	0.168 (0.134–0.202)
9	56.98 (52.67–61.29)	2.56×10^{-5} (-0.05×10^{-5}–5.18×10^{-5})	0.156 (0.119–0.212)
10	57.91 (56.11–59.71)	2.29×10^{-5} (-1.57×10^{-5}–6.16×10^{-5})	0.165 (0.119–0.211)
11	58.81 (55.63–61.98)	3.21×10^{-5} (-1.12×10^{-5}–7.53×10^{-5})	0.151 (0.107–0.195)
12	56.18 (54.80–57.55)	0.18×10^{-5} (-0.04×10^{-5}–0.41×10^{-5})	0.211 (0.163–0.259)
13	56.60 (53.91–59.29)	1.67×10^{-5} (0.12×10^{-5}–3.21×10^{-5})	0.155 (0.139–0.172)
14	56.81 (52.20–61.41)	0.26×10^{-5} (-0.07×10^{5}–0.60×10^{-5})	0.202 (0.170–0.234)
15	54.11 (50.82–57.39)	4.40×10^{-5} (-0.84×10^{-5}–9.64×10^{-5})	0.146 (0.118–0.174)
16	57.41 (54.27–60.55)	0.49×10^{-5} (-0.57×10^{-5}–1.54×10^{-5})	0.205 (0.138–0.272)
17	55.99 (54.14–57.84)	3.02×10^{-5} (-0.79×10^{-5}–6.82×10^{-5})	0.155 (0.109–0.201)
18	58.41 (56.65–60.18)	0.81×10^{-5} (-0.14×10^{-5}–1.75×10^{-5})	0.175 (0.128–0.223)
19	57.37 (54.35–60.38)	2.75×10^{-5} (0.14×10^{-5}–5.36×10^{-5})	0.144 (0.126–0.162)
20	55.86 (54.97–56.75)	1.88×10^{-5} (0.43×10^{-5}–3.33×10^{-5})	0.154 (0.133–0.175)
21	57.08 (54.54–59.63)	2.00×10^{-5} (0.25×10^{-5}–3.75×10^{-5})	0.163 (0.102–0.223)
22	55.64 (54.27–57.01)	0.80×10^{-5} (-0.76×10^{-5}–2.34×10^{-5})	0.188 (0.152–0.225)
23	58.56 (54.63–62.48)	3.69×10^{-5} (-2.34×10^{-5}–9.71×10^{-5})	0.141 (0.117–0.165)
24	54.26 (52.78–55.75)	0.43×10^{-5} (0.05×10^{-5}–0.82×10^{-5})	0.194 (0.160–0.227)
25	54.18 (50.56–57.81)	1.81×10^{-5} (-0.77×10^{-5}–4.40×10^{-5})	0.166 (0.121–0.210)

utable to cytoplasmic DNA (presumably mtDNA). The mt line was analyzed as a random effects variable with five replicates per line, where each replicate was the mean number of days until death for the 50 females within a single vial. REML is the recommended algorithm for analyses involving random effects to provide accurate estimates of variance components, and it can handle unbalanced designs (Shaw 1987). We used WinModest 1.0.2 (Pletcher 1999) to calculate rates of senescence. First, we evaluated which of four different models (Gompertz, Gompertz-Makeham, logistic, and logistic-Makeham) best described the temporal pattern of mortality in each vial, using log-likelihood tests. The Gompertz model describes an empirically observed pattern that mortality increases exponentially with age. Logistic models account for a frequently observed deceleration of the rate of mortality late in life; however, when such deceleration equals zero, logistic models are reduced to Gompertz (Pletcher, 1999). Finally, Makeham models add a constant to account for age-independent mortality. In most cases in our study, the mortality function was best described by the Gompertz model $\mu_x = \alpha e^{\beta x}$, where μ_x is the predicted instantaneous mortality rate at age x, α is the Gompertz intercept, or frailty, and β is the rate of increase in mortality with age, that is, the rate of senescence of the population (Finch 1990; Williams and Day 2003). This suggests that the fit of our data was not improved by adding the parameters that account for the effects of increased mortality early in life (Makeham models) or decelerated rate of senescence late in life (logistic models). We then performed sensitivity analyses to test for robustness of estimated model parameters for each vial using WinModest. WinModest employs a maximum likelihood estimation procedure and is recommended since it allows more accurate estimation of mortality parameters, especially with relatively low sample sizes (Promislow et al. 1999; Pletcher 1999). The Gompertz parameters for frailty and rate of senescence were estimated separately for each vial, and variance in frailty and rate of senescence was again analyzed as a random effects analysis of variance in PROC MIXED (SAS 9.1) with mt line as a random effects factor.

