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Nuclear–Mitochondrial Epistasis and Drosophila Aging: Introgression of *Drosophila simulans* mtDNA Modifies Longevity in *D. melanogaster* Nuclear Backgrounds

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ABSTRACT

Under the mitochondrial theory of aging, physiological decline with age results from the accumulated cellular damage produced by reactive oxygen species generated during electron transport in the mitochondrion. A large body of literature has documented age-specific declines in mitochondrial function that are consistent with this theory, but relatively few studies have been able to distinguish cause from consequence in the association between mitochondrial function and aging. Since mitochondrial function is jointly encoded by mitochondrial (mtDNA) and nuclear genes, the mitochondrial genetics of aging should be controlled by variation in (1) mtDNA, (2) nuclear genes, or (3) nuclear–mtDNA interactions. The goal of this study was to assess the relative contributions of these factors in causing variation in Drosophila longevity. We compared strains of flies carrying mtDNAs with varying levels of divergence: two strains from Zimbabwe (<20 bp substitutions between mtDNAs), strains from Crete and the United States (~20–40 bp substitutions between mtDNAs), and introgression strains of *Drosophila melanogaster* carrying mtDNA from *Drosophila simulans* in a *D. melanogaster* Oregon-R chromosomal background (>500 silent and 80 amino acid substitutions between these mtDNAs). Longevity was studied in reciprocal cross genotypes between pairs of these strains to test for cytoplasmic (mtDNA) factors affecting aging. The intrapopulation crosses between Zimbabwe strains show no difference in longevity between mtDNAs; the interpopulation crosses between Crete and the United States show subtle but significant differences in longevity; and the interspecific introgression lines showed very significant differences between mtDNAs. However, the genotypes carrying the *D. simulans* mtDNA were not consistently short-lived, as might be predicted from the disruption of nuclear–mitochondrial coadaptation. Rather, the interspecific mtDNA strains showed a wide range of variation that flanked the longevities seen between intraspecific mtDNAs, resulting in very significant nuclear × mtDNA epistatic interaction effects. These results suggest that even "defective" mtDNA haplotypes could extend longevity in different nuclear allelic backgrounds, which could account for the variable effects attributable to mtDNA haplogroups in human aging.

THE central role of mitochondria in metabolism makes mitochondrial genetics fundamentally important for understanding both the genetic basis and the evolution of aging. Mitochondrial function requires coordinated expression of hundreds of nuclear genes and a few dozen mitochondrial genes, many of which have been associated with either extended or shortened life span. Clearly, it is the alleles of genes and not just the genes themselves that extend or reduce longevity. The complex genetic architecture of mitochondrial function suggests that there should be an equally complex set of gene interactions (epistases) involving genetic variation in the nuclear and mitochondrial genomes that warrants further scrutiny in the context of the various mitochondrial theories of aging.

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The mitochondrial free radical theory of aging states that physiological decline with age is a result of the cumulative effects of damage from oxygen radical production (Harman 1957; Beckman and Ames 1998). Mitochondria are the primary source of reactive oxygen species (ROS) within the cell. During electron transport in the inner mitochondrial membrane, enzyme complexes I and III generate the majority of ROS as byproducts of the coenzyme Q cycle (Balaban *et al.* 2005). Mitochondria are also particularly sensitive targets for ROS damage during aging. The mtDNA molecule is not associated with histones and mtDNA has limited DNA repair mechanisms (Bogenhagen 1999). Consistent with these observations, mtDNA experiences greater damage from ROS than do nuclear proteins and DNA (Richter *et al.* 1988; Wei *et al.* 1998).

The basic principles of the mitochondrial theories of aging have gained wide support from biochemical, molecular genetic, and evolutionary studies (Agarwal and Sohal 1996; Beckman and Ames 1998; Finkel and

Holbrook 2000; Barja 2004). An extensive literature has documented age-specific increases in damage to mitochondrial proteins, membranes, and mtDNA (Sohal and Weindruch 1996; Wallace 1997; Yan *et al.* 1997), age-specific declines in the activity of mitochondrial enzyme complexes (Schwarze *et al.* 1998; Ferguson *et al.* 2005), and the accumulation of mutant mtDNAs within cells (heteroplasmy) (Cortopassi *et al.* 1992; Wallace 1995; Wang *et al.* 2001). But there is little direct experimental evidence that mtDNA mutations play a causal role in whole animal aging. The notable exception to this trend is the study by (Trifunovic *et al.* 2004) where knock-in mice with defective proofreading activity of mitochondrial polymerase lead to elevated mtDNA mutation rates and premature aging. Nevertheless, the distinction between the causes and consequences of mitochondrial decline in aging has been addressed by relatively few genetic experiments that manipulate mtDNA (de Gray 1999; Lightowlers *et al.* 1999; Golden *et al.* 2002; James and Ballard 2003).

While mitochondria are the primary source of ROS, nuclear-encoded antioxidant proteins defend against this damage. The primary antioxidant proteins are the mitochondrial Mn-superoxide dismutase (SOD), and the cytoplasmic CuZn-SOD, catalase, and glutathione peroxidase. This redox balance plays an important role in both cellular signaling and aging, and is determined by the relative levels of oxidant production *vs.* the activities of antioxidant pathways (Finkel and Holbrook 2000; Barja 2004). Thus, the free radical theory of aging predicts that longevity should be extended by (1) increasing the level of ROS defenses, or (2) decreasing the levels of ROS production. Overexpressing SOD has been shown to extend life span of Drosophila (Parkes *et al.* 1998; Sun and Tower 1999; Sun *et al.* 2002;), but this effect depends on genetic background (Sun and Tower 1999; Orr *et al.* 2003; Spencer *et al.* 2003). Overexpression of catalase in the mitochondria of mice also extends longevity, and shows significant variation between replicate overexpression constructs (Schriner *et al.* 2005). These studies support the biochemical argument that reducing ROS levels extends longevity, but reveal how sensitive these manipulations are to genetic background.

The extension of longevity by reducing ROS production is suggestive but remains indirect. Calorically restricted (CR) mice live longer and produce lower levels of ROS (Sohal *et al.* 1994; Masoro 2000), but CR increases production of stress response proteins and ROS scavenging enzymes (Sohal and Weindruch 1996; Weindruch *et al.* 2001). Flies selected for longevity show lower levels of hydrogen peroxide than control flies (Ross 2000). But again, the long-lived strain also shows increased expression of antioxidant defenses (Dudas and Arking 1995; Force *et al.* 1995), making it difficult to distinguish cause and effect.

Several studies in Drosophila have failed to support predictions that appear logical from these observations, suggesting additional complexity to nuclear–mitochondrial signaling. If mitochondria are responsible for aging, it would follow that some change in metabolic rate or ROS levels in mitochondria should be detected with increasing age. However, metabolic rates do not change with age in most Drosophila species (Promislow and Haselkorn 2002). ROS levels do not change with age in *D. melanogaster*, and neither CR nor overexpression of adenine nucleotide translocase (important in regulating ADP transport in mitochondria) decreases ROS production (Miwa *et al.* 2004). Metabolic rate is reduced neither by CR nor by reduced insulin signaling, both of which extend longevity (Hulbert *et al.* 2004). These observations do not falsify the mitochondrial theories of aging, but suggest that a diversity of pathways may be involved that influence the way nuclear–mitochondrial interactions modulate longevity.

