Report

Mitochondria, Maternal Inheritance, and Male Aging

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Summary

The maternal transmission of mitochondrial genomes invokes a sex-specific selective sieve, whereby mutations in mitochondrial DNA can only respond to selection acting directly on females [1–3]. In theory, this enables male-harming mutations to accumulate in mitochondrial genomes when these same mutations are neutral, beneficial, or only slightly deleterious in their effects on females [1–3]. Ultimately, this evolutionary process could result in the evolution of male-specific mitochondrial mutation loads; an idea previously termed Mother's Curse [2, 4–6]. Here, we present evidence that the effects of this process are broader than hitherto realized, and that it has resulted in mutation loads affecting patterns of aging in male, but not female *Drosophila melanogaster*. Furthermore, our results indicate that the mitochondrial mutation loads affecting male aging generally comprise numerous mutations over multiple sites. Our findings thus suggest that males are subject to dramatic consequences that result from the maternal transmission of mitochondrial genomes. They implicate the diminutive mitochondrial genome as a hotspot for mutations that affect sex-specific patterns of aging, thus promoting the idea that a sex-specific selective sieve in mitochondrial genome evolution is a contributing factor to sexual dimorphism in aging, commonly observed across species [7–9].

Results and Discussion

We present experimental evidence to support the contention that the maternal transmission of mitochondrial genomes has enabled sex-specific mutations to accumulate within them, which affect patterns of aging in males. We used thirteen naturally occurring mitochondrial haplotypes of *D. melanogaster* from around the globe, expressing each inside a completely isogenic nuclear background, w^{1118}. We then subjected the lines to an aging assay, in both males and females, to screen for genetic variation in longevity and the rate of senescence, and we sequenced the complete protein-coding regions of each of the thirteen mitochondrial haplotypes. All known environmental variables (e.g., food source, larval density, temperature, light, parental effects, age at mating, and mating status) were carefully controlled during the experiment, to minimize all other sources of variation.

Under this evolutionary process, it is predicted that the susceptibility of any given trait to the accumulation of male-specific mitochondrial mutation loads will be directly tied to the level of sexual dimorphism exhibited by that trait [1–3] (see Supplemental Information available online). This is because the benefits that males can salvage from relying on female-specific adaptation of mitochondrial DNA (mtDNA) will diminish as the level of sexual dimorphism increases and the intersexual genetic correlation erodes [10]. It is for this reason that most attention has focused on the vulnerability of the male reproductive tissues and gametes to this process, because these are sex-limited traits [1, 2, 10–12]. We found that longevity ($t_{109.87}$ = 16.90, p < 0.0001, X $_{males}$ = 49.21 ± 0.45 days, X $_{females}$ = 61.25 ± 0.55 days) and the rate of senescence (after controlling for frailty as a covariate, i.e., the frailty-corrected rate of senescence $F_{1,113}$ = 253.70, p < 0.0001) were each strongly sexual dimorphic across our fly lines. Each of these traits should thus, in principle, be vulnerable to the accumulation of male-specific mitochondrial mutation loads.

A second important prediction of this evolutionary process is that it should result in mitochondrial genomes that harbor mutation loads that are more pronounced in males and that these loads can be uncovered by demonstrating greater levels of functional mitochondrial genetic variance in males than in females [3]. Accordingly, we found significant genetic variance across mitochondrial haplotypes for longevity and the frailty-corrected rate of senescence in males (longevity: $F_{12,13.18}$ = 6.31, p = 0.0011; rate of senescence: $F_{12,14.25}$ = 3.71, p = 0.0106), but not in females (longevity: $F_{12,13.02}$ = 0.71, p = 0.7205; rate of senescence: $F_{12,12.91}$ = 0.50, p = 0.8803, Figure 1; Figure S1; Table S1). This is consistent with the underlying existence of male-specific mitochondrial mutation loads for these traits. Therefore, our results satisfy the core predictions of a sex-specific selective sieve in mitochondrial genome evolution and implicate it in the process of aging.

