

Mother's curse is pervasive across a large mitonuclear *Drosophila* panel

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The maternal inheritance of mitochondrial genomes entails a sex-specific selective sieve, whereby mutations in mitochondrial DNA can only respond to selection acting on females. In theory, this enables male-harming mutations to accumulate in mitochondrial genomes as long as they are neutral, beneficial, or only slightly deleterious to females. Ultimately, this bias could drive the evolution of male-specific mitochondrial mutation loads, an idea known as mother's curse. Earlier work on this hypothesis has mainly used small *Drosophila* panels, in which naturally sourced mitochondrial genomes were coupled to an isogenic nuclear background. The lack of nuclear genetic variation in these designs has precluded robust generalization. Here, we test the predictions of mother's curse using a large *Drosophila* mitonuclear genetic panel, comprising nine isogenic nuclear genomes coupled to nine mitochondrial haplotypes, giving a total of 81 different mitonuclear genotypes. Following a predictive framework, we tested the mother's curse hypothesis by screening our panel for wing size. This trait is tightly correlated with overall body size and is sexually dimorphic in *Drosophila*. Moreover, growth is heavily reliant on metabolism and mitochondrial function, making wing size an ideal trait for the study of the impact of mitochondrial variation. We detect high levels of mitonuclear epistasis, and more importantly, we report that mitochondrial genetic variance is larger in male than female *Drosophila* for eight out of the nine nuclear genetic backgrounds used. These results demonstrate that the maternal inheritance of mitochondrial DNA does indeed modulate male life history traits in a more generalisable way than previously demonstrated.

KEY WORDS: *Drosophila*, mitonuclear interactions, mother's Curse, mtDNA.

Impact Summary

Mitochondria are essential organelles in eukaryotic cells, which originally derived from free-living bacteria and still retain their own specialized "bioenergetic" genomes (mtDNA). The mitochondrial genome is necessary for cell respiration, which provides a large proportion of the energy required for most biological processes. Although important, mitochondria are not passed on to the next generation by both parents; instead, they exhibit strict maternal inheritance across most species, making males an evolutionary dead end. Not all adaptations that are beneficial in females are also good for males, so as mitochondria adapt to female needs males can be disadvantaged. This evolutionary bias predicts that

mitochondria will accumulate male harmful mutations, a hypothesis known as "mother's curse." Although there has been some experimental evidence for this hypothesis, how generally it applies across natural populations is still widely debated. We therefore tested the hypothesis in populations of *Drosophila melanogaster* drawn from around the world. We created a large *Drosophila* mitonuclear panel comprising nine nuclear genomes, each coupled to nine mtDNA haplotypes for a total of 81 strains. We screened for wing size across the whole array of strains, as this trait is known to differ between the sexes and exerts high metabolic demands. Our results show that mixing and matching mitonuclear genomes has marked consequences for wing size, providing evidence that interactions between these two genomes are critical for

phenotypic traits. Most importantly, we find evidence for mother's curse in eight out of the nine nuclear backgrounds, indicating that female inheritance of mtDNA does indeed have generalizable repercussions for male fitness and life history evolution.

Mitochondria are essential organelles for life in eukaryotes, taking center stage in the process of cell respiration. Respiration is unusual in that the respiratory complexes within mitochondria are composed of proteins encoded by two different genomes, the nuclear and the mitochondrial. These two genomes must work harmoniously not only to provide cellular energy but also the precursors for most macromolecule synthesis. Consequently, their interaction is vital for the maintenance of mitochondrial integrity and the viability of eukaryote life. Almost without exception, eukaryotes from protists to animals have retained these two genomes (Rand et al. 2004; Lane 2005; Wolff et al. 2014). It was long assumed that purifying selection would remove nonneutral genetic variation within the mtDNA, given the "haploid" nature of this genome and the crucial role they play in energy production (Ballard and Whitlock 2004; Dowling et al. 2008; Cooper et al. 2015). As such, the mitochondrial genome was harnessed as the exemplary molecular marker on which to base evolutionary and population genetic inferences, facilitated by its high mutation rate, maternal inheritance, and general lack of recombination (Avice 1986; Wallace 1994; Lynch 1997; Pesole et al. 2000; Saccone et al. 2000; Galtier et al. 2009). Over the past two decades, however, a rising number of studies has challenged this assumption of neutrality (Ballard and Kreitman 1994; Rand 2001; Dowling et al. 2008). In particular, numerous studies have revealed that mitochondrial genetic variance sourced from separate populations contributes to the expression of a wide range of life history traits (James and Ballard 2003; Maklakov et al. 2006; Melvin and Ballard 2006; Dowling et al. 2007; Wolff et al. 2014; Zhu et al. 2014; Camus et al. 2015; Jelic et al. 2015; Immonen et al. 2016; Salminen et al. 2017).

Mitochondria are maternally inherited in most species, and so natural selection acting on the mitochondrial genome is effective only in females (Rand 2001). Mutations in mtDNA that are beneficial, neutral, or even slightly deleterious to males can be selected for in the population, whereas mutations that are detrimental to females should be removed from the population via purifying selection (Frank and Hurst 1996; Gemmell et al. 2004). Uniparental inheritance means that males inherit mutations that are selected through the female lineage, even if these mutations are detrimental to them (Innocenti et al. 2011). Through evolutionary time, we expect males to accumulate mitochondrial mutation loads consisting of male-biased deleterious mutations. The process leading to sex-biased mutation accumulation has been

termed "sex-specific selective sieve" (Innocenti et al. 2011) or the "mother's curse hypothesis" (Gemmell et al. 2004).

The idea of the mother's curse was first described in the 1990s (Frank and Hurst 1996) with further theoretical support proposed in the following decade (Gemmell and Allendorf 2001; Gemmell et al. 2004). Efforts have been made to form testable predictions for this hypothesis, with a recent review highlighting a predictive framework to test for the curse (Dowling and Adrian 2019). The first prediction is that not all traits will be equally susceptible to the accumulation of male-biased mitochondrial mutation loads (Friberg and Dowling 2008; Innocenti et al. 2011). In particular, metabolically demanding traits that exhibit sexual dimorphism in expression are most likely to be targets of the mother's curse. This is because the mitochondrial genome underpins most metabolic traits, given the crucial role that mtDNA plays in energy production. When it comes to optimizing mitochondrial function for sexually homologous traits, male homologues will exhibit suboptimal expression as they will not be able to rely on the female-mediated adaptation of the mtDNA sequence. This is unless nuclear alleles arise that compensate for male-harming mtDNA mutations, which given the difference in evolution rates between mitochondrial and nuclear genes necessarily lags behind (Connallon et al. 2018). In other words, mitochondrial mutations that are metabolically selected for in females may be detrimental to male metabolic demands, given that metabolism is itself a highly dimorphic trait.

