ARTICLE

Mitochondrial genetic effects on reproductive success: signatures of positive intra-sexual, but negative inter-sexual pleiotropy

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Abstract

Theory predicts that maternal inheritance of mitochondria will facilitate the accumulation of mtDNA mutations that are male biased, or even sexually antagonistic, in effect. While there are many reported cases of mtDNA mutations conferring cytoplasmic male sterility in plants, historically it was assumed such mutations would not persist in the streamlined mitochondrial genomes of bilaterian metazoans. Intriguingly, recent cases of mitochondrial variants exerting male-biases in effect have come to light in bilaterians. These cases aside, it remains unknown whether the mitochondrial genetic variation affecting phenotypic expression, and in particular reproductive performance, in bilaterians is routinely comprised of sex-biased or sex-specific variation. If selection consistently favours mtDNA variants that augment female fitness, but at cost to males, this could shape patterns of pleiotropy and lead to negative intersexual correlations across mtDNA haplotypes. Here, we show that genetic variation across naturally occurring mitochondrial haplotypes affects components of reproductive success in both sexes, in the fruit fly *Drosophila melanogaster*. We find that intrasexual correlations across mitochondrial haplotypes, for components of reproductive success, are generally positive, while intersexual correlations are negative. These results accord with theoretical predictions, suggesting that maternal inheritance has led to the fixation of numerous mutations of sexually antagonistic effect.

43 ## Introduction

44 Eukaryotic cells are thought to have arisen from the ancient symbiotic union between two
45 prokaryote cells; one an α-proteobacterium that evolved into the mitochondrion, and the other
46 an archaean-like organism that evolved into the eukaryote [1]. Each of these ancestral entities
47 possessed their own genomes, and their symbiosis kick-started millions of years of inter-
48 genomic coevolution that delineates contemporary eukaryotes from the organisms of other
49 domains [2]. Almost without exception, eukaryotes have retained these two genomes – one
50 mitochondrial (comprised of mtDNA), the other nuclear, and interactions between genes
51 spanning each of these genomes coordinate closely to regulate critical biological processes tied
52 to cellular metabolism via oxidative phosphorylation (OXPHOS) [3-5].

53 Notwithstanding that large variation exists in both the size and content of the mitochondrial
54 genome across eukaryote taxa (e.g., large, with introns and generally low mutation rates in
55 plants [6]; to streamlined, with high mutation rates in bilaterian metazoans [7]), over the course
56 of evolutionary history most of the genome's protein-coding genes have been translocated to
57 the host nuclear genome. In bilaterians, this process of genome reduction was extreme, with
58 just thirteen protein-coding genes remaining [4]. Given these mitochondrial genes all encode
59 essential subunits of OXPHOS, evolutionary biologists long assumed that purifying selection
60 would generally prevent the accumulation of non-neutral (i.e., phenotype-modifying) genetic
61 variation within the mtDNA sequence. Accordingly, the mitochondrial genome was harnessed
62 as the go-to molecular marker upon which to base evolutionary and population genetic
63 inferences, facilitated by its maternal inheritance, presumed lack of pervasive recombination,
64 and, at least in bilaterians, its high mutation rate [8-12].

65 Over the past two decades, however, an increasing number of studies has challenged this
66 assumption of neutrality of mtDNA sequence variation, with examples from plants [13, 14] ,

67 fungi [15, 16] and animals [17-19]. In particular, numerous studies have used multigenerational
68 breeding schemes with the power to partition cytoplasmic genetic from nuclear genetic effects
69 [19]. For example, in plants, cytonuclear interactions (interactions involving polymorphisms
70 within the mitochondrial and/or chloroplast genome and those in the nuclear genome) were
71 shown to affect 23 of 28 phenotypes measured in *Arabidopsis thaliana*, with pervasive effects
72 on traits involved in germination, resource acquisition, phenology, height, fecundity and
73 survival [20], and also on regulation of the metabolome [21]. In bilaterian animals, from flies
74 to mice and humans, genetic polymorphisms that delineate distinct mitochondrial haplotypes
75 have been linked to the expression of traits tied to reproductive success, development, and
76 longevity [3, 22-29].

77 Maternal inheritance of mitochondrial genomes adds a further layer of complexity to the
78 dynamics of mtDNA evolution, because it means that selection can only act directly on non-
79 neutral mtDNA polymorphisms through the female lineage [30-32]. This hypothesis, which
80 has been called "Mothers Curse" [30, 31], predicts that mutations that are neutral, beneficial or
81 slightly deleterious to females may accumulate in the mtDNA sequence even if these same
82 mutations are harmful in their effects on males ("Mother's Curse mutations") [30, 32-34].
83 While Mother's Curse effects occur very commonly in plants [35], through mtDNA-mediated
84 Cytoplasmic Male Sterility, it was traditionally thought there was little scope for the
85 streamlined mtDNA sequence of bilaterians to harbour mutations of male-biased effect [31,
86 32]. Yet, within the past decade, several cases of individual Mother's Curse mutations
87 conferring male-specific fertility effects have been identified in *Drosophila* flies [36-38], mice
88 [39], hares [40] and humans [41]. Furthermore, in humans, emerging evidence suggests that
89 particular candidate mutations in the mtDNA sequence are responsible for male biases in the
90 penetrance of Leber's Hereditary Optical Neuropathy and rates of infant mortality [42]. These
91 examples raise the possibility that sex-specific variation might routinely build up, and be

maintained, within the mitochondrial genome of bilaterians that exhibit strict maternal inheritance of mtDNA.