We then asked whether the male-specific mitochondrial mutation loads affecting patterns of aging more likely comprise a few mutations of major effect or numerous mutations of smaller effect dispersed throughout the mitochondrial genome. We hypothesized that clear support for the latter scenario would come from the existence of a positive association between the nucleotide divergence and phenotypic divergence for male aging, across pairwise combinations of mitochondrial haplotypes. We found exactly that, both for longevity (r = 0.243, p = 0.029, Figure 2) and the corrected rate of senescence (r = 0.312, p = 0.007, Figure 2). These analyses were based only on the pool of nonsynonymous SNPs, because there was a strong positive correlation between the number of nonsynonymous and synonymous SNPs across mitochondrial haplotypes (r = 0.77, p < 0.001; Supplemental Experimental Procedures; Figure S2), which suggests that the number of nonsynonymous SNPs found across any given pair of mitochondrial haplotypes correlates with the evolutionary divergence separating these haplotypes. Thus, our analysis indicates that the greater the number of SNPs separating any two mitochondrial haplotypes, the more those haplotypes generally differed in their aging profiles, supporting the idea that there are many mtDNA-encoded loci that
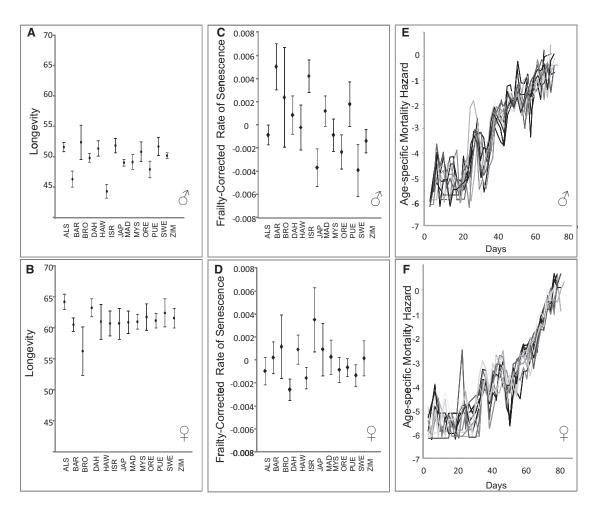
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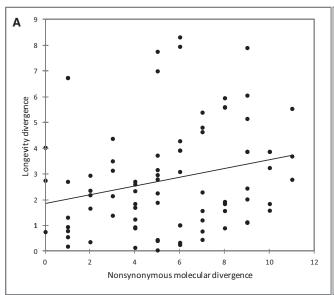Figure 1. Genetic Variance across Mitochondrial Haplotypes for Male and Female Aging Components

(A) Male longevity.

(B) Female longevity.

(C) Male frailty-corrected rate of senescence.

(D) Female frailty-corrected rate of senescence.

(E) Male age-specific mortality hazards for each mitochondrial haplotype.

(F) Female age-specific mortality hazards for each mitochondrial haplotype.

In (A)–(D), all data points denote means ± 1 SE. Rate of senescence values in (C) and (D) were calculated by taking the mean of the residuals of a linear regression of β on $\ln(\alpha)$, where each data point in the regression represented the β and α score of a cohort of on average 90 flies. Positive values of senescence indicate that the rate of senescence, β, associated with a given mitochondrial haplotype is greater than that expected based on the baseline mortality [$\ln(\alpha)$] value, whereas negative values indicate that that the rate of senescence is less than that expected based on the baseline mortality associated with a given haplotype. ALS denotes the Alstonville mtDNA haplotype; BAR, Barcelona; BRO, Brownsville; DAH, Dahomey; HAW, Hawaii; ISR, Israel; JAP, Japan; MAD, Madang; MYS, Mysore; ORE, Oregon; PUE, Puerto Montt; SWE, Sweden; and ZIM, Zimbabwe. In (E) and (F), the age-specific mortality hazards are calculated as [$\ln(\mu x)$], which is composed of the two aging components, $\ln(\mu x) = \ln(\alpha) + \beta x$. Each line represents a distinct mitochondrial haplotype.

affect male longevity and aging. Although our analyses cannot disentangle the relative importance of nonsynonymous versus synonymous SNPs in driving the differences in expression of aging phenotypes observed here, it is plausible that both contribute.

