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Experimental Evidence Supports a Sex-Specific Selective Sieve in Mitochondrial Genome Evolution

Paolo Innocenti,[1]* Edward H. Morrow,[1] Damian K. Dowling[2]*†

Mitochondria are maternally transmitted; hence, their genome can only make a direct and adaptive response to selection through females, whereas males represent an evolutionary dead end. In theory, this creates a sex-specific selective sieve, enabling deleterious mutations to accumulate in mitochondrial genomes if they exert male-specific effects. We tested this hypothesis, expressing five mitochondrial variants alongside a standard nuclear genome in *Drosophila melanogaster*, and found striking sexual asymmetry in patterns of nuclear gene expression. Mitochondrial polymorphism had few effects on nuclear gene expression in females but major effects in males, modifying nearly 10% of transcripts. These were mostly male-biased in expression, with enrichment hotspots in the testes and accessory glands. Our results suggest an evolutionary mechanism that results in mitochondrial genomes harboring male-specific mutation loads.

The mitochondrial-eukaryote union represents one of life's most important symbioses, equipping the eukaryotic cell with a highly efficient means of energy conversion. The upshot of this union is that contemporary metazoa harbor two obligate genomes. The genome located within the cell nuclei (nuclear genome) contains the vast majority of function-encoding genetic material (about 14,000 genes in *Drosophila melanogaster*) (*1*), whereas the mitochondrial genome (mtDNA) is diminutive in size, consisting of just 37 genes (13 protein-coding, 22 tRNA, and 2 ribosomal RNA) (*2*). Although few in number, mitochondrial genes serve an essential function, with both nuclear- and mtDNA-encoded proteins interacting within the mitochondria to perform cellular respiration. The tight coordination in gene expression necessary for organelle function is achieved by extensive signaling between mitochondrial and nuclear genomes, via anterograde and retrograde regulation (*3*).

Unlike their nuclear counterparts, mitochondrial genomes generally have solely maternal transmission, resulting in evolutionary dead ends for the mtDNA of males (*4*). As such, mtDNA should only be able to make a direct evolutionary response to selection through females [but see (*5*, *6*)]. This should pose no problem for traits with identical function across males and females. However, mutations in mtDNA that contribute to the expression of traits exhibiting different sex-specific optima (i.e., sexually dimorphic or sexually antagonistic traits) will be subject to a sex-specific selective sieve. This means that selection will fail to prevent the accumulation of mtDNA mutations that are deleterious when expressed in males, if the same mutations are only slightly deleterious (*4*), neutral (*7*), or beneficial (*8*, *9*) when expressed in females.

If a sex-specific selective sieve is important in mitochondrial genome evolution, then we predict that mitochondrial genomes should generally harbor a mutation load that is more pronounced in males. Also, the effect of the mutation load should increase with the degree of male-biased sexual dimorphism of the trait or tissue in question, because the benefits that males can salvage from relying on female-specific adaptation of mtDNA will diminish as the level of sex-biased expression increases and the intersexual genetic correlation erodes.

We tested these predictions on the basis of the facts that extensive signaling takes place between mitochondrial and nuclear genomes (*3*) and that polymorphisms in mtDNA typically exert their phenotypic effects through interactions with the nuclear genome (*10*). We explored genome-wide variation in nuclear gene expression across strains of *D. melanogaster* that differ only in the origin of their mitochondrial genomes by using GeneChip Drosophila Genome 2.0 microarrays (Affymetrix, Incorporated, Santa Clara, CA). We examined five distinct, naturally occurring mitochondrial genomes from around the globe [*Alstonville* (New South Wales, Australia), *Brownsville* (Texas, USA), *Dahomey* (Benin), *Japan*, and *Mysore* (India)]. An abundance of nonsynonymous and synonymous polymorphisms exists across the protein-coding sequences of these five genomes [supporting online material (SOM) text]. Each mitochondrial genome was coexpressed with the isogenic nuclear background, w^{1118}, in two replicates per strain. Replicates for each mtDNA strain were created as a safeguard to ensure that inferences regarding effects of mtDNA polymorphism were not confounded by cryptic genetic variation that might have accumulated in the nuclear background during the period in which the strains were created and assayed. No differential expression between replicates of any mtDNA strain was found ($P > 0.05$ after multiple testing corrections in two-tailed t tests for all the transcripts, for each pair of replicate introgressions). Other environmental variables (e.g., food source, temperature, light, age, and mating status) were carefully controlled to minimize sources of variation.

