

1 **Sex-specific effects of mitochondrial haplotype on metabolic rate in *Drosophila***  
2 ***melanogaster* support predictions of the Mother's Curse hypothesis**

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12 ***Short running title:*** Mitochondrial haplotype affects metabolic rate

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26 **Abstract**

27 Evolutionary theory proposes that maternal inheritance of mitochondria will facilitate the  
28 accumulation of mitochondrial DNA (mtDNA) mutations that are harmful to males but benign  
29 or beneficial to females. Furthermore, mtDNA haplotypes sampled from across a given species  
30 distribution are expected to differ in the number and identity of these “male-harming”  
31 mutations they accumulate. Consequently, it is predicted that the genetic variation that  
32 delineates distinct mtDNA haplotypes of a given species should confer larger phenotypic  
33 effects on males than females (reflecting mtDNA mutations that are male-harming, but female-  
34 benign), or sexually antagonistic effects (reflecting mutations that are male-harming, but  
35 female-benefitting). These predictions have received support from recent work examining  
36 mitochondrial haplotypic effects on adult life history traits in *Drosophila melanogaster*. Here,  
37 we explore whether similar signatures of male-bias or sexual antagonism extend to a key  
38 physiological trait - metabolic rate. We measured the effects of mitochondrial haplotypes on  
39 the amount of carbon dioxide produced by individual flies, controlling for mass and activity,  
40 across 13 strains of *D. melanogaster* that differed only in their mtDNA haplotype. The effects  
41 of mtDNA haplotype on metabolic rate were larger in males than females. Furthermore, we  
42 observed a negative intersexual correlation across the haplotypes for metabolic rate. Finally,  
43 we uncovered a male-specific negative correlation, across haplotypes, between metabolic rate  
44 and longevity. These results are consistent with the hypothesis that maternal mitochondrial  
45 inheritance has led to the accumulation of a sex-specific genetic load within the mitochondrial  
46 genome, which affects metabolic rate and that may have consequences for the evolution of sex-  
47 differences in life history.

48

49 **Keywords:** mitochondrial DNA; pleiotropy; rate of living; sex specific selective sieve; sexual  
50 conflict; sexually antagonistic selection

## 51 **Background**

52 Mitochondrial genes encode products that are key to the regulation of oxidative  
53 phosphorylation. Given the pivotal importance of oxidative phosphorylation in the conversion  
54 of chemical energy in eukaryotes, it was traditionally assumed that intense purifying selection  
55 would prevent the accumulation of non-neutral (i.e. functional) genetic variants within the  
56 coding sequence of the mitochondrial DNA (mtDNA). This assumption has, however, been  
57 challenged over the past two decades by studies harnessing experimental designs able to  
58 partition mitochondrial from nuclear genetic contributions to phenotypic expression [1-4].  
59 These studies have generally shown that mtDNA haplotypes routinely harbour functional  
60 polymorphisms that affect the expression of physiological and life history traits [5, 6].  
61 Furthermore, several studies have reported that levels of mitochondrial genetic variation  
62 underpinning phenotypic expression are often sex-specific, with the general pattern seemingly  
63 one of male-bias (whereby mtDNA haplotypes confer greater effects on phenotypic expression  
64 in males than in females) [7, 8].

65 Observations of male-bias in the degree to which divergent mtDNA haplotypes affect  
66 phenotypic trait expression are intriguing because they are consistent with an evolutionary  
67 hypothesis known as *Mother's Curse*. This hypothesis predicts that maternal inheritance of the  
68 mitochondria will render natural selection ineffective at purging mtDNA mutations that are  
69 male-biased in their phenotypic effects [9, 10]. An emerging theoretical framework predicts  
70 two possible manifestations of this process, which have been described as a *weak* and *strong*  
71 form of the Mother's Curse hypothesis [7, 11]. The 'weak' form of the hypothesis is derived  
72 from the original theory established by Frank and Hurst in 1996, who used a population genetic  
73 model to demonstrate that male-harming mtDNA mutations could be maintained within a  
74 population under the mutation-selection balance when these same mutations were benign or  
75 only slightly deleterious in effect in females [10]. Under this weak form, accumulation of

76 'male-harming, but female-benign' mutations would lead to a male-biased genetic load  
77 accumulating within the mitochondrial haplotypes of different populations. Furthermore, the  
78 identity, number and severity of the male-harming mutations would be expected to differ across  
79 haplotypes of different populations, given that each is evolving along its own independent  
80 trajectory. Thus, under the weak form of the hypothesis, it is predicted that the genetic variation  
81 that delineates distinct mitochondrial haplotypes (across the natural distribution of any given  
82 species of eukaryote) will confer greater effects on phenotypic expression in males than in  
83 females [8, 12, 13].

84 Yet, the maternal inheritance of mitochondria could, in theory, also facilitate the  
85 accumulation of mutations that are male harming, but directly beneficial to female fitness.  
86 Because these mutations would directly augment the fitness of females, if any such mutations  
87 were to appear within the mtDNA sequence, they would be expected to be under strong positive  
88 selection and thus quickly accumulate in frequency within populations [13-15]. This  
89 encapsulates the strong form of the Mother's Curse hypothesis [7, 11]. Furthermore, if these  
90 mutations formed an appreciable component of the genetic architecture of the mitochondrial  
91 genome, the outcome would be a negative intersexual genetic correlation for fitness across  
92 haplotypes; i.e., the haplotypes that confer the highest fitness in females would confer the  
93 lowest fitness in males [7, 16].

94 Ultimately, the evolutionary significance of the Mother's Curse hypothesis hinges on  
95 the capacity by which mutations, exhibiting sex-specific effects on the phenotype, can accrue  
96 within the mtDNA sequence. Several studies have now documented evidence for the presence  
97 of mutations conferring male-specific effects on components of adult life history—reproductive  
98 outcomes in vinegar flies (*Drosophila melanogaster*), mice (*Mus musculus*), chicken (*Gallus*  
99 *domesticus*), brown hares (*Lepus europaeus*) and humans [8, 16-23], and longevity [12, 24]  
100 and certain mitochondrial bioenergetic traits in vinegar flies [25, 26]. Other studies, however,

101 while reporting sex-differences in effects of mtDNA haplotype on various traits, failed to find  
102 consistent male-biases in the direction of these effects, with some reporting patterns of female  
103 bias in effects of mtDNA haplotype [27-32].

104 Notwithstanding, evidence for the widespread existence of mtDNA mutations with sex-  
105 specific effects on life history trait expression is noteworthy, since it supports the contention  
106 that genetic variation that accumulates within the mitochondrial genome could play a role in  
107 the dynamics of evolutionary conflict between the sexes and the expression of genetic trade-  
108 offs between life history traits [11]. Combined, these studies also raise the question of how just  
109 a small amount of sequence variation within a genome that is diminutive in size in comparison  
110 to its nuclear counterpart, can exert such broad-scale effects on the expression of components  
111 of adult life history, and mediate patterns of inter-sexual pleiotropy. In this regard, our  
112 understanding of the proximate basis underpinning the link between mitochondrial genotype  
113 and life history phenotype remains rudimentary [33].

