Report

Current Biology

Single Nucleotides in the mtDNA Sequence Modify Mitochondrial Molecular Function and Are Associated with Sex-Specific Effects on Fertility and Aging

Highlights

- Mitochondrial haplotypes affect mtDNA copy number and mitochondrial gene expression
- These effects can be traced backward to individual SNPs within the mtDNA sequence
- Effects can be traced forward to sex-specific effects on fertility and longevity
- Study thus uncovers molecular mechanisms linking mitochondrial genotype to phenotype

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In Brief

Camus et al. report that small changes to the mtDNA sequence affect mitochondrial gene expression and mtDNA copy number. They then map single-base-pair changes in mtDNA to the expression of mitochondrial protein-coding genes and show that these effects have sex-specific—even sexually antagonistic—consequences on fertility and longevity.

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Report

Single Nucleotides in the mtDNA Sequence Modify Mitochondrial Molecular Function and Are Associated with Sex-Specific Effects on Fertility and Aging

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SUMMARY

Mitochondria underpin energy conversion in eukaryotes. Their small genomes have been the subject of increasing attention, and there is evidence that mitochondrial genetic variation can affect evolutionary trajectories and shape the expression of life-history traits considered to be key human health indicators [1, 2]. However, it is not understood how genetic variation across a diminutive genome, which in most species harbors only about a dozen protein-coding genes, can exert broad-scale effects on the organismal phenotype [2, 3]. Such effects are particularly puzzling given that the mitochondrial genes involved are under strong evolutionary constraint and that mitochondrial gene expression is highly conserved across diverse taxa [4]. We used replicated genetic lines in the fruit fly, *Drosophila melanogaster*, each characterized by a distinct and naturally occurring mitochondrial haplotype placed alongside an isogenic nuclear background. We demonstrate that sequence variation within the mitochondrial DNA (mtDNA) affects both the copy number of mitochondrial genomes and patterns of gene expression across key mitochondrial protein-coding genes. In several cases, haplotype-mediated patterns of gene expression were gene-specific, even for genes from within the same transcriptional units. This invokes post-transcriptional processing of RNA in the regulation of mitochondrial genetic effects on organismal phenotypes. Notably, the haplotype-mediated effects on gene expression could be traced backward to the level of individual nucleotides and forward to sex-specific effects on fertility and longevity. Our study thus elucidates how small-scale sequence changes in the mitochondrial genome can achieve broad-scale regulation of health-related phenotypes and even contribute to sex-related differences in longevity.

RESULTS AND DISCUSSION

We quantified cellular mtDNA copy number and levels of mitochondrial gene expression (for 9 of 13 protein-coding mtDNA genes) across 13 *D. melanogaster* lines, each of which is characterized by a mitochondrial haplotype sourced from a distinct global locality and expressed alongside a completely isogenic nuclear background, w^{1118} [5, 6] (Table S1). The assays were replicated separately for each sex and at both younger (6 days) and older (35 days) ages (Experimental Procedures; Figure S1).

We uncovered an effect of mitochondrial haplotype on mtDNA copy-number variation and found that it was contingent on interactions involving the sex and age of the flies (Table S2A). Furthermore, we found mtDNA copy number to be sexually dimorphic (generally exhibiting higher values in females) for flies of most, but not all, haplotypes; older flies exhibited greater levels of dimorphism than younger flies (Figure 1 and Table S2A; age × sex × mtDNA haplotype, $p < 0.001$). This effect was dependent on haplotype, and in two cases (Dahomey and Hawaii mtDNA at an older age) the pattern of dimorphism was one of male bias (Figure 1 and Figure S2A).