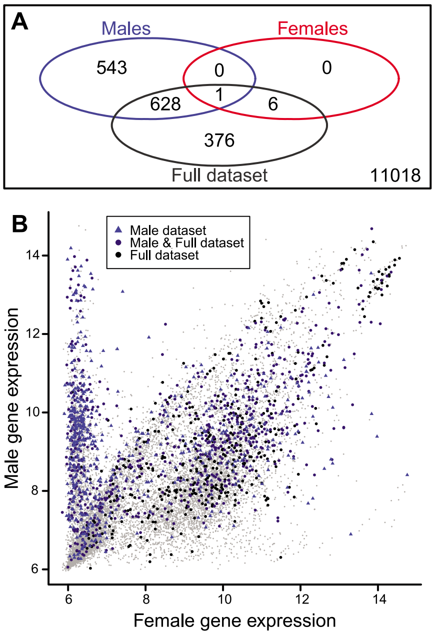

Fig. 1. Differentially expressed genes. (**A**) Venn diagram representing the intersections between the sets of nuclear genes showing a significant effect of mitochondrial strain in males (blue, top left), in females (red, top right), and in the full data set (black, bottom). (**B**) Expression levels (in $\log_2$ scale) of the same sets of genes (blue triangles and dark blue and black dots), in male and female adults of *D. melanogaster*. Gray dots in the background represent the expression level of non–differentially expressed genes.

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When male and female gene expression data were considered together, different mitochondrial strains significantly affected the expression of 1011 nuclear genes [8.0% of tested genes; $P < 0.05$ after a multiple testing correction was set as a cut-off for all contrasts (*6*)]. When male and female gene expression profiles were analyzed separately, the strain effect on males accounted for the differential expression of 1172 genes (9.3%), whereas only 7 genes (~0.06%) were significant for the strain effect on female gene expression. These results suggest that, in general, different mitochondrial strains have a large impact on nuclear gene expression and that this effect extends to a disproportionately larger number of genes in males than in females. The intersection of the three lists of genes (Fig. 1A) also indicates that most of the total variation in gene expression is driven by males (~62% of the genes showing an overall strain effect also show a significant strain effect in male gene expression). In total, 1554 nuclear genes were differentially expressed in at least one sex as a consequence of their coexpression with mitochondrial genomes of different origins.

We then examined the relationship between the significant genes and the levels of sexual dimorphism of the transcriptome. The nuclear genes with expression affected by the mitochondrial genomes were significantly more male-biased in expression than expected [two-tailed Fisher's exact test: odds ratio = 1.13, $P = 0.028$, mean-rank gene set enrichment test (*11*), MR-GSE, $P = 3.3 \times 10^{-22}$ (Fig. 1B)], and this effect was magnified when we considered only the transcripts that were differentially expressed in the male-only data set (Fisher's exact test: odds ratio = 1.56, $P = 1.1 \times 10^{-12}$, MR-GSE test, $P = 1.8 \times 10^{-52}$). To evaluate the likely impact of the mitochondrially affected genes on fitness, we tested their association with transcripts known to be associated with sex-specific fitness (*12*). Our set of 1554 significant genes was overrepresented among the genes associated with male fitness (MR-GSE test, $P = 4.19 \times 10^{-6}$) but not with female fitness (MR-GSE test, $P = 0.61$).

The pairwise correlations between the significant transcripts were used to compute modules of correlated expression and to cluster these genes into groups to determine whether they exhibit functional relationships. We identified 52 transcriptional modules containing from 1 to 275 genes. The largest modules [those containing more than 10 genes, $n = 18$ (Fig. 2)] were evaluated for their level of sexual dimorphism, tissue-specific enrichment, cross-tissue correlation, chromosomal distribution, association with functional categories (gene ontologies) and male fitness, female fitness and sexual antagonism (*12*) (SOM text, Table 1, tables S1 to S18, and figs. S1 to S18).