Some likely candidate pathways are oxidative phosphorylation (OXPHOS), insulin signaling, apoptosis, and uncoupling. Down-regulation of a number of nuclear- and mtDNA-encoded genes involved in OXPHOS extended longevity in *Caenorhabditis elegans* (Dillin *et al.* 2002). The insulin-signaling pathway is an evolutionary conserved modulator of longevity (Tatar *et al.* 2003), with a myriad of downstream metabolic effects that may intersect with mitochondrial function (Larsen and Clarke 2002; Tatar and Rand 2002). The role of apoptosis in aging warrants further dissection as it may be a sensitive response pathway to caloric restriction (Cohen *et al.* 2004) and oxidative stress (Kujoth *et al.* 2005). Uncoupling proteins represent an additional class of nuclear-encoded proteins that may modulate mitochondrial aging by permitting the leakage of protons across the inner mitochondrial membrane and reducing ROS production (Brand 2000; Speakman *et al.* 2004). Thus, mitonuclear cross talk has many pathways that can be modulated to reduce or extend longevity.

The molecular genetics of aging has been dissected using a variety of tools available in Drosophila such as transgenic and overexpression constructs (Parkes *et al.* 1998; Sun *et al.* 2002; Orr *et al.* 2005), and hypomorphic or knock-out alleles (Lin *et al.* 1998; Rogina *et al.* 2000; Clancy *et al.* 2001; Tatar *et al.* 2001). But selection for delayed aging has robust responses, so ample genetic variation exists for longevity (Luckinbill *et al.* 1984; Rose 1984; Sgro *et al.* 2000). Since variation in rates of aging is inherently a population-level problem, the role of genetic polymorphisms in modulating aging is an important challenge. As the number of candidate genes for aging grows, the role of polymorphism at these genes becomes increasingly important to the genetics of aging, and needs to be incorporated into genetic experiments.

If we consider mtDNA as a candidate gene (or genome) for aging, the high levels of polymorphism in mtDNA provide a large pool of potential variants affecting aging. It is well established that mildly deleterious

mtDNA polymorphisms are a general property of animal populations (Nachman 1998; Rand and Kann 1998; Weinreich and Rand 2000). In humans, there is statistical evidence that alternative mtDNA haplotypes are associated with variation in longevity (Ivanova *et al.* 1998; Tanaka *et al.* 1998; De Benedictis *et al.* 1999). However, some long-lived haplotypes carry mutations associated with disease suggesting that genetic background is an important modulator of mtDNA effects on aging (Rose *et al.* 2001). Studies in Drosophila have established that nuclear–mitochondrial interactions (epistases) are important in the fitness effects of mtDNA variation (Clark and Lyckegaard 1988; Kilpatrick and Rand 1995; Rand *et al.* 2001; James and Ballard 2003). While there has been an explosion of research on mitochondrial aspects of aging, there has been a general lack of studies that manipulate nuclear and mitochondrial genetic variation to explore nuclear–mitochondrial epistatic effects on longevity.

Here we present the results of such a study, using strains of Drosophila carrying mtDNAs on different nuclear backgrounds. We compare the longevities of wild-type strains of *D. melanogaster* carrying alternative mtDNAs from (1) within a Zimbabwe population, (2) between an Old World (Crete) and a New World (Rhode Island) strain, and (3) between mtDNA introgression strains carrying *D. simulans* mtDNA on *D. melanogaster* nuclear backgrounds. We test the predictions that variation in longevity is due to (1) mtDNA, (2) nuclear genetic background, and (3) an interaction between nuclear and mtDNA. A further prediction is that the effect of mtDNA on aging should increase in parallel with increasing levels of mtDNA divergence. While the results are consistent with this latter prediction, mtDNA haplotypes affect aging in a nuclear-background dependent manner. These mitonuclear epistases suggest that the main effects of mtDNA on aging could be masked, or even exaggerated, by significant interactions with ubiquitous nuclear allelic variation.

MATERIALS AND METHODS

Fly strains: Two *D. melanogaster* strains from Zimbabwe (Zim 2 and Zim 53), one strain each from Crete and Rhode Island, and two mtDNA introgression strains with *D. melanogaster* Oregon-R chromosomes and *D. simulans siII* mtDNA were used for longevity analyses. The strain from Iraklion, Crete (Crete 10) was provided by E. Zouros, and the strain from Rhode Island (Four Town Farm 100, FTF 100) was collected by DMR. All strains had been in laboratory culture for >5 years prior to analyses. Some data on longevity for Zim 2, Zim 53, and FTF 100 have been reported previously (Fry and Rand 2002; Fry *et al.* 2004).

The interspecific mtDNA introgression strains were obtained from K. Sawamura (Sawamura *et al.* 2000). In a search for *D. simulans* lines (hereafter *Dsim*) that would rescue the sterility of hybrids from crosses with *D. melanogaster* (hereafter *Dmel*), a *Dsim* line was discovered (C167.4) that produced fertile F_1 females in a *Dsim* female × *Dmel* male cross (Davis *et al.* 1996). Due to the maternal inheritance of mtDNA, these lines should have the *Dsim* mtDNA, and we confirmed this by DNA sequencing (see below). The *Dmel* male used in the initial cross was *In(1)AB*, while subsequent generations of backcrossing used *Dmel* Oregon-R males (Davis *et al.* 1996; Sawamura *et al.* 2000). The crossing scheme was as follows (K. Sawamura, personal communication): *Dsim* C167.4 females × *Dmel* In(1)AB, F_1 females × *Dmel* Ore-R males (F_1 cross), F_2 female (single) × *Dmel* Ore-R males (F_2 crosses). F_2 females were numbered, and the line number represents these females: no. 12 and no. 33 were from the same F_1 cross, no. 14 and no. 21 were from the same F_1 cross, no. 22 and no. 38 were from the same F_1 cross. In the F_3 generation, sib-mating was done to establish isofemale lines. In the current study we used descendants of only two of these lines: no. 21 and no. 38. Two sib-matings and four isofemale lines were set up from no. 21 female (on April 11, 2000) and only one isofemale was fertile. Four sib-matings were set from no. 38 female (April 14, 2000) and all four were fertile (one of which was obtained from K. Sawamura). We refer to these lines as "sim-mel 21," and "sim-mel 38" (hereafter sm21 and sm38) to indicate that they carry *Dsim* mtDNA on a *Dmel* nuclear background (see below).

When we obtained the lines from K. Sawamura, they had been backcrossed for 4–5 additional generations to *Dmel* Ore-R males, followed by maintenance in vial cultures for 2 years. On the basis of the conclusions from Sawamura *et al.* (2000), this should select against *Dsim* nuclear genes linked to fertility factors. Before conducting our demography assays, we treated all lines with tetracycline to remove Wolbachia. PCR assays confirmed that this was successful (data not shown). In addition, we subjected all lines to three additional generations of single-pair backcrossing to *Dmel* Ore-R males. With free recombination and no selection, these lines should have >99.2% Ore-R nuclear alleles (assuming seven generations of backcrossing, not including the 2 years of culture which should select against *Dsim* alleles). When not undergoing single-pair backcrossing, all lines were maintained in vial culture at ~50 pairs per generation prior to expansion of lines to collect sufficient adults for demography.

mtDNA haplotypes: The mtDNA of the Zim 53 strain has been completely sequenced (Ballard 2000). Nucleotide polymorphism surveys for 1500 bp of the NADH dehydrogenase subunit 5 (ND5) gene (Rand *et al.* 1994; Rand and Kann 1996) indicate that Zim 2 should differ from Zim 53 at <20 nucleotide sites across the coding sequence of the mtDNA (the ND5 data represent about 10% of the entire coding region of Drosophila mtDNA). The Crete 10 and FTF 100 differ by 4 bp in this ND5 region, implying ~40 total base pair differences between the coding regions of these two mtDNAs (considerably more than between Zim 2 and Zim 53). Nucleotide polymorphism for pooled samples of Crete and FTF (or either sample and Zimbabwe) are >10-fold higher than polymorphism within either of the samples (A. Fry and D. M. Rand, unpublished results), confirming that between-population variation in *Dmel* mtDNA (*e.g.*, Crete and FTF) is much greater than intrapopulation variation (between Zim 2 and Zim 53).