Similarly, mitochondrial gene expression exhibited strong signatures of sexual dimorphism, in this case generally male biased (Figure 2 and Figure S2A). Expression patterns were contingent on interactions involving the mtDNA haplotype and sex and age of the flies (Table S2B; age × sex × mtDNA haplotype, $p < 0.001$), and mtDNA-mediated effects on expression also varied across the sampled mitochondrial genes (Figure 2 and Table S2B; gene × mtDNA haplotype; $p < 0.001$). Notably, some genes exhibited strong differential expression across mtDNA haplotypes, whereas others did not (Figure 2).

These results are noteworthy for several reasons. First, they demonstrate not only that molecular phenotypes such as gene expression and copy number are affected by naturally occurring variation in mitochondrial haplotypes but also that these mtDNA-mediated effects are contingent on the context of sex and life stage. Second, it was previously believed that mtDNA copy-number regulation was purely under the control of the nucleus because the DNA polymerase γ responsible for mitochondrial replication is nuclear encoded [7]. Our finding adds to a recent study that showed that an mt-tRNA point mutation in *Drosophila* alters mtDNA copy and organelle number in a context-dependent manner; this alteration was found to be contingent on

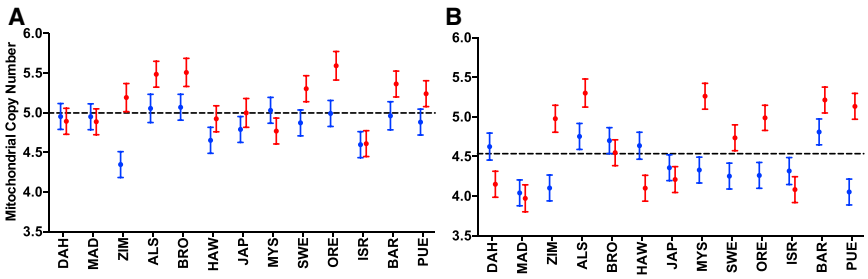

Figure 1. Mitochondrial Genetic Effects on mtDNA Copy-Number Variation

Least-square means (±1 SE) of $\log_{10}$-transformed mtDNA copy number across mtDNA haplotypes for males (blue points) and females (red points) at (A) younger (day 6) and (B) older (day 35) ages. The dashed line represents the mean for all lines and both sexes at that specific age category. See also Figures S1 and S2.

interactions between the mitochondrial and nuclear genome [8, 9]. The context-dependent effects, arising even in conditions of isogenicity of the nuclear background, demonstrate that the links between mitochondrial genotype and phenotype will be difficult to predict in many cases. This context dependency might also help explain why the epidemiology of mitochondrial diseases in humans is so complex and why simple links between candidate mtDNA mutations and disease penetrance often remain elusive [2, 10].

Notably, the effects of mitochondrial haplotypic variation on patterns of mitochondrial gene expression extended to cases in which the genes were nested within the same transcriptional units [11], and were therefore expected to exhibit the same pattern of expression. The most striking example pertains to the mt-*ND4* and mt-*ND5* genes, each of which is transcribed as part of a larger polycistronic precursor in *D. melanogaster* [11]. Expression of *ND4* was stable, but expression of *ND5* varied substantially across haplotypes (Figure 3A). Expression of *ND5* clustered into two groups of haplotypes, each delineated by an eightfold difference in expression. Males of the *ND5* "high-expression" group of haplotypes were associated with shorter longevity than males of the "low-expression" group, whereas female longevity did not differ across groups (sex × expression group, $p = 0.039$; Table S2C and Figure 3B). Furthermore, these groups correlate closely to the molecular phylogeny of the haplotypes (Figure 3A) and thus effectively represent mitochondrial clades differing in expression. The Mysore haplotype was the only exception. It shows high gene expression, yet it closely clusters with the low-expression Swedish haplotype. Together, these two haplotypes are more closely related to the clade of low-expression haplotypes (18 synonymous SNPs between these and the Japanese haplotype) than the clade of high-expression haplotypes (38 synonymous SNPs between these and the Oregon haplotype). The Mysore haplotype differs from the Swedish haplotype by one non-synonymous SNP (Ala-318-Asp) within the protein-coding region, and this SNP is located in *ND5* itself. This Ala-318-Asp SNP is thus putatively responsible for high *ND5* expression at the Mysore gene (mt-GWAS p value < 0.001; Table S2D), despite the observation that this haplotype appears to be phylogenetically aligned within the low-expression clade. Given that sequence polymorphism in *ND5* seems to directly affect its mRNA abundance, this indicates gene-level parallel evolution that was brought about by different underlying polymorphisms [12].