The second prediction is that, if populations harbor mitochondrial genomes comprising male-biased mitochondrial mutation loads, then we should observe greater levels of mitochondrial genetic variance underpinning male relative to female phenotypes. The context of such an interpopulation prediction is that mitochondrial haplotypes will evolve along their own population-specific trajectories and accumulate their own distinct pools of male-biased mtDNA mutations with deleterious effects. Purifying selection, however, should remove any such mutations from mtDNA haplotypes that exert deleterious effects on females. These mutations can be unmasked by placing mitochondrial genomes alongside a foreign nuclear genome, where males cannot rely on male-specific coadapted alleles. Thus, when sampling mtDNA haplotypes from distinct populations, we expect greater levels of mitochondrial haplotypic variance underlying the expression of male compared with female phenotypes.

The first conclusive empirical validation of mother's curse was obtained in a study that examined the effects of mitochondrial variation on genome-wide patterns of nuclear gene expression. Specifically, by placing five different mitochondrial haplotypes alongside an isogenic nuclear background, approximately 10% of the nuclear transcripts were found to be differentially expressed in males relative to females (Innocenti et al. 2011). Interestingly, these differentially expressed transcripts were mostly

localized to the male reproductive system (testes, accessory glands, and ejaculatory duct), while having no major effect on male nonreproductive or female tissues. More recently, the scope of mother's curse has broadened beyond reproductive traits (Smith et al. 2010) to other life history traits including ageing (Camus et al. 2012) and metabolic rate (Nagarajan-Radha et al. 2020).

Although support for the mother's curse hypothesis is growing, one of the limitations of current *Drosophila* work has been unbalanced experimental designs (Rand and Mossman 2020). Studies have either used a single nuclear background coupled to many mitochondrial genomes (Innocenti et al. 2011; Camus et al. 2012; Nagarajan-Radha et al. 2020) or several nuclear genomes coupled to only a few mitochondrial haplotypes (Mossman et al. 2016; Mossman et al. 2016). This creates a situation whereby it is difficult to assess how generally the hypothesis might hold across species with high levels of genetic variation.

Here, we use a new *Drosophila* panel, which comprises a full factorial matrix of nine worldwide-sourced nuclear genomes coupled to nine mtDNA haplotypes (81 mitonuclear genotypes in total). Using this panel, we can study both mitonuclear interactions and mitochondrial genetic variance across several nuclear backgrounds. For each genotype and sex combination, we obtain measurements of wing centroid size, which is a highly reliable proxy for *Drosophila* body size (Carreira et al. 2009). We chose this trait first because of its link to metabolism (Guertin and Sabatini 2007; Bryk et al. 2010), with studies showing a positive correlation between *Drosophila* size and resting metabolic rate (Videli er et al. 2019; Videli er et al. 2020). Moreover, there is direct and indirect evidence for divergent selection in the two sexes, as this trait is sexually dimorphic (Partridge et al. 1994; De Jong and Bochdanovits 2003). Although this trait has an indirect link to fitness, it is a trait that we can measure with high precision on a large number of individuals—unlike fitness components that are highly stochastic and environmentally labile. These points combined make a strong case for body size being an ideal candidate trait to test for mother's curse. Our results show complex interactions between both mitochondrial and nuclear genomes, which modulate wing centroid size. These interactions consistently generate greater variance in males, against all but one nuclear background, confirming that mother's curse is indeed pervasive across *Drosophila* populations.

Materials and Methods

DROSOPHILA STOCK AND MAINTENANCE AND MITONUCLEAR PANEL

The mitonuclear *Drosophila* panel was produced through the de novo full factorial crossing of nine isogenic lines (9 nuDNA

x 9 mtDNA). The nine isogenic lines were obtained from the "Global Diversity Panel," which originate from five different continents and vary in phylogeographic relatedness (Early and Clark 2013; Grenier et al. 2015). Genetic diversity in this panel is representative of that between fruit fly populations worldwide. More specifically, they are sourced from A/ZIM184 (Zimbabwe), B/B04 (Beijing), C/I16 (Ithaca), D/I23 (Ithaca), E/N14 (Netherlands), F/N15 (Netherlands), G/T01 (Tasmania), H/T23 (Tasmania), and i/N01 (Netherlands). Using a balancer chromosome crossing scheme (Zhu et al. 2014), we replaced mitochondrial and nuclear genomes. Consequently, the panel contains 81 mitonuclear genotypes, also known as lines, nine of which are coadapted and 72 are disrupted. Following the balancer chromosome crossing scheme, the complete panel was backcrossed to their respective nuclear genome for three generations. We note that one of the mitonuclear genotypes ($F_{\text{nuc}} \times A_{\text{mito}}$) was not viable due to a strong incompatibility, and so for all experiments we proceeded with 80 genotypes.

Lines were propagated by 4-day-old parental flies, with approximate densities of 80–100 eggs per vial. Flies were kept at 25°C and 50% humidity, on a 12:12 h light:dark cycle, and reared on 8 mL of cornmeal-molasses-agar medium per vial (see Table S1 for recipe), with ad libitum live yeast added to each vial to promote female fecundity. All lines had been cleared of potential bacterial endosymbionts, such as *Wolbachia*, through a tetracycline treatment at the time the lines were created. Clearance was verified using *Wolbachia*-specific PCR primers (O'Neill et al. 1992).

WING CENTROID SIZE MEASURE

Focal flies from all genotypes were propagated via two sets of two sequential 4-h lays. The each lay contained 10–50 flies from each genotype. After a second round of oviposition, all vials were cleared. For each lay, vials were density controlled to contain 80–100 eggs. Focal flies were left to develop in their vials for 14 days by when all flies had eclosed and were sexually mature. On this day, flies of each vial were split by sex and flash frozen in Eppendorf tubes for subsequent wing analysis.