We sequenced >10 kb of the mtDNA for each of the sim-mel introgression strains (sm21 and sm38), and as expected they carry the *Dsim siII* mtDNA haplotype (Solignac *et al.* 1986; Ballard 2000). The *Dsim si*II mtDNA differs from *Dmel* Ore-R mtDNA by 539 synonymous mutations and 86 amino acid mutations (Ballard 2000). This should provide a strong set of mtDNA "alleles" with which to probe mitonuclear interactions in *D. melanogaster*.

Demography assays: All longevity assays were done on F_1 flies from reciprocal crosses between specific strains carrying the different mtDNAs. While backcrossing is commonly used

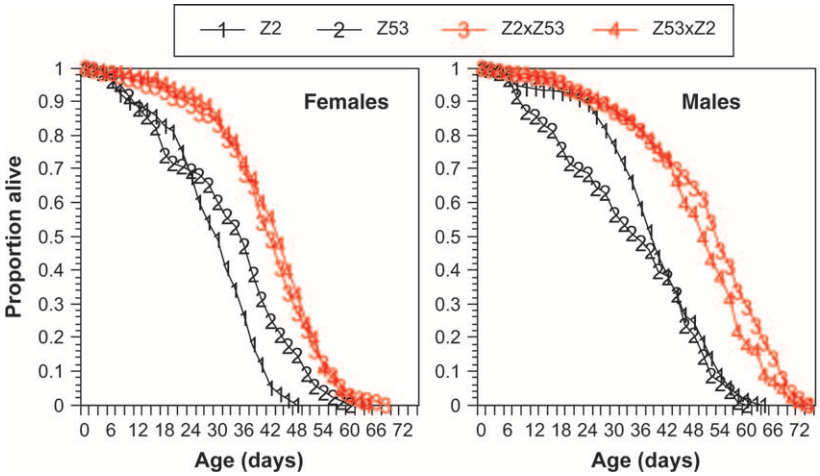

FIGURE 1.—Survival of parental and reciprocal cross genotypes of two Zimbabwe strains of *D. melanogaster*. The parental strains (Zimbabwe 2 and Zimbabwe 53) are significantly different from each other, and from either reciprocal cross ($P < 0.001$, log-rank test). The two reciprocal genotypes are not significantly different for females, and are significantly different for males ($P < 0.01$, log-rank test).

to remove nuclear genetic variation, subtle allelic variation can be retained, and single male backcrossing leads to inbreeding. Moreover, the interspecific introgression lines could retain nuclear alleles of *Dsim* that were selected for in backcrosses due to coadaptation with the *Dsim* mtDNA. These potential nuclear allelic effects were controlled using reciprocal crosses. Reciprocal F_1 females (*e.g.* sm21 female × Crete 10 male *vs.* Crete 10 female × sm21 male) will be identically heterozygous for any segregating alleles between the two parental strains, but will carry alternative mtDNAs, due to the maternal inheritance of mtDNA. Reciprocal F_1 males will be identical for autosomes, but will differ in mtDNA, X, and Y chromosomes. We compared longevities of F_1 flies from the following crosses (female × male), which generated different combinations of nuclear and mitochondrial genes (hereafter mitonuclear genotypes):

Zim 2 *vs.* Zim 53 (intrapopulation parental strains)
Zim 2 × Zim 53 *vs.* Zim 53 × Zim 2 (intrapopulation mitonuclear comparison)
FTF 100 × Crete 10 *vs.* Crete 10 × FTF 100 (interpopulation mitonuclear comparison)
FTF 100 × sm 21 *vs.* sm 21 × FTF 100 (interspecific mitonuclear comparison)
FTF 100 × sm 38 *vs.* sm 38 × FTF 100 (interspecific mitonuclear comparison)
Crete 10 × sm 21 *vs.* sm 21 × Crete 10 (interspecific mitonuclear comparison)
Crete 10 × sm 38 *vs.* sm 38 × Crete 10 (interspecific mitonuclear comparison)

All crosses were performed at 25° on a 12:12 light:dark cycle using 25 pairs of parents for each cross. Parental strains had been acclimated under the same conditions at controlled density for at least one generation prior to crossing.

Longevity was measured by placing 100 males and 100 females (F_1 offspring of a single genotype listed above) into each of three replicate demography cages. Approximately 8400 deaths were recorded in the study (14 genotypes × 3 cages × 2 sexes × 100 flies of each sex). The demography cages were made from one-quart plastic containers with a screened lid and a side coupling for attaching a food vial. The cages were kept in a walk-in incubator on a 12:12 light:dark photoperiod at 25° and 40% relative humidity. The food vials were replaced every other day and dead flies were removed with an aspirator, sexed, and counted. Survival was analyzed with a semi-parametric proportional hazards statistical model using day of death as the dependent variable, and cross (reciprocals above), mtDNA, and their interactions as predictors. Mortality analyses were done on pooled data across the three replicate demography cages for each genotype. Survival curves were compared with log-rank tests, and tested for significance using JMP statistical software.

Age-specific mortality (μ_x) was reported as $\mu_x = -\ln(1 - q_x)$ where q_x is the proportion of individuals that die in age class x (number of individuals that die, divided by the number of individuals that enter, age class x). We estimated age-specific mortality models for various lines using maximum likelihood (PLETCHER and GEYER 1999; PLETCHER *et al.* 2000). The Gompertz (G) model describes an exponential increase in mortality rate with age and is given by $\mu_x = \alpha e^{-\beta x}$, where $\mu_{(x)}$ is the mortality rate at age x, α is the initial mortality rate or intercept, and β is the slope or age-dependent increase in mortality. The Gompertz–Makeham (GM) model is the Gompertz plus a parameter, C, that describes age-independent mortality. The Logistic (L) model is the Gompertz plus a frailty parameter, s, to describe late-life mortality rate deceleration. The Makeham and Logistic models can be combined with the Gompertz to produce a four-parameter Logistic–Makeham (LM) model. These models are nested, so likelihood ratio tests can be used to compare model parameters between different lines by allowing parameters to vary, or constraining them to be the same. Significance is determined using twice the difference in likelihood scores, distributed as a chi-square with degrees of freedom equal to the difference in the number of parameters. All such analyses were done with the Winmodest program (PLETCHER *et al.* 2000).

RESULTS

Longevities of intrapopulation crosses: The longevity patterns for strains from within the Zimbabwe population (Zim 2, Zim 53) are shown in Figure 1. There are very significant differences between the inbred parental strains (Zim 2, Zim 53), but the longevities of the reciprocal F_1 genotypes are very similar. The survivorship curves reveal a clear effect of heterosis, as the two F_1 genotypes are significantly longer lived than the two parental strains. Reciprocal F_1 females are not significantly different from one another ($P > 0.1$, log-rank tests), while males are ($P < 0.001$; reciprocal cross males also differ in any X-linked or Y-linked polymorphisms).

