doi:10.1111/evo.12788

Intergenomic interactions between mitochondrial and Y-linked genes shape male mating patterns and fertility in *Drosophila melanogaster*

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Received April 22, 2015
Accepted September 21, 2015

Under maternal inheritance, mitochondrial genomes are prone to accumulate mutations that exhibit male-biased effects. Such mutations should, however, place selection on the nuclear genome for modifier adaptations that mitigate mitochondrial-incurred male harm. One gene region that might harbor such modifiers is the Y-chromosome, given the abundance of Y-linked variation for male fertility, and because Y-linked modifiers would not exert antagonistic effects in females because they would be found only in males. Recent studies in *Drosophila* revealed a set of nuclear genes whose expression is sensitive to allelic variation among mtDNA- and Y-haplotypes, suggesting these genes might be entwined in evolutionary conflict between mtDNA and Y. Here, we test whether genetic variation across mtDNA and Y haplotypes, sourced from three disjunct populations, interacts to affect male mating patterns and fertility across 10 days of early life in *D. melanogaster*. We also investigate whether coevolved mito-Y combinations outperform their evolutionarily novel counterparts, as predicted if the interacting Y-linked variance is comprised of modifier adaptations. Although we found no evidence that coevolved mito-Y combinations outperformed their novel counterparts, interactions between mtDNA and Y-chromosomes affected male mating patterns. These interactions were dependent on male age; thus male reproductive success was shaped by G × G × E interactions.

KEY WORDS: **Adaptation, genomic conflict, mitonuclear, mtDNA, male fertility, sexual conflict.**

The evolutionary trajectories of alleles at one gene locus can be entwined with the trajectories of alleles at other loci, promoting coevolution via epistasis. Epistasis is embedded in several core evolutionary concepts, such as Wright's Shifting Balance Theory (Wright 1931), the evolution of reproductive isolation via Bateson-Dobzhansky-Muller Incompatibilities (Bateson 1909; Dobzhansky 1936; Muller 1942), and the evolution of recombination rates (Kouyos et al. 2006; Dapper and Lively 2014). Despite this, the role of epistasis as a factor underlying evolutionary adaptation was traditionally either overlooked (Carlborg and Haley 2004; Hansen 2013), or otherwise underestimated due to limitations in experimental design or power (Flint and Mackay 2009). Recent findings, however, indicate that epistasis might well underpin the expression of numerous complex traits, and that the magnitude of epistatic effects can be as large as the additive effects of alleles at individual loci (Jasnos and Korona 2007; St Onge et al. 2007; Flint and Mackay 2009; Huang et al. 2012; Dobler et al. 2014).

Indeed, epistasis might well have been fundamental in shaping the evolution of complex life (Rand et al. 2004; Dowling et al. 2008; Wolff et al. 2014). Energy conversion in eukaryotes hinges on coordinated interactions between genes that span two

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obligate genomes—mitochondrial and nuclear (Wallace 2013; Wolff et al. 2014). Of the approximate 500 genes that comprise the core and evolutionary-conserved mitochondrial proteome (Lotz et al. 2014), only 37 are encoded by the mitochondrial DNA (mtDNA), but these play a disproportionately large role in assembly and function of the polypeptide subunits involved in oxidative phosphorylation, with 13 of the approximate 86 subunits being mtDNA-encoded (McKenzie et al. 2007; Dowling et al. 2008). While the allelic variation harbored within these mtDNA-encoded genes was traditionally assumed to be selectively neutral (Galtier et al. 2009), experimental studies over two decades have challenged this assumption by linking mitochondrial allelic variation to patterns of phenotypic expression (Ballard and Whitlock 2004; Dowling et al. 2008). Several of these have indicated that the link between the mitochondrial genotype and key life-history phenotypes, such as the metabolic rate (Arnqvist et al. 2010), developmental rate (Dowling et al. 2007aa), fertility (Dowling et al. 2007cc, d), and longevity (Rand et al. 2006; Clancy 2008; Dowling et al. 2010; Zhu et al. 2014) is mediated by epistatic interactions between loci spanning mitochondrial and nuclear genomes. These findings thus suggest that mitochondrial-nuclear (mito-nuclear) coevolution plays a prominent role in shaping the evolutionary trajectories of populations (Dobler et al. 2014; Wolff et al. 2014).

One model of mito-nuclear coevolution has gained increased attention in recent years. It centers on perpetual mutation accumulation in the mitochondrial genome driving selection for nuclear compensatory adaptations that offset the mtDNA-mediated negative effects (Rand et al. 2004; Burton et al. 2006; Ellison and Burton 2006; Dowling et al. 2008; Ellison and Burton 2008; Burton and Barreto 2012; Burton et al. 2013). Mitochondrial genomes, in metazoans, generally have (i) higher mutation rates than nuclear genomic regions, (ii) lower effective population sizes that will in theory dampen the efficiency of selection acting upon mtDNA-encoded genes, and are (iii) constrained in their capacity to purge deleterious mutations from their DNA because of an absence of recombination (Lynch 1996, 1997; Ballard and Whitlock 2004; Dowling et al. 2008). These factors should lead to the accumulation of mutation loads within mitochondrial genomes (Lynch 1996, 1997). Given the critical function of the gene products associated with mitochondrial function, mtDNA-induced mutational pressure should therefore place intense selection on the nuclear genome for compensatory counter-adaptations that restore and maintain mitochondrial integrity (Rand et al. 2004; Dowling et al. 2008; Burton and Barreto 2012). This hypothesis has been supported by experimental evidence showing reductions in performance upon experimental disruption of coevolved mito-nuclear allelic combinations, at both interpopulation and interspecies levels (Burton et al. 2013; Wolff et al. 2014), in wasps (Niehuis et al. 2008) and copepods (Burton et al. 2006; Ellison and Burton 2008; Barreto and Burton 2013), as well as primate (Kenyon and Moraes 1997; Barrientos et al. 1998) and murid (Yamaoka et al. 2000; McKenzie et al. 2003) cell lines.

Furthermore, the mutation loads that accumulate within mitochondrial genomes are expected to be manifested most acutely in males, as a consequence of maternal inheritance of mitochondria. Thus, the compensatory model of mito-nuclear coevolution is particularly relevant to males (Frank and Hurst 1996; Yee et al. 2013; Beekman et al. 2014). Maternal inheritance renders males evolutionary cul-de-sacs for the mtDNA, such that natural selection is only able to directly act on the mtDNA sequence when it is carried by females. This invokes an evolutionary sieve in selection on mtDNA mutations that exert male-biased effects (Frank and Hurst 1996). Under this sex-specific selective sieve, mtDNA mutations that are benign, or only slightly deleterious, to females can linger and accumulate within a population, even if these same mutations are outright deleterious in their effects to males (Frank and Hurst 1996; Gemmell et al. 2004; Innocenti et al. 2011). As such, mitochondrial genomes can theoretically harbor an extra set of deleterious mutations, over-and-above those that are expressed by both sexes, which are specifically male-biased in expression (Frank and Hurst 1996; Gemmell et al. 2004). This hypothesized process has been colloquially termed "Mother's Curse" (Gemmell et al. 2004). Recent empirical evidence has provided evidence for the process (Smith et al. 2010; Innocenti et al. 2011; Camus et al. 2012), by showing greater levels of mitochondrial haplotypic variation for male relative to female phenotypes (Innocenti et al. 2011; Camus et al. 2012), or by linking mitochondrial genetic polymorphisms, or whole haplotypes, to cases of male-specific infertility (St John et al. 2005; Clancy 2008; Smith et al. 2010; Clancy et al. 2011; Yee et al. 2013). This extra set of male-biased mitochondrial mutations should then confer selection for compensatory modifier adaptations that specifically offset the effects of male-harming mtDNA mutations, with such selection acting most strongly on the nuclear genome when carried by males (Frank and Hurst 1996; Yee et al. 2013; Beekman et al. 2014), and leading to male-mediated trajectories of mito-nuclear coevolution.