Fifteen right wings from each sex of each genotype were pulled and placed on a glass slide using double-sided tape. Along with a 10-mm scale bar, each wing was photographed at 2.5× magnification using an INFINITY Stereo Microscope attached to an Apple computer. The programs "tpsUtil," "tpsDIG2," and "COORD GEN 8" were consecutively used to determine the wing centroid size of each photographed wing from eight standard landmarks.

STATISTICAL ANALYSES

Our data followed a normal distribution and we used linear models to analyze the data. We first created a model to examine

all factors. Centroid size was a response variable with sex, mitochondrial genome, and nuclear genome (plus all their interactions) modeled as fixed factors. To further probe this three-way interaction, we divided the dataset by sex and analyzed the male and female data separately. For this, we used the same model framework as mentioned before: wing centroid size as a response variable with mitochondrial genome, and nuclear genome (plus their interaction) as fixed factors. All models were performed using the *lm/lmer* function in R version 3.3.2 (R Core Team 2016).

To visualize signatures of mother's curse, we standardized all datapoints by their respective sex- and nuclear-specific mean. This way we are able to better compare mitochondrial genetic variance across all nuclear genomes and both sexes (Fig. S1). To statistically test for mother's curse predictions, we calculated the mitochondrial coefficient of variation for each nuclear and sex combination using untransformed data. This was done by computing bootstrapped mitochondrial coefficients of variation (CV_m), in which trait means were resampled with replacement (1000 replicates). This procedure was performed using the *boot* package implemented in R. We used a two-sided sign test implemented in the *BDSA* package in R version 3.3.2 (R Core Team 2016) to test for a significant bias in the differences between male and female mitochondrial coefficients across nuclear genotypes.

Given that sequences are available for the protein coding genes of all mitochondrial haplotypes used in our study (Early and Clark 2013), we also tested if there was a correlation between the genetic distance between strains and their phenotypic divergence. To this end, we created matrices of genetic and phenotypic distances between strains (Table S2). Genetic distance was quantified as the total number of SNPs difference between lines—excluding the hypervariable region (D-loop). This is because the hypervariable region *Drosophila melanogaster* is a 4.7-kb region with >85% AT richness, making it very difficult to accurately map reads. Phenotypic matrices were specific to each experimental treatment (performed per sex and nuclear genotype) and phenotypic trait (centroid size). We used a Mantel test for matrix correlation between two dissimilarity matrices, with 10,000 permutations. Mantel test was implemented with the “*mantel.rtest*” function from the R package *ade4* (Dray and Dufour 2007).

Results

MITONUCLEAR EPISTASIS

Our models show sexual dimorphism for wing centroid size, with females having larger centroid size than males ($F = 13,328.2098$, $P < 0.001$; Fig. 1A; Table S3). Our full model also revealed effects of both mtDNA and nuDNA, with a significant three-way interaction between all main factors (mtDNA \times nuDNA \times sex:

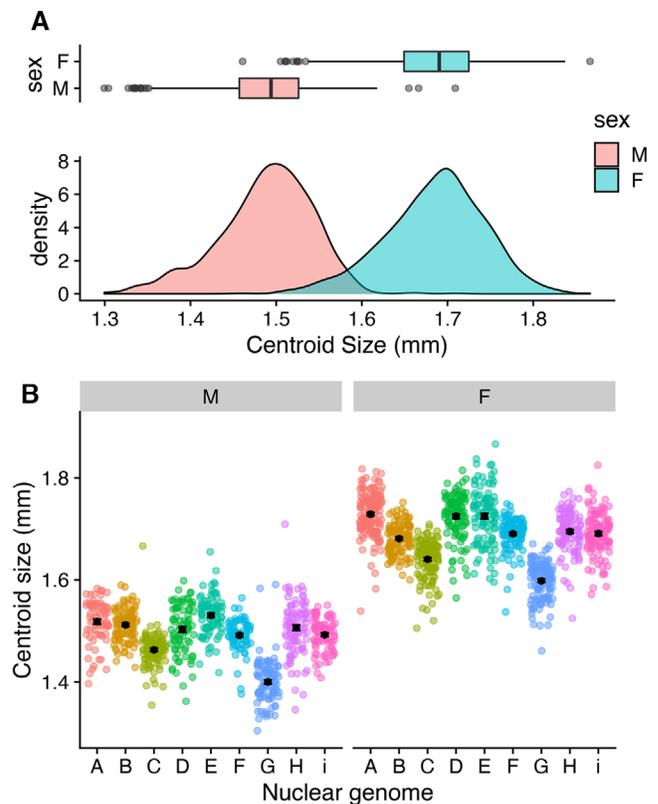


Figure 1. Wing centroid size measurements. (A) Broad patterns of sexual dimorphism for this trait with females being larger on average than males. (B) patterns of nuclear genetic variation for each sex, showing significant nuclear genetic effects driving centroid size. Individual datapoints are colored by nuclear genome.

$F = 1.5639$, $P = 0.003481$). When splitting the models by sex, we find significant mtDNA by nuDNA interactions for both males and females (male: $F = 5.1517$, $P < 0.001$; female: $F = 4.4962$, $P < 0.001$; Fig. 2; Table S3). We were interested in the effect size of each genetic component to wing size. For this, we calculated the proportion of variance explained by each effect using sums squared derived from the models. Our results show that for both sexes, a large proportion of the variance in wing size is explained by the nuclear genome (female: 51%, male: 49%; Table S4). Although we found mitochondria to contribute less to the overall variance in wing size observed in our data, we found it to be higher in males (2.3%) than females (0.9%). Similarly, we found the males to have a higher proportion of variance explained by the interaction (15.6%) term than females (10.8%).