RESULTS

There was an additive effect of mt line on female longevity ($Z = 2.89$, $P = 0.0019$). Mean grand longevity for all flies used in this experiment was 56.18 days. The coefficient of variation (CV) of longevity across mt line mean values was 4.3%. The mean longevities and 95% confidence intervals for the each of the mt lines are presented in Table 1. We also found a significant effect of mt line on Gompertz intercept $\ln(\alpha)$ (frailty) ($Z = 1.94$, $P = 0.026$, $CV_\alpha = 121\%$; Tables 1 and 2) as well as on Gompertz rate-of-senescence β ($Z = 1.78$, $P = 0.038$, CV = 13.1%, Tables 1 and 2). Because $\ln(\alpha)$ and β tend to be negatively phenotypically correlated (Hughes 1995), we performed a separate analysis for the rate of senescence while taking into account the Gompertz intercept $\ln(\alpha)$ as a fixed covariate. Although $\ln(\alpha)$ was indeed negatively correlated to β (type III F-test, $F_{1,115} = 2722.21$, $P < 0.0001$), the effect of mt line on the rate-of-senescence β remained significant ($Z = 2.77$, $P = 0.0028$). We also analyzed the effects of mt line on the mortality of flies surviving to the age of 56 days (mean grand longevity) and found a significant effect of mt line ($Z = 2.83$, $P = 0.002$, CV = 24%).

We note that one of our mt lines (line 7) had a much lower mean value for longevity (46.37 days) compared with the rest of the lines, which ranged between 54.11 and 58.81 days

TABLE 2. The variance component estimates, including lower and upper (absolute) 95% confidence intervals (CI) and scaled variance components of the effect of mt lines on longevity, Gompertz intercept (ln[α]) and Gompertz rate of senescence (β). Note that the confidence intervals and the scaled variance are based on five replicates per mt line, each consisting of 50 flies. We present the estimates for all 25 mt lines as well as the estimates calculated after excluding line 7. See text for the details of the estimation procedure.

Dependent variable	Covariance parameter	Variance estimate	Lower 95% CI	Upper 95% CI	Scaled variance (%)
All lines					
Longevity	mt line	5.00	2.8	11.32	51
	residual	4.81	3.7	6.49	49
Frailty	mt line	0.87	0.39	3.37	21
	residual	3.26	2.51	4.39	79
Rate of senescence	mt line	2.5×10^{-5}	1.1×10^{-5}	11.3×10^{-5}	18.4
	residual	11.1×10^{-5}	8.5×10^{-5}	15×10^{-5}	81.6
Excluding line 7					
Longevity	mt line	0.83	0.3	6.21	14.2
	residual	5.01	3.8	6.82	85.8
Frailty	mt line	0.53	0.19	4.15	13.6
	residual	3.37	2.59	4.58	86.4
Rate of senescence	mt line	2.2×10^{-5}	0.9×10^{-5}	12.5×10^{-5}	16.2
	residual	11.4×10^{-5}	8.7×10^{-5}	15.5×10^{-5}	83.8

(Table 1). We re-analyzed our data, excluding line 7 from the dataset, and found that the effect of mt line on longevity and Gompertz parameters was marginally nonsignificant (longevity: $Z = 1.46$, $P = 0.072$, CV = 2.4%; ln(α): $Z = 1.44$, $P = 0.075$, $CV_\alpha = 91.2\%$; β: $Z = 1.62$, $P = 0.052$, CV = 12.7%). There was, however, a significant effect of mt line on Gompertz slope after controlling for Gompertz intercept ($Z = 2.08$, $P = 0.019$; ln(α): type III F-test, $F_{1,114} = 2867.55$, $P < 0.0001$). The effect of mt line on mean grand longevity was also significant when line 7 was excluded from the analysis ($Z = 2.15$, $P = 0.016$, CV = 18.1%).

DISCUSSION

The goal of this study was to test the possibility that variation in mitochondrial genes contributes to within-population variation in aging. To evaluate this hypothesis, we used a laboratory population that had adapted to a discrete generation life cycle for over 300 generations before the start of these experiments. In the LH_M population, flies are discarded after 14 days (five days of adulthood) and only eggs laid within the last 18 h are used to found the next generation. This rearing protocol should increase the potential for the nuclear and mitochondrial genomes to accumulate mutations with deleterious effects late in life (i.e., starting from the sixth day of adulthood; Medawar 1952; for detailed discussion of such effects in laboratory cultures of *Drosophila*, see Promislow and Tatar 1998), as well as to accrue mutations that enhance performance early in life at the expense of decreased performance late in life (Williams 1957). Because of this lack of selection after day five of adulthood, genetic variation for traits expressed late in life is likely to be elevated relative to natural populations and therefore easier to detect. The observed levels of genetic variation presented here may therefore be inflated. Nevertheless, the fact that mtDNA explained such a large proportion of the phenotypic variation clearly points to an important role of mtDNA for variation in life span and aging. It should also be noted that the fact that females are expected to express less mtDNA based phenotypic variation than males (Frank and Hurst 1996) would tend to reduce the effects seen in our experiment (i.e., making our test more conservative).