Indeed, recent studies in *D. melanogaster* have supported this contention by showing that genetic variation across a pool of naturally occurring mtDNA haplotypes is associated with male-biased effects on genome-wide patterns of gene expression [43] and longevity [24, 44]. Nonetheless, the extent to which mitochondrial haplotypes exhibit sex-biases in their effects on the expression of life history phenotypes in metazoans remains generally unclear, because few studies have measured phenotypic effects across sets of naturally occurring mtDNA haplotypes in both males and females, respectively [24-26, 43-48]. The sparsity of studies reporting sex-specificity in effects is particularly evident when it comes to traits tied to reproductive performance [49]. Indeed, only a single study to date has sought to measure the effects associated with natural mtDNA haplotypes on components of reproductive success in both males and females. In that study, Immonen et al. (2016) examined the expression of components tied to reproductive success in each of the sexes across orthogonal combinations of mitochondrial and nuclear genotype sourced from three distinct populations, in the seed beetle, *Callosobruchus maculatus*. The nuclear genomic backgrounds, against which the three different mtDNA haplotypes were placed were not isogenic, but rather represented by large pools of segregating nuclear allelic variance that were sourced from each of three global populations. The authors reported mitochondrial genetic, and mito-nuclear interactions for female fecundity, and male ejaculate weight, and also an effect on female egg size that was traceable to an interaction involving the age and mito-nuclear genotype of the sire. Correlations in the reported mitochondrial, or mito-nuclear, genetic effects across the measured traits were, however, not examined [25].

Currently, little information exists as to the capacity for genetic variants in the mitochondrial genome to exert pleiotropic effects on multiple fitness traits, and whether the directions of

117 pleiotropy might change within and across the sexes. On the one hand, it might reasonably be
118 expected that the sign of mitochondrial genetic correlations for key phenotypic traits will
119 routinely be positive, assuming that mutations that accumulate within the mtDNA sequence
120 are likely to modify the performance of core metabolic processes, with cascading effects on a
121 range of energy-reliant phenotypes. But on the other hand, under the Mother's Curse hypothesis
122 it is plausible that the direction of these correlations will be negative across the sexes.
123 Assuming strict maternal inheritance, female-harming but male-benefiting mtDNA mutations
124 that appear in the mtDNA sequence should be efficiently purged by purifying selection. In
125 contrast, if mtDNA mutations appear that are female-benefiting, but male-harming, they will
126 be under positive selection and potentially increase in frequency [32]. Furthermore, the pool of
127 sexually antagonistic mutations accumulating within the mitochondrial genomes will differ
128 across populations – in terms of the identity of the mutation sites at which they occur, the
129 associated nucleotides, and total number of mutations accrued. Accordingly, we should expect
130 to observe a negative genetic correlation across haplotypes, with haplotypes that harbour
131 numerous female-benefiting but male-harming mutations (or alternatively harbouring a few
132 mtDNA mutations of major sexually antagonistic effect) conferring higher relative female, but
133 lower male, reproductive success. Conversely, those haplotypes harbouring few such mutations
134 (or alternatively mutations of only minor effect) will confer lower female reproductive success
135 relative to other haplotypes, but relatively higher success in males.

136 Studies that have tested for mitochondrial haplotype effects on multiple traits, and screened for
137 the presence of mitochondrial genetic correlations between the traits, have confirmed that
138 correlations frequently exist, and that they can be either positive or negative in direction. In
139 2009, Dowling *et al.* reported a strong positive association in effects of two mtDNA haplotypes,
140 segregating within a laboratory population (LH_M) of *D. melanogaster*, on two life history traits
141 in females - reproductive performance and longevity [50]. The haplotype conferring higher

142 female reproductive success also conferred higher female lifespan. Rand *et al.* (2001) reported
143 a negative correlation between the sexes for a measure of juvenile viability in *D. melanogaster*
144 (based on a chromosome segregation assay), across two of three mtDNA haplotypes measured
145 (these haplotypes were broadly clustered into 3 groups: Old World 1, Old World 2 and New
146 World). Camus et al (2015) reported that a Single Nucleotide Polymorphism (SNP) within the
147 mtDNA-encoded cytochrome B (Ala-278-Thr in *mt:Cyt-b*) gene of *D. melanogaster*, which is
148 found on a haplotype sourced from Brownsville USA, is associated with low male fertility [38],
149 but high male lifespan and short female lifespan, relative to twelve other haplotypes harbouring
150 other variants of this gene [24]. This SNP is therefore associated with antagonistic pleiotropic
151 effects both within and across the sexes, consistent with the prediction that mtDNA SNPs can
152 accumulate under positive selection in females, even if they are associated with suboptimal
153 male phenotypes [32], leading to sexually antagonistic trajectories of mtDNA evolution [33,
154 51-53].

155 To address patterns of sex-specificity and pleiotropy within the mitochondrial genome, here
156 we measured components of reproductive success in each sex, across a fully replicated panel
157 of thirteen naturally occurring mitochondrial haplotypes in *D. melanogaster*, in which each
158 haplotype is expressed alongside a standard, isogenic nuclear background [44, 54, 55]. Given
159 that the nuclear background of the panel is strictly controlled and isogenic, this experimental
160 approach provides an accurate means to home in on true mitochondrial genetic effects on
161 reproductive trait expression, and test for the magnitude and direction of mitochondrial genetic
162 correlations underpinning these traits. Such an approach provides a powerful proof-of-concept,
163 but also comes with a general caveat. Mitochondrial genetic effects on phenotypic trait
164 expression are likely to be routinely mediated via epistatic interactions between mitochondrial
165 and nuclear genotype [3, 5]. By constraining the number of nuclear backgrounds in our study
166 to just the one isogenic variant, we are unable to assess levels of mito-nuclear epistasis for the

167 traits under study, nor investigate whether effects or correlations across haplotypes are
168 dependent on the nuclear genetic context. However, while a recent meta-analysis by Dobler et
169 al. (2014) confirmed that effect sizes associated with cyto/mito-nuclear interactions generally
170 exceeded those associated with additive cytoplasmic/mitochondrial genetic effects across plant
171 and animal kingdoms, their analyses nonetheless revealed the additive effects were moderate
172 to strong in magnitude. This therefore suggests that despite the ubiquity of mito-nuclear
173 epistasis, a substantial pool of the genetic polymorphisms maintained within the mitochondrial
174 genome are expressed at least to some degree additively, and will be uncovered using our
175 approach.