Overall, there were positive genetic correlations between the sexes ($r_{mf} = 0.828$, $P < 0.001$; Fig. S2), indicating that the directionality for each mitonuclear genotype was concordant between the sexes. Within each nuclear genome, we found significant genetic correlations for most nuclear genomes (Fig. S2; Table S5). We did not find significant genetic correlations for nuclear genomes C, F, and I, although the directionality of the

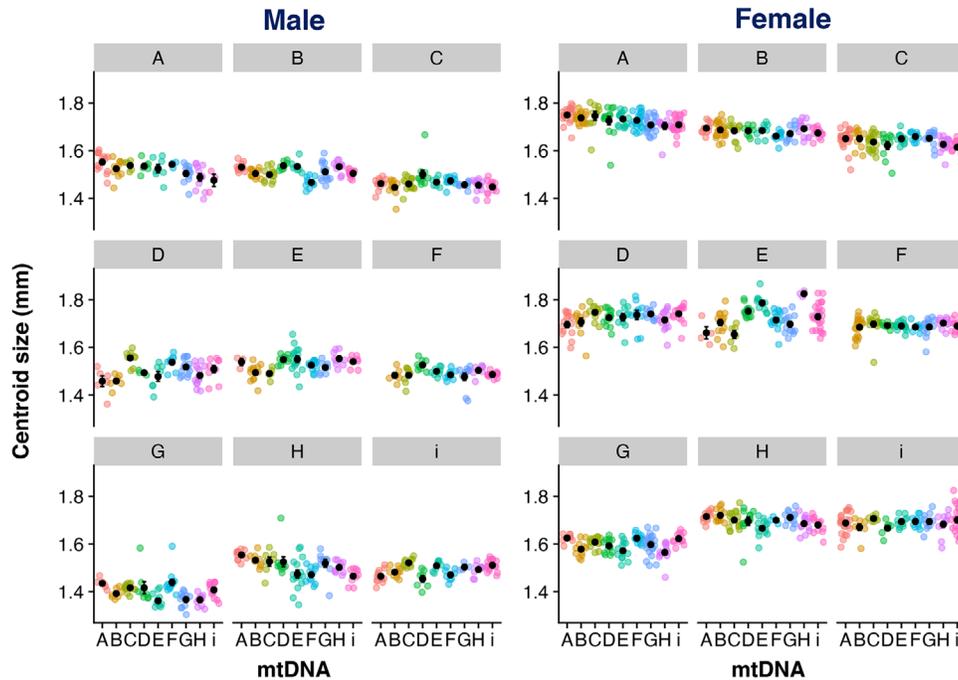


Figure 2. Wing centroid size across all treatments (mean \pm SE). Centroid size for males (left) and females (right). Each box corresponds to a specific nuclear genome, with mitochondrial genetic variation shown within each nuclear genome on the x-axis. Individual datapoints are colored according to the mtDNA genome. Note that the genotype $F_{\text{nuc}} \times A_{\text{mito}}$ was not sampled in this experiment as this mitonuclear combination is completely incompatible.

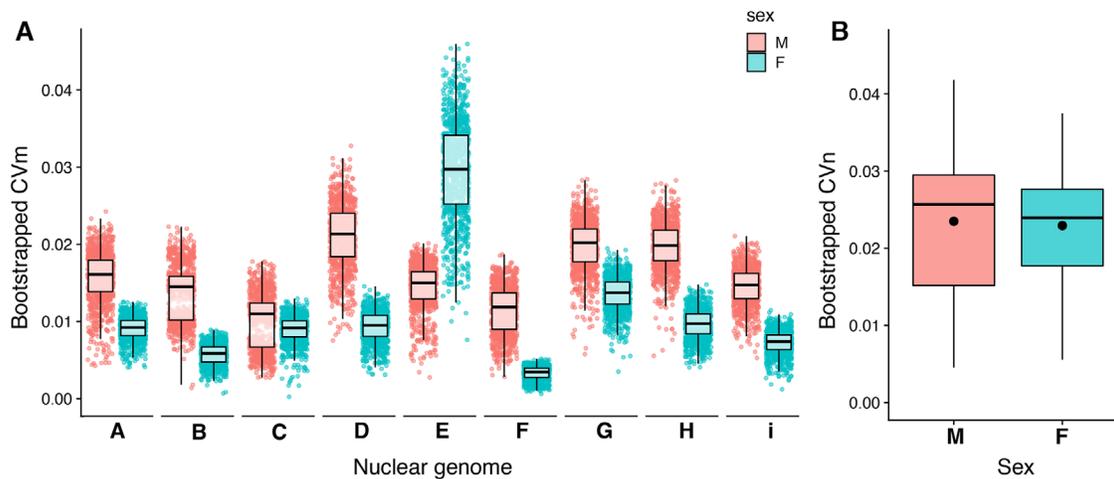


Figure 3. Coefficients of variation. (A) Bootstrapped mitochondrial coefficients of variation within each nuclear genome for both sexes. (B) Bootstrapped nuclear coefficients of variation for males and females. Dot in the middle of boxplot is the mean of each distribution.

relationship was positive in all three cases ($r_C = 0.1652$, $r_F = 0.4303$, $r_i = 0.6449$; Table S5).

TESTING MOTHER'S CURSE PREDICTIONS

An important prediction of mother's curse 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. To this end, we calculated coefficients of variation for each nuclear genome and sex combination, and our results show that overall males have significantly higher levels of mitochondrial genetic variance than females ($P = 0.03906$ [0.0015, 0.0097]; Fig. 3A; Table S6). Upon closer inspection, this rule is true for all but one nuclear genome ("E" nuclear genome). Moreover, we find that for six of the eight genotypes that have greater mitochondrial coefficients of

variation in males even raw variance is higher—despite males being smaller (Table S6).

MALE VARIABILITY HYPOTHESIS

The above sex difference in variation across mitonuclear combinations could be confounded by general sex differences in genetic variation for trait values, independent of mitonuclear interactions. For species with XY sex determination systems, males are predicted to show higher levels of genetic variation due to being the heterogametic sex (such as *Drosophila* and mammals) (Reinhold and Engqvist 2013). Consequently, we wanted to test if our dataset followed the general pattern of male-biased variability, or if the effects we observed were due to mother's curse. For this, we calculated the coefficients of variation between nuclear genomes for each sex separately. We extracted only the coevolved lines and use those mean values to calculate bootstrapped CVs. We first found that the nuclear CVs for both sexes were higher than mitochondrial CVs, as expected (Fig. 3B), and we additionally found no difference in nuclear CVs between the sexes ($F = 2.6186$, $P = 0.1058$).

