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

Venkatesh Nagarajan-Radha[1*]; Ian Aitkenhead [1]; David J. Clancy[2]; Steven L. Chown[1] and Damian K. Dowling[1*]

[1]School of Biological Sciences, Monash University, VIC 3800, Australia

[2]Division of Biomedical and Life Sciences, Faculty of Health and Medicine, Lancaster University, Lancaster, UK

Short running title: Mitochondrial haplotype affects metabolic rate

[*]Addresses for Correspondence

Venkatesh Nagarajan-Radha, School of Biological Sciences, Monash University, Clayton campus, VIC 3800, Australia.

Email: venkatesh.nagarajan.radha@gmail.com

Damian K. Dowling, School of Biological Sciences, Monash University, Clayton campus, VIC 3800, Australia.

Email: damian.dowling@monash.edu

Abstract

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

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

Background

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

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

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

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

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

However, evidence for this prediction remains limited. Many previous studies examining the capacity for mitochondrial genotypic regulation of physiology have considered only one or other of the sexes, or pooled both sexes in their analyses [39-44], precluding inferences of sex-specificity. Some studies have, however, tested for effects in both sexes. For example, Aw *et al.,* (2017) screened for sex biases in effects on mitochondrial function (oxidative phosphorylation (OXPHOS) functioning measured from complex I activity, mtDNA copy number, maximum reactive oxygen species production, and superoxide dismutase activity) across two mtDNA haplotypes in *D. melanogaster*, reporting a male-bias in effects on three of the four traits measured. Yet, other studies have not revealed consistent signatures of male-bias in the magnitude of mitochondrial genetic effects on physiological traits [26, 27, 30, 45]. For example, in one recent study, Wolff *et al*. [26] observed male-biases in levels of mitochondrial genetic variation for mitochondrial quantity, but not for respiratory rate of the individual OXPHOS complexes, across a panel of thirteen mitochondrial haplotypes of *D. melanogaster*. In another study, Novičić *et al.* (2015) reported that as much as 20% of the variation in whole-organism metabolic rate (measured as CO_2 production), across adult *D. subobscura* could be mapped to genetic variation across three mtDNA haplotypes. Yet, although these mitochondrial genetic effects exhibited some degree of sex-specificity, the general pattern was not one of clear male-bias [45].

Thus, it currently remains unclear whether mitochondrial haplotypic variation affects the expression of metabolic rate in a pattern similar to previously-reported effects on longevity and reproductive success [12, 16]; and if so, whether such mitochondrial effects on metabolic rate are involved in sex-specific trade-offs between physiology and life history phenotypes. To address this question, we screened for effects of mitochondrial haplotypic variation on the metabolic rate (measured by indirect calorimetry as CO_2 production, [46]) of each sex, across a panel of thirteen genetic strains in *D. melanogaster*, which differ only in their mtDNA

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

Methods

Mitochondrial panel

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

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

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

Experimental design

The experiment was designed to assay the *in vivo* metabolic rate ($\dot{V}CO_2$) of individual adult males and females from each of the thirteen strains. The experiment was conducted over three temporally-separated sampling blocks, each of which was separated by a single 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 ($\dot{V}CO_2$) 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_2 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_2 anaesthesia on the metabolic rate had dissipated by the time of the assay [50].

222 A standard Sable Systems International (SSI, www.sablesys.com, Las Vegas, USA)
223 flow-through CO_2 respirometry system, connected to four LI-COR 7000 infrared CO_2/H_2O gas
224 analysers (LICOR, Lincoln, USA), was used to measure carbon dioxide production as a proxy

of metabolic rate ($\dot{V}CO_2$) of adult flies. Two identical setups were created, each underpinned by two LI-COR 7000s (SSI, www.sablesys.com, Las Vegas, NV, USA). For each configuration, compressed air was directed through Bev-A-Line tubing to three scrubber columns (silica gel, soda lime, 1/3 Drierite 2/3 soda-lime respectively), where the air was scrubbed of atmospheric CO_2 and water vapour (H_2O) to facilitate a dry, CO_2 free-flow. The airstream was then split using a PVC T-piece to direct the flow to one of two LI-CORs in the set-up, with a flow rate of 25 ml/min using a mass flow controller (Sierra 840 series). Each LI-COR was connected to a MUX2 intelligent multiplexer (Sable Systems), which housed eight 5×65 mm^2 polycarbonate chambers (Trikinetics, Waltham, USA). We placed one focal fly within each chamber, the ends of which were sealed with 5 mm of foam, such that each fly was left with a 5×55 mm^2 maneuverable space. Seven of the chambers contained flies while the eighth chamber remained empty and served as a baseline to account for machine drift throughout the experiment.