114 Recent studies suggest that mitochondrial genetic variation could regulate life history  
115 functioning, at least in part, through modifications to patterns of gene expression both within  
116 the mitochondrial [34, 35] and nuclear transcriptomes [13, 36]. Moreover, life history theory  
117 predicts that physiological traits, such as metabolic rate, will underpin energy allocation  
118 patterns across various components of life history and are thus, candidate mediators of  
119 pleiotropic trade-offs between life history traits [37, 38]. Accordingly, we predict that  
120 previously-reported mitochondrial genotypic effects on adult life history are likely to be  
121 mediated by mitochondrial genotypic regulation of metabolic rate. If so, mitochondrial  
122 genotypic effects on the metabolic rate could plausibly shape the entire organismal life history,  
123 with mitochondrial genetic variation potentially mediating trade-offs between metabolic rate  
124 and longevity, or other key components of life history, such as fertility, in each sex.

125           However, evidence for this prediction remains limited. Many previous studies  
126 examining the capacity for mitochondrial genotypic regulation of physiology have considered  
127 only one or other of the sexes, or pooled both sexes in their analyses [39-44], precluding  
128 inferences of sex-specificity. Some studies have, however, tested for effects in both sexes. For  
129 example, Aw *et al.*, (2017) screened for sex biases in effects on mitochondrial function  
130 (oxidative phosphorylation (OXPHOS) functioning measured from complex I activity, mtDNA  
131 copy number, maximum reactive oxygen species production, and superoxide dismutase  
132 activity) across two mtDNA haplotypes in *D. melanogaster*, reporting a male-bias in effects on  
133 three of the four traits measured. Yet, other studies have not revealed consistent signatures of  
134 male-bias in the magnitude of mitochondrial genetic effects on physiological traits [26, 27, 30,  
135 45]. For example, in one recent study, Wolff *et al.* [26] observed male-biases in levels of  
136 mitochondrial genetic variation for mitochondrial quantity, but not for respiratory rate of the  
137 individual OXPHOS complexes, across a panel of thirteen mitochondrial haplotypes of *D.*  
138 *melanogaster*. In another study, Novičić *et al.* (2015) reported that as much as 20% of the  
139 variation in whole-organism metabolic rate (measured as CO<sub>2</sub> production), across adult *D.*  
140 *subobscura* could be mapped to genetic variation across three mtDNA haplotypes. Yet,  
141 although these mitochondrial genetic effects exhibited some degree of sex-specificity, the  
142 general pattern was not one of clear male-bias [45].

143           Thus, it currently remains unclear whether mitochondrial haplotypic variation affects  
144 the expression of metabolic rate in a pattern similar to previously-reported effects on longevity  
145 and reproductive success [12, 16]; and if so, whether such mitochondrial effects on metabolic  
146 rate are involved in sex-specific trade-offs between physiology and life history phenotypes. To  
147 address this question, we screened for effects of mitochondrial haplotypic variation on the  
148 metabolic rate (measured by indirect calorimetry as CO<sub>2</sub> production, [46]) of each sex, across  
149 a panel of thirteen genetic strains in *D. melanogaster*, which differ only in their mtDNA

150 haplotype and which have been previously used to study sex-specific patterns of mitochondrial  
151 variation mediating the expression of life history phenotypes [12, 16]. We tested whether  
152 signatures of mitochondrial genetic variation were consistent with predictions of the weak  
153 (male-biases in size of effect across haplotypes) or strong (negative intersexual correlation  
154 across haplotypes) forms of the Mother's Curse hypothesis. We then leveraged trait means for  
155 longevity from Camus *et al.*, (2012) and reproductive fitness from Camus and Dowling (2018)  
156 of each sex-by-haplotype combination, to test whether mitochondrial variation for metabolic  
157 rate is involved in sex-specific trade-offs between physiology and life history phenotypes.

158

## 159 **Methods**

### 160 **Mitochondrial panel**

161 To statistically partition mitochondrial haplotype effects from those of the nuclear  
162 genetic background, it is necessary to place a set of mtDNA haplotypes alongside a  
163 standardized (controlled) nuclear background. Furthermore, it is expected that the  
164 accumulation of male-harming mutations within the mitochondrial genome will place selection  
165 on the standing nuclear variation in the populations in which these mtDNA mutations  
166 accumulate, for counteradaptations that offset the effects of these mitochondrial mutations [14].  
167 Thus, uncovering the phenotypic effects associated with these mutations requires that mtDNA  
168 haplotypes are placed alongside an evolutionary novel nuclear background that lacks the  
169 requisite counteradaptations required to offset the negative effects of these mutations [7].

170 We utilised a panel of thirteen strains of *D. melanogaster*, each of which is  
171 characterised by a distinct and naturally-occurring mtDNA haplotype, placed alongside an  
172 isogenic nuclear background w<sup>1118</sup> (Bloomington stock number: 5905) [12, 47]. The strains are  
173 labelled according to the location from which the mtDNA haplotypes were initially collected  
174 (**ALS** - Alstonville, Australia; **BAR** - Barcelona, Spain; **BRO** - Brownsville, USA; **DAH** -

175 Dahomey, Benin, **MAD** - Madang, Papua New Guinea; **MYS** - Mysore, India; **HAW** -  
176 Hawai'i, USA, **ISR** - Israel; **JAP** - Japan; **ORE** - Oregon, USA; **PUE** - Puerto Montt, Chile;  
177 **SWE** - Sweden and **ZIM** - Zimbabwe) [12]. The strains were obtained from David Clancy in  
178 2007, at which point we created a duplicate copy of each, such that each haplotype has been  
179 maintained in independent replicate for over a decade. These replicates are denoted as  
180 “mitochondrial strain duplicates”. The strain duplicates are maintained by back-crossing five  
181 virgin females from each duplicate to five males of the  $w^{1118}$  strain. The  $w^{1118}$  strain is itself  
182 propagated each generation via a solitary full-sibling mating pair. Thus, any new mutations in  
183 the nuclear genome that appear in the  $w^{1118}$  strain should be quickly purged, or if fixed would  
184 be immediately donated to each of the mitochondrial strain duplicates, thereby ensuring the  
185 nuclear background of these strains is maintained as nearly-isogenic. Each of the mitochondrial  
186 strains and their respective duplicates had undergone at least 80 generations of backcrossing at  
187 the time of the respirometry experiments described below. Back-crosses were always  
188 conducted at low adult densities (5 pairs), and only eggs produced by parents that were four  
189 days old at the time of egg-laying were used to propagate the next generation. All strains were  
190 treated with tetracycline hydrochloride (0.3 mg/mL) to eliminate *Wolbachia* infections before  
191 their receipt from David Clancy in 2007. We confirmed the absence of *Wolbachia* by screening  
192 Illumina sequencing data from each of the strains for the presence of *Wolbachia*-specific reads  
193 [48] in Geneious v9.0.4 [49].