RELATIONSHIP BETWEEN MITOCHONDRIAL GENETIC DISTANCE AND PHENOTYPIC DIFFERENTIATION

We asked whether the degree of coadaptation influenced phenotypic differentiation. Specifically, we wanted to know whether flies with more divergent mtDNA genomes also had higher phenotypic divergence. For this, we used Mantel tests to correlate two matrices: one comprising the mitochondrial genetic differences between the haplotypes (total SNPs—Table S3), with the other consisting of the mean phenotypic divergence from the coevolved strain. This analysis was performed for each nuclear genome separately. Given that we were unable to collect phenotypic data for one mitonuclear genotype (due to a severe incompatibility), the analysis for the F nuclear background was run using eight genotypes rather than nine. This analysis shows that there is no relationship between genetic and phenotypic divergence for females (Fig. 4). Males showed similar results to females; however, for two out of the nine nuclear genomes there was a significant positive association between genotype and phenotypic divergence. More specifically, these were nuclear genome A ($r = 0.543$, $P = 0.00391$) and H ($r = 0.228$, $P = 0.0158$). Such weak or absent correlation between mitonuclear genotype and phenotype has been reported before (Camus et al. 2020) and reflects the stochastic nature of small numbers of interactions between closely related populations, combined with the requirement for metabolic plasticity between tissues and sexes, which can buffer the effects of mutations.

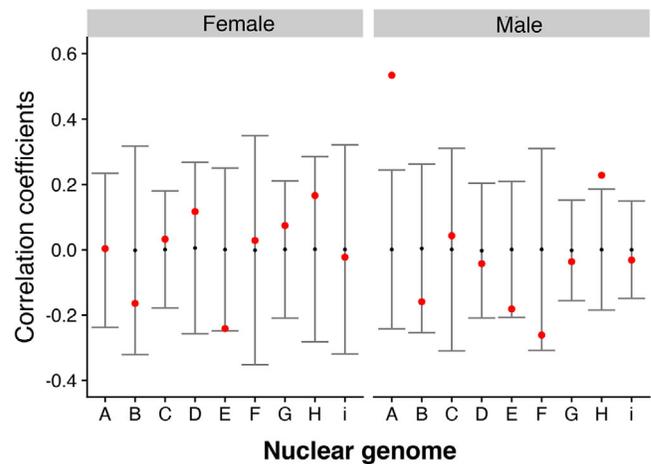


Figure 4. Association between genotypic and phenotypic divergence. Mantel test correlation of two matrices showing the null distribution of the permutations in gray with the mean correlation value for each test in red. Significant associations are when the correlation statistic falls outside of the null distribution. Mitochondrial genetic differences were calculated as total number of SNPs difference (not including the hypervariable region) between all the strains. For the phenotypic matrix, each nuclear genome was considered separately, creating a matrix of the average size difference between the coevolved strain and all other eight strains. Note that for the “F” nuclear genome, this analysis was performed with eight genomes as one of our genotypes was completely incompatible.

Discussion

The maternal inheritance of the mtDNA causes selection to favor mutations that are beneficial in females, even if these mutations are harmful in males. Ultimately, this evolutionary process has the potential to drive the accumulation of male-specific mitochondrial mutation loads, a hypothesis known as mother's curse. Although there is some experimental evidence for this hypothesis, one of the most pertinent criticisms is that the limited use of nuclear genetic backgrounds raises the possibility that mother's curse is not generalisable (Rand and Mossman 2020). Here, we aim to address this criticism by using a new panel of *Drosophila melanogaster* comprising nine nuclear backgrounds, each coupled to nine mtDNA haplotypes, resulting in 81 new mitonuclear genotypes. In line with the predictions of mother's curse (Dowling and Adrian 2019), we measured wing centroid size because the trait is sexually dimorphic and its development exerts high metabolic demands. We find complex interactions between all three factors (sex \times nuDNA \times mtDNA), indicating important epistatic effects in our dataset. Moreover, we find that mtDNA produces greater variance in males than in females across eight out of the nine nuclear backgrounds used in this study. Our findings thus demonstrate that the mother's curse is more pervasive than previously appreciated.

We first confirmed the sexual dimorphic patterns for wing centroid size, a pattern that has been observed in many earlier studies (Carreira et al. 2009; Gidaszewski et al. 2009). We also observed high levels of nuclear genetic variance within each sex for this trait, with this variation being mostly sexually concordant (Poissant et al. 2010; Connallon and Matthews 2019). Previous work that considered the contribution of nuclear genetic variance for wing size/shape using the DGRP strains also found very similar results (Pitchers et al. 2019). Our analyses demonstrate that a large proportion of this variation can be explained by the nuclear genome. Given that the nuclear backgrounds used in this study are representative of five different worldwide populations, we could have investigated whether this variation is due to the different geographical regions; however, our sample sizes per location were not adequate. During the creation of the panel, we selected fly strains that maximized both nuclear and mitochondrial genetic diversity, so it is possible that we captured many rare genomes from each population. The fact that each nuclear genome is inbred also complicates population-specific comparisons, so we deemed it best to treat them as independent genetic units.

Not all studies that looked for signatures of Mother's Curse have found male-biased effects (Mossman et al. 2016a,b, Dordevic et al. 2017). For example, mixed results have been obtained when examining mitochondrial genetic effects on nuclear gene expression. More specifically, although Innocenti et al. (2011) found male-biased genes being affected by replacing the mtDNA genome, work by Mossman et al. (2016) did not find these patterns. There are many differences between the two studies, with one of the main differences being that the latter study used mitonuclear flies that combined genomes from different species (*D. melanogaster* and *D. simulans*) and limited the number of mtDNA genomes sampled. Recent efforts have been made to create robust experimental guidelines to systematically test the predictions of mother's curse (Dowling and Adrian 2019). One of the main recommendations was that adequate levels of mitochondrial genetic variation be tested, as small numbers of mitochondrial genomes can easily under- or overrepresent variance. With this in mind, when creating our mitonuclear panel for our study, we aimed to maximize the number of mtDNA genomes. Although we do not have as many mtDNA genomes as some previous studies (Camus et al. 2012, 2015), we have captured far more nuclear genetic variation, enabling us to test for mother's curse across multiple nuclear environments.

Our results support the mother's curse hypothesis in two ways. First, the variance accounted for by mtDNA in our linear models is greater in males than females. Although the contribution of the mitochondrial genome to wing size variation is clearly much less than that of the nuclear genome, we also see an equivalent pattern when looking at the mito \times nuclear interaction

term. Previous work has found similar results when considering the contribution of the two genomes to the transcriptome, with the nuclear genome having a much greater effect than mtDNA (Mossman et al. 2016, 2017). We then measured the mitochondrial coefficients of variation and found similar patterns, whereby for most nuclear genomes, males showed higher levels of variation than females (Fig. 3). The only nuclear background where this was not the case was "E" (strain N14), where changing the mtDNA genome had a large effect on females. Although we do not know the exact mechanisms driving this response, we note that this nuclear background was particularly sick during rearing. We also noticed that mtDNA replacement for this nuclear background tends to decrease wing size, suggesting that the "E" nuclear background could be particularly susceptible to changes in physiology (Table S7). In sum, our experimental design was formulated taking into account the framework proposed to test the predictions of mother's curse: we have generated a robust dataset in favor of the hypothesis.