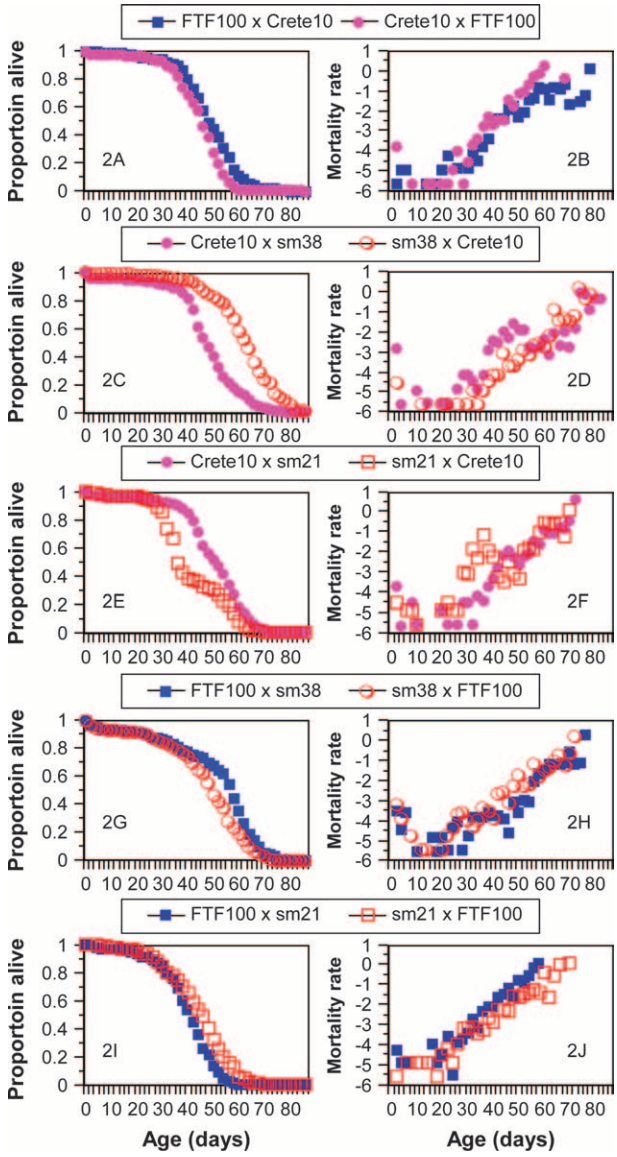

FIGURE 2.—Survival and mortality plots for females. Data for reciprocal crosses between two strains (female × male) are shown. (A and B) Reciprocal crosses between the two *D. melanogaster* wild strains FTF 100 and Crete 10. (C–J) Crosses between one *D. melanogaster* strain and a sim-mel introgression strain (sm21 or sm38). Blue squares, FTF 100 cytoplasm; pink dots, Crete 10 cytoplasm; open circles, sm38 cytoplasm; open squares, sm21 cytoplasm. Red signifies a *D. simulans* mtDNA. Each pair of genotypes is significantly different using log-rank tests of survivorship after correcting for multiple tests ($P < 0.001$), but data in A and B and I and J show no significant difference in mortality parameters (see RESULTS and Table 1).

The small mtDNA difference between these two strains (<20 bp substitutions; see MATERIALS AND METHODS) suggests that mtDNA haplotype effects cannot override the clear heterosis effects that result from reciprocal crosses between these two strains, which may have accumulated deleterious recessive mutations during laboratory culture (PROMISLOW and TATAR 1998; MACK *et al.* 2000; YAMPOLSKY *et al.* 2000).

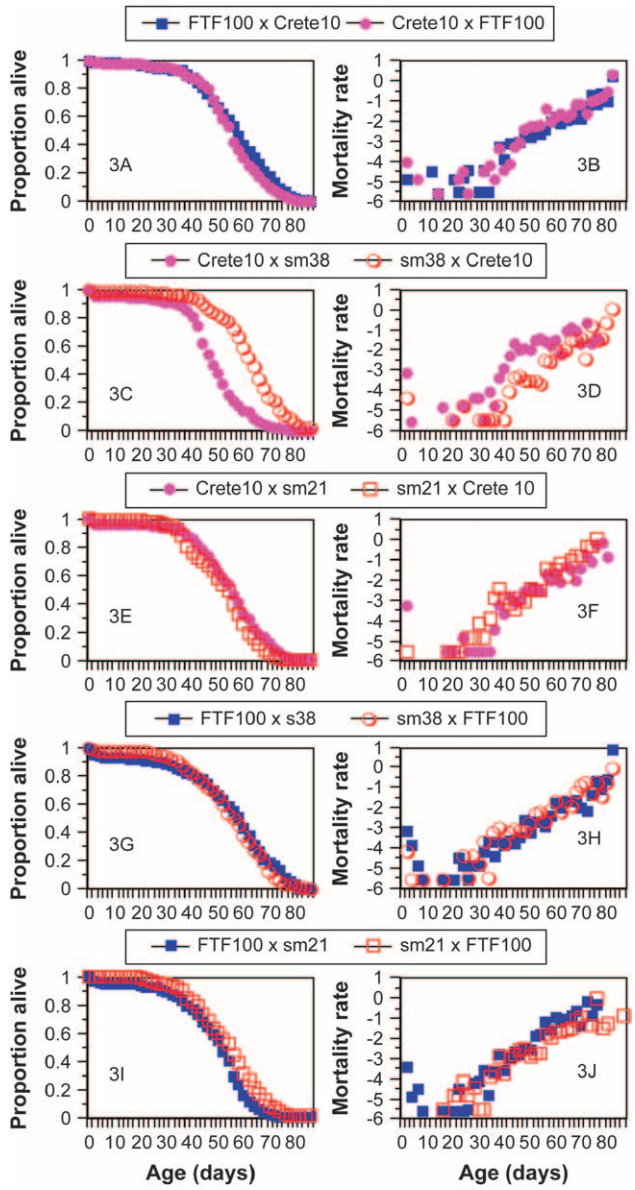

FIGURE 3.—Survivorship and mortality plots for males. Same reciprocal cross format as Figure 2, but note that reciprocal cross males can differ in X- and Y-linked polymorphisms. Only genotype pairs in C and I are significantly different using log-rank tests of survivorship after correcting for multiple tests ($P < 0.001$).

Longevities of interpopulation crosses: Survivorship and mortality plots for the interpopulation comparison of the two *D. melanogaster* strains from Crete and Four Town Farm, Rhode Island (Crete 10 and FTF 100) are shown in the top panels of Figures 2 (females) and Figures 3 (males). Similar data for the reciprocal crosses of these two strains to each of the two sim-mel interspecies introgression strains (sm21 and sm38) are shown in the lower panels of Figures 2 and 3, and will be discussed below. These data are presented in the same figure to facilitate comparison among the interpopulation (Crete 10 and FTF 100) and interspecies crosses (sm21

or sm38 crossed to either Crete 10 or FTF 100). The descriptive statistics of survival and mortality for all strains are presented in Table 1.