194

## 195 **Experimental design**

196 The experiment was designed to assay the *in vivo* metabolic rate ( $\dot{V}CO_2$ ) of individual  
197 adult males and females from each of the thirteen strains. The experiment was conducted over  
198 three temporally-separated sampling blocks, each of which was separated by a single  
199 generation of fly propagation (fourteen days).



200

### 201 ***Generating focal flies***

202 All focal flies (i.e. those used in the experiment) were produced by parents and  
203 grandparents that were four days of adult age at the time of egg-laying. In the two generations  
204 leading up to the assay, all flies were reared under carefully controlled densities (10 pairs of  
205 adult flies per vial, and egg numbers per vial reduced to 80), at constant laboratory conditions  
206 (25°C). We ensured we had a steady daily supply of standard-aged focal flies for the metabolic  
207 rate ( $\dot{V}\text{CO}_2$ ) measurements, by allowing the great-grandparents of the focal flies to lay eggs  
208 that produced the grandparental flies over several successive days (five days in sampling blocks  
209 one and two, and eight days in block three). Thus, although all focal flies had parents and  
210 grandparents of precisely standardised age, they had been produced by great-grandparents that  
211 differed in age by up to seven days.

212

### 213 **Metabolic rate assay**

214 The focal flies were collected under mild CO<sub>2</sub> anaesthesia within six hours of their  
215 eclosion into adulthood, thus ensuring their virginity, and then housed in single-sex groups of  
216 ten flies per vial. These flies remained in these vials for four days before measurement of their  
217 metabolic rate. For any given sampling day, we maintained one vial of ten focal flies per strain  
218 duplicate per sex within each block. The use of virgin flies removed any physiological effects  
219 on metabolic rate caused by mating *per se* and post-mating inter-sexual harassment.  
220 Additionally, the four-day recovery period following collection of the focal flies ensured that  
221 impact of CO<sub>2</sub> anaesthesia on the metabolic rate had dissipated by the time of the assay [50].

222 A standard Sable Systems International (SSI, [www.sablesys.com](http://www.sablesys.com), Las Vegas, USA)  
223 flow-through CO<sub>2</sub> respirometry system, connected to four LI-COR 7000 infrared CO<sub>2</sub>/H<sub>2</sub>O gas  
224 analysers (LICOR, Lincoln, USA), was used to measure carbon dioxide production as a proxy

225 of metabolic rate ( $V\text{CO}_2$ ) of adult flies. Two identical setups were created, each underpinned  
226 by two LI-COR 7000s (SSI, [www.sablesys.com](http://www.sablesys.com), Las Vegas, NV, USA). For each  
227 configuration, compressed air was directed through Bev-A-Line tubing to three scrubber  
228 columns (silica gel, soda lime, 1/3 Drierite 2/3 soda-lime respectively), where the air was  
229 scrubbed of atmospheric  $\text{CO}_2$  and water vapour ( $\text{H}_2\text{O}$ ) to facilitate a dry,  $\text{CO}_2$  free-flow. The  
230 airstream was then split using a PVC T-piece to direct the flow to one of two LI-CORs in the  
231 set-up, with a flow rate of 25 ml/min using a mass flow controller (Sierra 840 series). Each LI-  
232 COR was connected to a MUX2 intelligent multiplexer (Sable Systems), which housed eight  
233  $5 \times 65 \text{ mm}^2$  polycarbonate chambers (Trikinetics, Waltham, USA). We placed one focal fly  
234 within each chamber, the ends of which were sealed with 5 mm of foam, such that each fly was  
235 left with a  $5 \times 55 \text{ mm}^2$  maneuverable space. Seven of the chambers contained flies while the  
236 eighth chamber remained empty and served as a baseline to account for machine drift  
237 throughout the experiment.

238 The MUX2 was interfaced with a computer using a UI-2 universal interface (Sable  
239 Systems, NV, USA) and was programmed to sequentially measure each chamber using the  
240 software ExpeData (Sable Systems, NV, USA). Each chamber was measured once for 10  
241 minutes, with a two-minute pause period between every measurement to allow time for the  
242  $\text{CO}_2$  readings to stabilise. The assaying chambers were flushed with a humidified airflow (80%  
243 RH) in the pause-period of 2-min between  $V\text{CO}_2$  measurements, to reduce potential detrimental  
244 effects of desiccation. This was achieved using a LICOR-610 portable dew point generator.  
245 The assay was conducted within a light-controlled constant temperature cabinet (Panasonic  
246 MLR-352H-PE environmental growth cabinet, Panasonic Healthcare Co., Ltd, Sakata, Japan).  
247 The temperature of the cabinet was set to  $25^\circ\text{C}$  and was continuously recorded in the baseline  
248 chamber using a type-T thermocouple (Omega Engineering Inc., Stamford, USA) attached to  
249 a TC-2000 thermocouple meter (Sable Systems).

250 The respirometry assays were run over five consecutive days in blocks one and two,  
251 and over eight consecutive days in block three. We ran four “experimental trials” per day at  
252 approximately 09h00, 11h30, 14h00 and 16h30. We were able to assay 26 flies per  
253 experimental trial, with every possible combination of one mitochondrial strain duplicate × sex  
254 represented once per trial. In total, we measured the metabolic rate of 72 focal flies for each  
255 combination of mitochondrial strain × sex (36 per strain duplicate), over the three blocks.

256 The mean metabolic rate ( $VCO_2$ ) data from the 10-min assay for each fly was extracted  
257 using ExpeData (Sable Systems). All data were “nearest-neighbour smoothed” to remove noise  
258 from the  $VCO_2$  trace, and baseline corrected to account for machine drift over time [51]. We  
259 also extracted data on the intensity of activity of each fly from the  $VCO_2$  trace file, which was  
260 measured as the cumulative sum of absolute differences in deflection (ADS) of  $VCO_2$  signal  
261 [52-54]. In essence, the ADS was calculated by adding the absolute differences between  
262 adjacent data points in the  $VCO_2$  trace file [55, 56]. Although ADS is not an absolute  
263 quantification of locomotor activity [52], the measure has been used to correct for overall  
264 variability in metabolic rate due to the activity intensity of the assayed organism [52-54, 56-  
265 58]. Thus, from the  $VCO_2$  traces, high ADS values were indicative of flies being more active  
266 during the assay; and *vice versa*, small values of ADS indicative of the flies being less active.  
267 The ADS was extracted for each focal fly, and this served as a measure of activity intensity in  
268 the subsequent statistical analysis.