Mitochondria are an important source of reactive oxygen species (ROS) that can damage both DNA and other molecules in the cell, and it is this process that has been implicated in aging (reviewed in Mandavilli et al. 2002; Ballard and Whitlock 2004). MtDNA is more prone to damage than nuclear DNA, and mtDNA damage can lead to further production of ROS, ultimately resulting in cell death (Mandavilli et al. 2002). The hypothesis that mtDNA affects aging was recently supported by two experimental studies (Trifunovic et al. 2004; Kujoth et al. 2005), although neither provided evidence for mitochondria causing aging through increasing production of ROS. In addition to the above two studies that created ''mutator phenotypes'' to directly test the effect of mtDNA mutations on aging, a recent study by James and Ballard (2003) explored the possibility that naturally occurring levels of variation in mtDNA haplotypes may differentially affect aging in male *D. simulans*. They found that mtDNA haplotypes that were derived from different, geographically isolated populations indeed had different effects on life span in males. We extend upon this result by showing that cytoplasmic variation within a single panmictic population may differentially affect the pattern of aging. To our knowledge, this is the first within-population test for genetic variation in cytoplasmic genes on aging. It provides strong support for the idea that there is sufficient genetic variation in mtDNA within a single population to differentially affect patterns of aging. However, although an accumulating number of studies has provided compelling evidence that mitochondria are indeed involved in the process of aging, the direct mechanism behind this process is still obscure.

Empirical studies that have tested for genetic variation in mtDNA in relation to phenotypic traits have largely confirmed theoretical expectations: that stable polymorphisms in mtDNA genes related to fitness within a panmictic population can only be maintained under restricted conditions (see Clark and Lyckegaard 1988; Rand et al. 2001). Although these studies were powerful in terms of sample size and imple-

mentation, they were restricted to juvenile performance (egg-to-adult survival) in *D. melanogaster*. We note that only about 15% of the nuclear genetic variation for fitness is attributable to the juvenile stage in *D. melanogaster* (see Chippindale et al. 2001), and we see no reason to believe that variation in mitochondrial genes should be disproportionably manifested during the juvenile phase.

Variation in longevity across populations is commonly attributed to differences in frailty (Gompertz intercept) rather than rate of senescence (Gompertz slope) (e.g., Promislow et al. 1996; Pletcher et al. 2000; Bronikowsky et al. 2002). In line with these studies, we found that variation for frailty was much higher than variation for the rate of senescence between mt lines derived from a single panmictic population. Houle (1992) calculated a median coefficient of variation for longevity of 9.89% from seven studies on *D. melanogaster*. Based on our data, mitochondrial genetic variation may explain at least 6 (excluding line 7) to 37 (including line 7) percent of the total genetic variation for longevity. We note that an mt-type that distinctly reduces longevity late in life is unlikely to confer a fitness disadvantage to its bearers in this population and therefore can be maintained, unless it has a negative pleiotropic effect early in life. Alternatively, this large impact may be caused some other cytoplasmic factor. Some earlier studies have pointed to the effects of maternally transmitted cytoplasmic bacteria (*Wolbachia* spp.) on longevity in *D. melanogaster* (e.g., Driver et al. 2004). Because we carefully controlled for this potentially confounding factor by first testing for the presence of *Wolbachia*, using diagnostic PCR analysis, in all lines without finding anything and then treating the lines with antibiotics (tetracycline hydrochloride) as an extra precaution, we suggest that the effects reported here are indeed due to sequence polymorphism in mtDNA. We do, however, acknowledge that other cytoplasmic components, such as cytoplasmic viruses (e.g., Clark 1985; Rand et al. 2001), could in theory contribute to the reported effects.

The intrinsic effects of cytoplasmic variation on life span and aging proposed here could potentially have an alternative extrinsic explanation. When larvae hatch and start to feed, their activities liquefy the medium, which may increase the death rate of adult flies that become stuck to the medium. If the mitochondrial lines varied in fecundity, different larval densities could differentially affect the stickiness of the food surface, resulting in a negative association between fecundity and aging and/or life span, and potentially explaining the variation found here. However, we think that this possibility is unlikely in this case, for two reasons. First, the flies were transferred to fresh vials every second day. The larvae, which hatch after 24 h (Ashburner et al. 2005), therefore only had a short time to affect the medium, and during this time they were very small and did not noticeably influence the quality (stickiness) of the medium. Second, the females were only mated once and then provided with live yeast every second day, which guarantees a high daily egg production and, in this particular population of flies, results in females running out of sperm after approximately 10 days (A. D. Stewart and W. R. Rice pers. comm.). Differential fecundity could thus only affect mortality through larval activity during the first 10 days, a time at which no measurable effects on variation in longevity and aging were recorded (17 flies of 6104 died up until day 10 and these were distributed randomly across the lines).

In conclusion, we found sizeable cytoplasmic genetic variation for life span and age-dependent mortality in females, within a single population. This finding is particularly interesting because LH_M is a North American population of *D. melanogaster*, and such populations have previously been reported to contain very low levels of mtDNA sequence polymorphism (see Solignac 2004). Our results provide important support for the mitochondrial theory of aging and highlight the need for further research regarding the role of the mitochondrial genome in the evolution of senescence.

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