Significance tests of survivorship curves were done using log-rank tests, while significance tests of different mortality parameters were done using likelihood ratio tests (see MATERIALS AND METHODS). These tests can have different outcomes that reflect basic differences in survival *vs.* age-specific mortality. Survivorship curves display cumulative data so differences in survivorship between genotypes in one age interval are carried forward to other ages, which can inflate the effects of log-rank tests. Mortality parameters are a more accurate description of true aging effects since they focus on differences in the slope, intercept, or linearity of the mortality rate between genotypes. The data for all genotypes were contrasted using four mortality models [G, GM, L, and LM (see MATERIALS AND METHODS)]. Comparisons of alternative mortality models using the Winmodest package (PLETCHER *et al.* 2000) established that the four-parameter LM model was preferred over others for all genotypes presented in Table 1. We then compared each genotype to its reciprocal genotype to determine if any of the four parameters of the LM model differed between flies carrying alternative mtDNAs. This was done by determining the likelihood of a model that allowed each genotype to have a unique set of parameters (eight independent parameters; see Table 1, "unique" column) *vs.* a model that constrained one of the parameters to be the same for the two genotypes and allowed the remaining six parameters to vary (Table 1, "fixed α," "fixed β," "fixed s," and "fixed C" columns).

Reciprocal F_1 genotypes from crosses between two wild strains of *Dmel*, Crete 10, and FTF 100 show subtle but significant differences (Figures 2, A and B and 3, A and B). Log-rank tests show a significant difference between these two genotypes ($P < 0.001$), but likelihood ratio tests of mortality parameters do not show any significant differences. For Crete 10 and FTF 100 females, these likelihoods could not be distinguished (Table 1). In males there is a marginally significant difference in survivorship using a log-rank test ($P < 0.02$), and additional differences in mortality parameters ($P < 0.01$), neither of which is significant after correcting for multiple tests (see Table 1). In addition, it is not certain that this reciprocal cross effect in males can be attributed to mtDNA *vs.* X, or Y-chromosomal factors.

Comparisons between *Dmel* and *Dsim* mtDNAs: Genotypes carrying either a *Dmel* or a *Dsim* mtDNA are shown in Figure 2, C–J (females), and Figure 3, C–J (males), representing reciprocal crosses between either Crete 10 or FTF 100, and the two "sim-mel" mtDNA introgression lines, sm21 and sm38. In general, the differences in longevity among flies carrying *Dmel vs. Dsim* mtDNA are much more pronounced than those carrying alternative *Dmel* mtDNAs (compare survivorship curves in Figure 2A to those in Figure 2, C, E, G, and I). However, the data clearly show that genotypes carrying the *Dsim* mtDNA are not consistently shorter lived than those carrying the *Dmel* mtDNA, as might be expected if *Dsim* mtDNA disrupts coadapted nuclear–mtDNA interactions in a *Dmel* nuclear background. Reciprocal crosses involving the "disrupted" mitonuclear sim-mel strains sm21 and sm38 are either shorter or longer lived than the reciprocal crosses between the two "native" mitonuclear *Dmel* strains FTF 100 and Crete 10 (see Figure 4). When the "sim-mel" introgression strains are crossed to the *Dmel* strain Crete 10, the sm38 (*Dsim*) mtDNA shows extended longevity, and sm21 mtDNA shows reduced longevity, relative to the Crete 10 (*Dmel*) mtDNA. The reverse is true when *Dmel* FTF 100 is used: sm21 mtDNA lives longer, and the sm38 mtDNA shows reduced longevity, relative to the FTF 100 mtDNA. The direction of these effects was the same in both sexes, although the magnitude of the effects was generally reduced in males (compare Figures 2 and 3; summarized in Figures 4 and 5).

Among the four pairs of reciprocal crosses involving Crete 10, FTF 100, sm38, and sm21, the effect of mtDNA haplotype on longevity is entirely epistatic, *i.e.,* it depends on the allelic constitution of the F_1 heterozygous nuclear background (Figure 5). This was quantified in a two-way ANOVA with nuclear background (reciprocal crosses) and mtDNA (*Dmel vs. Dsim*) as main effects, plus the interaction term. The nuclear × mtDNA interaction terms of these ANOVAs are all highly significant (see Table 2). Table 2 presents three different two-way ANOVAs: one involving the two pairs of reciprocal crosses of Crete to sm21 or to sm38, a second ANOVA involving the two pairs of reciprocal crosses of FTF to sm21 or sm38, and a third involving all four pairs of reciprocal crosses involving Crete, FTF, sm21, and sm38. Data from the three replicate demography cages were pooled for each genotype and a Cox proportional hazard model was used with day of death as the dependent variable and nuclear background and mtDNA plus interaction as the predictors. This treats the two reciprocal crosses with *Dsim* mtDNA as replicates (*e.g.*, sm21 female × Crete male and sm38 female × Crete male). Thus, there is one degree of freedom for each factor in Crete or FTF ANOVAs, and three degrees of freedom for the nuclear reciprocal factors with all four reciprocals. The three replicate demography cages were generally in close agreement; ANOVA keeping them separate produced the same highly significant nuclear × mtDNA interaction terms (data not shown). To confirm the results of the ANOVAs, the analyses were done ignoring (1) the first 10 days, (2) the last 10 days, and (3) both the first and last 10 days of death events, and the results were qualitatively identical to the data reported in Table 2.

In females, the ANOVA shows strong variation among nuclear genotypes and in the nuclear × mtDNA interaction term, but no main effect of mtDNA. In males, the design violates a basic assumption of ANOVA since

TABLE 1

Demographic parameters for reciprocal F_1 genotypes

	Descriptive statistics				Mortality parameters				Likelihood scores				
	N	Median	Mean	Max.	α	β	s	*C*	Unique	Fixed α	Fixed β	Fixed s	Fixed *C*
Females													
FTF × Crete	282	48	48.43	80	0.0000	0.1808	0.9970	0.0014	−2184.30	−2184.33	−2184.31	−2185.30	−2184.34
Crete × FTF	300	46	44.42	86	0.0000	0.1839	0.5485	0.0016		NS	NS	NS	NS
Crete × sm38	288	48	48.72	84	0.0000	0.4305	6.3336	0.0036	−2272.02	−2281.11	−2288.59	−2298.61	−2279.82
sm38 × Crete	281	64	59.74	82	0.0001	0.1176	0.0057	0.0006		<0.0001	<0.0001	<0.0001	<0.0001
Crete × sm21	291	52	49.93	74	0.0001	0.1396	0.4158	0.0017	−2218.66	−2229.02	−2236.42	−2238.36	−2218.68
sm21 × Crete	277	36	41.39	72	0.0000	0.5611	7.3891	0.0019		<0.0001	<0.0001	<0.0001	NS
FTF × sm38	269	58	51.76	80	0.0000	0.2128	0.7590	0.0056	−2131.68	−2135.13	−2133.85	−2132.37	−2132.37
sm38 × FTF	257	50	47.06	74	0.0002	0.1193	0.2531	0.0039		<0.05	<0.05	NS	NS
FTF × sm21	282	42	40.05	60	0.0002	0.1426	0.1949	0.0019	−2138.14	−2138.97	−2139.68	−2138.14	−2138.99
sm21 × FTF	277	46	44.45	74	0.0007	0.1000	0.1657	0.0001	NS	NS	NS	NS	NS
Males													
FTF × Crete	272	58	56.50	86	0.0004	0.0867	0.1401	0.0003	−2259.81	−2264.19	−2264.96	−2263.41	−2260.9293
Crete × FTF	289	56	54.57	86	0.0000	0.1708	1.1759	0.0014		<0.01	<0.01	<0.01	NS
Crete × sm38	270	48	47.76	78	0.0000	0.3953	3.8210	0.0027	−2058.39	−2064.04	−2069.88	−2068.62	−2063.0638
sm38 × Crete	255	64	62.08	88	0.0000	0.1238	0.4554	0.0006		<0.01	<0.0001	<0.0001	<0.01
Crete × sm21	268	56	53.96	82	0.0001	0.1248	0.5459	0.0016	−2117.27	−2119.08	−2118.35	−2118.50	−2121.4039
sm21 × Crete	263	54	52.23	78	0.0004	0.0962	0.0896	0.0000		NS	NS	NS	<0.01
FTF × sm38	298	60	54.53	88	0.0002	0.0952	0.1190	0.0034	−2352.04	−2352.39	−2352.08	−2352.05	−2355.4449
sm38 × FTF	267	57	54.85	86	0.0004	0.0896	0.1607	0.0007		NS	NS	NS	<0.05
FTF × sm21	287	52	48.53	78	0.0001	0.1431	0.4281	0.0030	−2188.78	−2191.13	−2191.32	−2188.79	−2196.8517
sm21 × FTF	261	56	53.85	88	0.0003	0.1016	0.4013	0.0000		<0.05	<0.05	NS	<0.0001