269 Finally, we measured the body mass of each focal fly immediately after the metabolic  
270 rate assay, to the nearest 0.0001 mg (Cubis series MSA2.7s-000-DM microbalance, Sartorius  
271 AG, Goettingen, Germany).

272

## 273 **Statistical analyses**

### 274 *Linear mixed effect modelling of the global data*

275           The data analyses were performed in the R statistical environment (v3.4 [59]), and  
276 graphs were plotted in *ggplot2* package [60] in R and GraphPad Prism software v8.1.0. We  
277 analysed the mean metabolic rate data using a linear mixed-effect model in the *lme4* package  
278 [61] in R. The mean metabolic rate (extracted from VCO<sub>2</sub> trace file) of each focal fly was  
279 modelled as the response variable, with mtDNA haplotype (13 levels), sex of the fly (2 levels),  
280 time of day of the assay (4 levels), and the higher-order interactions between these factors as  
281 fixed effects. Other variables that accounted for the hierarchical structure of the data were  
282 included as random effects. These included the mitochondrial strain duplicates (13 strains × 2  
283 replicates = 26 levels), experimental blocks (3 levels), assay-day (8 levels; note that this  
284 variable was also an indicator of the great grandparental age), assay-day nested within  
285 experimental block (18 levels) and experimental trial nested within assay-day and block (70  
286 levels). We included body mass and ADS of the individual fly as fixed covariates in the model.  
287 A full model was thus built with fixed effects that included factors and covariates; higher-order  
288 interactions involving the fixed factors and between fixed factors and covariates; along with  
289 random effects that included random factors, higher-order interactions between the random  
290 factors, and interactions between fixed and random factors.

291           We then derived a final reduced model by performing a step-wise model reduction  
292 process using Log-likelihood ratio tests to assess the change of deviance associated with  
293 progressively simplified models, eliminating higher-order interactions that accounted for  
294 negligible effects on the metabolic rate. We first simplified the list of random effects using the  
295 restricted maximum likelihood estimation method and then the fixed effects component of the  
296 model using the maximum likelihood method. Ultimately, once we converged on the ‘final  
297 model’, parameter values of fixed effects and their significance were estimated using the Type  
298 III Kenward Roger’s method in *lmerTest* package of R [62].

299 We calculated the estimated marginal means (emmeans) of mean metabolic rate  
300 (referred as emmeans metabolic rate in Figures) for the final set of higher-order fixed effects  
301 interactions associated with statistically significant effects on metabolic rate, using the package  
302 *emmeans* [63]. These marginal means provide mean metabolic rate of our key contrasts  
303 estimated from the final statistical model, adjusted for variation in body mass and activity  
304 (ADS). These estimated marginal means are highly concordant with means calculated  
305 following manual correction of mean metabolic rate for body mass (ESM, Manual correction  
306 of mean metabolic rate for body mass, Figures S1, S2).

307 Because we identified a statistically significant interaction between mtDNA haplotype  
308 and sex on mean metabolic rate, we further probed the nature of this interaction by running two  
309 separate models, one for each sex. We followed the same protocol of building a full model with  
310 the mean metabolic rate as response variable; all possible fixed, random effects and covariates  
311 as described above for the model of the complete dataset but excluding the term ‘sex’ in these  
312 models. We then derived a final model for each sex separately by performing the same step-  
313 wise model reduction process and parameter estimation procedure as described above.  
314 Furthermore, we used these two models to estimate the marginal R-squared values (95%  
315 confidence intervals (CI)) for each model using the ‘nsj’ method in the *r2glmm* package [64];  
316 and further estimated the effect size attributed by the mtDNA haplotype on mean metabolic  
317 rate in each sex separately, using a method to calculate Cohen’s *d* parameter that involved mean  
318 and standard deviation estimated from the sex-specific datasets (the formula can be found in  
319 option 7 in [https://www.psychometrica.de/effect\\_size.html](https://www.psychometrica.de/effect_size.html)).

320

### 321 ***Estimating inter-sexual correlations, across haplotypes, for metabolic rate***

322 We performed a correlation test between the emmeans metabolic rate of male flies and  
323 female flies (estimated from the final model of the full dataset – i.e. that containing both sexes

324 – for the fixed effect interaction term ‘sex × haplotype’) across thirteen haplotypes to  
325 determine the magnitude and direction of inter-sexual correlation for metabolic rate. From this  
326 test, we estimated the Pearson’s correlation coefficient and then 95% confidence intervals for  
327 the correlation coefficient through a non-parametric bootstrapping approach in the *boot*  
328 package [65] in R. The trait means were resampled with replacement across 10 000 replicates  
329 and the confidence intervals of the correlation coefficient were estimated from the bias  
330 corrected and accelerated (BC<sub>a</sub>) method in the *boot* package.

331

### 332 *Estimating inter-trait correlations across haplotypes*

333 The full panel of thirteen mtDNA haplotypes used in this study has also been used in  
334 earlier studies that have tested effects of mitochondrial haplotype on longevity [12] and  
335 components of reproductive success [16] across both sexes. We obtained haplotype-specific  
336 trait means for each sex from these earlier studies and combined these with the trait means for  
337 emmeans metabolic rate and body mass from our study. We then tested for correlations, across  
338 haplotypes, between pair-wise combinations of traits within and between the two sexes. We  
339 estimated the Pearson’s correlation coefficient and 95% confidence intervals for the correlation  
340 coefficient independently for each pairwise comparison of trait means, through a non-  
341 parametric bootstrapping approach in *boot* package [65] in R. In each correlation test, trait  
342 means were resampled with replacement across 10 000 replicates and the confidence intervals  
343 of the correlation coefficient were estimated from the bias corrected and accelerated (BC<sub>a</sub>)  
344 method in the *boot* package.

345

## 346 **Results**

### 347 *Mitochondrial genetic variation for metabolic rate is male-biased*

348 A significant interaction between mtDNA haplotype and sex on metabolic rate was  
349 found (Table 1). This indicates that the identity of the mtDNA haplotype affects metabolic rate,  
350 but that the pattern of effects across haplotypes differs across the sexes (Figure 1A-C).  
351 Furthermore, analyses of the sex-specific datasets revealed the mtDNA haplotype effect on  
352 metabolic rate was significant only in males (Table 2). A male-bias in the magnitude of the  
353 mtDNA haplotypic effect was further supported by the examination of standardized effect sizes  
354 (marginal R-squared values (95% CI)) estimated from the sex-specific models ( $R^2_{\text{males}}$  estimate  
355  $\pm$  95% C.I.s = 0.236 [0.292, 0.202],  $R^2_{\text{females}} = 0.167$  [0.223, 0.138]; and Cohen's  $d$  ( $d_{\text{males}} =$   
356  $0.647$ ,  $d_{\text{females}} = 0.461$ ).