Tests of the mother's curse that rely on comparisons in trait variance between the sexes can be confounded by increased variance in males due to hemizygoty of the X chromosome (Reinhold and Engqvist 2013) or due to other factors (Wyman and Rowe 2014). Although a recent meta-analysis shows that male-biased variability is trait dependent (Zajitschek et al. 2020), we performed analyses to explicitly rule out sexual dimorphism in trait variability. Nuclear coefficients of variation were much greater than mtDNA CVs, which is expected as the nuclear genome has a larger contribution to this trait. Nevertheless, we did not find any differences in the CVs between the sexes when looking at the complete dataset and the coevolved genotypes, indicating that both sexes were equally variable for wing size in our experimental environment.

Given our experimental design, we were also able to investigate predictions for mitonuclear coadaptation, whereby coadapted genomes outperform disrupted combinations. We therefore examined whether phenotypic divergence from the coadapted mitonuclear combination correlated with genetic divergence. In other words, does greater genetic disruption result in larger phenotypic differences? Although we could see no association in females, we did find a significant positive association in two out of the nine nuclear genomes in males. These results suggest that mitonuclear interactions can exert significant, albeit inconsistent, effects on life history traits in different contexts. Previous work has likewise reported inconsistent results when looking at mitochondrial genetic relationships with fecundity and longevity (Camus et al. 2012, 2020). It could be that the effect and magnitude of different mtDNA SNPs depend on the environment that the nuclear background produces, so a SNP with a small effect in one nuclear background could have a large effect in another. Relatedly, the functional nuclear background varies from tissue

to tissue and between sexes, reflecting differences in gene expression and metabolic demands. This means that the mitochondrial genome must work effectively across multiple backgrounds, which likely limits the cumulative impact of SNPs, undermining correlations with genetic distance (Camus et al. 2020). Another caveat when considering coadaptation using this trait is that we are assuming that a larger wing reflects higher fitness. Although this tends to be true in females, it is less clear with males (Lefranc and Bundgaard 2000). That is because optimal male body size appears to be under stabilizing selection (Lefranc and Bundgaard 2000), with larger males more likely to harm to females (Pitnick and Garcia-Gonzalez 2002). So although there is a lot of evidence that metabolic deficits result in smaller flies (Bryk et al. 2010), there might also be an upper fitness optimum to how large *Drosophila* can get before size becomes a metabolic burden.

There have been several studies that have looked at the effects of the environment on mitonuclear interactions (more commonly reported as $G \times G \times E$) (Zhu et al. 2014; Aw et al. 2018; Drummond et al. 2019; Camus et al. 2020). Although we do not directly test this three-way interaction in our current study, we do predict for these patterns to be modulated by different environments. For instance, Montooth and colleagues showed that a mitonuclear incompatible genotype affects male fertility only when developed at an increased temperature (Montooth et al. 2019). Curiously, they were able to partially rescue the male fertility phenotype by changing their diet, indicating that the environment is of great importance when it comes to understanding the evolution of male-harming mutations. These studies open new avenues to explore how mother's curse is modulated by different environmental stressors. One can predict that increasing metabolic stress would reveal greater levels of variation, as individuals with mitonuclear incompatibilities are not able to fully cope with the increased metabolic burden.

In conclusion, we provide robust evidence for the mother's curse hypothesis. Our experimental design aimed to address some of the weaknesses of earlier work, notably the use of single nuclear backgrounds, and allowed us to demonstrate greater levels of mitochondrial genetic variance affecting male traits in all but one of the nuclear backgrounds. The panel will now allow us to test further predictions of the mother's curse hypothesis, notably the relationship between the sex-difference in trait variance and the degree of dimorphism across traits, as well as the degree to which the signature of the mother's curse depends on the metabolic burden imposed by developing or maintaining a trait.

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AUTHOR CONTRIBUTIONS

MFC, KF, and MR created the mitonuclear *Drosophila* panel. LC ran the experiments, collected all data, and performed preliminary analyses. MFC designed the experiment, assisted with data collection, and performed analysis. MR helped with statistical analyses. MFC lead in manuscript writing with helpful contributions from NL.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

DATA ARCHIVING

All data are available on DRYAD repository: <https://doi.org/10.5061/dryad.2280gb5r2>.

LITERATURE CITED

- Avise, J. C. 1986. Mitochondrial DNA and the evolutionary genetics of higher animals. *Philos. Trans. R. Soc. Lond. B Biol. Sci.* 312:325–342.
- Aw, W. C., S. G. Towarnicki, R. G. Melvin, N. A. Youngson, M. R. Garvin, Y. Hu, et al. 2018. Genotype to phenotype: diet-by-mitochondrial DNA haplotype interactions drive metabolic flexibility and organismal fitness. *PLoS Genet.* 14:e1007735.
- Ballard, J. W. and M. Kreitman. 1994. Unraveling selection in the mitochondrial genome of *Drosophila*. *Genetics* 138:757–772.
- Ballard, J. W. O. and M. C. Whitlock. 2004. The incomplete natural history of mitochondria. *Mol. Ecol.* 13:729–744.
- Bryk, B., K. Hahn, S. M. Cohen, and A. A. Teleman. 2010. MAP4K3 regulates body size and metabolism in *Drosophila*. *Dev. Biol.* 344:150–157.
- Camus, M. F., D. J. Clancy, and D. K. Dowling. 2012. Mitochondria, maternal inheritance and male ageing. *Curr. Biol.* 22:1717–1721.
- Camus, M. F., J. B. W. Wolf, E. H. Morrow, and D. K. Dowling. 2015. Single nucleotides in the mtDNA sequence modify mitochondrial molecular function and are associated with sex-specific effects on fertility and ageing. *Curr. Biol.* 25:2717–2722.
- Camus, M. F., J. Moore, and M. Reuter. 2020. Nutritional geometry of mitochondrial genetic effects on male fertility. *Biol. Lett.* 16:20190891.
- Camus, M. F., M. O'Leary, M. Reuter, and N. Lane. 2020. Impact of mitonuclear interactions on life-history responses to diet. *Philos. Trans. R. Soc. Lond. B Biol. Sci.* 375:20190416.
- Carreira, V. P., J. Mensch, and J. J. Fanara. 2009. Body size in *Drosophila*: genetic architecture, allometries and sexual dimorphism. *Heredity* 102:246–256.
- Connallon, T. and G. Matthews. 2019. Cross-sex genetic correlations for fitness and fitness components: connecting theoretical predictions to empirical patterns. *Evol. Lett.* 3:254–262.
- Connallon, T., M. F. Camus, E. H. Morrow, and D. K. Dowling. 2018. Coadaptation of mitochondrial and nuclear genes, and the cost of mother's curse. *Proc. R. Soc. B Biol. Sci.* 285:20172257.
- Cooper, B. S., C. R. Burrus, C. Ji, M. W. Hahn, and K. L. Montooth. 2015. Similar efficacies of selection shape mitochondrial and nuclear genes in both *Drosophila melanogaster* and *Homo sapiens*. *G3* 5:2165–2176.
- De Jong, G. and Z. Bochdanovits. 2003. Latitudinal clines in *Drosophila melanogaster*: body size, allozyme frequencies, inversion frequencies, and the insulin-signalling pathway. *J. Genet.* 82:207–223.
- Dordevic, M., B. Stojkovic, U. Savkovic, E. Immonen, N. Tucic, J. Lazarevic, et al. 2017. Sex-specific mitonuclear epistasis and the evolution of