The MUX2 was interfaced with a computer using a UI-2 universal interface (Sable Systems, NV, USA) and was programmed to sequentially measure each chamber using the software ExpeData (Sable Systems, NV, USA). Each chamber was measured once for 10 minutes, with a two-minute pause period between every measurement to allow time for the CO_2 readings to stabilise. The assaying chambers were flushed with a humidified airflow (80% RH) in the pause-period of 2-min between $\dot{V}CO_2$ measurements, to reduce potential detrimental effects of desiccation. This was achieved using a LICOR-610 portable dew point generator. The assay was conducted within a light-controlled constant temperature cabinet (Panasonic MLR-352H-PE environmental growth cabinet, Panasonic Healthcare Co., Ltd, Sakata, Japan). The temperature of the cabinet was set to 25°C and was continuously recorded in the baseline chamber using a type-T thermocouple (Omega Engineering Inc., Stamford, USA) attached to a TC-2000 thermocouple meter (Sable Systems).

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

The mean metabolic rate ($\dot{V}CO_2$) data from the 10-min assay for each fly was extracted using ExpeData (Sable Systems). All data were "nearest-neighbour smoothed" to remove noise from the $\dot{V}CO_2$ trace, and baseline corrected to account for machine drift over time [51]. We also extracted data on the intensity of activity of each fly from the $\dot{V}CO_2$ trace file, which was measured as the cumulative sum of absolute differences in deflection (ADS) of $\dot{V}CO_2$ signal [52-54]. In essence, the ADS was calculated by adding the absolute differences between adjacent data points in the $\dot{V}CO_2$ trace file [55, 56]. Although ADS is not an absolute quantification of locomotor activity [52], the measure has been used to correct for overall variability in metabolic rate due to the activity intensity of the assayed organism [52-54, 56-58]. Thus, from the $\dot{V}CO_2$ traces, high ADS values were indicative of flies being more active during the assay; and *vice versa*, small values of ADS indicative of the flies being less active. The ADS was extracted for each focal fly, and this served as a measure of activity intensity in the subsequent statistical analysis.

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

Statistical analyses

Linear mixed effect modelling of the global data

The data analyses were performed in the R statistical environment (v3.4 [59]), and graphs were plotted in *ggplot2* package [60] in R and GraphPad Prism software v8.1.0. We analysed the mean metabolic rate data using a linear mixed-effect model in the *lme4* package [61] in R. The mean metabolic rate (extracted from VCO_2 trace file) of each focal fly was modelled as the response variable, with mtDNA haplotype (13 levels), sex of the fly (2 levels), time of day of the assay (4 levels), and the higher-order interactions between these factors as fixed effects. Other variables that accounted for the hierarchical structure of the data were included as random effects. These included the mitochondrial strain duplicates (13 strains × 2 replicates = 26 levels), experimental blocks (3 levels), assay-day (8 levels; note that this variable was also an indicator of the great grandparental age), assay-day nested within experimental block (18 levels) and experimental trial nested within assay-day and block (70 levels). We included body mass and ADS of the individual fly as fixed covariates in the model. A full model was thus built with fixed effects that included factors and covariates; higher-order interactions involving the fixed factors and between fixed factors and covariates; along with random effects that included random factors, higher-order interactions between the random factors, and interactions between fixed and random factors.

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

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

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

Estimating inter-sexual correlations, across haplotypes, for metabolic rate

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

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

Estimating inter-trait correlations across haplotypes

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

Results

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_{males} estimate
355 ± 95% C.I.s = 0.236 [0.292, 0.202], $R^2_{females}$ = 0.167 [0.223, 0.138]; and Cohen's *d* (d_{males} =
356 0.647, $d_{females}$ = 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