357

### 358 ***Metabolic rate of males is sensitive to circadian effects***

359 Additionally, the interaction between sex and time of day of the assay affected the mean  
360 metabolic rate (Table 1). This circadian variation resulted from high levels of plasticity in  
361 metabolic rate across the day in males. In contrast, metabolic rate values in females were  
362 largely stable across the four-time periods at which we ran the experiments. These time-  
363 dependent sex differences resulted in a sign shift in the direction of sexual dimorphism for  
364 metabolic rate between morning (male-biased) and afternoon (female-biased) measurements  
365 (Figure 2).

366

### 367 ***Intersexual correlation for metabolic rate, across haplotypes, is negative***

368 We found a negative correlation between the sexes in metabolic rate across the thirteen  
369 mtDNA haplotypes, controlling for body mass and activity (i.e. based on the emmeans of the  
370 final model in Table 1, Pearson's correlation coefficient  $r_p = -0.64$ ; bootstrapped 95%  
371 confidence intervals = -0.84, -0.31). That is, haplotypes which conferred greater trait means in  
372 females conferred lower trait means in male flies (Figure 1D).

373

### 374 *Intrasexual correlations involving metabolic and life history traits*

375 We observed a negative correlation between emmeans metabolic rate and longevity  
376 across the thirteen haplotypes in males (Pearson's correlation coefficient  $r_p = -0.63$ ;  
377 bootstrapped 95% confidence intervals = -0.88, -0.021), but not in females ( $r_p = -0.38$ ; 95%  
378 CIs = -0.78, 0.26) (Figure 3). The correlation between body mass and longevity was positive  
379 in males ( $r_p = 0.63$ , 95% CIs = -0.03, 0.84) but negative in females ( $r_p = -0.70$ , 95% CIs = -  
380 0.98, 0.09; ESM, Figure S3), but the confidence intervals of each overlapped with zero. We  
381 did not observe any signatures of pleiotropy between the metabolic rate or body mass and  
382 components of fertility outcomes, in either of the sexes (ESM, Figure S4).

383

### 384 **Discussion**

385 Here we aimed to determine whether the nucleotide differences that delineate a panel  
386 of 13 mtDNA haplotypes in *D. melanogaster* affect the expression of metabolic rate in vinegar  
387 flies; whether any such effects are sex-biased, and whether mtDNA haplotypes confer  
388 pleiotropic effects across different physiology and life history traits, resulting in genetic  
389 correlations across the haplotypes.

390

### 391 *Sex-specific effects of mtDNA haplotype on metabolic rate*

392 Not all studies that have tested for mtDNA haplotype effects on phenotypic expression  
393 have uncovered evidence for a male-bias in the magnitude of effects [27-31]. However, studies  
394 that leveraged this same panel of thirteen mtDNA haplotypes, or subsets of haplotypes from  
395 the panel, have reported male-biases in the magnitude of haplotype effects on traits such as  
396 longevity and survival, patterns of nuclear gene expression, early-life mitochondrial  
397 abundance, mtDNA copy number, maximum reactive oxygen species production and activity



398 of key mitochondrial enzymes [12, 13, 24-26]. Our results extend these observations by  
399 showing that the haplotype effect on metabolic rate (measured as  $\dot{V}CO_2$ ) is similarly male-  
400 biased in effect. These results are consistent with the prediction of the weak form of the  
401 Mother's Curse hypothesis, which predicts the accumulation of a pool of male-harming but  
402 female neutral mutations within the mtDNA sequence as a consequence of the maternal  
403 transmission of mitochondria [7, 10, 11]. Furthermore, the results support the contention that  
404 previously reported sex-differences in the magnitude of mitochondrial haplotypic effects on  
405 the expression of life history traits might be mediated through the intermediary effects of the  
406 mtDNA haplotype on metabolic rate. More generally, the findings suggest that the genetic  
407 architecture of metabolic rate is complex. Recent studies have uncovered additive or epistatic  
408 contributions of the mtDNA haplotype to this trait [39, 43, 44], and our study adds to these by  
409 providing evidence that the mtDNA sequence variants that affect metabolic rate can  
410 consistently confer effects that are larger in males than in females.

411

#### 412 *Negative intersexual correlation for metabolic rate across haplotypes*

413 Furthermore, the effects of mtDNA haplotype on metabolic rate differed not only in  
414 their magnitude across males and females but also in their rank order. We observed a negative  
415 intersexual correlation across the 13 haplotypes for metabolic rate. This result is consistent  
416 with the strong form of the Mother's Curse hypothesis, which predicts that mtDNA mutations  
417 conferring sexually antagonistic effects – specifically those that are female-beneficial but male-  
418 harmful – would be shaped by positive selection as a consequence of maternal transmission of  
419 mitochondria. Similar signatures of sexual antagonism in the rank order of effects on a  
420 component of juvenile fitness have previously been reported across mtDNA haplotypes in *D.*  
421 *melanogaster* [66], while Camus and Dowling (2018) have similarly reported negative  
422 intersexual correlations across the same set of haplotypes as we have used here for various

423 components of reproduction. Intriguingly, the haplotypes conferring relatively higher and  
424 lower sex-specific reproductive success in the study of Camus and Dowling (2018) do not  
425 correspond with those conferring relatively higher and lower metabolic rates in our study  
426 (ESM, Figure S4). Furthermore, another difference between the two studies is that Camus and  
427 Dowling (2018) uncovered statistically significant mtDNA haplotype effects on most of the  
428 reproductive traits they studied, in males and females alike; whereas we did not find evidence  
429 for a statistically significant effect of mtDNA haplotype on metabolic rate in females. This  
430 raises the important question of whether the negative intersexual correlation we observed for  
431 metabolic rate is likely to reflect a true genetic correlation across haplotypes, or whether it is  
432 driven by some other confounding source of variances, such as a vial-sharing effect or a  
433 phenotypic correlation. Such phenotypic correlations for metabolic rate are possible here, since  
434 males and females used in the experiments of a given mitochondrial strain duplicate, within a  
435 given block, were collected from the same sets of vials, and thus spent their juvenile  
436 development (from egg to eclosion) within these vials prior to being stored separately by sex  
437 for four days once adults, prior to the  $VCO_2$  measurements. Indeed, the presence of an  
438 intersexual genetic correlation for any given trait depends on there being genetic variation  
439 underpinning the trait in each sex, but we have not found support for a haplotype effect in  
440 females. Notwithstanding, visual inspection of Figure 1B is suggestive of a possible signature  
441 of variation across haplotypes in females, if so, at an effect size that was too small to detect at  
442 the sample sizes used in this study. Furthermore, our standardised effect size calculations,  
443 presented in the results, suggest the haplotypic effect in females is likely to be smaller than  
444 males, but greater than zero. Yet, on this point, we note that the effect sizes for metabolic rate  
445 that we have reported here (about a 14% difference between the most different emmeans) could  
446 have pervasive effects for organismal life history, given demonstrations of strong relationships  
447 between metabolic rate, personalities and both survival and reproductive outcomes [67-70],

448 and the importance of even small variation in metabolic rates or other traits over extended  
449 periods (e.g. [71, 72]). Ultimately, further work is required to test whether the intersexual  
450 correlations for metabolic rate, observed here, are genetic or phenotypic in origin.