The two largest modules (12 and 16), together accounting for ~30% of the significant transcripts, also show the most male-biased expression. Module 12, containing 275 genes, is almost exclusively male-limited transcripts [mean intensity (x_m) = 9.7, SD = 1.2, x_f = 6.4, SD = 0.6; range of the whole data set, 5.7 to 15.1; Wilcoxon test: $W = 74553$, $P = 1.6 \times 10^{-86}$ (Fig. 3, A and B, and fig. S12B)], with expression largely limited to the male testes [Fisher's exact test: odds ratio = 107, $P = 2 \times 10^{-183}$; not significant in any other tissue (Fig. 3D and fig. S12F)]. Module 16 is represented by 185 genes

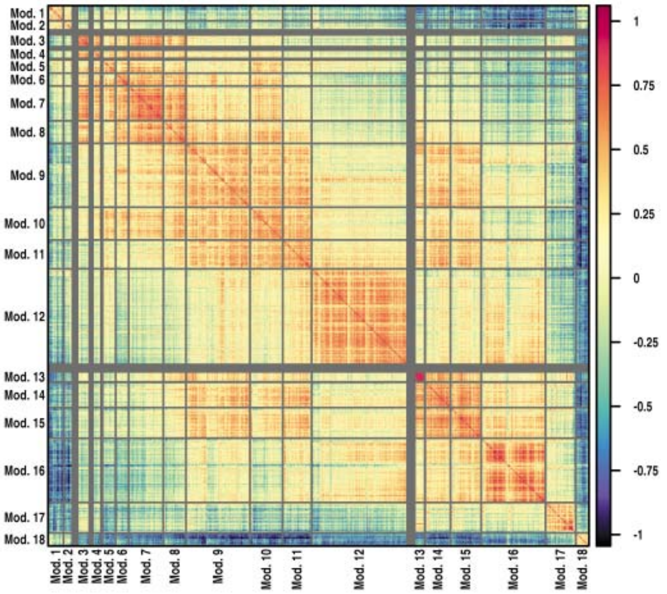

Fig. 2. Transcriptional modules. Level plot representing pairwise correlations between the 1554 differentially expressed genes. The correlation matrix has been used to compute modules of correlated expression. Modules are separated by gray lines (very small modules can be masked by overlapping dividers). The 18 modules with size greater than 10 are labeled.

Table 1. Main transcriptional modules. See (*6*) for details on the tests performed. Size indicates number of genes; Chr. dist., chromosomal distribution bias; GO, Gene Ontology; Associated with, module is overrepresented in lists of candidate male/female fitness-associated genes, published in (*12*); dash entries, not significant.

Module	Size	Overall sex bias	Tissue specificity	Chr. dist.	GO terms	Associated with
1	44	–	Spermatheca, midgut, fat body	–	Table S1	Male fitness, sexually antagonistic selection
2	26	–	–	–	Table S2	–
3	34	Male biased	Head, brain, eyes, thoracic ganglion, crop	–	Table S3	–
4	24	–	Brain, eyes, thoracic ganglion	4 (enriched)	Table S4	–
5	38	–	–	4 (enriched)	Table S5	Male fitness
6	37	Female biased	Ovary	–	Table S6	–
7	100	Female biased	Brain, eyes, thoracic ganglion, heart, ovary	X (enriched)	Table S7	–
8	65	–	–	–	Table S8	–
9	184	Female biased	–	–	Table S9	–
10	94	Female biased	Ovary	–	Table S10	–
11	83	Female biased	Ovary	–	Table S11	–
12	275	Male biased	Testes	2L (enriched)	Table S12	–
13	29	Female biased	–	–	Table S13	–
14	73	Female biased	–	–	Table S14	–
15	88	–	Accessory gland, ejaculatory duct, spermatheca, salivary gland	–	Table S15	–
16	185	Male biased	Accessory gland, ejaculatory duct	X (poor)	Table S16	Male fitness, sexually antagonistic selection
17	87	–	Heart, carcass	–	Table S17	Male fitness
18	38	–	–	–	Table S18	–