176 Unlike previous screens of mitochondrial variation for longevity that had uncovered strong
177 male-biases in effects [44], we found that both male and female reproductive traits were
178 affected by the mitochondrial genetic variation harboured across our panel of haplotypes.
179 Furthermore, we found signatures of pleiotropy across haplotypes in effects on the reproductive
180 traits. Intriguingly, mitochondrial genetic correlations were generally positive for different
181 reproductive traits measured within a given sex, but negative for traits of the different sexes.

182

183 Materials and Methods

184 Mitochondrial strains

185 Our experimental design is informed by the evolutionary prediction that nuclear compensatory
186 variants that offset the negative effects of Mother's Curse mutations are likely to routinely arise
187 and be selected for [56]. That is, if surveying natural populations, Mother's Curse mutations
188 should remain cryptic and masked by their rescuing nuclear modifiers. Indeed, this is the
189 scenario we see with Cytoplasmic Male Sterility in plants [35]. Therefore, our strain

190 construction is based on the premise that in order to detect Mother's Curse effects, we must
191 first unmask them by placing them alongside an evolutionary novel nuclear background.
192 Perhaps the strongest evidence for this premise to date in bilaterians, comes from Yee et al.
193 2013 [57], who reported that fertility outcomes were higher when mtDNA haplotypes were
194 expressed alongside their putatively coevolved nuclear backgrounds than alongside an
195 evolutionary novel nuclear background. Accordingly, thirteen *Drosophila melanogaster* strains
196 were used, which have been previously described [24, 38, 55]. In brief, the isogenic nuclear
197 background from the w^{1118} strain (Bloomington stock number: 5905) was coupled to
198 mitochondrial haplotypes from thirteen distinct geographic locations using a crossing scheme
199 that is outlined in Clancy (2008). These strains have each been maintained in duplicate since
200 2007, with the duplicates propagated independently, to enable us to partition mitochondrial
201 genetic effects from cryptic nuclear variance that might have accumulated among the strains,
202 as well as from other sources of environmental variation. Each generation, virgin females are
203 collected from each duplicate of each mitochondrial strain (hereafter *mitochondrial strain*
204 *duplicate*) and backcrossed to males of the w^{1118} strain, to maintain isogenicity of the nuclear
205 background. Furthermore, w^{1118} is itself propagated by one pair of full-siblings per generation.
206 Thus, if mutations arise in the w^{1118} strain, they will be swiftly fixed and passed to all
207 mitochondrial strain duplicates, thus maintaining the critical requirement of isogenicity of the
208 nuclear genome.
209
210 One of the mitochondrial haplotypes (Brownsville) included in our panel incurs complete male
211 sterility in the w^{1118} nuclear background used here, and low male fertility in all other nuclear
212 backgrounds surveyed to date [57, 58], whereas females who harbour this haplotype remain
213 fertile [38]. This strain was therefore excluded from assays of male reproductive success (n=12
214 haplotypes in these assays), but included in assays of female reproductive success (n=13

215 haplotypes). All mitochondrial strains and w^{1118} flies were reared at 25˚C, under a 12h: 12h
216 light: dark photoperiod regime, on potato-dextrose-agar food medium and with *ad libitum*
217 access to live yeast. All strains had been cleared of any potential bacterial endosymbionts, such
218 as *Wolbachia*, through tetracycline treatment at the time that the strains were created [59].
219 Diagnostic PCR with *Wolbachia*-specific primers confirmed all lines are free of *Wolbachia*
220 [60].
221
222 **Male Reproductive Success**

223 <u>*Male reproductive success following exposure to a single female (short-burst offspring*</u>
224 <u>*production)*</u>

225 This experiment measured offspring produced by a single male after a one-off mating
226 opportunity with a virgin female at 4 days of adult age. This assay measures the ability of a
227 male to convince a virgin female to mate, and then measures the number of offspring produced
228 from sexual interaction with that female, which is likely to be a function of the male ejaculate
229 quality (number and quality of sperm, and content and quality of reproductive proteins,
230 transferred). The assay was run in two blocks, each separated in time by one generation. For
231 three generations leading up to the experiment, each mitochondrial strain duplicate was
232 propagated across 3 vials, with each vial containing 10 pairs of flies of standardised age (4-day
233 old), and at controlled larval densities (approximately 80 eggs per vial). Then, ten virgin males
234 from each mitochondrial strain duplicate (total 20 male flies per haplotype) were collected
235 randomly from the 3 vials that propagate the line, and each stored individually in separate 40
236 ml vials containing 5mL of food medium. At the same time, virgin females were collected from
237 the isogenic w^{1118} strain to be used as "tester" flies in the experiment. These females were
238 sourced from 10 separate vials, which had been propagated and stored under the same

239 experimental conditions as described for the mitochondrial strain focal males, and they were
240 stored in groups of 10 females per vial.

241 When four days old, each focal male was then combined with an equivalently-aged "tester"
242 female, and these flies then cohabited the same vial for a 24 h period. Following this, focal
243 males were removed from the mating vial and discarded. Females were then transferred into
244 fresh vials with food substrate every 24 h over a 4-d period. The total number of offspring
245 eclosing across these four vials was recorded for each focal male.