Numbered crosses refer to the same numbers in Figure 4. Values were estimated using Winmodest program (Pletcher *et al.* 2000). The mortality parameters are the intercept (α), slope (β), late-life mortality deceleration term (s), and an age-independent mortality term (*C*) for age-specific mortality rate (see Figures 2 and 3). The preferred model for all genotypes was the Logistic-Makeham (data not shown). Likelihood ratio tests were conducted by contrasting the likelihood of model where each line in a pair of lines was allowed to have unique values for the four parameters ("unique") *vs.* a model where one term was constrained to a fixed value for both lines (fixed α, fixed β). The intercept terms (α) often differed by several orders of magnitude despite being very low; only four digits are displayed. See materials and methods for details.

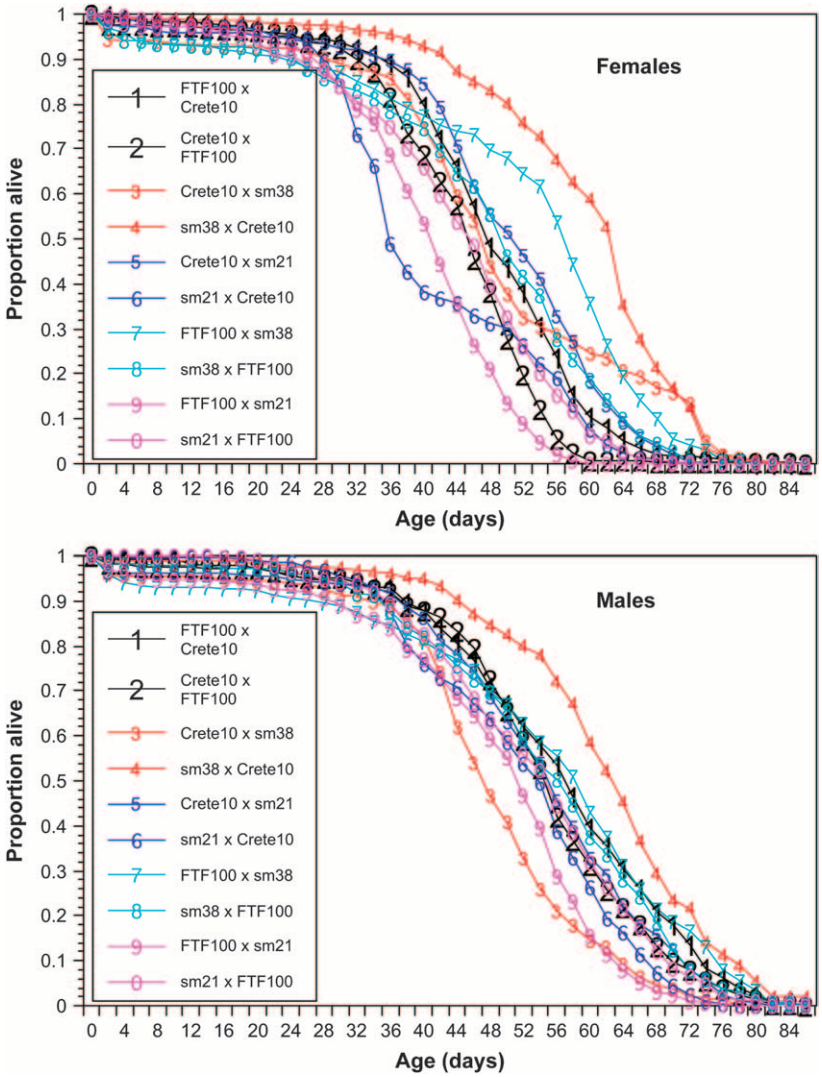

FIGURE 4.—Survival plots for all reciprocal genotypes. The two reciprocal crosses carrying *D. melanogaster* mtDNAs (labeled 1 and 2) are intermediate to the reciprocal genotypes carrying either *D. melanogaster* or *D. simulans* mtDNA (reciprocal cross genotypes are numbered sequentially and are shown in the same color).

reciprocal crosses cannot really be considered replicated effects, so the results are presented for comparison only. Note that in males there are significant main effects of mtDNA, but this incorporates X- and Y-chromosome variation as well. As is evident in the figures, there is more nuclear and nuclear × mtDNA variation among genotypes in females than in males (female $r^2 = 13.1\%$, male $r^2 = 6.6\%$), suggesting that X- or Y-linked factors are reducing apparent effects of cytoplasmic term even if they are confounding it.

Demographic patterns: Some additional comments are warranted about differences in mortality patterns among genotypes. Table 1 shows descriptive statistics for each of the reciprocal crosses involving the sim-mel introgression strains, with estimates of the LM terms that characterize mortality. The right side of the table presents the likelihood ratio tests of mortality parameters between reciprocal genotypes that quantify the differences shown in the right-hand panels of Figures 2 and 3 (mortality plots). In clear contrast to the results from the Crete × FTF cross (*Dmel* mtDNA only), the crosses with *Dsim* mtDNA strains show many significant differences in mortality parameters. The intercept (α), slope (β), and late-life mortality deceleration terms (s) are all different for crosses to Crete 10. The effect of the latter term (s) can be seen in the Crete 10 crosses where a reduction of mortality occurs in mid to late life (Figure 2, C–F). It is also clear that crosses to FTF show fewer significant effects. This may be due to the fact that the sim-mel strains have largely Ore-R nuclear chromosomes, and these should be less differentiated from other North American strains such as FTF. Females show more significant differences than males.