Such a male-mediated compensatory model of mito-nuclear coevolution would, however, be inherently susceptible to overt sexual antagonism. Firstly, if the male-harming mtDNA mutations acted to enhance female fitness, then such mutations would be under positive selection and likely to increase in frequency in a population, despite any harm that these same mutations would cause to male fitness (Unckless and Herren 2009; Innocenti et al. 2011). Secondly, the putative nuclear modifier alleles that are selected in males to restore male fitness could be subject to intralocus sexual conflict if they were to exert negative pleiotropic effects on female fitness (Bonduriansky and Chenoweth 2009). The sexual antagonism inherent to compensatory mito-nuclear

interactions could, however, be resolved if the nuclear genes involved in the compensatory response were male-limited, either in their genomic location or otherwise in their expression, because in such a scenario the compensatory genes would only be expressed in males and not interfere with female function. The Y chromosome has recently emerged as a promising candidate to harbor such modifier alleles given it is found only in males (Bachtrog 2013), and despite hosting a paucity of protein-coding genes, harbors an abundance of genetic variance for male components of fitness (Chippindale and Rice 2001).

Indeed, counter to traditional expectation, recent observations have linked naturally occurring genetic variation harbored within both the mitochondrial genome (Innocenti et al. 2011) and the Y chromosome (Lemos et al. 2008; Lemos et al. 2010) to widespread effects on genome-wide patterns of nuclear gene expression in *D. melanogaster*. Concordant with the predictions of Mother's Curse, Innocenti et al. (2011) showed that mtDNA-mediated effects on patterns of nuclear gene expression are heavily male-biased. Specifically, they reported that more than 1000 nuclear genes in male *D. melanogaster* were sensitive to the identity of the mtDNA sequence (*mito-sensitive* nuclear genes), relative to just seven genes in total in females. These patterns are consistent with the notion that purifying selection has removed function-modifying allelic variance from accumulating within the mitochondrial genome that affects components of female function, but has failed to prevent mtDNA mutations from accumulating when male-biased in their expression (Frank and Hurst 1996; Gemmell et al. 2004). Similarly, Lemos et al. (2008, 2010) showed that Y chromosomes harbor cryptic regulatory variation that exerts sizable effects on patterns of nuclear gene expression, including to genes involved in mitochondrial function. Indeed, the mito-sensitive nuclear genes identified by Innocenti et al. (2011) are overrepresented in nuclear genes identified by Lemos et al. (2008) to be sensitive to Y-linked regulatory variation (Rogell et al. 2014). Another recent study indicated that the Y chromosome plays a key role in modulating the effects of X-linked sex ratio distortion in *D. simulans*, and that these Y-mediated effects involve interactions with genes encoding mitochondrial-related functions, including genes that are encoded by the mtDNA (Branco et al. 2013). Combined, these patterns are consistent with the hypothesis that mtDNA-induced pertubation of nuclear gene expression in the male transcriptome might provoke an evolutionary counter-response in the paternally inherited Y chromosome to compensate for the effects of male-harming mtDNA mutations. That is, the mito-sensitive nuclear genes identified by Innocenti et al. (2011) might be the stage on which an intergenomic conflict between maternally -inherited mitochondrial genomes and paternally inherited Y chromosomes is played out.

In this study, we set out to investigate the capacity for compensatory mito-Y coevolution, by testing whether components of male reproductive success are affected by epistatic interactions between mitochondrial haplotypic and Y-linked polymorphisms. Furthermore, we probed whether population-specific coevolved combinations of mtDNA and Y chromosome conferred greater reproductive performance in males relative to evolutionarily novel combinations. To achieve this, we used strains of *D. melanogaster* that differed only in the origin of the mitochondrial haplotypes and the Y chromosomes. One mitochondrial haplotype and one Y chromosome was sourced from each of three populations spanning different continents, and then expressed alongside each other in all nine possible mtDNA-Y combinations, in an otherwise isogenic nuclear genetic background. Each of these mitochondrial haplotypes differs from the others by at least 30 single nucleotides (Jonci Wolff, Florencia Camus, pers. comm.). We then assayed male ability to successfully mate, and associated fertility, for each mtDNA-Y genotype, during which each male was provided with a virgin female every 24 h for 10 consecutive days. While male fertility hinged largely on the additive effects of the mtDNA haplotype, we found that mito-Y interactions affected the likelihood of a male successfully mating throughout the experiment. The magnitude and pattern of these mito-Y interactions, however, changed across the 10 days of the experiment. Although we detected mito-Y interactions for male mating success, "coevolved" mito-Y combinations were not observed to outperform evolutionarily novel combinations. Thus, we found no evidence that the mito-Y interactions we detected were characterized by compensatory epistasis, involving male-harming mtDNA mutations and modifier Y-linked adaptations.

Methods

CREATION OF MITOCHONDRIAL AND Y LINES

We harnessed lines in which the mitochondrial haplotypes of three globally distinct isofemale lines of *D. melanogaster* (DAHOMEY, derived from Benin, Africa; ISRAEL derived from Middle East, and MADANG, derived from Papua New Guinea) were placed alongside an isogenic nuclear background (Fig. 1A) (Clancy 2008). While the Dahomey and Madang haplotypes are delineated by 30 SNPs in total, only one of these is found in the protein-coding region (J.N. Wolff, M.F. Camus, pers. comm.). This is, however, a nonsynonymous SNP at site 4853 of the COX3 gene, which confers an amino acid transition from aspartic acid [Asp] to asparagine [Asn]. The Israel haplotype is much more divergent from the other two, differing by 148 and 166 SNPs with respect to the Dahomey and Madang haplotypes (J. N. Wolff, M. F. Camus, pers. comm.), 45 and 46 of which are located in the protein coding region, 8 and 7 of which are nonsynonymous (Camus et al. 2012). The isogenic background was provided by the w^{1118} line (Bloomington stock no. 5905, isogenic for chromosomes X/Y, A2, and A3, constructed by John Roote, Cambridge,