At mt-*CYTB*, a haplotype sourced from Brownsville, USA, exhibited a 4-fold decrease in expression relative to that of the other haplotypes, an effect observed in both sexes (Figure 3C). This effect was traced to a unique non-synonymous SNP (Ala-278-Thr) in *CYTB* itself, which delineates Brownsville from the other haplotypes (mt-GWAS, $p < 0.001$). The downstream effects of this SNP are far reaching, and the Brownsville haplotype is associated with sex specificity in the expression of the core life-history phenotypes: fertility and longevity. The Brownsville haplotype, which harbors the SNP, confers complete male sterility when expressed alongside w^{1118} [13], representing to our knowledge the only known case of mitochondrially induced cytoplasmic male sterility in metazoans. It is, however, associated with greater male longevity than the other haplotypes (Figure 3D), suggesting the Ala-278-Thr SNP has antagonistic pleiotropic effects such that sterile males are effectively released from the costs of producing highly viable sperm and enjoy longer lives. Females who harbor this haplotype remain fertile (at least under the standardized and non-competitive conditions in which we culture the females), but they exhibit shorter longevity than females harboring other haplotypes (sex × SNP, $p = 0.002$; Table S2E, Table S2F, and Figure 3D). This SNP therefore represents the first documented example of a candidate sexually antagonistic polymorphism segregating within the mitochondrial genome of a metazoan. Currently, it remains unclear whether the mitochondrial genome might generally be enriched for sexually antagonistic fitness variation, and this question deserves further experimental attention. Evolutionary theory predicts that maternal inheritance of mitochondria will render the mitochondrial genome prone to accumulation of mutations of sex-biased effect [14–17]. In particular, any de novo mtDNA sequence mutations that are overtly sexually antagonistic—that explicitly benefit females but harm males—would seem particularly likely to accrue quickly given that these mutations can only directly respond to selection through females [16, 17].

Intriguingly, the CYTB Ala-278-Thr SNP is associated with negatively pleiotropic effects on fertility and aging, both within and between the sexes. These effects have the potential to complicate tests of evolutionary theory based on the mitochondrial maternal inheritance, which predicts that mitochondrial genomes will accrue mutations that are explicitly male harming. Although in our case it is clear that the negative male-sterilizing effects would clearly outweigh any benefits recouped by enhanced male longevity, our findings nonetheless highlight the need for future studies to take several phenotypes into account in assessments of evolutionary predictions tied to mitochondrial maternal inheritance [18].

It was previously thought there was little capacity for individual mitochondrial genes to respond to particular SNPs, as observed in the case of *ND5* and *CYTB*, given that these genes are transcribed as part of broader transcriptional units [11]. Remarkably, the candidate SNPs we identified were located at