246 *Male reproductive success across 8 days (sustained offspring production)*

247 This assay represents a measure of male reproductive stamina (a function of male mating rate
248 across time, and ability to replenish sperm and ejaculate stores). Sustained offspring production
249 was assayed following the method described in Yee et al. (2015). In brief, individual males
250 collected from each mitochondrial strain duplicate were provided with the opportunity to mate
251 with eight different virgin females over eight consecutive 24 h long exposures [61]. To initiate
252 the assay, twenty virgin males were collected from each mitochondrial strain duplicate, and
253 each placed in a separate vial (total of 40 flies per mitochondrial haplotype). Twenty-four hours
254 later, one 4-day-old virgin w^{1118} female was added to each vial, and the focal male and tester
255 female then cohabited for 24 h. Following this 24 h exposure, males were removed and placed
256 with another 4-day-old virgin w^{1118} female for another 24 h period. This process was repeated
257 until day eight of the experiment (8 separate exposures). After each exposure, the w^{1118} females
258 were retained and themselves transferred into fresh vials every 24 h for a total period of 4
259 consecutive days (including the 24 h cohabitation period), thus providing each female with up
260 to 96 h to oviposit. Thirteen days following the 96 h oviposition period, the number of eclosed
261 adult offspring emerging from each vial was counted.

262 **Female reproductive success**

263 *Female components of short-burst offspring production, and short-burst 'egg-to-adult'*
264 *viability*

265 The first experiment gauged "short-burst" components of success, in which the number of eggs
266 produced per female (fecundity), number of adults (reproductive success) produced, and
267 proportion of eggs that ultimately eclosed into adulthood (an index of short-burst viability)
268 were scored, following a 24 h laying opportunity early in life (4 days of age). The assay was
269 run in five blocks, each separated in time by one generation. Female focal flies from each
270 mitochondrial strain duplicate were collected as virgins, and stored individually. These were
271 collected over numerous 40 mL vials, each of which had been propagated by 10 pairs of age-
272 controlled parents (4 day old), and at controlled larval densities (approximately 80 eggs per
273 vial). When 4 days of age, each female was exposed to one 4 d old tester virgin male, collected
274 from the w^{1118} strain, for a period of 12 hours and then the females transferred to a fresh vial
275 for 24 h to oviposit. Following this 24 hour ovipositioning period, females were discarded. We
276 counted the eggs oviposited per female over this 24 h period (an index of short-burst fecundity),
277 plus the offspring that emerged from these eggs (an index of short-burst offspring production).
278 Furthermore, we calculated the proportion of eggs laid by each female that were converted into
279 adult offspring (short-burst viability).

280 *Female offspring production across 13 days (sustained offspring production)*

281 This experiment measured female reproductive success over a 13-day period, thus representing
282 a measure of reproductive stamina. Forty females from each mitochondrial strain duplicate
283 were collected as virgins, and placed in individual vials. One day later, two 4 d old virgin w^{1118}
284 males were placed into each female vial. Females, and the two males with which each female
285 cohabited, were then transferred into fresh vials every 24 hours, for 13 days. The accompanying
286 males were discarded every fourth day, and two 4 d old virgin males of the w^{1118} strain were

287 added. This ensured that females were not sperm-limited throughout the duration of the
288 experiment. At the end of day 13, all flies across all vials were discarded, and vials were kept
289 for eggs to develop. Female reproductive success was determined by counting the total number
290 of adult offspring produced by each female, per vial, over the 13-day assay.

291 **Statistical Analysis**

292 General linear mixed models, using a Gaussian distribution, were fitted to the male short-burst
293 offspring production data. Female short-burst fecundity data and female short-burst offspring
294 production were modelled by fitting a generalized linear mixed model, using a Poisson
295 distribution. For data that conformed to a Poisson distribution, we checked for over-dispersion
296 using the function "*dispersion_glmer*" in the package *blmeco* [62]. Short burst viability data
297 was modelled as a binomial vector, composed of the number of adults and number of eggs that
298 failed to hatch (eggs-adults), using a binomial distribution and logit link. For each analysis,
299 mitochondrial strain, the duplicate nested within mitochondrial strain, and the sampling block
300 (for assays of short-burst components, which were assayed over multiple blocks) were
301 modelled as random effects in the *lme4* package [63] in R [64]. Finally, female short-burst
302 offspring production and fecundity had the addition of a random dummy variable to account
303 for over-dispersion. To test for mitochondrial genetic variance for each trait, we used
304 parametric bootstrap analysis to compare a full model to a reduced model which lacked the
305 mitochondrial strain term. The parametric bootstrap was performed using the *PBmodcomp*
306 function implemented in the package *pbkrtest* [65]

307

308 For the experiments gauging sustained offspring production, the overall total number of
309 offspring (for both male and female models) was zero-inflated, and the resulting models over-
310 dispersed. We therefore analysed both datasets using a negative binomial distribution [66], in
311 which the zero values are a blend of sampling and structural effects (negative binomial