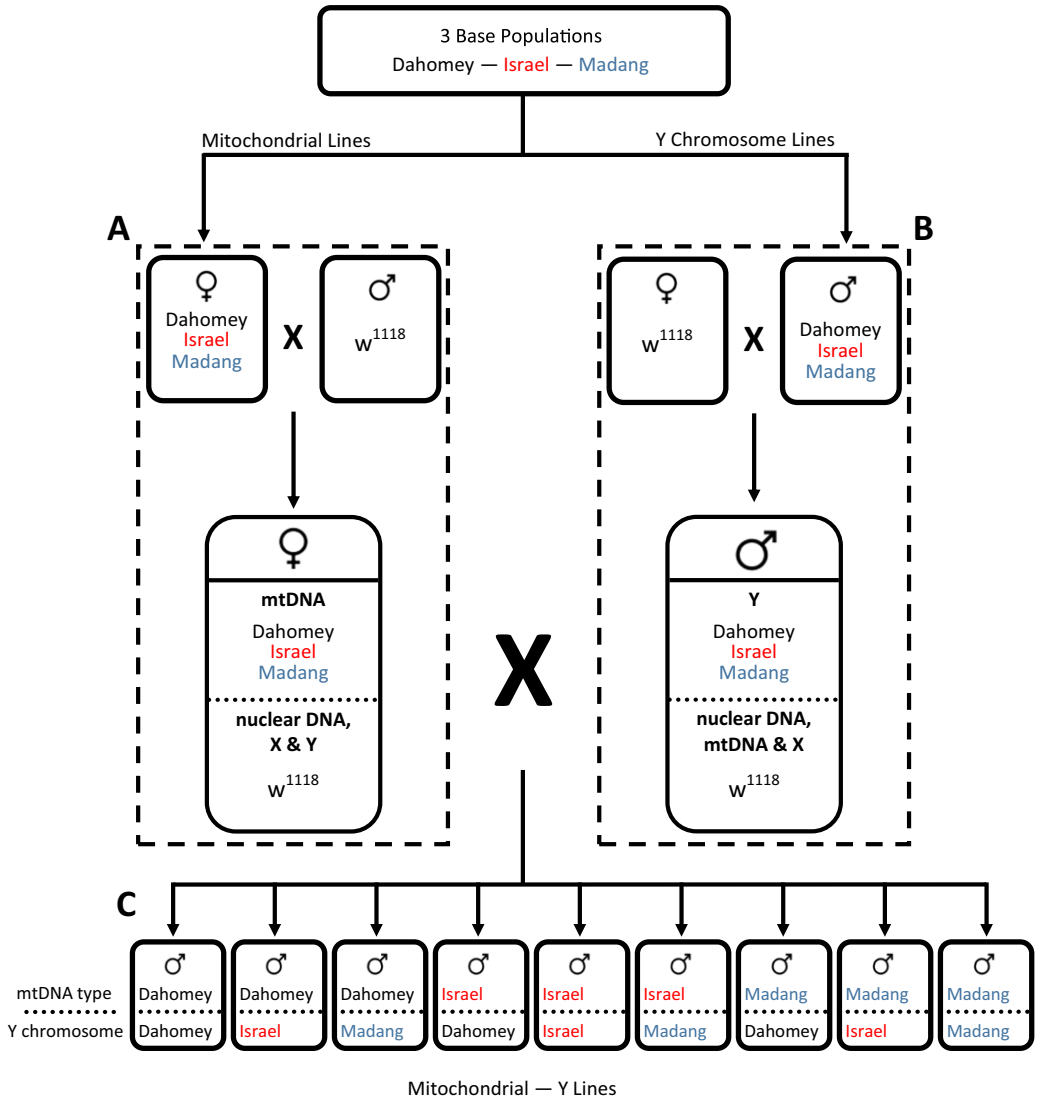

Figure 1. **Experimental breeding scheme. Three distinct isofemale lines; Dahomey, Israel, and Madang were used to create the mitochondrial (A) and Y chromosome lines (B). (A) Mitochondrial lines consisted of each of three mtDNA haplotypes derived from each of the three isofemale lines, expressed against an isogenic nuclear background, represented by the line *w*^{1118}. These lines were maintained by crossing virgin females from each line to males from *w*^{1118} each generation. (B) Y chromosome lines consisted of each of three Y chromosomes derived from each of the three isofemale lines, expressed against the isogenic nuclear background and mtDNA haplotype provided by *w*^{1118}. (C) By crossing females from each mitochondrial line with males of the Y chromosome lines, nine fully crossed combinations of mitochondrial DNA haplotype and Y chromosome, expressed alongside the *w*^{1118} background, were created.**

UK, Canton-S mitochondria). We maintained the isogenicity of *w*^{1118} (across all four nuclear chromosomes) by propagating it via a solitary full-sibling pair, each generation. The crossing scheme originally used to place the three mtDNA haplotypes onto the *w*^{1118} background, is depicted in Clancy (2008).

In 2007, we split each of the mitochondrial lines into duplicates, each of which was then independently propagated via an additional 50 generations of back-crossing of mitochondrial line virgin females to *w*^{1118} males. This back-crossing procedure, when coupled with the full-sibling culturing of *w*^{1118}, effectively eliminates the possibility of cryptic allelic variance accumulating within the isogenic backgrounds of the mitochondrial lines. Duplication of each mitochondrial line provides a further safeguard, by enabling us to statistically partition phenotypic effects attributable to the mtDNA haplotype per se from effects linked to any cryptic nuclear variance that might have nonetheless accumulated across the mitochondrial lines despite the safeguards.

We then created a second set of lines, in which Y chromosomes of the same three global populations were placed alongside the same *w*^{1118} isogenic nuclear background, as per the mitochondrial lines, and in duplicate (two independent crosses per Y chromosome line) (Fig. 1B). This was achieved over a series of crosses.

In the first generation, males from each isofemale line (the same isofemale lines from which the mitochondrial lines were derived) were crossed to females of an isogenic nuclear strain (Bloomington stock no. 4361), containing recessive markers on all four chromosomes: yellow [y^1; X chromosome], brown [bw^1; chromosome 2], ebony [e^4; chromosome 3], and cubitus interruptus and eyeless [ci^1, ey^R; chromosome 4] (Lemos et al. 2008). Sons of this cross were themselves crossed to females of 4361. We were able to ensure that all chromosomes associated with the original isofemale lines had been fully replaced by the chromosomes of stock number 4361, by checking for the expression of each recessive phenotype (caused by alleles on each of the four chromosomes). At this point, Y chromosomes of Dahomey, Israel, and Madang sat alongside the 4361 strain. Males of each of these lines were then backcrossed to females of w^{1118} for four generations, to replace the 4361 chromosomes with those of the isogenic w^{1118} background, again relying on the lack of recombination in male fruit flies. We ensured that chromosomal replacement had been achieved in full, by crossing numerous males drawn from each of the resultant lines to females of 4361 and ensuring that no recessive phenotypes were observed. This procedure was conducted in duplicate, per line.

All lines were maintained at a constant temperature of 25°C on a 12:12 hour light: dark cycle in 40 ml plastic vials containing a substrate of 8 ml potato-yeast-agar, with ad libitum live yeast added to the substrate surface. Lines were treated with substrate-laced tetracycline hydrochloride (0.3 mg/ml) many generations prior to the experiments, to eliminate potential *Wolbachia* infections.

We then generated nine fully crossed combinations of mtDNA haplotype and Y chromosome, all expressed alongside the w^{1118} background, simply by crossing females from each of the mitochondrial lines to males of the Y lines (Fig. 1C). The crossing scheme was independently duplicated (thus there were 18 independent crosses in total), simply by matching the Duplicate identities of each mitochondrial and Y chromosome line to each other (i.e. Duplicate 1 of a given mitochondrial line to Duplicate 1 of a given Y line, and 2 to 2). Each cross was represented by 60 pairs across three vials.

MALE REPRODUCTIVE SUCCESS

Twenty virgin males of each mito-Y line duplicate were collected under light CO_2 anesthesia. These males were the focal males in the assay of male reproductive success. Each male was housed individually with access to substrate and ad libitum live yeast, for five days, prior to entering the experiment.