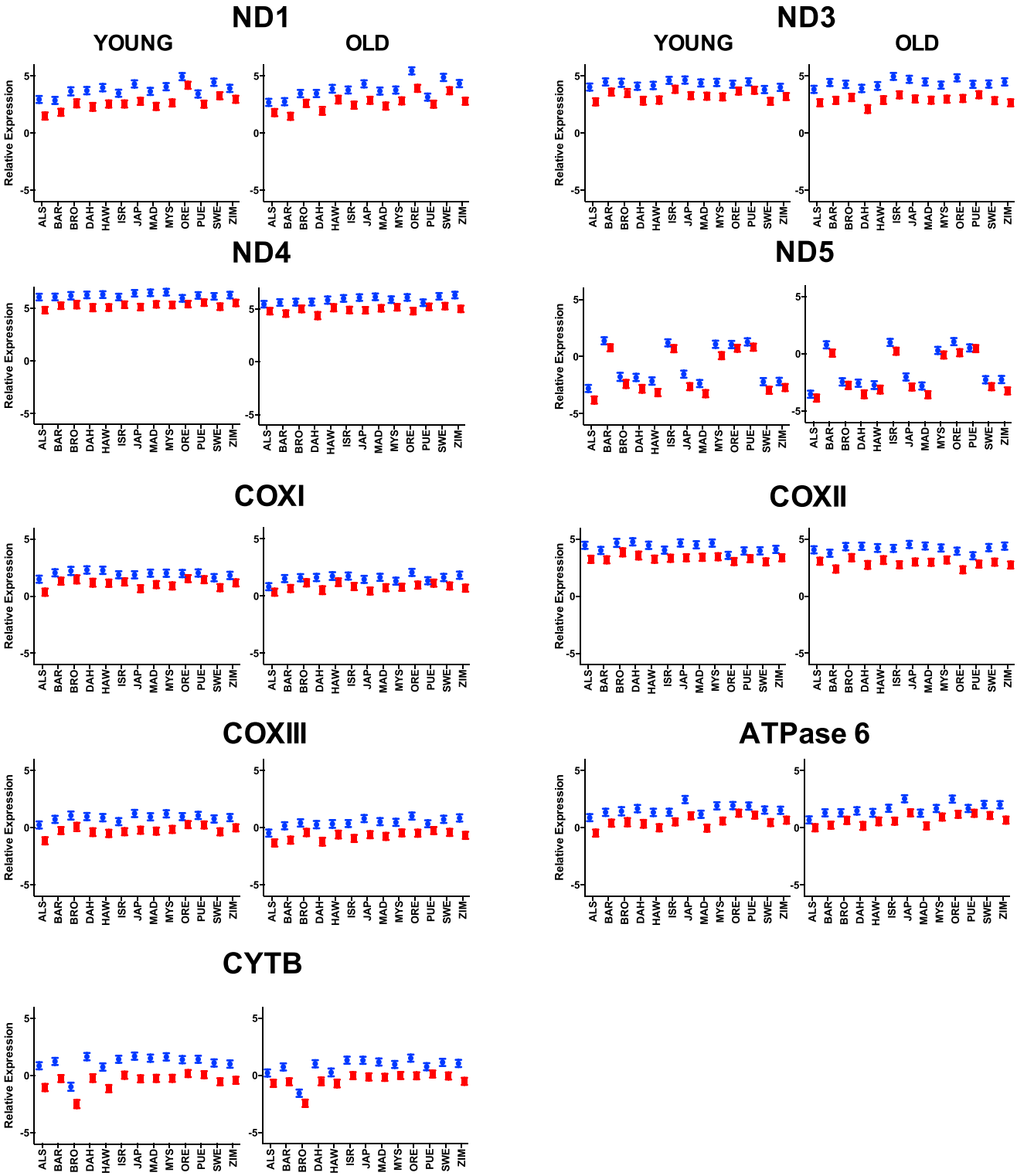

Figure 2. Mitochondrial Genetic Effects on Mitochondrial Gene Expression

Least-square means (±1 SE) of $\log_{10}$-transformed gene-expression values of nine mtDNA protein-coding genes across 13 mtDNA haplotypes in males (blue points) and females (red points) of both young (6d) and old (35d) flies. See also Figure S1.