and is also mostly male-limited [$x_m = 9.6$, SD = 1.8, $x_f = 6.8$, SD = 1.2, Wilcoxon test: $W = 31608$, $P = 2.8 \times 10^{-46}$ (Fig. 3, E and F, and fig. S16B)], with expression primarily in the accessory gland (Fisher's exact test: odds ratio = 72, $P = 5 \times 10^{-144}$) and ejaculatory duct (Fisher's exact test: odds ratio = 7.8, $P = 1 \times 10^{-21}$) and relatively low or absent in the other tissues (Fig. 3H and fig. S16F).

Remarkably, the correlation in expression between these two clusters of genes in their respective male-limited tissues and other tissues is on average zero or even negative (Fig. 3, C and G), in stark contrast to the high levels of correlation typically observed among tissues for the whole transcriptome and subsets thereof (see, for example, figs. S7D, S8D, and S9D). Taken together, these data support the hypothesis that a large proportion of the observed differential effects attributable to the mitochondrial variants are driven by the accumulation of mutations in mtDNA that exert male-specific effects. That is, selection on mtDNA in females will presumably fail to screen genetic polymorphisms that are strongly male-biased, active only in male-limited tissues, and uncorrelated in their expression with other "shared" tissues.

A mitochondrial mutation load in the male reproductive tissues is expected to lead to a sharp decline in male fertility. This has been termed "mother's curse" (*13*) and has gained empirical support from studies in humans (*14*), hares (*15*), and beetles (*16*), which have documented associations between particular mitochondrial genetic variants and components of male fertility and reproductive fitness (*13*, *15*). Genetic polymorphisms within the cytoplasm leading to reductions or elimination of male fertility, via cytoplasmic male sterility in plants (*17*, *18*) and yeast (*19*) and via cytoplasmic incompatibility induced by intracellular parasites (e.g., *Wolbachia*) in arthropods (*20*, *21*), have long been documented. We purged the studied *Drosophila* lines of bacterial infection by using antibiotics and can conclude that the differential gene expression witnessed in our study is most likely driven by mtDNA polymorphisms (*6*). Indeed, males carrying one of the five mitochondrial haplotypes (*Brownsville*) used here are sterile when that haplotype is expressed alongside the w^{1118} isogenic background (*22*) but are fertile when the same haplotype is expressed alongside the coevolved natal background (*6*). The findings involving mtDNA polymorphism, and cytoplasmic genomes in general, indicate the profound scope by which cytoplasmic genomes affect male fertility. Existence of a sex-specific selective sieve in mtDNA is likely to exert strong selection on counteradaptations in the nuclear genome that restore lost male function (*10*, *23*, *24*).

Among the other clusters, we identified two broad groups whose characteristics suggest that they are involved in retrograde regulation of nuclear DNA transcription (*3*): genes in modules 6, 7, 10, and 11 (and to some extent 8 and 9) that are generally moderately expressed in every tissue but that show a peak in the ovary, suggesting a weak female bias in expression, and genes with a tendency to be highly correlated across tissues (figs. S6 to S11). Furthermore, Gene Ontology (www.geneontology.org)–enriched molecular functions mostly refer to proteins with enzymatic, adenosine triphosphate (ATP)–binding and trans-membrane transporter activity (tables S6 to S11), involved in metabolic and biosynthetic processes, macromolecule localization, and post-transcriptional modifications. The second group is characterized by transcripts specifically involved in mitochondrial functions (modules 1, 12, 14, and 17; see tables S1, S12, S14, and S17). Enriched gene ontologies for these modules explicitly refer to mitochondrial cellular structures (inner membrane, intermembrane space, matrix and ATP respiratory chain complex) and molec-