We collected 3600 virgin females, over 10 consecutive days (360 per day), from the w^{1118} line, to be used as the "tester" females in the male fertility assay. Despite hatching over 10 consecutive days, these tester females were all produced by parents of the same age (5 d), and were reared in vials that had been density controlled to 80 eggs (Supporting Information S1). These females were stored in groups of 10 with access to ad libitum live yeast, also for 5 days prior to their entry into the experiment. From this point forward, CO_2 anesthesia was not used when transferring flies during the experiment.

When 5 days old, each focal male was placed into a fresh vial, containing no live yeast, along with a single 5-day old virgin tester female with which he had the opportunity to mate. After 24 h, each male was transferred to a fresh vial with a new 5 day old virgin female. This step was repeated for 10 consecutive 24 h periods, thus each focal male was able to mate with 10 different females—all of whom were of standard age and genotype—over a 240 h opportunity (Fig. 2).

Following each 24 h opportunity, each tester female was immediately transferred to a second vial for 24 hours, and then a third vial for 72 hours, to enable ovipositioning. Thus, each female had 120 h in which to oviposit across vials that contained no live yeast. The females were then discarded, and the number of offspring eclosing from each of the three vials per female scored 12 days later (Fig. 2).

In total, 360 males were assayed across each of 10 days of adult life (day 5–14), and thus data was collected from 3600 tester females.

STATISTICAL ANALYSIS

The number of eclosing flies was initially modeled using a Generalized Linear-Mixed Model with Poisson errors and an observation-level random effect to account for over-dispersion. However, the dataset contained many zero values and simulations of the upper 95% confidence interval of the number of zeroes expected in our dataset based on the final Poisson model confirmed that the data were indeed zero-inflated (number of zeroes in our data = 4600, 97.25% quantile of expected number of zeroes = 4474). We therefore analyzed the data using a Hurdle model, in which the zero values are modeled separately from the nonzero values (Mullahy 1986). The zero analysis models the proportion of males (those that produced zero offspring vs. produced at least one offspring) that successfully mated on a given day, per mtDNA and Y haplotype. The nonzero "count" analysis models the number of offspring produced per day, across each experimental unit, including only nonzero data points.

The model contained the fixed effects of focal male mitochondrial haplotype (three levels: "Dahomey," "Israel," and "Madang"), Y chromosome haplotype (three levels: "Dahomey," "Israel," and "Madang"), and the day of the mating assay (days), as well as all possible interactions between the fixed effects. Focal male identity, tester female identity, mitochondrial duplicate identity, and Y duplicate identity were added as random effects to the model. There was a clear mean-variance relationship associated

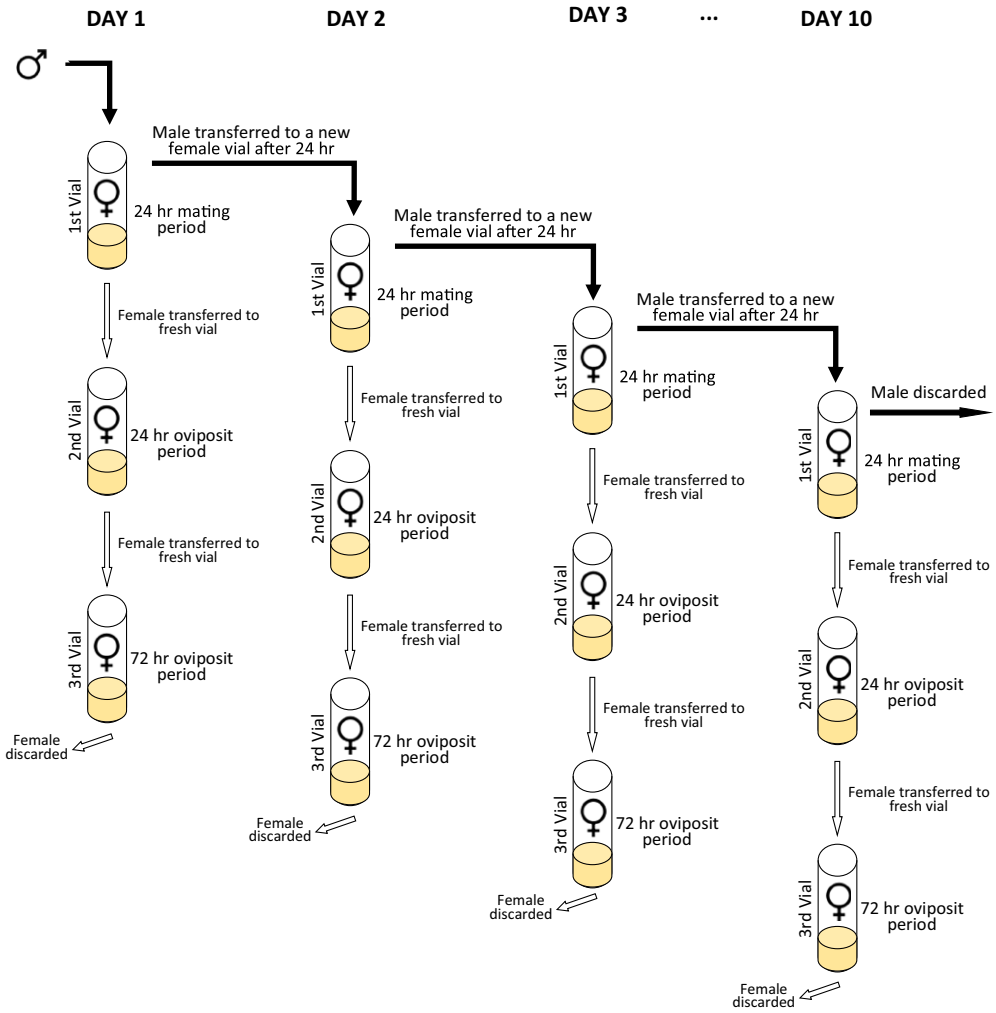

Figure 2. **Schematic illustration of the assay of male reproductive success. During all stages of the assay, the tester females used were always 5 days of age when mated to the focal male. Black arrows represent the transfer of the focal male to a new vial containing a tester female while white arrows represent the transfer of the tester females to fresh vials in which to oviposit.**

with the covariate "day" between the intercepts and slopes of the model (Fig. 3), and this would have induced strong biases among the parameter estimates in the event that the default contrast matrix in R—nonorthogonal "contrast treatments"–-had been used. Although interpretations of regression coefficients derived from orthogonal contrasts are less intuitive, they are less prone to biases in "ANCOVA" type models that include interactions, than their nonorthogonal counterpart (Schielzeth 2010). Hence, we used orthogonal Helmert contrasts, and the covariate "day" was mean centered and scaled to a variance of 1, to enable interpretation of the regression coefficients (Schielzeth 2010).

When using Helmert contrasts, the first regression coefficient estimated denotes an overall mean. Given a factor with k levels, Helmert contrasts compare the mean of all levels up to $k + 1$ to the mean of all levels up to k. For example, the factor Mitochondrial Haplotype has three levels ("Dahomey," "Israel," and "Madang"). The first regression coefficient associated with mitochondrial haplotype ($k = 1$), mtDNA.1, from our model denotes the difference between the mean of the first and the second levels, in alphabetical order ($k = 1 =$ "Dahomey" and $k + 1 = 2 =$ "Israel") and the mean of the first level ($k = 1 =$ "Dahomey"). The second regression coefficient associated with mitochondrial haplotype ($k = 2$), mtDNA.2, from our model denotes the difference between the means of the first three alphabetical levels ("Dahomey," "Israel," and "Madang") and the mean of the first and the second alphabetical levels ("Dahomey" and "Israel"). Helmerts contrasts are thus difficult to associate with changes to a particular level (i.e., haplotype), and the table should be viewed as a test of significance within the hierarchical factors (such as the overall effects of mitochondrial haplotype), and their interactions, rather than used to estimate means of levels within factors, in a similar manner to classic ANOVA tables.