312 parameter; variance = ϕμ). These models were performed using the R (v. 3.0.2) package
313 glmmADMB (http://glmmadmb.r-forge.r-project.org/glmmADMB.html). The response
314 variable was total number of offspring produced, with day of sampling being a fixed factor.
315 The random effects in the model were mitochondrial strain, mitochondrial duplicate nested
316 within mitochondrial strain, and the interactions between mitochondrial haplotype with day of
317 sampling. Similar to the previous analyses for components of fitness, we used a model
318 comparison approach whereby we compared the full model with a reduced model that lacked
319 the mitochondrial strain term. Model comparisons were performed using likelihood-ratio tests.
320
321 A matrix of mitochondrial genetic correlations (Pearson's correlation coefficients and 95%
322 Confidence Intervals) was created by obtaining mtDNA haplotype-specific means for each
323 reproductive trait across all mitochondrial strains (Table S1). Thus, we had 13 means (mean of
324 all individual datapoints within one haplotype) for each female measure of short burst
325 (including short-burst viability) and sustained offspring production, and 12 means for the male
326 measures (since the Brownsville haplotype was excluded from the male assays). Inter-sexual
327 correlations across haplotypes were thus based on 12 means. Correlation coefficients of all
328 pairwise combinations of traits were then further assessed using a bootstrapping procedure, in
329 which trait means were resampled with replacement (10,000 replicates), and 95% confidence
330 intervals were calculated using the Adjusted Percentile (BCa) Confidence interval method, as
331 recommended by Puth et al. (2015) given its high performance across a broad range of
332 situations [67]. Bootstrapping the confidence intervals appeared appropriate, given that we had
333 captured a representative sample of the total global mtDNA haplotype variation present in *D.*
334 *melanogaster* (Figure S2) [55], given the modest number (n=12) of data points in each
335 correlation, and given that not all of the underlying distributions for each sampled trait were

336 Gaussian. Bootstrapped correlation coefficients plus their confidence intervals were calculated
337 using the functions "*boot*" and "*boot.ci*" in the R package *boot* [68].
338

339 Results

340 *Male Mitochondrial Reproductive Success Assays*

341 We found statistically significant mitochondrial genetic variance for male short-burst offspring
342 production (parametric bootstrap stat: 2.63, $p < 0.05$, Table 1A). We also uncovered
343 statistically significant mitochondrial variance for male sustained offspring production
344 (haplotype, deviance: 5.53, $p < 0.05$), levels of which were in part contingent on the day of the
345 mating assay (haplotype × day, deviance: 6.54, $p < 0.05$, Table 1B, Figure 1A, Figure S1).
346 Male offspring production tended to increase up to day 4 of adult age, and then incrementally
347 decrease to day 8. However, the magnitude of increase was contingent on the mtDNA
348 haplotype, with only two haplotypes exhibiting a clear peak in reproductive success at day 4
349 (MYS and ORE, Figure S1). The reaction norms per haplotype crossed-over across the eight
350 days of the experiment, with several haplotypes that exhibited the highest relative reproductive
351 success at the peak of the assay (day 4) generally associated with low reproductive success
352 relative to the other haplotypes at Day 1 and 8 of the experiment (Figure 1A).
353

354 *Female Mitochondrial Reproductive Success, and Short-burst Viability Assays*

355 We found mitochondrial genetic variance for egg-to-adult viability of a female's clutch
356 (parametric bootstrap stat: 3.51, $p < 0.05$, Table 1C), short-burst offspring production
357 (parametric bootstrap stat: 3.7506, $p < 0.05$, Table 1D), but not short-burst fecundity
358 (parametric bootstrap stat: 0.0132, $p = 1$, Table 1E). We found statistically significant
359 mitochondrial genetic variance for sustained female reproductive success (haplotype, deviance:

360 6.04, $p < 0.001$), levels of which were again partly contingent on an interaction between
361 mitochondrial strain and day of the mating assay (haplotype × day, deviance: 21.4, $p < 0.001$,
362 Table 1F, Figure 1B, Figure S1). All haplotypes exhibited a similar trend, with reproductive
363 success incrementally increasing up until day 4 of the assay, following which point,
364 reproductive success began to decline albeit with slight upticks at days 8 and 12 that coincided
365 with the addition of fresh tester males to the female vials (Figure S1). Again, however, these
366 patterns were contingent on the mtDNA haplotype, with norms of reaction crossing per
367 haplotype across Days 1, 4 and 8 of the assay (Figure 1B).

368

369 *Mitochondrial Genetic Correlations*

370 Intra-sexual correlations between reproductive traits tended to be positive in direction,
371 including a positive correlation between short-burst viability and short-burst offspring
372 production in females, across haplotypes (Figure 3). In contrast, inter-sexual correlations
373 tended to be negative in direction (Figure 3).

374

375 **Discussion**

376 We explored mitochondrial genetic variance, across distinct and naturally occurring
377 mitochondrial haplotypes, on components of reproductive success in male and female *D.*
378 *melanogaster*, using an approach that enabled us to unambiguously trace genetic variation to
379 the level of the mtDNA sequence. Notably, genetic polymorphisms located across these
380 haplotypes affected almost all components of reproductive success measured – in females and
381 in males. For measures of sustained reproductive success, we found that the level of
382 mitochondrial genetic variation changed with the age of the focal flies (across the days of the
383 experiment), and such genotype-by-age effects might be one means by which genetic variance
384 within mitochondrial genomes might be maintained within and between populations.

385 Furthermore, we uncovered a signature of pleiotropy in the reported effects. These patterns of
386 pleiotropy were positive for intra-sexual correlations across haplotypes (e.g. for associations
387 between short-burst and sustained [when calculating means of total reproductive success across
388 all days] components of reproductive success in each of the sexes), but negative for several of
389 the inter-sexual correlations. While individual mutations conferring male sterility are well
390 known in plants [35], and have recently been documented in metazoans [36, 38, 40], the
391 signature of intersexual negative correlations across mtDNA haplotypes detected here,
392 suggests that sexual antagonism might be a pervasive force under which genetic variation in
393 the mitochondrial genome accumulates.