The mitochondrial mutations responsible for the male-specific variance in longevity and aging could in theory accumulate if they were neutral [13], nearly neutral [1], or positive [3] in effect when expressed in females. The lack of any significant mitochondrial genetic variance for female longevity and rates of senescence indicates that the mutations involved exerted only slight effects, if any, on female components of aging. These mutations might then have accrued under mutation-selection balance augmented by drift (if slightly deleterious to females), drift alone (if strictly neutral in females), or under positive selection (if beneficial to females). Although we are unable to distinguish between these three possibilities, it is plausible that all three contributed to the mtDNA-induced effects on males. Our results allow us to rule out the possibility that the mutations involved were consistently strongly sexually antagonistic in their effect on aging (in particular, a scenario whereby mutations that were of larger benefit to females were of larger harm to males). In such a case, we would have expected to have observed mitochondrial genetic variance for female longevity and senescence in the first instance, coupled with negative intersexual correlations for these traits across the mitochondrial haplotypes. This was not found (longevity: $r_{mf} = -0.0081$, $p = 0.978$, corrected rate of senescence: $r_{mf} = -0.205$, $p = 0.481$). Nonetheless, the mutations might have had positive effects on other components of
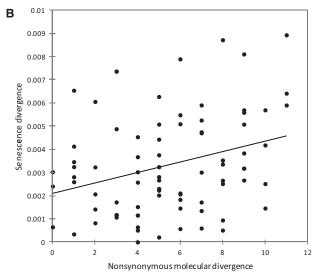
Figure 2. Associations between Molecular and Phenotypic Divergence across the Mitochondrial Haplotypes

Shown is the relationship between the number of nonsynonymous nucleotide differences and the differences in male mean longevity (A) and male mean frailty-corrected rates of senescence (B), across all pairwise combinations of mtDNA haplotypes.

female life history (e.g., fecundity and fertility) that went unmeasured in this study, with antagonistic effects on male components of aging.

Mitochondrial genetic variance for components of male fitness [4, 12, 14, 15], and in particular male aging [16–18], has previously been reported. However, the core predictions of a mitochondrial sex-specific selective sieve were not validated until recently, when a study reported that mitochondrial mutation loads affect the expression of hundreds of nuclear genes within the male, but not female, reproductive tissues of *D. melanogaster* [3], with putative effects on male fertility. Here, we have advanced understanding of mitochondrial genome evolution, by showing that mitochondrial genomes harbor variation that affects male-specific patterns of aging, thereby demonstrating the dramatic and hitherto unappreciated consequences of maternal inheritance of mtDNA to male life history evolution. Furthermore, we point out that although sexual dimorphism is predicted to be the initial trigger that enables the instigation of this evolutionary process within a population [1, 3, 10], once in operation it seems entirely plausible that the sex-specific selective sieve will then drive further increases in dimorphism across the sexes. If so, then we suggest that genetic variance harbored within the mitochondria might be a significant contributor to the patterns of sexual dimorphism observed in longevity and aging across the animal kingdom.

Experimental Procedures

Mitochondrial Lines
Thirteen populations of *D. melanogaster* were sourced from around the globe. These were as follows: Alstonville (New South Wales, Australia, collected 2002), Japan (Jume, Japan from DM Rand), Madang (Papua New Guinea, derived from massbred stock [19]), Mysore (India, Tucson StockCentre), Dahomey (now Benin, Africa, derived from massbred population collected in 1970), Zimbabwe (Zimbabwe, Zim53 from JWO Ballard [20]), Barcelona (Barcelona, Spain, isofemale derived from stock started founded by four females, 1954. Source: Bloomington Stock Center), Sweden (Swedish-C, Bloomington Stock Center), Israel (Israel, isofemale

derived from stock founded by four females, 1954. Source: Bloomington Stock Center), Puerto Montt (Puerto Montt, Chile, isofemale derived from massbred [21]), Brownsville (Brownsville, Texas, USA, from David Rand [22]), Oregon (Oregon-R-C, Oregon, USA, Bloomington Stock Center), and Hawaii (USA, Tucson Stock Center).