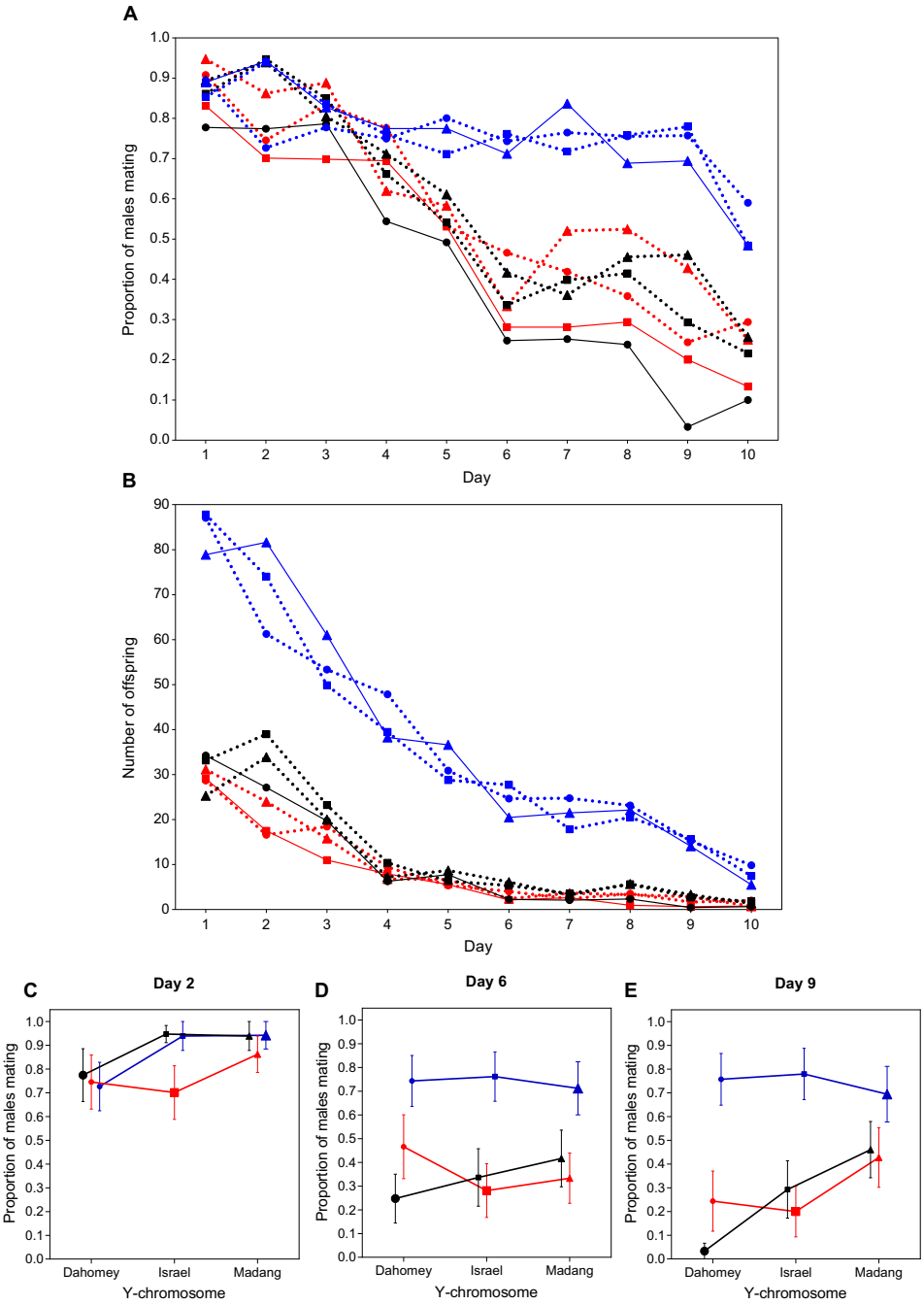

Figure 3. Components of male fertility across mtDNA and Y haplotypes. Each day, the focal males were provided with a new virgin tester female with which he could mate. (A) Denotes the proportion of focal males that produced at least one offspring (i.e., indicates the proportion of successful matings), per mtDNA and Y chromosome combination, for each of the 10 days of the experiment. (B) Denotes the mean number of offspring resulting from male matings per mtDNA and Y chromosome combination for each of the 10 days of the experiment. (C–E) Represent snapshots of (A), showing proportion of focal males mating per mito-Y combination at particular time points—(C) at Day 2, (D) at Day 6, and (E) at Day 9 of the experiment. In A–E, the three different mtDNA haplotypes are denoted in different colors—Dahomey by black, Israel by red, and Madang by blue. The Y haplotypes are denoted symbols of differing shape—circles represent Dahomey Y, squares represent Israel, and triangles Madang. In (A) and (B), means for a given mito-nuclear combination are connected by lines across the days of the experiment; solid lines represent coevolved mito-Y combinations, while dotted lines represent evolutionarily novel mito-Y combinations. In C–E, the Y chromosome haplotype of the focal male is denoted on the horizontal axis, and each of the three lines represents a distinct mitochondrial haplotype, denoted by the same colors as described above. In these panels (C–E), larger sized data points highlight the coevolved mito-Y combinations. Sample sizes for each data point per day range from 25 to 39 across the 10 days of the experiment; Day 2, $n = 29–37$; Day 6, $n = 28–36$; Day 9, $n = 26–35$ per experimental unit.

Table 1. Effects of mtDNA and Y chromosome haplotype, and day at measurement on male fertility.

(A) Zeroes parameter	Postmean	l-95%	u-95% CI	pMCMC
Fixed effects				
μ	−1.28055	−1.976902	−0.55691	0.009286
mtDNA.1	2.843543	2.004303	3.708183	0.003
mtDNA.2	2.779526	1.923139	3.704632	0.002
y1	−0.115751	−0.645504	0.35181	0.61
y2	−0.174759	−0.477784	0.071357	0.16
Day	1.980325	1.820462	2.142824	<0.001
mtDNA.1 × y1	0.516565	0.103215	0.930589	0.02
mtDNA.2 × y1	0.002659	−0.220773	0.259225	0.99
mtDNA.1 × y2	0.002451	−0.217064	0.241607	0.98
mtDNA.2 × y2	0.0883	−0.040208	0.213633	0.2
mtDNA.1 × Day	−0.093723	−0.243582	0.084831	0.28
mtDNA.2 × Day	−0.279915	−0.369993	−0.185227	<0.001
y1 × Day	0.041239	−0.133029	0.212906	0.64
y2 × Day	−0.060279	−0.147112	0.030726	0.19
mtDNA.1:y1 × Day	0.266416	0.051023	0.47731	<0.01
mtDNA.2 × y1 × Day	0.090395	−0.034846	0.19977	0.13
mtDNA.1 × y2 × Day	0.111958	−0.012787	0.215048	0.06
mtDNA.2 × y2 × Day	0.11081	0.045797	0.172586	<0.001
Random effects				
mtDNA duplicate	0.1326	0.0001594	0.3619	
Y Duplicate	0.167	0.0001799	0.6882	
Male ID × Day	5.332	4.471	6.17	
Male ID	4.44	3.42	5.532	
Residual variance	1	Fixed		