Likelihood ratio tests between other pairs of genotypes further support the notion of complex nuclear–mtDNA interactions. For example, contrasts between the reciprocal crosses with sm38 females show no significant differences in α, β, or s (sm38 × Crete *vs.* sm38 × FTF), but contrasts between the reciprocal crosses with sm21 females show highly significant differences in α, β, and s (sm21 × Crete *vs.* sm21 XFTF; data available on request). Similarly, reciprocal crosses where FTF

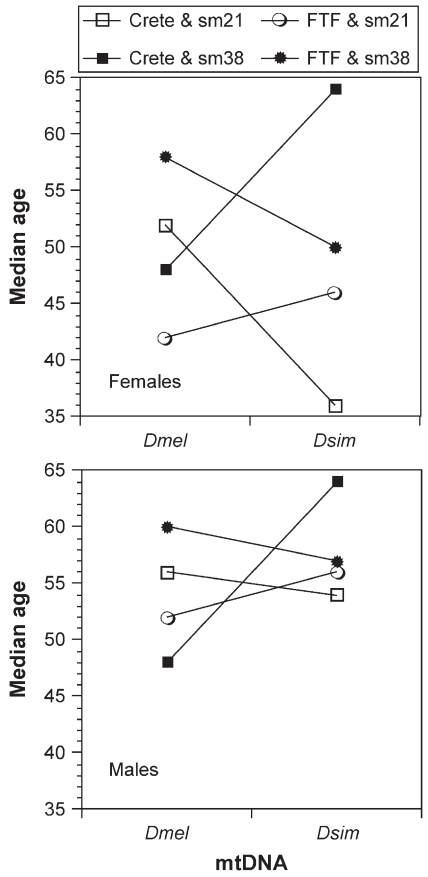

FIGURE 5.—Nuclear–mitochondrial epistasis for longevity. Median longevity, in days, is plotted for pairs of reciprocal crosses. Reciprocal genotypes have the same symbol connected by a line, analogous to reaction norms for hybrid nuclear genotypes in alternative mtDNA environments. The X-axis shows the mtDNA present in each genotype (*Dmel* signifies either Crete or FTF; *Dsim* signifies either sm21 or sm38). A plot with mean values is very similar. Error bars are omitted, but all interaction effects are highly significant (see Table 2).

TABLE 2

ANOVAs for nuclear–mtDNA interactions in longevity

	DF	SSQ	*F* ratio	*P*
Females: Crete, sm21, sm38				
Nuclear	1	21007.39	96.69	<0.0001
mtDNA	1	460.46	2.12	0.1457
Nuclear × mtDNA	1	27327.53	125.78	<0.0001
Females: FTF, sm21, sm38				
Nuclear	1	12669.21	55.16	<0.0001
mtDNA	1	197.57	0.86	0.3539
Nuclear × mtDNA	1	4903.11	21.35	<0.0001
All females				
Nuclear	3	52224.77	117.64	<0.0001
mtDNA	1	376.09	2.54	0.111
Nuclear × mtDNA	3	27239.20	61.36	<0.0001
Males: Crete, sm21, sm38				
Nuclear	1	1183.72	8.40	0.0038
mtDNA	1	5639.72	40.01	<0.0001
Nuclear × mtDNA	1	17435.89	123.68	<0.0001
Males: FTF, sm21, sm38				
Nuclear	1	6254.24	35.33	<0.0001
mtDNA	1	18.96	0.11	0.7435
Nuclear × mtDNA	1	1888.23	10.67	0.0011
All males				
Nuclear	3	7539.74	15.77	<0.0001
mtDNA	1	3213.01	20.16	<0.0001
Nuclear × mtDNA	3	21799.14	45.60	<0.0001

Nuclear refers to the two reciprocal crosses between pairs of strains carrying alternative mtDNAs. mtDNA refers to *D. melanogaster* (Crete 10 or FTF 100) or *D. simulans* mtDNA (carried in two mtDNA introgression strains with *D. melanogaster* Oregon-R chromosomes and *D. simulans siII* mtDNA; sm21 or sm38). Three different comparisons are made: one *D. melanogaster* strain crossed to both *D. simulans* mtDNA introgression strains (*e.g.,* Crete with sm21 or sm38), and both *D. melanogaster* wild lines with both introgression strains (*e.g.,* All Females). SSQ, sum of squares. See MATERIALS AND METHODS and RESULTS for details.

provided the mtDNA are significantly different for α and C, but reciprocal crosses where Crete provided mtDNA are highly significantly different for all four parameters α, β, s, and C. Thus, the same *Dsim* mtDNA haplotype can have very different mortality patterns when crossed to alternative *Dmel* wild lines, and the same wild *Dmel* mtDNA has different mortality patterns when crossed to different sim-mel mtDNA introgression strains. These analyses show that nuclear–mitochondrial epistatic interactions can modulate age-specific survival in a variety of ways.

DISCUSSION

More than 90% of the functional mitochondrial genome is encoded in the nucleus. These nuclear-encoded mitochondrial genes (mitonuclear genes) arise either by transfer events from the mitochondrial to the nuclear genome or by recruitment of nuclear genes to a novel mitochondrial function through the acquisition of mitochondrial targeting sequences (RAND *et al.* 2004). This genetic architecture suggests that the mitochondrial phenotypes should be governed by (1) mitochondrial genes (mtDNA), (2) mitonuclear genes, or (3) genetic interactions between mtDNA and mitonuclear genes. Moreover, coadaptation among mitochondrial and mitonuclear genes predicts that the phenotypic effects of alternative mtDNAs should increase with increasing levels of DNA sequence divergence between native and foreign mtDNAs (*e.g.*, mtDNA from different populations or species; RAND *et al.* 2004). The current study seeks to test these alternatives in the context of Drosophila aging by studying longevity among reciprocal crosses of strains carrying alternative mtDNAs.

Our results are qualitatively consistent with the coadaptation hypothesis: the inferred effects of alternative mtDNAs on Drosophila longevity increase in parallel with the degree of DNA sequence divergence. Differences in longevity were weak or absent between reciprocal F_1 flies carrying alternative mtDNAs from within a

Zimbabwe population, a subtle but significant difference was observed between an Old World and New World strain, and highly significant differences were observed among genotypes carrying mtDNAs from either *D. melanogaster* (*Dmel*) or *D. simulans* (*Dsim*). However, the results are not consistent with the hypothesis that flies carrying foreign mtDNA from a different species are generally disrupted and show reduced longevity. Our results clearly show that the effects of the foreign *Dsim* mtDNA on longevity depend strongly on the nuclear genetic background, *i.e.,* there is nuclear–mtDNA epistasis for longevity.

Nuclear–mitochondrial epistasis and longevity: Why are nuclear–mitochondrial epistatic effects stronger than mtDNA main effects on Drosophila longevity? Presumably main effects of mtDNA would be seen if a highly divergent mtDNA (say, from *D. pseudoobscura*) were carried on a *Dmel* nuclear background. One possibility is that the roughly 500 silent and 80 amino acid changes that exist between the *Dsim* and *Dmel* mtDNAs used in this study represent neutral or nearly neutral substitutions of nucleotide positions that are free to vary, rendering the two haplotypes nearly neutral variants with respect to longevity. Several lines of evidence argue against this. First, deleterious evolution of mtDNA is well established (Nachman 1998; Rand and Kann 1998), and non-neutral patterns of evolution have been documented for divergence of *Dmel* and *Dsim* mtDNAs (Ballard and Kreitman 1994; Rand *et al.* 1994; Ballard 2000). Second, population cage studies with these mtDNAs indicate that *Dsim* mtDNA is indeed selected against relative to *Dmel* mtDNA (D. Rand, unpublished results). Moreover, less-diverged mtDNA haplotypes within *D. simulans* show repeatable differences in fitness effects of mtDNA (James and Ballard 2003; Ballard and James 2004). While these arguments do not establish that *Dmel* and *Dsim* mtDNA are non-neutral for longevity, it seems plausible that if mtDNA effects are detectable at an early age, there should be some additive mtDNA effects for these haplotypes at a later age when selection is less likely to have influenced their evolution.