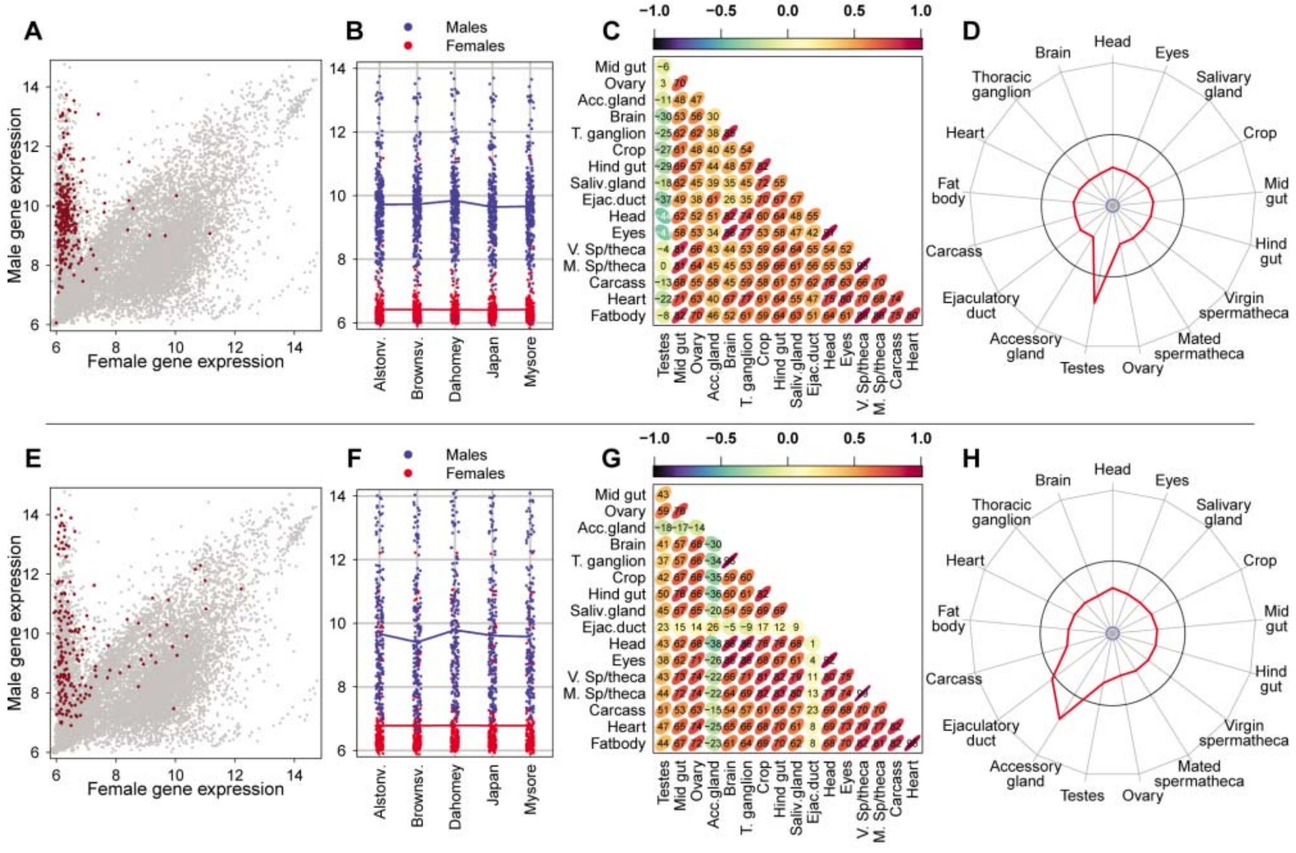

Fig. 3. Module 12 and 16. (**A** and **E**) Scatter plot representing sex bias in expression of the 275 genes in module 12 (A) and the 185 genes in module 16 (E). (**B** and **F**) Sex-specific expression of genes in module 12 (B) and module 16 (F) across the five mitochondrial haplotypes. Red and blue lines represent the within-line module average. (**C** and **G**) Correlation of the genes in module 12 (C) and module 16 (G) across the major tissues of *D. melanogaster*. (**D** and **H**) Average levels of tissue-specificity in expression for genes in module 12 (D) and 16 (H). The range of the data set is delimited by the center (minimum) and the external perimeter (maximum). The circular black line indicates the average expression in the whole body. The red line represents the expression in each tissue. Data on tissue-specific expression were obtained from FlyAtlas (*30*).

ular processes (oxidative phosphorylation, respiratory electron transport chain, ATP synthesis).