394 Negative inter-sexual correlations are striking because they indicate that, at the level of whole
395 haplotypes, those haplotypes that confer relatively high reproductive success in one sex,
396 generally confer low success in the other. Furthermore, we note that our estimate of this
397 negative correlation is conservative, because it excluded the Brownsville mtDNA haplotype,
398 which is completely male-sterile in the nuclear background assayed here (w^{1118}), and which we
399 have previously reported to host a sexually antagonistic polymorphism located in the mt:*Cyt-b*
400 gene [24, 58, 69]. The negative correlation between male and female reproductive success is
401 consistent with evolutionary theory first developed by Frank and Hurst (1996), and which is
402 routinely called "Mother's Curse" [31], which proposes that maternal inheritance of the
403 mitochondria will lead to the accumulation of male-biased mutation loads within the mtDNA
404 sequence [43]. Specifically, however, while Frank and Hurst (1996) envisaged that such
405 mutations would accumulate under mutation-selection balance (i.e. the mutations would be
406 largely benign, or slightly deleterious, in their effects on females), our results suggest a role for
407 sexually antagonistic selection [32, 33], with mutations accumulating in the mtDNA sequence
408 that augment female reproductive success, but that come at cost to male reproductive
409 performance.

410 In our study, we included egg-to-adult viability of the female clutch in our analyses; a measure
411 that lies at the interface between a maternal and an offspring trait [70-75]. It is well established
412 that maternal effects shape this trait in *D. melanogaster* [70-72], in alignment with predictions
413 of classic life-history theory, in which maternal resource provisioning into the ova lies at the
414 heart of the classic evolutionary trade-off between gamete size and number [73]; a trade-off
415 that extends to *Drosophila* [74, 75]. While ultimately it is not possible for us to delineate
416 whether any mitochondrial haplotype effects on short-burst viability are manifested primarily
417 through mothers (as mtDNA-mediated maternal effects) or primarily on the offspring
418 themselves (via the direct effects of mtDNA mutations on survival through juvenile
419 development), it was nonetheless informative to examine patterns of mitochondrial haplotypic
420 variation affecting this trait.

421 Indeed, we found two intriguing and complementary patterns involving mitochondrial effects
422 on egg-to-adult viability. Firstly, the Brownsville haplotype was associated with high viability,
423 despite its association with male fertility impairment in adult life (Figure 1A&C). The
424 Brownsville haplotype thus harbours a candidate mutation in the *mt:Cyt-b* gene associated with
425 reduced adult male fertility [38, 58], and sexually antagonistic effects on longevity [24], but
426 which is associated with high fitness in the juvenile phase of life. This result is consistent with
427 a recent study, which showed that despite being associated with population suppression via its
428 effects on male fertility impairment, when seeded into large experimental populations of *D.*
429 *melanogaster* harbouring high levels of segregating nuclear allelic variance, population
430 frequencies of the Brownsville haplotype were stably maintained, and indeed tended to increase
431 across 10 generations of evolution [69]. In combination, these results suggest that this male
432 sterilising mtDNA mutation has been maintained under positive selection on adult female and
433 juvenile fitness. Secondly, the correlations we observed across the other twelve haplotypes
434 further support this contention. The mitochondrial genetic correlation between short-burst

435 viability and female sustained offspring production was positive, while the correlation between
436 short-burst viability and male short-burst offspring production was negative. These patterns
437 reinforce the case of the Brownsville haplotype, by suggesting that the direction of selection
438 on mitochondrial mutations might not only be routinely antagonistic between adult males and
439 adult female reproductive traits, but also between juvenile components of fitness and
440 components of adult male fitness; thus acting to exacerbate the rate at which male-biased
441 mitochondrial mutation loads could accumulate within populations.

442 The possibility exists that our results might have been affected by the existence of heteroplasmy
443 across our genetic strains. Heteroplasmy refers to the occurrence of multiple mtDNA
444 haplotypes co-occurring within the same individuals, often brought about following instances
445 of paternal leakage. While such cases have been reported in *Drosophila*, these have typically
446 occurred between interspecific crosses involving individuals of divergent species [76, 77], or
447 intraspecific crosses in species exhibiting much higher levels of divergence across the mtDNA
448 haplotypes than those found in *D. melanogaster* [78]. One study, however, using intraspecific
449 crosses in *D. melanogaster*, reported that as many as 14% of individuals are heteroplasmic,
450 which would suggest the capacity for widespread paternal leakage in this species [79]. Another
451 study indicated higher rates of leakage in males than females [77], but these cases all came
452 from interspecific crosses between distinct species. Clearly, paternal leakage, leading to
453 heteroplasmy, could potentially complicate our inferences, if present across our panel of
454 mitochondrial strains, or if sex-specific in occurrence. The protein-coding sequences of the
455 mtDNA haplotypes of each strain used in this study were originally sequenced by Clancy
456 (2008). Since 2007, we have intermittently confirmed the genotype of each using haplotype-
457 specific diagnostic SNPs, and we have also recently re-sequenced the haplotypes of each strain
458 at high power to detect low frequency heteroplasmies (~1000× coverage). Throughout this
459 time, we have never detected any instances of paternal leakage, or heteroplasmy, within any of

460 our strain duplicates. We acknowledge that we have only genotyped and sequenced females
461 from each strain to date, leaving open the possibility that heteroplasmy might occur in males
462 among our strains. However, if so, cases of male heteroplasmy should nonetheless be reset
463 each and every generation, given generally strict maternal inheritance of the mtDNA and the
464 predicted rarity of paternal leakage. Thus, we suggest that it is unlikely our results will be
465 influenced by sex differences in levels of mtDNA heteroplasmy in our study.