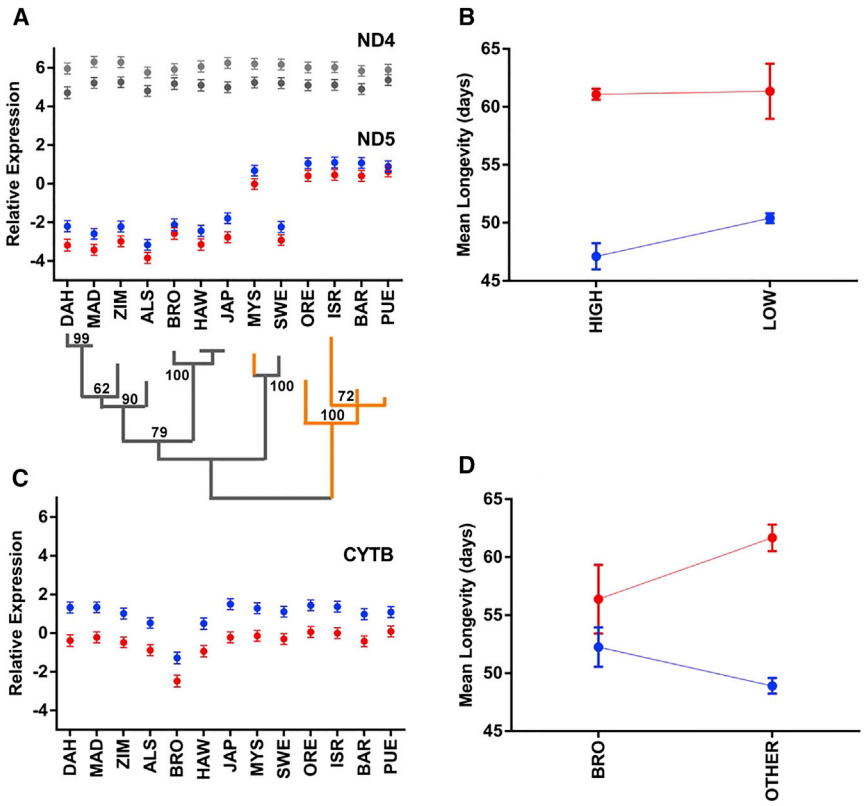

Figure 3. Mitochondrial Genetic Effects on Gene-Expression Map to Patterns of Sex-Specific Longevity

(**A**) Least-square means (±1 SE) of $\log_{10}$-transformed gene expression across mtDNA haplotypes for *ND4* and *ND5*. For *ND4*, males are denoted by light gray data points, and females are denoted by dark gray. For *ND5*, males are denoted by blue points, and females by red. Drawn below the axes is the mitochondrial phylogeny (neighbor-joining) derived from the complete genome (excluding D-loop) of all thirteen mtDNA haplotypes. Least-square means for all plots are derived from the multilevel models, which take into account mtDNA copy number as a covariate (see also Table S2B).

(B) Mean longevity comparisons between the low and high *ND5* expression groups of haplotypes; females are denoted in red, and males in blue.

(C) Least-square means (±1 SE) of $\log_{10}$-transformed *CYTB* expression across mtDNA haplotypes in males (blue points) and females (red points).

(D) Comparisons of mean longevity (±1 SE) between the Brownsville mtDNA haplotype (BRO) and the other (OTHER) haplotypes in females (red) and males (blue).

See also Figures S1 and S2.

non-synonymous sites and nested within the very same genes whose expression they affected. These candidate SNPs are therefore likely to exert their effects post-transcriptionally, potentially by altering the stability of transcripts [11]. Furthermore, although the effects of these SNPs on patterns of gene expression were generally of similar magnitude across the sexes, their effects on longevity and fertility were strikingly sex specific.

In conclusion, we have been able to trace effects of naturally segregating single nucleotides to the expression of protein-coding mitochondrial genes, then link these effects to patterns of longevity and fertility. Our study documents previously unrealized levels of context dependence in the expression of mitochondrial molecular phenotypes, which are plausible mechanistic mediators of the link between mitochondrial genotype and life-history phenotype. Furthermore, our study provides novel insights into the contribution of the mitochondrial genome to life-history trait evolution and suggests that mitochondrial polymorphisms can contribute to the sex differences observed in traits such as longevity, in which the females of many animal species generally outlive the males [18, 19].