Population genetic models (*4*) suggest that mtDNA mutations that are slightly deleterious to females can be maintained at low frequencies under mutation-selection balance even when highly deleterious to males. Genetic drift will fix some of these mutations over time, as well as male-harming mutations that are totally neutral in females. This is because the efficiency of selection acting on mitochondrial genomes should be dampened, owing to their haploidy, maternal transmission, and a lack of recombination (*10*, *25*). In this sense, they are destined to accrue mutations in the same way that asexual organisms will via Muller's ratchet (*26*). Sexually antagonistic selection may also contribute to this mutation load, given that mutations of even slight benefit to female fitness will be selectively favored even if of catastrophic consequence to males. Under this scenario, the signal in nuclear gene expression induced by these mutations might simply be too subtle to detect within the nuclear transcriptome of females, if the phenotypic effects tied to the different mtDNA strains are much larger in males. Alternatively, the nuclear transcriptome might house modifiers (*17*, *27*), expressed within the male reproductive tissues, that have evolved to counter the negative effects of mtDNA mutations and that responded to the variance in mitochondrial genomes in our study. Although we cannot confidently ascribe which of the above processes is the major contributor to the male-specific mutation load that we have detected, they are all likely to have played some role. Lastly, we raise the possibility that the existence of large male-specific differences in nuclear gene expression across our experimental lines might occur if modifications to the expression of these nuclear transcripts are neutral to selection. However, this possibility seems unlikely given that (i) the differentially expressed transcripts are associated with male (but not female) fitness, (ii) one experimental mtDNA haplotype renders males sterile when expressed in the w^{1118} genetic background, whereas it does not have this effect in the nuclear background of origin, and (iii) mitochondrial genetic variation for male fertility has been previously documented (*14*–*16*). Moreover, if widespread differences in gene expression are neutral to selection, this phenomenon should also have been observed in females, short of assuming a generalized sex-specific difference in sensitivity to transcriptional levels.

In sum, mitochondrial genetic effects on male gene expression within the nuclear transcriptome were strong, affecting about 10% of all genes tested. Notably, these affected transcripts stretched well beyond those involved directly in bioenergetic processes of the mitochondria (oxidative phosphorylation and ATP production), demonstrating that retrograde signaling between the genomes is much more pervasive than previously considered. In this sense, there are parallels with the Y chromosome (*28*), which also houses a paucity of protein-coding genes and shows asymmetric transmission, but is nonetheless entwined in widespread regulatory control of the nuclear transcriptome (*28*, *29*). In contrast to the effects on males, mitochondrial effects on female-specific expression of the transcriptome were weak in magnitude and negligible in number. This discrepancy between the sexes is entirely consistent with the idea that the mitochondrial genetic polymorphisms involved have accrued under a sex-specific selective sieve.

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Supporting Online Material

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Role for piRNAs and Noncoding RNA in de Novo DNA Methylation of the Imprinted Mouse *Rasgrf1* Locus

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Genomic imprinting causes parental origin–specific monoallelic gene expression through differential DNA methylation established in the parental germ line. However, the mechanisms underlying how specific sequences are selectively methylated are not fully understood. We have found that the components of the PIWI-interacting RNA (piRNA) pathway are required for de novo methylation of the differentially methylated region (DMR) of the imprinted mouse *Rasgrf1* locus, but not other paternally imprinted loci. A retrotransposon sequence within a noncoding RNA spanning the DMR was targeted by piRNAs generated from a different locus. A direct repeat in the DMR, which is required for the methylation and imprinting of *Rasgrf1*, served as a promoter for this RNA. We propose a model in which piRNAs and a target RNA direct the sequence-specific methylation of *Rasgrf1*.

Imprinted genes show parental origin–specific monoallelic expression, which is controlled by DNA methylation in the differentially methylated regions (DMRs) (*1*–*5*). Differential methylation of the DMRs is established in the parental germ line, passed to the zygote, and maintained in the somatic cells, which eventually leads to monoallelic expression. During fetal testis development, the DMRs are demethylated (imprint erasure) in primordial germ cells at 10.5 to 12.5