of key mitochondrial enzymes [12, 13, 24-26]. Our results extend these observations by showing that the haplotype effect on metabolic rate (measured as $\dot{V}CO2$) is similarly male-biased in effect. These results are consistent with the prediction of the weak form of the Mother's Curse hypothesis, which predicts the accumulation of a pool of male-harming but female neutral mutations within the mtDNA sequence as a consequence of the maternal transmission of mitochondria [7, 10, 11]. Furthermore, the results support the contention that previously reported sex-differences in the magnitude of mitochondrial haplotypic effects on the expression of life history traits might be mediated through the intermediary effects of the mtDNA haplotype on metabolic rate. More generally, the findings suggest that the genetic architecture of metabolic rate is complex. Recent studies have uncovered additive or epistatic contributions of the mtDNA haplotype to this trait [39, 43, 44], and our study adds to these by providing evidence that the mtDNA sequence variants that affect metabolic rate can consistently confer effects that are larger in males than in females.

Negative intersexual correlation for metabolic rate across haplotypes

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

components of reproduction. Intriguingly, the haplotypes conferring relatively higher and lower sex-specific reproductive success in the study of Camus and Dowling (2018) do not correspond with those conferring relatively higher and lower metabolic rates in our study (ESM, Figure S4). Furthermore, another difference between the two studies is that Camus and Dowling (2018) uncovered statistically significant mtDNA haplotype effects on most of the reproductive traits they studied, in males and females alike; whereas we did not find evidence for a statistically significant effect of mtDNA haplotype on metabolic rate in females. This raises the important question of whether the negative intersexual correlation we observed for metabolic rate is likely to reflect a true genetic correlation across haplotypes, or whether it is driven by some other confounding source of variances, such as a vial-sharing effect or a phenotypic correlation. Such phenotypic correlations for metabolic rate are possible here, since males and females used in the experiments of a given mitochondrial strain duplicate, within a given block, were collected from the same sets of vials, and thus spent their juvenile development (from egg to eclosion) within these vials prior to being stored separately by sex for four days once adults, prior to the $\dot{V}CO_2$ measurements. Indeed, the presence of an intersexual genetic correlation for any given trait depends on there being genetic variation underpinning the trait in each sex, but we have not found support for a haplotype effect in females. Notwithstanding, visual inspection of Figure 1B is suggestive of a possible signature of variation across haplotypes in females, if so, at an effect size that was too small to detect at the sample sizes used in this study. Furthermore, our standardised effect size calculations, presented in the results, suggest the haplotypic effect in females is likely to be smaller than males, but greater than zero. Yet, on this point, we note that the effect sizes for metabolic rate that we have reported here (about a 14% difference between the most different emmeans) could have pervasive effects for organismal life history, given demonstrations of strong relationships 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

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

Mitochondrial genetic effects should be tested across broader contexts

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

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

Conclusions

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

Acknowledgements

We thank Violeta Lopez and Winston Yee for their help during the experiment; Tom Allison and Craig White for their help with analyses; and three anonymous reviewers for their

constructive comments on an earlier version of this manuscript. DKD conceived the experiment; VNR, DKD, SLC, IA and DJC designed the experiment; VNR and IA conducted the metabolic rate measurements; VNR conducted data analyses; DKD advised on data analyses; VNR and DKD wrote the first draft of the manuscript; SLC, IA and DJC contributed to subsequent drafts and the final version of the manuscript.

Funding

This research was funded by the Australian Research Council (DP170100165 & FT160100022 to DKD, LE150100083 to DKD and SLC). VNR was supported by a Monash Graduate Scholarship.

Conflict of interest: None.

Data accessibility: Raw data and electronic supplementary material are available through the Monash Figshare repository DOI: https://doi.org/10.26180/5dae68bb85221.