451

452 *Negative correlation between metabolic rate and lifespan, across haplotypes, is limited to*  
453 *males*

454 Metabolic rate has been routinely proposed as a major currency on which the expression  
455 of life history traits and trade-offs depends [38, 73, 74]. Given the products of the mitochondrial  
456 genome are all involved in encoding core components of OXPHOS respiration, metabolic rate  
457 has also been viewed as a nexus trait linking mitochondrial bioenergetics to life history function  
458 [26, 27, 75]. Yet, very few studies to date have empirically explored whether mitochondrial  
459 genotypic effects on components of life history are underpinned by effects on the metabolic  
460 rate [44, 70, 76, 77], and thus the mechanistic factors that link mitochondrial genotype to life  
461 history phenotype remain unclear. Furthermore, previous studies to test for effects of mtDNA  
462 haplotype variation on physiological traits have generally confined their tests to one sex only  
463 [43, 44], with only few exceptions [26, 27, 30, 45]. Thus, the capacity by which the mtDNA  
464 haplotype can confer sex differences in organismal physiology remains elusive.

465 Here, we identified a negative correlation across haplotypes for metabolic rate and  
466 longevity in males, which suggests a possible male-specific genetic trade-off between the  
467 optimal expression of these traits. Indeed, a negative correlation between these two traits is the  
468 key prediction of the “rate of living” hypothesis [78-82], which contends that organisms  
469 exhibiting high metabolic rates should have shorter lifespans. Despite its widespread appeal,  
470 little evidence exists for this prediction at either an interspecific or intraspecific scale [76, 80,  
471 83]. Our observation of a male-specific negative mitochondrial correlation between metabolic  
472 rate and longevity is striking because it suggests that genetic variation might accumulate within

473 the mitochondrial genome in a manner consistent with the rate of living hypothesis, albeit with  
474 a twist. Maternal inheritance of the mitochondrial genome will, in theory, render selection  
475 efficient at removing the pool of mutations that reduce female metabolic rate and longevity,  
476 but less efficient in removing the pool of mutations that exert male-specific effects on each of  
477 these two traits. The male-specificity of the mitochondrial correlation between metabolic rate  
478 and longevity would, therefore, suggest that any mtDNA mutations that underpin this  
479 correlation are likely to be non-adaptive, accumulating under a selection shadow, and therefore,  
480 not associated with fitness benefits to males. This contention is supported by the lack of a  
481 positive genetic correlation, across haplotypes, between metabolic rate and reproductive fitness  
482 in males (ESM, Figure S4).

483

#### 484 *Sex- and circadian contexts of metabolic rate expression in vinegar flies*

485 Our study also provided new insights into the magnitude and context-dependency of  
486 sexual dimorphism in the metabolic rate of *D. melanogaster*. The mean mass- and activity-  
487 adjusted metabolic rate (i.e., emmeans of the final model in Table 1) across the haplotypes was  
488 generally higher in females than in males (females: mean = 1.89, SD = 0.04; males: mean =  
489 1.84, SD = 0.07). However, the magnitude of the sex difference in metabolic rate changed  
490 across the day due to high levels of time-mediated plasticity in metabolic rate in males. The  
491 existing literature confirms that sexual dimorphism in the expression of metabolic rate in  
492 vinegar flies is context-dependent [84, 85]. In this regard, the genotype of flies, number of flies  
493 assayed in the respirometer (single fly vs group of flies), type of respirometry setup (open vs  
494 flow-through), mating status of the focal flies (virgins vs mated), age of the focal fly (young vs  
495 old), and the type of assaying area (confined vs unconfined) have all been shown to influence  
496 patterns of sexual dimorphism in metabolic rate of vinegar flies [84]. Notwithstanding, while  
497 circadian effects are known to affect mating behaviours of male and female *Drosophila* and

498 moths [86-88], as far as we are aware, this is the first study to report sex differences that depend  
499 on circadian effects; a result that potentially has design implications when it comes to planning  
500 and implementation of experiments aimed at testing for sex differences in physiology.

501

### 502 *Mitochondrial genetic effects should be tested across broader contexts*

503         The panel of haplotypes used here provides an excellent toolkit in which to examine  
504 the role of mitochondrial haplotypic variation in driving sex differences in trait expression and  
505 life history trade-offs. The panel consists of thirteen haplotypes that represent the entire global  
506 distribution of *D. melanogaster*, and that therefore capture much of the mitochondrial genetic  
507 variation present in the species [48]. Thus, inferences from this panel are likely to be robust to  
508 the effects of sampling error. Furthermore, because the nuclear background in which the  
509 haplotypes are expressed is completely isogenic, and each of the haplotypes is replicated across  
510 independent duplicates, the panel offers a powerful means to unambiguously partition true  
511 mitochondrial haplotypic effects from effects of cryptic and residual nuclear variation or other  
512 sources of environmental variance [7]. However, like any resource – the panel comes with its  
513 limitations – namely the approach of replicating our strains within a solitary nuclear  
514 background ( $w^{1118}$ ) carries a caveat. From a theoretic standpoint, mitochondrial genes must  
515 work in intimate coordination with nuclear genes to encode key processes such as OXPHOS,  
516 and thus it is likely that mitochondrial haplotypic effects on the phenotype will be at least in  
517 part shaped by epistatic mitochondrial-nuclear interactions [89, 90]. Yet, while a recent meta-  
518 analysis across animal and plant kingdoms suggested that effect sizes associated with cyto  
519 (mitochondrial and/or chloroplast)-nuclear epistasis are generally larger than those associated  
520 with additive cytoplasmic effects, the additive effect size is nonetheless moderate to large [6].  
521 This suggests that the sex-differences in mitochondrial effects we have uncovered here are  
522 likely to extend across more than just the one nuclear background used here. Nonetheless, our

523 study and previous studies conducted to date on this panel of flies should, at this stage, be seen  
524 as providing proof-of-concept for the weak and strong forms of Mother's Curse hypothesis. It  
525 is important that future studies screen patterns of sex-specific mitochondrial genetic effects on  
526 physiology and life history traits, across a range of nuclear genetic backgrounds to determine  
527 whether patterns of male-bias or sexual antagonism are upheld across a broad array of nuclear  
528 genotypes. Further studies would also benefit by testing whether the key predictions of the  
529 Mother's Curse hypothesis are upheld when mtDNA haplotypes are all sourced from the one-  
530 and-the-same natural population.