EXPERIMENTAL PROCEDURES

Drosophila Lines

We used 13 "mitochondrial lines" of *Drosophila melanogaster*, reflecting the global diversity of mtDNA genetic variation [5, 6] (Supplemental Experimental Procedures). These lines were created by Clancy [5] via a chromosomal substitution procedure (Table S1) that replaced the nuclear backgrounds associated with each of the 13 mtDNA haplotypes with that of a standardized isogenic, homozygous nuclear background (w^{1118}). Since their generation, the lines have been maintained through the backcrossing of virgin females from each line to males of the isogenic w^{1118} line for a further 70 generations. The w^{1118} line is itself propagated by only one full-sibling-pair mating, per generation, ensuring that any cryptic mutations that accrue within w^{1118} will be swiftly purged or otherwise transmitted to all of the mitochondrial lines and thus maintaining the critical requirement for nuclear isogenicity across the lines. As a final safeguard of isogenicity, each mitochondrial line has been independently maintained and propagated in duplicate since 2007, which enables effects attributable to allelic variation across mtDNA haplotypes to be statistically partitioned from effects of residual cryptic nuclear genetic variance or other environmental sources of variance (Figure S1). Thus, placement of the mtDNA haplotypes alongside the isogenic w^{1118} nuclear background provides a powerful model in which we can probe for mitochondrial genetic variance for traits such as copy number and gene expression. A caveat is that by controlling for the effects of the nuclear background, we curtail the ability to test for the role of mito-nuclear epistasis in affecting the traits under study [20]. Thus, future studies should be designed to test for mito-nuclear interactions and also to test for the additive mitochondrial genetic effects under a broader range of nuclear genetic backgrounds.

All lines had been cleared of any potential bacterial endosymbionts, such as *Wolbachia* [21], as confirmed by diagnostic PCR [22].

Experimental Design

We tested for mitochondrial genetic variation in mtDNA copy number (an indicator of mitochondrial DNA abundance) and gene expression across nine mitochondrial protein-coding genes spanning all four mitochondrial polycistronic transcripts [11]. Copy number and gene expression were measured at two age classes (6 and 35 days) separately in males and females. The first age class represents flies in their reproductive prime, whereas the latter age represents the age at which population-level mortality rates start to increase exponentially when flies are kept under single-sex conditions and thus indicates the onset of physiological senescence [6, 23].

The experimental design was fully factorial (13 mito-lines × 2 sexes × 2 age classes = 52 experimental units), and each experimental unit was represented by three biological replicates of eight flies (Supplemental Experimental Procedures; Figure S1). All known environmental variables (e.g., food source, larval density, temperature, light, age, parental effects, and mating status) were carefully standardized during rearing of the experimental flies.

Total RNA and DNA Extraction and cDNA Synthesis

Total RNA was jointly extracted from all eight flies of each biological replicate with a combination of TRIzol Reagent and the Roche *HighPure* RNA extraction kit (Roche Applied Science) according to the manufacturer's instructions. By using TRIzol Reagent, we were able to separate and independently store DNA and RNA from the one sample. This resulted in 40 μl of purified RNA per sample and 30 μl of purified DNA, which we quantified by using a Nanodrop Spectrophotometer (NanoDrop Technologies). We assessed RNA and DNA integrity with 1% agarose gel electrophoresis. We determined the purity of total RNA as the 260/280 ratio; expected values were between 1.8 and 2.0. We synthesized cDNA from 1 μg RNA with the Roche Transcriptor First Strand cDNA Synthesis Kit and used a mixture of random hexamers and oligodT primers to capture mitochondrial transcripts both in the transitory polycistronic stage and as individual polyadenylated single transcripts [24].