The prevailing mitochondrial haplotypes (GenBank accession numbers: AF200828 and AF200829, FJ190106–FJ190110, JX266575–JX266580) associated with each of these populations were then extracted and placed inside a completely isogenic nuclear background, w^{1118} (Bloomington stock no. 5905, isogenic for chromosomes 1, 2, and 3, constructed by John Roote, Cambridge, UK), using the crossing scheme depicted in Table S2. We put w^{1118} through 58 sequential generations of full-sib mating (i.e., the line was propagated by only one full-sib pair) prior to the commencement of the assays in 2011, to ensure the ongoing isogenicity of the whole nuclear genome, including the fourth chromosome.

In 2007, each of the mitochondrial lines was split in duplicate, and these duplicates were then propagated as separate entities. This measure was taken as a safeguard in the statistical analyses, to make sure that cryptic genetic variation had not accumulated within the nuclear background during the time that the lines were being constructed and prepared for analysis. Virgin females of each duplicate per mitochondrial line were backcrossed each generation to males of the isogenic w^{1118}. At least 30 generations of sequential backcrosses were performed on each line following the creation of the duplicates. This effectively ensured that all four chromosomes in the w^{1118} nuclear background were isogenic across the mitochondrial lines, in preparation for the eventual aging assays.

During the generation of the mitochondrial lines, all flies were reared at 25°C, on a 12:12 hr light:dark cycle, in 10 dram plastic vials, on a potato-dextrose-agar medium, with ad libitum live yeast added to each vial.

All strains used were cured for *Wolbachia* using 0.164 mg mL^{-1} tetracycline in food prior to 2007 [18]. In March 2010, each line was screened for the presence of *Wolbachia* [23], with mtDNA sequences used as positive controls for DNA quality. All lines were clear of infection.

Aging Assays
We scored the longevity of replicated cohorts of flies from each duplicate within each mitochondrial line. The sexes were assayed separately, in replicated cohorts of 30 flies per vial. Focal flies were collected from each duplicate, as virgins (within 6 hr of eclosion) under light CO_2 anesthesia. The flies of each duplicate were collected from numerous vials that had been trimmed back to contain 150 eggs (a moderate larval density), laid by mothers that were 4 days of age at the time of oviposition. Focal flies of each duplicate were then stored separately by sex, in vials of 32 individuals, for 4 days, with ad libitum access to live yeast.

When they were 4 days old, focal adults were mated en masse to tester flies of the opposite sex. These tester flies had been collected from the w^{1118} line as virgins, propagated under controlled densities (150 eggs per vial), hatched from eggs laid by mothers that were 4 days of age, and then themselves stored as adults for 4 days before mating to the focal flies. Each mating vial contained 32 pairs, and flies were given a 120 min window of opportunity to mate. Our pilot experiments on these mitochondrial lines show that almost all flies mate once and only once under these conditions. Tester flies were then separated from the focal flies, under light anesthesia, and discarded. Thirty focal flies were retained per vial and immediately entered into the aging assay.

Each group of 30 flies was then transferred to a fresh vial, containing food medium and 0.001 g live yeast, every second day, and the number of dead flies recorded at the time of transfer. Throughout the experiment, a small number of flies remained stuck on the surface of the previous vial at the time of transfer. These flies were always near death, and when this happened, we carefully transferred such flies manually with the aid of a spatula, taking precaution not to damage the fly. In total, 384 vials were scored, comprising a total of 11,049 flies, across sequential trials.

Molecular Data

PCR, product purification, and DNA sequencing were performed from genomic DNA by Macrogen Inc. (Seoul, South Korea). All 13 mtDNA-encoded protein genes were sequenced, with each section sequenced in both directions. Sequences were assembled and aligned using CodonCode Aligner (CodonCode Corp., Dedham, MA, USA) with the Zimbabwe 53 strain complete coding sequence for reference (AF200829). SNPs were detected, using DNAsp v.5 [24]. These SNPs were then classified as either synonymous or nonsynonymous, using the *D. melanogaster* mitochondrial DNA genetic code (Table S3).