531

## 532 **Conclusions**

533 In summary, our study uncovers sex-specific effects of the mtDNA haplotype on  
534 metabolic rate, showing a male-bias consistent with the prediction of the weak form of  
535 Mother's Curse hypothesis. Furthermore, we have presented evidence for a negative  
536 intersexual correlation for metabolic rate across haplotypes. This correlation is consistent with  
537 the key prediction of the strong form of the Mother's Curse hypothesis, which predicts that  
538 maternal inheritance of mitochondria has enabled mutations to accrue that augment female  
539 fitness and are therefore shaped under positive selection but at the expense of male fitness.  
540 Future research should now explore whether the signatures of male-bias, and sexual  
541 antagonism, detected across haplotypes in our study, are upheld, across a broader range of  
542 nuclear genetic and environmental contexts, and whether they can be similarly detected in  
543 studies of other metazoan species.

544

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560

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563

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## 814 **Tables**

815 **Table 1:** Results from the *lmer* model of the full dataset, which included metabolic rate of both  
816 sexes. In this model, haplotype and sex were modelled as fixed effects, and centred body mass  
817 and ADS as fixed covariates. The final model was derived by sequentially eliminating non-  
818 significant higher-order interaction terms across both fixed and random effects using log-  
819 likelihood ratio tests, retaining only the final list of fixed and random effects, and any higher-  
820 order interactions that were statistically significant at  $p < 0.05$ . In this table, the random effects

821 – Trial[Day[Block]] denotes “experimental trial nested within day nested within block” and  
 822 Day[Block] denotes “experimental day nested within block”.

823

<i>Fixed effects</i>	<i>Sum sq</i>	<i>Mean sq</i>	<i>NumDF</i>	<i>DenDF</i>	<i>F value</i>	<i>P - value</i>
Haplotype	1.250	0.104	12	13.05	0.782	0.662
Sex	0.282	0.282	1	1701.41	2.115	0.146
Time of assay	4.365	1.455	3	50.33	10.913	<0.0001
Body mass	17.728	17.728	1	1531.64	132.982	<0.0001
ADS	11.605	11.605	1	1740.55	87.05	<0.0001
Haplotype × Sex	3.997	0.333	12	1696.57	2.499	0.0029
Sex × time of assay	3.755	1.252	3	1699.49	9.389	<0.0001
Sex × ADS	5.114	5.114	1	1769.22	38.358	<0.0001
<i>Random effects</i>	<i>Variance</i>	<i>p-value</i>				
Strain duplicate	0	1				
Day[Block]	0.0035	<0.0001				
Trial[Day[Block]]	3.59e-11	1				
Residual	0.133					

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825 **Table 2:** Results from sex-specific *lmer* models. Here, haplotype was modelled as a fixed  
 826 effect. The centred body mass and ADS were retained as covariates in the final model. The  
 827 final model was derived by sequentially eliminating non-significant higher-order interaction  
 828 terms across both fixed and random effects using log-likelihood ratio test.

829 ***Male-specific lmer model***

<i>Fixed effects</i>	<i>Sum sq</i>	<i>Mean sq</i>	<i>NumDF</i>	<i>DenDF</i>	<i>F value</i>	<i>P - value</i>
Haplotype	4.102	0.342	12	822.07	2.0354	0.019
Time of assay	7.236	2.412	3	51.1	14.360	<0.0001
Body mass	3.843	3.843	1	612.2	22.88	<0.0001
ADS	20.099	20.099	1	883.51	119.67	<0.0001
<i>Random effects</i>	<i>Variance</i>					
Day[Block]	0.0073					

Trial[Day[Block]]	4.93e-09
Residual	0.168

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830

831 ***Female-specific lmer model***

<i>Fixed effects</i>	<i>Sum sq</i>	<i>Mean sq</i>	<i>NumDF</i>	<i>DenDF</i>	<i>F value</i>	<i>P - value</i>
Haplotype	1.068	0.089	12	822.41	0.953	0.493
Time of assay	0.2536	0.0845	3	49.89	0.906	0.445
Body mass	12.72	12.72	1	865.17	136.253	<0.0001
ADS	0.309	0.309	1	871.15	3.307	0.069

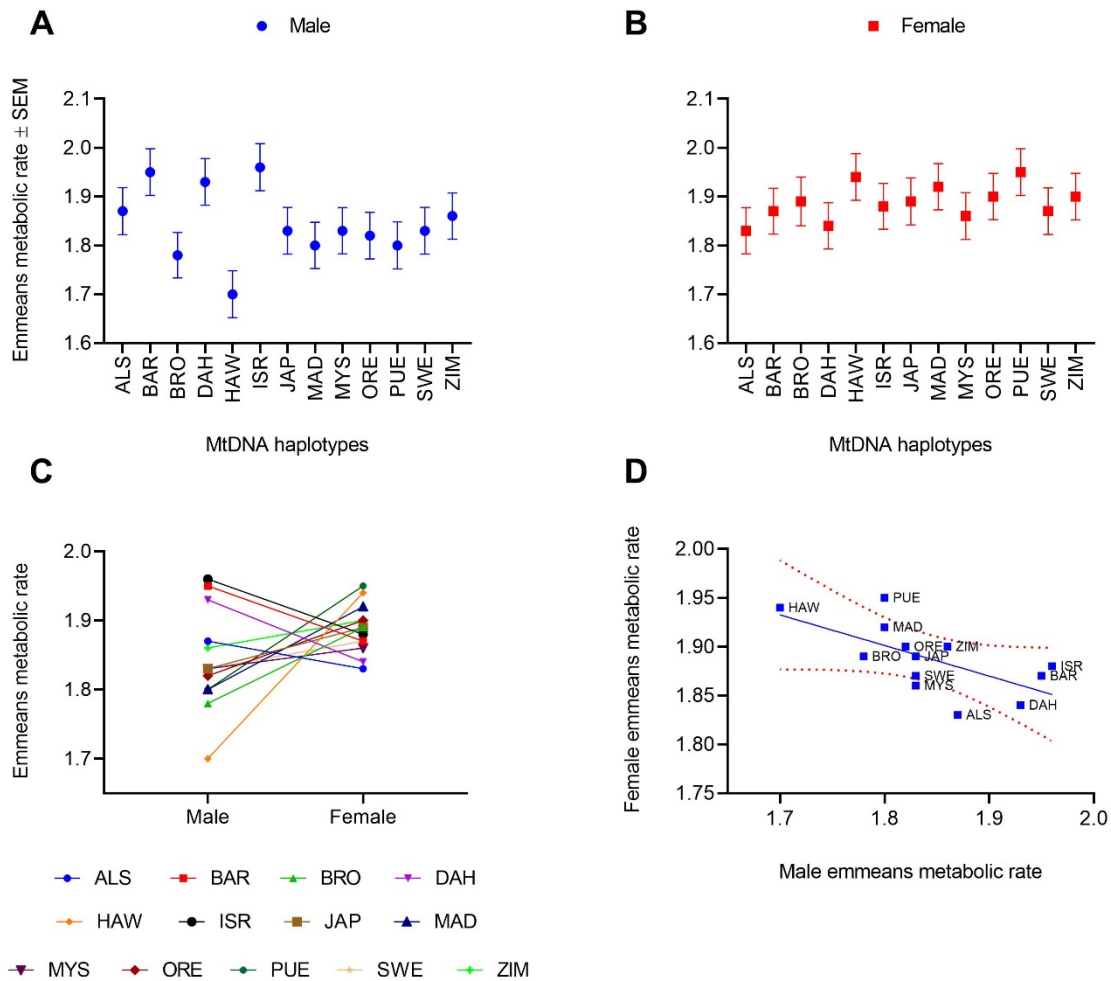
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<i>Random effects</i>	<i>Variance</i>
Day[Block]	0.0045
Trial[Day[Block]]	0.0011
Residual	0.093