Gene-Expression Quantification

Nine out of the thirteen mitochondrial protein-coding genes were amplified: *COXI*, *COXII*, *COXIII*, *ATPase6*, *ND1*, *ND3*, *ND4*, *ND5*, and *CYTB* (see Table S2G for complete list of primers used). The other four genes were either too small to amplify using quantitative real time (qRT) PCR (*ND6*, *ND4L*, *ATPase8*) or were too A-T rich to make suitable primers (*ND2*). Gene expression of each biological replicate was measured with a Roche Lightcycler 480 (Roche Applied Science, Indianapolis, IN). Reactions occurred in a 384-well plate, which was designed with a sample maximization layout [25]. Each plate measured the expression of a single mitochondrial gene (nine genes corresponding to nine plates in total), such that all 52 experimental units and their three biological replicates (156 independent reactions) were assayed per plate, and each independent reaction itself was subjected to a further level of replication—i.e., a technical replicate (Supplemental Experimental Procedures).

Reactions were performed with the Roche SYBRGreen I Mastermix (Roche Applied Science). Each well contained 5 μl SYBR buffer, 4 μl 2.5 μM primer mix, and 1 μl diluted cDNA. The amplification regime was as follows: 90°C (10 s), 60°C (10 s), and 72°C (10 s) for 45 cycles, followed by a melt-curve analysis to verify the specificity of the primer pair (See Supplemental Experimental Procedures).

For standardization, three nuclear housekeeping genes (HKGs) were chosen from an initial candidate list of 50 commonly used HKGs in *Drosophila* [26] (See Supplemental Experimental Procedures). For each experimental sample, the expression values of the mitochondrial target genes were standardized as follows:

$$2^{-\Delta Ct}$$

in which the cycle threshold '$\Delta Ct = Ct_{GOI} - Ct_{GEOM}$' is a relative measure of gene expression, GOI is the gene of interest, and GEOM is the geometric mean of the three housekeeping genes. The values provided from this equation indicate relative gene expression for each experimental sample in relation to expression of the housekeeping genes.

We obtained gene expression levels of all nine mitochondrial genes by determining the ΔCt, measured at the maximum acceleration of fluorescence, per sample by using the "second derivative maximum" method [27] in Roche Lightcycler software V1.5.0 (Roche Applied Science).

mtDNA Copy-Number Quantification

mtDNA copy number is a factor known to differ across sexes [28, 29] and may be expected to covary with transcript abundance [30]. We saved the DNA fraction from all RNA extractions and purified the DNA by using the Gentra Puregene Tissue Kit (QIAGEN). We then measured mtDNA copy number per biological replicate, relative to a single-copy gene in the nuclear genome [31]. The parameter thus reflects the average number of mitochondrial DNA copies per cell (or nucleus). We performed mitochondrial quantification by amplifying a 113 bp region of the large ribosomal subunit (CR34094, FBgn0013686) by quantitative real-time PCR. No nuclear copies of this gene are found in the *Drosophila melanogaster* genome. We quantified Nuclear DNA by amplifying a 135 bp region of the single-copy [32] subunit of the RNA polymerase II gene (CG1554, FBgn0003277).

Longevity Analyses

Mean longevity data were obtained for the mitochondrial lines from Camus et al. [6]. Longevity data points were extracted as mean longevity estimates for a "cohort" of on average 90 flies per mitochondrial line (as per the original analysis in Camus et al. [6]); thus we had several longevity data points per mitochondrial haplotype.

SUPPLEMENTAL INFORMATION

Supplemental Information includes Supplemental Experimental Procedures, including statistical analyses, two figures, and two tables and can be found with this article online at http://dx.doi.org/10.1016/j.cub.2015.09.012.

AUTHOR CONTRIBUTIONS

M.F.C., D.K.D., J.B.W.W., and E.H.M. designed the study; M.F.C. conducted the experiment and led the analyses; and M.F.C., D.K.D., J.B.W.W., and E.H.M. wrote the paper.

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