(B) Nonzeroes Parameter	Postmean	l-95% CI	u-95% CI	pMCMC
Fixed effects				
μ	2.452652	2.275377	2.641277	<0.001
mtDNA.1	−0.909225	−1.127409	−0.667244	<0.001
mtDNA.2	−1.164278	−1.393524	−0.931612	<0.001
y1	−0.024219	−0.138058	0.081551	0.61
y2	−0.001818	−0.064241	0.054818	0.94
Day	−0.519365	−0.560674	−0.475686	<0.001
mtDNA.1 × y1	−0.066772	−0.15764	0.023489	0.14
mtDNA.2 × y1	−0.00699	−0.048421	0.03351	0.75
mtDNA.1 × y2	0.003401	−0.042241	0.049102	0.87
mtDNA.2 × y2	−0.004329	−0.02695	0.017474	0.68
mtDNA.1 × Day	−0.016008	−0.068432	0.044213	0.58
mtDNA.2 × Day	−0.007549	−0.032898	0.018341	0.57
y1 × Day	−0.010217	−0.065852	0.04498	0.7
y2 × Day	0.005753	−0.022985	0.034001	0.68
mtDNA.1 × y1 × Day	−0.046297	−0.125534	0.026191	0.24
mtDNA.2 × y1 × Day	−0.004032	−0.038103	0.028879	0.83
mtDNA.1 × y2 × Day	−0.004652	−0.04053	0.033346	0.81
mtDNA.2 × y2 × Day	−0.005063	−0.024139	0.0117	0.56

(*Continued*)

Table 1. Continued.

(B) Nonzeroes parameter	Postmean	l-95%	u-95% CI	pMCMC
Random effects				
mtDNA Duplicate	0.01194	0.0001891	0.03917	
Y Duplicate	0.008592	0.0001788	0.02869	
Male ID × Day	0.1989	0.1568	0.239	
Male ID	0.04646	0.02391	0.07017	
Residual variance	0.5231	0.481	0.5646	

A Hurdle model was used, with zero values (a) of fertility modeled separately from nonzero (i.e., count) values (b). The modeling approach is outlined in the *Statistical Analysis* section of the paper. "Postmean" is the posterior mean of the parameter estimate; "l-95%" and "u-95%" denote its associated 95% credibility intervals for each fixed effect parameter. "pMCMC" is the Bayesian *P*-value. For the random effects, variance components are accompanied by upper and lower CIs. "mtDNA Duplicate" is the variance explained by mtDNA replicate, "Y Duplicate" is the variance explained by Y-chromosome replicate, "Male ID" is the variance explained by male identity, "Male ID × Day" is the interaction variance explained by the combination of male identity and day identity. Residual variance is fixed at 1 for the binary part of the analysis. Note that the default contrast matrix in R ("contrast treatments") yields parameter estimates that are easier to interpret, but that these would have been highly collinear and impossible to use for statistical evaluation. The table should hence be viewed as a mean of significance testing, and should be put into biological context using Figure 1.

Table 2. Analysis of deviance table of the effects of coevolutionary status (coevolved) of the mtDNA and Y chromosome combination on male mating success.

Fixed effects	Chi-sq	df	*P*-value
(Intercept)	1.4107	1	0.2349351
Coevolved	5.6325	1	0.0176302
Day	574.4493	1	$<2.2 \times 10^{-16}$
Coevolved × Day	11.6303	1	0.0006489

Random effects	Variance
mtdna × Y × male ID × f day	3.37834
mtdna × Y × male ID	3.07076
Ydup[Y]	0.03748
mitodup[mtdna]	1.14229

Day represents the mean centered effect of day of the experiment (1–10). Chi-sq is the chi-square value associated with the parameter. Mtdna/Y/male ID/fday represents the interaction variance between mitochondrial haplotype, Y-chromosome haplotype, male ID, and day (scored as a factorial variable). Mtdna/Y/male ID is the interaction variance between mitochondrial haplotype, Y-chromosome haplotype, and male ID. Y:ydup denotes the Y chromosome duplicate nested within the Y-chromosome haplotype and mtdna/mitodup denotes the mitochondrial duplicate nested within the mitochondrial haplotype.

The model was fit using a Gibbs sampler implemented in the R package MCMCglmm (Hadfield 2010). Flat priors were used for the fixed effects and locally uninformative priors were used for random effects, both representing little prior knowledge. After a burnin of 4×10^5, a sample of the posterior distribution of 5.6×10^6 was made with a thinning interval of 2000, yielding a total posterior sample of 2800. All autocorrelations across successively stored posterior samples were in the interval <0.1 and >-0.1.

The data were analyzed using flat priors for the fixed effects and locally uninformative priors on the random effects. Such priors are weakly informative and were used because more informative priors are likely to affect the results, in which case we would not know if the results were data-driven or driven by the a priori expectation. To confirm that our choice did not affect the results, four additional sets of priors were examined and found to give close to identical results. The additional models are presented in the Supporting Information file (S4).

Then, to examine if any interactions between mtDNA and Y chromosomes that we observed in the previous model were consistent with a signature of compensatory mito-nuclear coevolution, we assigned each mitochondria-Y chromosome combination as either "coevolved" or "evolutionary foreign" based on whether the combination was sourced from the same population, or not. "Coevolutionary status" (coevolved, foreign mito-Y combination) and day of the mating assay (continuous, mean centered, and scaled to a variance of 1) were fitted as fixed effects. The interaction between mitochondrial haplotype, Y-chromosome haplotype, male ID, and the day of the mating assay, the interaction between mitochondria, Y-chromosome, and male ID, the Y chromosome duplicate nested within Y-chromosome haplotype, and the mitochondrial duplicate nested nested within mitochondrial haplotype, were added as random effects to the model. Because the replication of mitochondrial haplotypes and Y-chromosome haplotypes was low (three of each), this model was heavily overparameterized. We could hence only fit mating rate (proportion of matings, indicated by analysis of Zeroes, Table 1) as a response variable with a Laplace algorithm implemented in the package *lme4* (Bates et al. 2012) in R. The model was tested for significance using an analysis of deviance on a chi-square distribution (ANOVA, type III sums of squares) implemented in the R package

car (Fox and Weisberg 2011). We note, however, that this model must be interpreted with caution because the limited replication of mitochondrial and Y-chromosome haplotypes means that we were unable to test the assumption that the effects of the mitochondrial and Y-chromosomal variants on mating rate are realized draws from a normal distribution.

Results

Males exhibited a general decrease in mating rate (proportion of matings, indicated by analysis of Zeroes, Table 1A), as well as the number of offspring produced per day (Count analysis in Table 1B), across the 10 days of the experiment (Fig. 3A and B, Supporting Information S2 and S3). The identity of the mitochondrial haplotype affected both the mating rate of males, and the number of offspring produced, with males harboring the Madang haplotype procuring more matings and exhibiting higher fertility relative to the other haplotypes (Table 1, Fig. 3A and B). In addition, the rate at which the mating rate decreased over the 10 days of the experiment was slower for males harboring the "Madang" haplotype relative to males harboring the other haplotypes, as indicated by an interaction between mtDNA haplotype and Day (Table 1, Fig. 3A).