A second possible explanation for the inconsistent main effects of mtDNA on longevity could stem from genomic imprinting of nuclear chromosomes. To infer an mtDNA effect on longevity, we compared reciprocal F_1 genotypes carrying alternative mtDNAs. Reciprocal F_1 females will be identically heterozygous and carry alternative mtDNAs, but could also differ in parent-of-origin effects on the nuclear alleles transmitted into the F_1 flies studied. While imprinting has been reported for the Drosophila Y chromosome (Maggert and Golic 2002), this should not affect females. Notably, our results show that the differences in longevity among reciprocal F_1 males was less than that for sibling females, so if imprinting was active in our lines it appears to have reduced, not increased, reciprocal cross effects (*i.e.*, inferred mtDNA effects).

A third possible source of the strong epistatic effects is the retention of *Dsim* nuclear alleles in the mtDNA introgression lines carrying *Dsim* mtDNA. Introgression studies for a region of the second chromosome reveal that fertility and viability factors accounting for the isolation between *Dmel* and *Dsim* are densely distributed (Sawamura *et al.* 2000). During the initial generation of backcrossing between the *Dsim* C167.4 and *Dmel* In(1)AB, there may have been *Dsim* segments of the nuclear chromosomes that escaped the purging effects of hybrid unfitness in the increasingly *Dmel* nuclear background. Perhaps the presence of a *Dsim* mtDNA preferentially selected for individual *Dsim* alleles that are retained in some, but not all, of our sim-mel introgression strains (*e.g.*, in sm21 but not sm38). While spurious selection effects during the backcrossing may also have contributed to the results, the strong reciprocal cross effects in females would require that the selection altered dominance patterns of certain nuclear alleles for a strictly nuclear effect to explain our results. The alternative mtDNAs carried in reciprocal cross females seems like a more parsimonious explanation.

Sequence analyses of 70 nuclear encoded subunits of mitochondrial OXPHOS subunits in all six of our sim-mel introgressions strains show them to have the *Dmel* Oregon-R allele (as expected from Oregon-R male backcrossing). However, there are hundreds of known mitonuclear genes that remain to be sequenced for these strains, plus many genes of unknown mitochondrial function that could retain putative *Dsim* alleles that alter longevity. Such loci could have epistatic interactions with the FTF 100 or Crete 10 alleles used in our hybrid crosses. Alternatively, the epistatic effects of these putative nuclear × nuclear interactions could be sensitized by the presence of the alternative mtDNAs in their respective F_1 genotypes (Zeyl *et al.* 2005; Wade and Goodnight 2006).

A further source of the nuclear–mtDNA epistatic effects we observed may stem from alterations of gene expression that result from the crossing of wild strains. Transcript profiling of reciprocal F_1 genotypes from crosses between strains of *D. melanogaster* revealed that up to 33% of genes had significantly different expression patterns from the parental strains, and most of these effects were non-additive (Gibson *et al.* 2004). This misregulation effect is also pronounced in interspecific hybrids (Ranz *et al.* 2004), with some genes showing expression outside the range of either parent.

If the two introgression strains (sm21, sm38) have retained distinct *Dsim* alleles during the backcrossing process, this could add further variation to the patterns of mis-regulated genes in reciprocal crosses of these strains to the two wild *Dmel* strains (Crete 10 and FTF 100). For this to account for our longevity data, these misregulation effects would have to be altered by the mtDNA haplotype present in the reciprocal F_1 flies. Indeed, for this to occur in reciprocal F_1 females, it

suggests that mtDNA haplotype might alter the dominance of alternative alleles present in these hybrids. Mitochondrial-to-nuclear (retrograde) signaling modulates gene expression (Butow and Avadhani 2004) and affects longevity in yeast (Kirchman *et al.* 1999). In this context mitochondrial genotypes should be considered as novel "environments" for alleles and genotypes of nuclear loci. Since both nuclear × nuclear epistatic interactions and genotype × environment interactions affect Drosophila longevity (Leips and Mackay 2000; Vieira *et al.* 2000), the effects of alternative mtDNA haplotype on the misregulation of genes in hybrid crosses is a potentially important component of variation in longevity among individuals in outbred populations, and warrants further study.

Nuclear–mitochondrial coadaptation: The coadaptation hypothesis predicts that disrupted mitonuclear genotypes (*e.g.*, *Dmel* nuclear chromosomes with *Dsim* mtDNA) should have reduced performance, and there is compelling evidence for this in a variety of systems (Edmands and Burton 1999; Rawson and Burton 2002; McKenzie *et al.* 2003; Sackton *et al.* 2003). It might follow that such genotypes would have reduced longevity due to disrupted OXPHOS functions, possibly resulting in elevated ROS production. However, the relationship between metabolic rates, electron transport and ROS production are not at all clear (Speakman *et al.* 2004).

In most species of Drosophila, metabolic rates do not change with age (Promislow and Haselkorn 2002). There is no correlation between age and ROS production, and over-expression of the adenine nucleotide translocase (ANT) decreases ROS levels but shortens longevity (Miwa *et al.* 2004). Moreover, caloric restriction and reduced insulin signaling have no effect on metabolic rates or ROS production. It remains possible that the disruption of mitonuclear coadaptation could generate cellular states or gene expression patterns that extend longevity. An RNAi screen in *C. elegans* showed that extended longevity was associated with down-regulation of a number of mitochondrial genes, both nuclear and mtDNA encoded (Dillin *et al.* 2002). In mice, extended longevity was associated with higher metabolism due to higher levels of uncoupling (Speakman *et al.* 2004).

Thus, it may be simplistic to assume that different sets of heterozygous flies carrying the same divergent mtDNA should show reduced longevity simply because an interspecific coadapted gene complex is disrupted. These genotypes may vary in the levels of expression of OXPHOS or uncoupling proteins that result in reduced or extended longevity in an mtDNA-dependent manner. For example, one pair of reciprocal crosses (sm38 × Crete 10) showed the greatest longevity extension, while a different pair showed the most reduced longevity (FTF 100 × sm21). These kinds of reciprocal crosses among mtDNA introgression lines provide excellent material for dissecting the mechanistic bases of altered longevity as they generate strong genetic effects that should have biochemical and cellular explanations.

Our results demonstrate that even with relatively divergent mtDNA alleles (mtDNA differing at over 700 point mutations including >80 amino acid altering sites), epistatic interactions with nuclear genetic background are a significant component of the mitochondrial genetics of aging. This kind of epistasis could explain why some mtDNA mutations can have very different phenotypic effects in different individuals, possibly obscuring the mtDNA effects in human aging and disease (Jacobs and Holt 2000; Rose *et al.* 2001). Studies that seek to identify mitochondrial genotype effects in aging should not seek to remove nuclear genetic variation, but should embrace it by replicating mitochondrial genotypes across several different nuclear genetic backgrounds. While the human species has relatively low nuclear genetic variation relative to Drosophila, two average people differ by ~3 million nuclear base pairs, and ~50 mtDNA point mutations. The significance of this vast array of possible mitonuclear genotypes should be considered in the context of the unpredictable nature of mitonuclear epistasis.

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