466 Our results are based on a panel of 13 mtDNA haplotypes. Given each haplotype is replicated
467 and expressed alongside an isogenic background, this enabled us to unambiguously partition
468 mitochondrial genetic variance underpinning phenotypic trait expression. Furthermore, the
469 panel of haplotypes is large enough to be broadly representative of the total levels of
470 mitochondrial genetic diversity present within the global distribution of *D. melanogaster* [55]
471 (Figure S2), and large enough to overcome the risk of sampling error leading to erroneous
472 inferences that is likely to arise when sampling just a small subset of haplotypes that might
473 have coincidentally similar breeding values. A caveat of our panel, however, is that it remains
474 possible that patterns of mitochondrial genetic variation that we have detected in this study,
475 might be specific to the particular nuclear background in which we have sampled the
476 haplotypes; that is if the mitochondrial genetic variation screened here is only manifested via
477 mito-nuclear interactions [3, 5], and not expressed additively. Indeed, in a broad meta-analysis
478 of the magnitude of cytoplasmic genetic effects, Dobler et al (2014) reported that the effect
479 size associated with cyto-/mito-nuclear interactions across taxa tended to be larger than the
480 additive cytoplasmic/mitochondrial genetic effect size [80]. Importantly, however, the additive
481 mitochondrial genetic effect was nonetheless moderate to large in metazoans, indicating that
482 despite the ubiquity of mito-nuclear epistasis, a substantial pool of the mitochondrial genetic
483 polymorphisms are expressed at least to some degree additively. Furthermore, we note that
484 inferences based on the expression of focal genotypes alongside otherwise isogenic

485 backgrounds is by no means a limitation specific to our study, but a pervasive design feature
486 of many genetic studies, for instance chromosomal substitution studies of the sex chromosomes
487 [81, 82]. Ultimately, while we cannot conclude that the patterns observed here would be evident
488 across the complete pool of nuclear backgrounds in which they are tested, at its root our study
489 provides an important proof of concept that sexually antagonistic fitness variation can be
490 maintained within the mitochondrial genome, thus substantiating predictions of well-
491 established population genetic theory that have previously remained elusive.
492
493 Future studies are, however, now needed to assess the generality of our findings, not only
494 across a broader array of nuclear backgrounds within a species, but across a broad sample of
495 metazoans. Indeed, almost everything we know about metazoan mitochondrial genomes,
496 comes from the bilaterians and their streamlined genomes, but it now clear that non-bilaterian
497 mitochondrial genomes are much different in their gene content and arrangement [83].
498 Assessment of the capacity for sexually antagonistic mitochondrial variation should be
499 extended to these taxa. Furthermore, while our approach assumed that Mother's Curse
500 mutations will routinely lie hidden within natural populations, being offset by co-adapted
501 nuclear modifiers that rescue male fitness [5, 32], this assumption also requires further
502 theoretical and empirical attention. Indeed, a recent population genetic model suggests that
503 even when nuclear genetic variation for compensatory evolution is abundant, the negative
504 impact of Mother's Curse substitutions on male fitness can still be large, particularly in species
505 with intermediate effective population sizes [56]. This suggests that the dynamics of sexually
506 antagonistic mitochondrial evolution will differ across species, providing strong motivation for
507 broadening the emerging platform of research into Mother's Curse effects beyond the few
508 model species currently studied.
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521

522

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735 # Tables and Figures

736 **Table 1:** Mitochondrial genetic variance for male (**A**) short-burst offspring production and (**B**)
737 sustained offspring production, and female (**C**) short-burst viability, (**D**) short-burst offspring
738 production, (**E**) short-burst fecundity, and (**F**) sustained offspring production. Haplotype
739 denotes the effect of mitochondrial strain (hence mtDNA haplotype), and
740 Duplicate[Haplotype] denotes the mitochondrial strain duplicate. In the short-burst assays,
741 each experiment was conducted over consecutive sampling blocks (Block), and required a
742 dummy variable to account for overdispersion. In the sustained offspring production assays,
743 each experiment was conducted over a number of consecutive days (Day; 8 in males, 13 in
744 females). For all models, statistical significance of levels of mitochondrial genetic variance is
745 based either on a parametric bootstrap model comparison (for the short burst traits), or
746 Likelihood-ratio test (for the sustained traits). We also present variance (Var) and standard
747 deviation (SD) for random effects.

A) Male short-burst offspring production			
	PB stat	**df**	**P**
Haplotype	2.625	1	0.044
	var		
duplicate[Haplotype]	8.795		
Haplotype	23.125		
Block	0		
Residual	464.21		

B) Male sustained offspring production			
		deviance	**P**
Haplotype		5.53	0.038
Haplotype × day		6.54	0.010
	var		
Haplotype	1.13E-07		
duplicate[Haplotype]	0.066		

Haplotype x day	0.01995	0.1412	

C) Female short-burst viability

	PB stat	**df**	**P**
Haplotype	3.518	1	0.047
	var		
duplicate[Haplotype]	0.013		
Haplotype	0.017		
Block	0.122		

D) Female short-burst offspring production

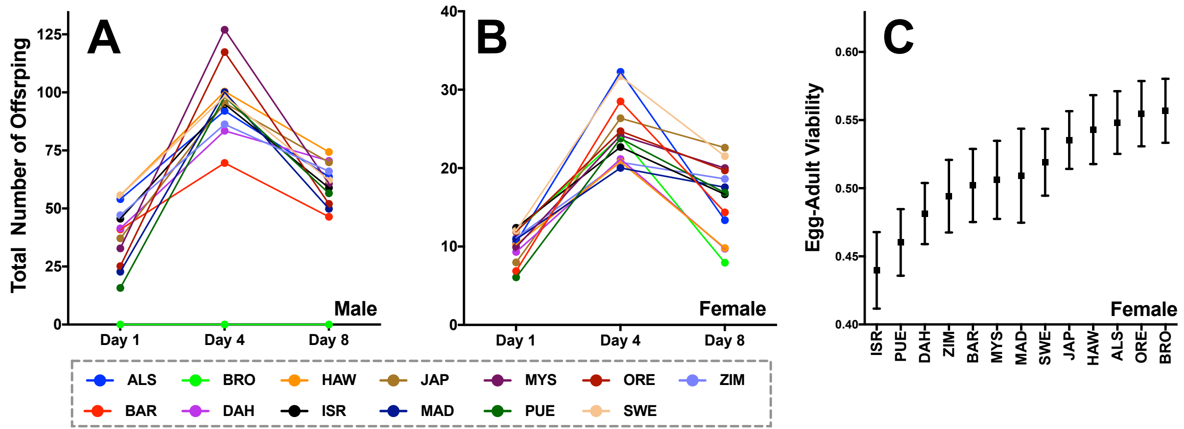
	PB stat	**df**	**P**
Haplotype	3.751	1	0.012
	var		
dummy	0.301		
duplicate[Haplotype]	0		
Haplotype	0.006		
Block	0.016		