There were no additive Y chromosome-linked effects on the male mating rate or number of offspring produced. However, patterns of male mating across time were affected by interactions between Y chromosomes and mtDNA haplotypes (mtDNA × Y × Day interaction, Table 1). Specifically, variance in the proportion of males mating attributable to mito-Y interactions (Fig. 3A, C–E) was greater over the first days of the experiment (Fig. 3A, C), and the latter days (Fig. 3A, D, E), with less variance between days 3 and 5.

Visual inspection of Figure 3 indicated no support for the prediction that coevolved mito-Y combinations outperformed their evolutionarily novel counterparts (Fig. 3). In fact, the proportion of matings across the coevolved mito-Y combinations was significantly lower than for the evolutionarily novel combinations (Fig. 3A, C–E, Table 2, but note these analyses must be interpreted with caution). These differences were most apparent for mito-Y combinations involving the Dahomey and Israel mtDNA haplotypes, in which the coevolved combinations exhibited particularly low mating success in the later stages of the experiment (days 9 and 10, Fig. 3A).

Discussion

We tested whether epistasis between alleles spanning mitochondrial genomes and Y chromosomes affected two components of male reproductive success; male mating success, as gauged by the proportion of matings a male successfully procured over a 10 day period, and fertility, assessed as the resulting number of offspring sired over this period. We detected interactions between mtDNA haplotypes and Y chromosomes for male mating success, but not fertility. Although the existence of such interactions would in general support the idea that genetic variation spanning mitochondrial genomes and Y chromosomes might coevolve, in the case of our study the magnitude and pattern of these interactions changed across the 10 days of the experiment (as evidenced by the crossing over of the reaction norms for mating success in Fig. 3). Furthermore, generally the population-specific coevolved mito-Y combinations conferred lower mating success, across the 10 days of the experiment, than did the evolutionarily novel combinations; an observation that ran counter to our predictions. Thus, we found no compelling evidence for compensatory mito-Y interactions for male fertility. However, additive mitochondrial genetic effects on each of the male reproductive traits were strong, attributable to superior performance of males carrying the Madang mtDNA haplotype. These effects reinforce previous observations that the mitochondrial genome is a hotspot for genetic variation affecting the outcomes of male fertility (Froman et al. 2002; St John et al. 2005; Nakada et al. 2006; Dowling et al. 2007dd; Innocenti et al. 2011; Yee et al. 2013).

The mito-Y interactions for male mating success, recorded here, were contingent on the day of the experiment. Nonetheless, this study is to our knowledge, the first to present evidence for epistatic allelic interactions between these two genomic regions. This finding is notable given that the mitochondrial genome and the Y chromosome never cotransmit together, the former being maternally and the latter paternally inherited (Rand et al. 2004). On the one hand, the absence of cotransmission between these gene regions will increase the number of novel mito-Y genotypic combinations, segregating within a population, on which selection is available to act (Hill 2014). On the other hand, the absence of cotransmission in theory constrains the potential for beneficial, coadapted allelic pairings located on mtDNA and Y chromosome to be maintained across generations (Rand et al. 2004; Drown et al. 2013), and might favor a scenario in which the allelic interactions are more likely to be characterized by antagonism (Rand et al. 2004).

Indeed, increases in genetic cotransmission can invoke sexual antagonism, when the cotransmission is sex biased. The mitochondrial genome is maternally inherited, rendering it prone to the accumulation of sex specific, even sexually antagonistic fitness variation (Frank and Hurst 1996; Unckless and Herren 2009). The female-bias in cotransmission of X-linked nuclear genes with the mitochondrial genome (mito-X allelic pairings cotransmit in two-thirds of cases in male heterogametic XY systems such as *Drosophila* and humans, relative to 50% of cases for mito-autosomal pairings) should facilitate the evolution of coadapted mito-X allelic combinations that are overtly sexually

antagonistic—benefiting females at the expense of males (Rand et al. 2001). Accordingly, Drown et al. (2013) and Rogell et al. (2014) presented evidence this antagonism might have been resolved by translocation of mitochondrial-interacting nuclear genes off the X chromosome, and onto other nuclear chromosomes. This would hence facilitate nuclear compensatory responses that offset mtDNA-mediated harm to male fitness (Frank and Hurst 1996; Yee et al. 2013; Beekman et al. 2014). If the compensatory modifier mutations were located on the Y chromosome, then they would be present only in males, and thus not interfere with female function, thus offering a resolution to sexual antagonism inherent to maternal transmission of the mtDNA (Rogell et al. 2014). However, while theoretically appealing, our findings provide no tangible evidence that the Y chromosome harbors such modifiers, a scenario in which we would have expected population-specific mito-Y combinations to outperform the novel combinations. This was not observed (Fig. 3), and in fact our findings suggested that when it comes to male mating success, if anything the evolutionarily novel mito-Y combinations performed better.

Mito-Y interactions were transient in their effects throughout the experiment, being contingent on an interaction with the day of the experiment. The variance attributable to such interactions did not simply increase or decrease in magnitude over the course of the experiment. Rather, the phenotypic variance attributable to mito-Y interactions was greatest early and late into the experiment (Fig. 3C–E). These mito $\times$ Y $\times$ day effects are likely attributed to the somatic age of the focal males, since each male was offered a new virgin female (each of the same standardized genotype and age) over a 10-day period that commenced when they were 5 days of age, and concluded when they were 14 days old. All other somatic and environmental sources of variance were controlled in our experiment. Yet, the effects might also be explained by mito-Y genotypic effects on male sexual motivation, which might plausibly erode or fluctuate across successive matings. A further experiment, which disentangled the mating history of the males from their age, would be required to delineate between these two possibilities.

Our finding of environmentally sensitive mito-Y effects is consistent with previous studies to have provided evidence for complex gene-by-gene-by-environment interactions involving mito-nuclear epistasis (Dowling et al. 2007a, c; Arnqvist et al. 2010; Dowling et al. 2010; Zhu et al. 2014), and which together indicate that the genetics underlying the expression of metabolically dependent traits will often be complex. However, our study provides several novel extensions in terms of understanding the dynamics of mito-nuclear interactions. First, most studies investigating the scope for mito-nuclear $\times$ environment interactions have examined the effects of abiotic sources of environmental variance, such as temperature or diet, while our interactions are mediated by intrinsic sources of environmental variance—either focal male somatic age or by age-dependent changes in sexual motivation. In this regard, we know of one other study to outline evidence that the outcomes of mito-nuclear effects might change according to an intrinsic trait. Dowling et al. (2010) provided evidence for mito-nuclear interactions that affected female longevity in the seed beetle (*Callosobruchus maculatus*), the magnitude and pattern of which were contingent on the mating status of the females. Indeed, we believe that somatic age-effects on the outcomes of mito-nuclear interactions are likely to be common, given the strong theoretic and empirical links between mitochondria and aging (Harman 1972; Trifunovic et al. 2004; Maklakov et al. 2006; Rand et al. 2006; Clancy 2008; Dowling et al. 2009; Park and Larsson 2011; Camus et al. 2012; Zhu et al. 2014), and given previous observations of clonal expansion of mtDNA mutations with somatic age across different species (Baines et al. 2014; Greaves et al. 2014). The age-sensitivity of mito-nuclear effects on key fitness-related traits thus needs to be further evaluated in future studies. Second, our study is able to trace interacting nuclear alleles involved in the mito-nuclear $\times$ environment interactions on male fertility, to a specific nuclear gene region–-the Y chromosome–that shares no trans-generational cotransmission with the mtDNA. Third, many studies to have previously examined the phenotypic consequences of mito-nuclear allelic interactions across environments have typically suffered from a key inferential limitation that precludes the epistatic genetic effects to be separated from other environmental sources of variance; a lack of independent genotype replication at the level of the mito-nuclear interaction. The combination of mito-Y genotypes we used in this study were all independently duplicated, enabling us to partition epistatic genetic from other confounding sources of variance.