- mitochondrial bioenergetics, ageing, and life history in seed beetles. *Evolution* 71:274–288.
- Dowling, D. K. and R. E. Adrian. 2019. Challenges and prospects for testing the mother's curse hypothesis. *Integr. Comp. Biol.* 59:875–889.
- Dowling, D. K., U. Friberg, F. Hailer, and G. Arnqvist. 2007. Intergenic epistasis for fitness: within-population interactions between cytoplasmic and nuclear genes in *Drosophila melanogaster*. *Genetics* 175:235–244.
- Dowling, D. K., U. Friberg, and J. Lindell. 2008. Evolutionary implications of non-neutral mitochondrial genetic variation. *Trends Ecol. Evol.* 23:546–554.
- Dray, S. and A. B. Dufour. 2007. The ade4 package: implementing the duality diagram for ecologists. *J. Stat. Softw.* 22:1–20.
- Drummond, E., E. Short, and D. Clancy. 2019. Mitonuclear gene X environment effects on lifespan and health: how common, how big? *Mitochondrion* 49:12–18.
- Early, A. M. and A. G. Clark. 2013. Monophyly of *Wolbachia pipientis* genomes within *Drosophila melanogaster*: geographic structuring, titre variation and host effects across five populations. *Mol. Ecol.* 22:5765–5778.
- Frank, S. A. and L. D. Hurst. 1996. Mitochondria and male disease. *Nature* 383:224.
- Friberg, U. and D. K. Dowling. 2008. No evidence of mitochondrial genetic variation for sperm competition within a population of *Drosophila melanogaster*. *J. Evol. Biol.* 21:1798–1807.
- Galtier, N., B. Nabholz, S. Glemin, and G. D. D. Hurst. 2009. Mitochondrial DNA as a marker of molecular diversity: a reappraisal. *Mol. Ecol.* 18:4541–4550.
- Gemmell, N. J. and F. W. Allendorf. 2001. Mitochondrial mutations may decrease population viability. *Trends Ecol. Evol.* 16:115–117.
- Gemmell, N. J., V. J. Metcalfe, and F. W. Allendorf. 2004. Mother's curse: the effect of mtDNA on individual fitness and population viability. *Trends Ecol. Evol.* 19:238–244.
- Gidaszewski, N. A., M. Baylac, and C. P. Klingenberg. 2009. Evolution of sexual dimorphism of wing shape in the *Drosophila melanogaster* subgroup. *BMC Evol. Biol.* 9:110.
- Grenier, J. K., J. R. Arguello, M. C. Moreira, S. Gottipati, J. Mohammed, S. R. Hackett, et al. 2015. Global diversity lines - a five-continent reference panel of sequenced *Drosophila melanogaster* strains. *G3* 5:593–603.
- Guertin, D. A. and D. M. Sabatini. 2007. Defining the role of mTOR in cancer. *Cancer Cell* 12:9–22.
- Immonen, E., M. Collet, J. Goenaga, and G. Arnqvist. 2016. Direct and indirect genetic effects of sex-specific mitonuclear epistasis on reproductive ageing. *Heredity* 116:338–347.
- Innocenti, P., E. H. Morrow, and D. K. Dowling. 2011. Experimental evidence supports a sex-specific selective sieve in mitochondrial genome evolution. *Science* 332:845–848.
- James, A. C. and J. W. O. Ballard. 2003. Mitochondrial genotype affects fitness in *Drosophila simulans*. *Genetics* 164:187–194.
- Jelic, M., G. Arnqvist, Z. Kurbalija Novicic, B. Kenig, M. Tanaskovic, and M. Andelkovic, et al. 2015. Sex-specific effects of sympatric mitonuclear variation on fitness in *Drosophila subobscura*. *BMC Evol. Biol.* 15:135.
- Lane, N. 2005. Power, sex, suicide: mitochondria and the meaning of life. Oxford Univ. Press, Oxford, U.K.
- Lefranc, A. and J. Bundgaard. 2000. The influence of male and female body size on copulation duration and fecundity in *Drosophila melanogaster*. *Hereditas* 132:243–247.
- Lynch, M. 1997. Mutation accumulation in nuclear, organelle, and prokaryotic transfer RNA genes. *Mol. Biol. Evol.* 14:914–925.
- Maklakov, A. A., U. Friberg, D. K. Dowling, and G. Arnqvist. 2006. Within-population variation in cytoplasmic genes affects female life span and aging in *Drosophila melanogaster*. *Evolution* 60:2081–2086.
- Melvin, R. G. and J. W. O. Ballard. 2006. Intraspecific variation in survival and mitochondrial oxidative phosphorylation in wild-caught *Drosophila simulans*. *Aging Cell* 5:225–233.
- Montooth, K. L., A. S. Dhawanjewar, and C. D. Meiklejohn. 2019. Temperature-sensitive reproduction and the physiological and evolutionary potential for mother's curse. *Integ. and Comp. Biol.* 59:890–899.
- Mossman, J. A., L. M. Biancani, C. T. Zhu, and D. M. Rand. 2016a. Mitonuclear epistasis for development time and its modification by diet in *Drosophila*. *Genetics* 203:463–484.
- Mossman, J. A., J. G. Tross, N. Li, Z. Wu, and D. M. Rand. 2016b. Mitochondrial-nuclear interactions mediate sex-specific transcriptional profiles in *Drosophila*. *Genetics* 204:613–630.
- Mossman, J. A., J. G. Tross, N. A. Jourjine, N. Li, Z. Wu, and D. M. Rand. 2017. Mitonuclear interactions mediate transcriptional responses to hypoxia in *Drosophila*. *Mol. Biol. Evol.* 34:447–466.
- Nagarajan-Radha, V., I. Aitkenhead, D. J. Clancy, S. L. Chown, and D. K. Dowling. 2020. Sex-specific effects of mitochondrial haplotype on metabolic rate in *Drosophila melanogaster* support predictions of the Mother's Curse hypothesis. *Philos. Trans. R. Soc. Lond. B Biol. Sci.* 375:20190178.
- O'Neill, S. L., R. Giordano, A. M. E. Colbert, T. L. Karr, and H. M. Robertson. 1992. 16s ribosomal-RNA phylogenetic analysis of the bacterial endosymbionts associated with cytoplasmic incompatibility in insects. *Proc. Natl Acad. Sci.* 89:2699–2702.
- Partridge, L., B. Barrie, K. Fowler, and V. French. 1994. Evolution and development of body size and cell size in *Drosophila melanogaster* in response to temperature. *Evolution* 48:1269–1276.
- Pesole, G., C. Gissi, D. Catalano, G. Grillo, F. Licciulli, S. Liuni, et al. 2000. MitoNuc and MitoAln: two related databases of nuclear genes coding for mitochondrial proteins. *Nucleic. Acids. Res.* 28:163–165.
- Pitchers, W., J. Nye, E. J. Marquez, A. Kowalski, I. Dworkin, and D. Houle. 2019. A multivariate genome-wide association study of wing shape in *Drosophila melanogaster*. *Genetics* 211:1429–1447.
- Pitnick, S. and F. Garcia-Gonzalez. 2002. Harm to females increases with male body size in *Drosophila melanogaster*. *Proc. Biol. Sci.* 269:1821–1828.
- Poissant, J., A. J. Wilson, and D. W. Coltman. 2010. Sex-specific genetic variance and the evolution of sexual dimorphism: a systematic review of cross-sex genetic correlations. *Evolution* 64:97–107.
- Rand, D. M. 2001. The units of selection in mitochondrial DNA. *Annu. Rev. Ecol. Syst.* 32:415–448.
- Rand, D. M. and J. A. Mossman. 2020. Mitonuclear conflict and cooperation govern the integration of genotypes, phenotypes and environments. *Philos. Trans. R. Soc. Lond. B Biol. Sci.* 375:20190188.
- Rand, D. M., R. A. Haney, and A. J. Fry. 2004. Cytonuclear coevolution: the genomics of cooperation. *Trends Ecol. Evol.* 19:645–653.
- Reinhold, K. and L. Engqvist. 2013. The variability is in the sex chromosomes. *Evolution* 67:3662–3668.
- Saccone, C., C. Gissi, C. Lanave, A. Larizza, G. Pesole, and A. Reyes. 2000. Evolution of the mitochondrial genetic system: an overview. *Gene* 261:153–159.
- Salminen, T. S., M. T. Oliveira, G. Cannino, P. Lillsunde, H. T. Jacobs, and L. S. Kaguni. 2017. Mitochondrial genotype modulates mtDNA copy number and organismal phenotype in *Drosophila*. *Mitochondrion* 34: 75–83.