Statistical Analysis

Raw mortality data were analyzed with *Winmodest 1.0.2* [25]. We took two separate approaches to analyze the data, which were driven by decisions made at the outset of the study (prior to data collection). Because the estimation of aging parameters is highly sensitive to sample sizes [25], for the purposes of our analyses we pooled replicate vials of each duplicate together into biologically relevant cohorts, at two separate levels. The first level was cohorts that averaged 90 individuals (i.e., each cohort constituted an average of three replicated vials), with a temporal structure that associates cohorts to the level of trials (vials of a cohort therefore shared overlapping "temporal" and "spatial" random environmental variance). The second level comprised cohorts that constituted all vials within a duplicate (i.e., each duplicate comprised a data point in the subsequent analysis, with all individuals within that duplicate contributing to the aging estimates). In the main text of the manuscript, we present our analyses based on cohorts corresponding to the first biological level of organization, whereas we present the analyses at the level of the duplicate within the Supplemental Information (Table S1). The important point is that each method produces qualitatively identical results. Similarly, whether or not we accounted for the exact number of deaths recorded per cohort (by including "total deaths" as a weight within the analyses) was inconsequential to the results. Hence, all results reported in the manuscript do not include this weighted term.

For each level of analysis (cohorts of 90, and entire duplicates as cohorts), we first explored which of four different models best described the temporal pattern of mortality in each cohort (Gompertz, Gompertz-Makeham, logistic and logistic-Makeham), using log-likelihood tests. In brief, the Gompertz model describes an exponential increase in mortality with age [25], denoted $\mu_x = \alpha\, e^{\beta x}$, where μ_x is the predicted instantaneous mortality rate at age x, α is the Gompertz intercept (also called age-independent mortality rate, or frailty), and β is the rate of increase in mortality with age (also called age-dependent mortality rate, or the rate of senescence of the population) [26]. Logistic models account for a deceleration in the rate of mortality with age, and when deceleration equals zero, logistic models are reduced to Gompertz. Finally, Makeham models include a constant that accounts for age-independent mortality [25].

We fitted Gompertz models for both sexes in our study, which were consistently the best fit to the data, as confirmed via sensitivity analyses that tested for the robustness of our parameter estimates (per cohort) using a maximum likelihood estimation procedure in *Winmodest*. The Gompertz parameters for frailty and the rate of senescence were estimated separately for each cohort. We also calculated mean longevity per cohort.

A negative association typically exists between the rate-of-senescence and frailty [27], and this was the case in our data set (regression coefficient = −0.014, n = 116, r^2 = 0.90, p < 0.001). Hence, rate of senescence was modeled with frailty (*ln* transformed) as a covariate in all of the subsequent analyses.

We tested for sexual dimorphism in longevity and in the rate of senescence using a t test for longevity, and an ANOVA for the rate of senescence. We then analyzed whether mitochondrial genetic variation exists for longevity and the rate of senescence in separate ANOVAs for each sex, to fulfill the key model assumption that variances across groups were homogeneous. In these analyses, the mitochondrial line and duplicate were treated as random effects and frailty as a fixed covariate in the senescence model, in Type III sum of squares ANOVA models, with Satterthwaite's approximation of denominator synthesis, in the PROC MIXED module of *SAS v.9.2*.

Given a high, positive correlation between the number of nonsynonymous and synonymous SNPs across mitochondrial haplotypes (Mantel test: r = 0.77, p < 0.001), here we analyze patterns across haplotypes involving only the pool of nonsynonymous SNPs, noting that these are substitutions of unambiguous evolutionary significance given that they result in a change in the amino acid sequence. We compared a matrix of differences in the number of nonsynonymous SNPs across all pairwise combinations of mitochondrial line, to matrices of phenotypic differences in male mean longevity and the male mean rate of senescence (corrected for frailty, by taking the residuals of β on lnα) across the same lines using Mantel tests in *XLSTAT 2011.4.01*. We also present gene by gene correlations between nonsynonymous and synonymous SNPs and mean male longevity and mean rate of senescence in Figure S2.

Accession Numbers

The GenBank accession numbers for the new prevailing mitochondrial haplotype sequences reported in this paper are JX266575–JX266580.

Supplemental Information

Supplemental Information includes two figures, three tables, and Supplemental Experimental Procedures and can be found with this article online at http://dx.doi.org/10.1016/j.cub.2012.07.018.

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