E) Female short-burst fecundity

	PB stat	**df**	**P**
Haplotype	0.0132	1	1
	var		
dummy	0.190		
duplicate[Haplotype]	0.001		
Haplotype	0		
Block	0.035		

F) Female sustained offspring production

		deviance	**P**
Haplotype		6.04	<0.001
Haplotype × day		21.4	<0.001
	var		
Haplotype	0.005		
duplicate[Haplotype]	0.021		
Haplotype x day	0.004		



750 **Figure 1:** Mean number of offspring produced (reproductive success) for (**A**) males and (**B**) females across the mitochondrial strains, at 3 different age points of the sustained offspring production experiment. **C**) Female egg-adult viability (mean ± SE) (short-burst viability) across all mitochondrial lines.

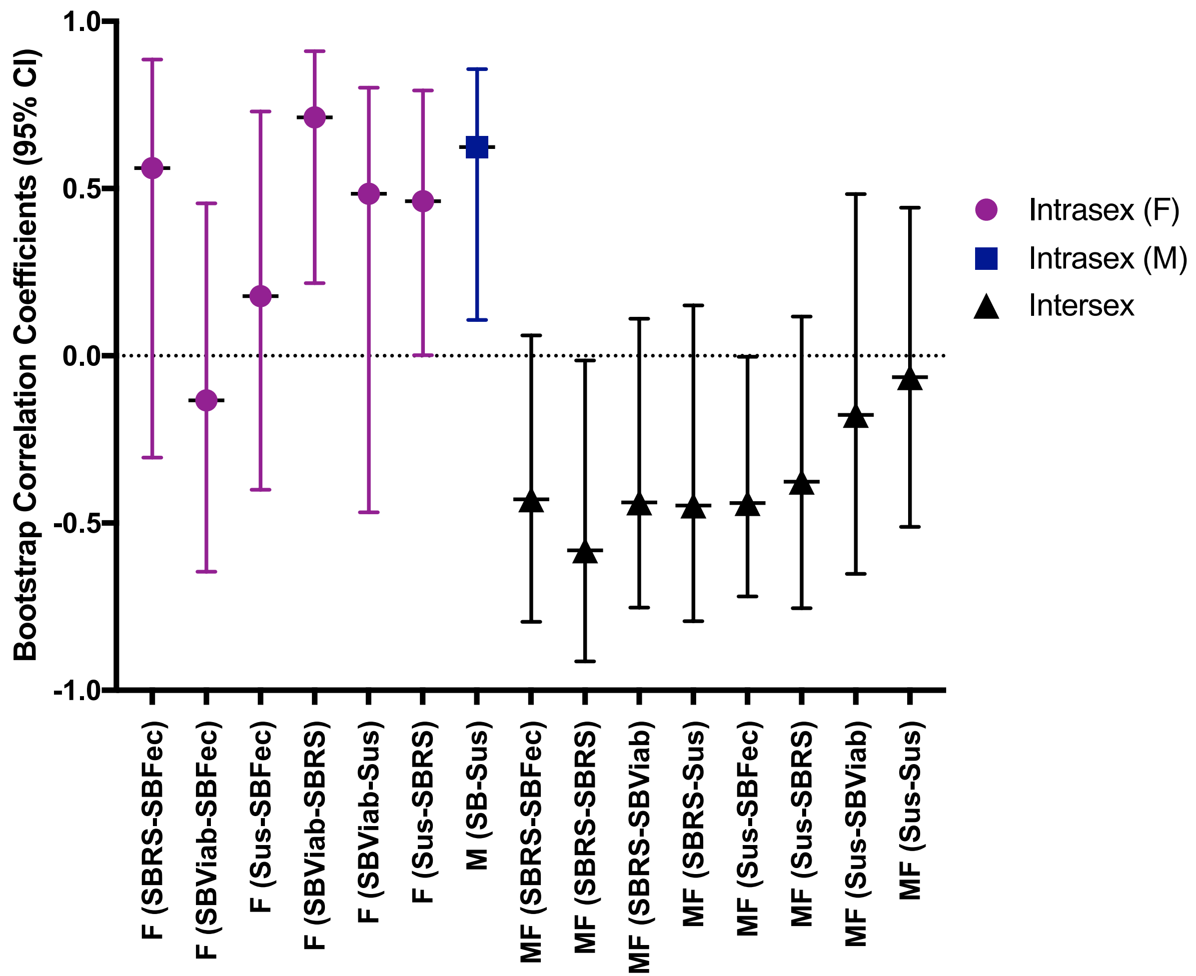


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Figure 2: Bootstrapped correlation coefficients (± 95% confidence intervals) estimates of intra- and inter-sexual genetic correlations for male and female reproductive traits across mitochondrial haplotypes. Acronyms refer to: SBRS (short-burst reproductive success), SBFec (short-burst fecundity), SBViab (short-burst viability), Sus (sustained reproductive success).

760 For instance, F(SBRS-SBFec) denotes the mitochondrial correlation between short-burst reproduction success and short-burst fecundity in females.