- Smith, S., C. Turbill, and F. Suchentrunk. 2010. Introducing mother's curse: low male fertility associated with an imported mtDNA haplotype in a captive colony of brown hares. *Mol. Ecol.* 19:36–43.
- R Core Team. 2016. R: a language and environment for statistical computing. R Foundation for Statistical Computing, Vienna.
- Videliere, M., H. D. Rundle, and V. Careau. 2019. Sex-specific among-individual covariation in locomotor activity and resting metabolic rate in *Drosophila melanogaster*. *Am. Nat.* 194:E164–EE76.
- Videliere M., Careau V., Wilson A. J., and Rundle H. D. 2020. Quantifying selection on standard metabolic rate and body mass in *Drosophila melanogaster*. *Evolution* 75:130–140.
- Wallace, D. C. 1994. Mitochondrial DNA sequence variation in human evolution and disease. *Proc. Natl. Acad. Sci. USA* 91:8739–8746.
- Wolff, J. N., E. D. Ladoukakis, J. A. Enriquez, and D. K. Dowling. 2014. Mitonuclear interactions: evolutionary consequences over multiple biological scales. *Philos. Trans. R. Soc. of Lond. Ser. B. Biol. Sci.* 369:20130443.
- Wyman, M. J. and L. Rowe. 2014. Male bias in distributions of additive genetic, residual, and phenotypic variances of shared traits. *Am. Nat.* 184:326–337.
- Zajitschek, S. R., F. Zajitschek, R. Bonduriansky, R. C. Brooks, W. Cornwell, D. S. Falster, et al. 2020. Sexual dimorphism in trait variability and its eco-evolutionary and statistical implications. *elife* 9:e63170.
- Zhu, C. T., P. Ingelmo, and D. M. Rand. 2014. GxGxE for lifespan in *Drosophila*: mitochondrial, nuclear, and dietary interactions that modify longevity. *PLoS Genet.* 10:e1004354.

Supporting Information

Additional supporting information may be found online in the Supporting Information section at the end of the article.

Table S1: *Drosophila* rearing medium recipe. This recipe makes for 2L worth of food.

Figure S1: Mitochondrial genetic variation for each nuclear genome and sex combination.

Table S2: Mitochondrial genetic differentiation matrix.

Table S3: Outputs from linear models where centroid size was modelled with mtDNA, nuDNA and sex as fixed factors.

Table S4: Proportion of variance explained by explanatory variables for both male and female models.

Figure S2: Centroid size correlations.

Table S5: Mitochondrial genetic correlations per nuclear genome

Table S6: Coefficient of variation (CV), variance and standard deviation estimates for both sexes and within each nuclear genome.

Table S7: Degree of coadaptation for each nuclear genome