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832

833 **Figures**



834

835 **Figure 1.** Effects of mtDNA haplotype on the metabolic rate of A. male; and B. female flies.

836 In panels A and B, the estimated marginal means (emmeans)  $\pm$  1 Standard Error of metabolic

837 rate for each mtDNA haplotype-by-sex combination were derived from the final model built

838 on global data, using the *emmeans* package in R. The emmeans accounted for variation

839 attributable to body mass and ADS in the final global model. C. Interaction plot showing

840 variation in emmeans metabolic rate between the sexes, across the thirteen mtDNA haplotypes.

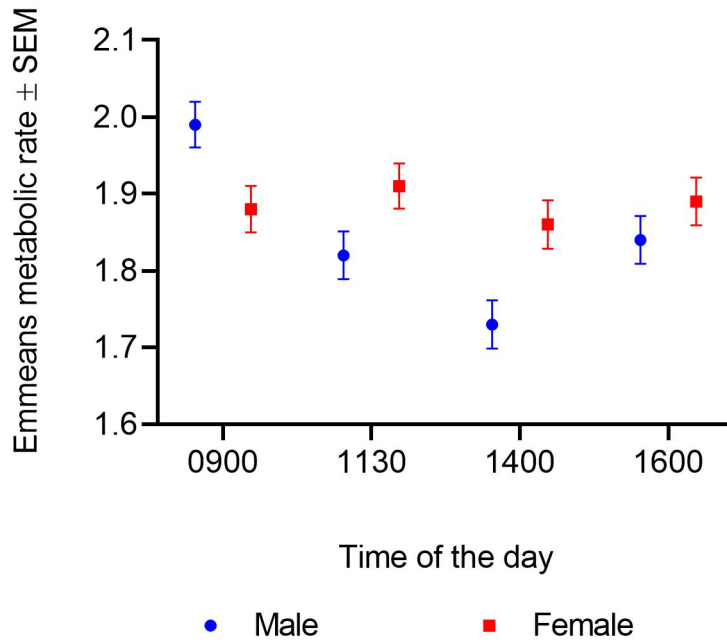
841 D. Negative inter-sexual mitochondrial correlation for emmeans metabolic rate (Pearson's

842 correlation coefficient  $r_p = -0.64$ , bootstrapped 95% CIs = -0.84, -0.31). The scales in both axes

843 are adjusted across the panels to elucidate the magnitude of variation and relationship between

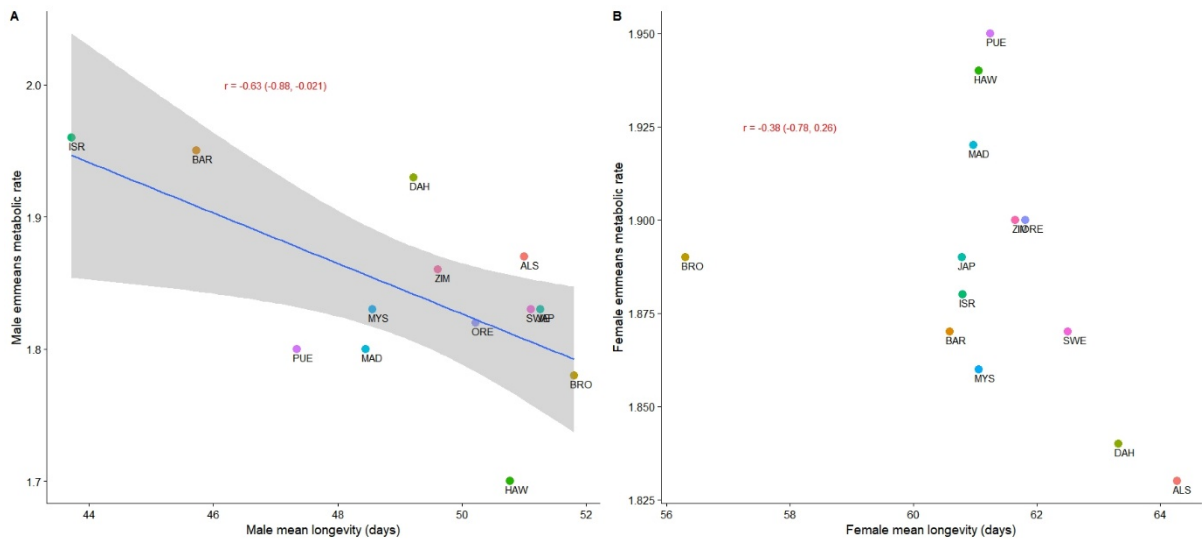
844 the trait means. For annotations of the mtDNA haplotypes, refer to the Methods section.

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**Figure 2.** Circadian effects on the metabolic rate of each sex. The emmeans metabolic rate (adjusted for body mass and ADS) for each sex-by-time of the day combination was estimated from the model in Table 1 using the *emmeans* package in R.



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**Figure 3.** Intra-sexual mitochondrial correlation between longevity and emmeans metabolic rate in A) males and B) females. The scales in both axes are adjusted to show the direction of the relationship between the traits in each sex. For annotations of the mtDNA haplotypes, refer



855 to the Methods section. The mean longevity scores for each sex-by-haplotype combination was  
856 sourced from Camus *et al.*, (2012).