Our results suggest that the coevolved combinations of mito-Y genotype did not outperform the evolutionarily novel combinations. This result is inconsistent with the general evolutionary prediction, backed by a growing number of empirical studies, that experimental mismatching of coevolved combinations of mitochondrial and nuclear genotype will lead to fitness depression, under a compensatory model of mito-nuclear coevolution (Dowling et al. 2008; Burton and Barreto 2012; Burton et al. 2013; Dowling 2014; Reinhardt et al. 2013; Wolff et al. 2014). Indeed, this compensatory model is expected to be particularly relevant to male life-history trait evolution, given that maternal transmission of the mitochondria renders the mtDNA prone to the accumulation of male-biased mutations (Frank and Hurst 1996; Sackton et al. 2003; Yee et al. 2013; Beekman et al. 2014; Dowling 2014). Specifically, our study investigated the potential for compensatory mutations to accrue in one nuclear genic region only–the Y chromosome; all other nuclear regions were held isogenic across the mitochondrial and Y lines. The results do not support the

hypothesis that the Y chromosome harbors modifier mutations that offset the effects of male-biased mutation loads (Rogell et al. 2014). In fact, when it came to the ability of males to garner successful matings, coevolved mito-Y combinations generally performed worse than the evolutionarily novel counterparts.

We also found an additive effect of the mtDNA haplotype, with males that harbored the Madang haplotype outperforming males with other haplotypes, both in terms of the ability to mate and fertilize a female over each of 10 consecutive days, and ultimately the offspring numbers sired. This effect was large—the Madang mtDNA conferred a greater than twofold increase in each component of reproductive success over the duration of the experiment, relative to the two other haplotypes. This reinforces the sizable contribution that allelic variants within the mitochondrial haplotype make to the expression of male fertility. The paradox here, however, is that these mitochondrial genetic effects on male fertility are inherited exclusively from mothers to sons (Dowling et al. 2007b, d; Friberg and Dowling 2008). Sons never transmit their mtDNA onto their offspring, and therefore any mtDNA alleles that affect male components of fertility might escape selection, unless they have pleiotropic effects on females, or are otherwise segregating in an inbred population or population in which the fitness of sons shapes the fitness of their female relatives (Wade and Brandvain 2009). Thus, these mtDNA alleles might accumulate within populations regardless of whether their effects on males are beneficial or deleterious (Frank and Hurst 1996; Gemmell et al. 2004; Beekman et al. 2014). Experimental evidence suggests that the mitochondrial genome is a hotspot for male-biased mutation accumulation in *D. melanogaster*, with these mutations exerting profound effects on patterns of gene expression throughout the nuclear genome in males, but not in females (Innocenti et al. 2011). Indeed, one particular mutation has been found in a naturally occurring mtDNA haplotype of *D. melanogaster* sourced from Texas, USA, which confers male in- or subfertility without any known negatively pleiotropic effects on the fertility or fecundity of females who harbor the same mutation (Clancy et al. 2011; Yee et al. 2013). This mtDNA mutation represents the first known case of mtDNA-induced Cytoplasmic Male Sterility in animals—a phenomenon common across plant taxa (Budar and Fujii 2012).

While around 45 SNPs separate the protein-coding regions of the Israel mtDNA haplotype from the Madang and the Dahomey haplotypes used in this study (and about 166 SNPs in total), the Madang and Dahomey haplotypes differ by just a solitary SNP (G to A) within the protein-coding region at site 4853 of the COX3 gene (and by 30 SNPs in total, J. N. Wolff, M. F. Camus, pers. comm.). Yet, surprisingly it is the Madang—not Israel—haplotype that stands apart from the other two in its effects on male fertility. Furthermore, the nonsynonymous SNP in the protein-coding region that delineates the Madang from the Dahomey mtDNA haplotype (and which confers an amino acid transition from aspartic acid [Asp] to asparagine [Asn]) is shared by the Israel haplotype (i.e., it is Dahomey with the unique SNP), and thus cannot be the candidate SNP that drives enhanced fertility of the Madang haplotype. This therefore implicates polymorphisms within the noncoding region of the mitochondrial genome in the fertility effects observed here. Our preliminary work has identified 30 polymorphisms outside of the protein coding regions, which separate the Madang and Dahomey haplotypes (Jonci Wolff, Florencia Camus, Damian Dowling unpublished data, in press), and thus there are numerous polymorphisms (or combinations thereof) in the noncoding regions that could in theory drive the observed effects between the mtDNA haplotypes observed here.

In conclusion, here we have provided proof-of-concept evidence that allelic variation harboured within the mtDNA can interact with that linked to the Y chromosome to affect components of male fertility, even though these two gene regions never cosegregate together. The nature of these inter-genomic associations appears to be complex, however, and sensitive to age of the focal flies. While this indicates scope by which mitochondrial genomes and Y chromosomes could be entwined in an evolutionary tug-of-war over overexpression within the male transcriptome (Rogell et al. 2014), our results suggest that the coevolved mito-Y combinations generally conferred lower male mating success than their evolutionarily novel counterparts. Furthermore, here we have shown that the additive effects of genes within the mtDNA might well make a larger contribution to male reproductive fitness than additive Y effects. This is consistent with previous observations that have shown that much of the Y-linked effects on male fitness are regulated by epistatic interactions with the genetic background (Chippindale and Rice 2001; Jiang et al. 2010). Nonetheless, it is an intriguing finding when considered from the evolutionary standpoint because these mtDNA-linked genes are never transmitted through males, and might therefore constrain an optimal response by males to sexual selection for enhanced fertility (Dowling et al. 2007b, d). se mtDNA-linked genes, across the haplotypes used.

ACKNOWLEDGMENTS

This study was funded by the Australian Research Council (DP1092897) to D.K.D, and a Monash University Faculty of Science Dean's postgraduate scholarship to W.K.W.Y. We thank Florencia Camus and Jonci Wolff for discussions on the molecular sequences of the mtDNA haplotypes.

DATA ARCHIVING

Dryad: doi:10.5061/dryad.gh350

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Associate Editor: J. Hunt
Handling Editor: M. Servedio

Supporting Information

Additional Supporting Information may be found in the online version of this article at the publisher's website:

Supporting Information S1. Experimental plan illustrating how tester females of a standard age (5 days) were produced over the course of 10 consecutive days. Arrows represent egg to adult development periods for parents (thick arrow) and tester flies (thin arrows).

Supporting Information S2. Mean (± standard error) proportion of successful matings, per day, for males of each mtDNA haplotype, Y haplotype, and mito-Y genetic combination.

Supporting Information S3. Mean (± standard error) number of offspring produced per day for males of each mtDNA haplotype, Y haplotype, and mito-Y genetic combination.

Supporting Information S4. MCMCglmm models with four additional sets of priors V= a) 1, b) 0.00001, c) 2 & d) 10.

a) V = 1 (presented in the main manuscript)

b) V = 0.00001

c) V = 2

d) V = 10