References

[1] Ballard, J. W. O. & Kreitman, M. 1995 Is Mitochondrial-DNA a Strictly Neutral Marker. *Trends in ecology & evolution* **10**, 485-488.
[2] Ballard, J. W. O. & Whitlock, M. C. 2004 The incomplete natural history of mitochondria. *Mol Ecol* **13**, 729-744. (DOI:10.1046/j.1365-294X.2003.02063.x).
[3] Dowling, D. K., Friberg, U. & Lindell, J. 2008 Evolutionary implications of non-neutral mitochondrial genetic variation. *Trends Ecol Evol* **23**, 546-554. (DOI:10.1016/j.tree.2008.05.011).
[4] Rand, D. M. 2001 The units of selection on mitochondrial DNA. *Annual Review of Ecology and Systematics* **32**, 415-448. (DOI:DOI 10.1146/annurev.ecolsys.32.081501.114109).
[5] Ballard, J. W. O. & Pichaud, N. 2014 Mitochondrial DNA: more than an evolutionary bystander. *Funct Ecol* **28**, 218-231. (DOI:10.1111/1365-2435.12177).
[6] Dobler, R., Rogell, B., Budar, F. & Dowling, D. K. 2014 A meta-analysis of the strength and nature of cytoplasmic genetic effects. *Journal of Evolutionary Biology* **27**, 2021-2034. (DOI:10.1111/jeb.12468).
[7] Dowling, D. K. & Adrian, R. E. 2019 Challenges and Prospects for Testing the Mother's Curse Hypothesis. *Integr Comp Biol*. (DOI:10.1093/icb/icz110).
[8] Vaught, R. C. & Dowling, D. K. 2018 Maternal inheritance of mitochondria: implications for male fertility? *Reproduction* **155**, R159-R168. (DOI:10.1530/Rep-17-0600).

582 [9] Cosmides, L. M. & Tooby, J. 1981 Cytoplasmic inheritance and intragenomic conflict. *J Theor Biol*
583 **89**, 83-129.
584 [10] Frank, S. A. & Hurst, L. D. 1996 Mitochondria and male disease. *Nature* **383**, 224.
585 (DOI:10.1038/383224a0).
586 [11] Havird, J. C., Forsythe, E. S., Williams, A. M., Werren, J. H., Dowling, D. K. & Sloan, D. B. 2019
587 Selfish Mitonuclear Conflict. *Curr Biol* **29**, R496-R511. (DOI:10.1016/j.cub.2019.03.020).
588 [12] Camus, M. F., Clancy, D. J. & Dowling, D. K. 2012 Mitochondria, maternal inheritance, and male
589 aging. *Curr Biol* **22**, 1717-1721. (DOI:10.1016/j.cub.2012.07.018).
590 [13] Innocenti, P., Morrow, E. H. & Dowling, D. K. 2011 Experimental evidence supports a sex-specific
591 selective sieve in mitochondrial genome evolution. *Science* **332**, 845-848.
592 (DOI:10.1126/science.1201157).
593 [14] Beekman, M., Dowling, D. K. & Aanen, D. K. 2014 The costs of being male: are there sex-specific
594 effects of uniparental mitochondrial inheritance? *Philosophical Transactions of the Royal Society B-*
595 *Biological Sciences* **369**. (DOI:Unsp 20130440

596 Doi 10.1098/Rstb.2013.0440).
597 [15] Unckless, R. L. & Herren, J. K. 2009 Population genetics of sexually antagonistic mitochondrial
598 mutants under inbreeding. *Journal of Theoretical Biology* **260**, 132-136. (DOI:DOI
599 10.1016/j.jtbi.2009.06.004).
600 [16] Camus, M. F. & Dowling, D. K. 2018 Mitochondrial genetic effects on reproductive success:
601 signatures of positive intrasexual, but negative intersexual pleiotropy. *Proceedings of the Royal*
602 *Society B: Biological Sciences* **285**.
603 [17] Clancy, D. J., Hime, G. R. & Shirras, A. D. 2011 Cytoplasmic male sterility in Drosophila
604 melanogaster associated with a mitochondrial CYTB variant. *Heredity (Edinb)* **107**, 374-376.
605 (DOI:10.1038/hdy.2011.12).
606 [18] Froman, D. P. & Kirby, J. D. 2005 Sperm mobility: Phenotype in roosters (Gallus domesticus)
607 determined by mitochondrial function. *Biology of Reproduction* **72**, 562-567.
608 [19] Milot, E., Moreau, C., Gagnon, A., Cohen, A. A., Brais, B. & Labuda, D. 2017 Mother's curse
609 neutralizes natural selection against a human genetic disease over three centuries. *Nature Ecology &*
610 *Evolution* **1**, 1400-1406. (DOI:10.1038/s41559-017-0276-6).
611 [20] Nakada, K., Sato, A., Yoshida, K., Morita, T., Tanaka, H., Inoue, S. I., Yonekawa, H. & Hayashi, J. I.
612 2006 Mitochondria-related male infertility. *P Natl Acad Sci USA* **103**, 15148-15153.
613 (DOI:10.1073/pnas.0604641103).
614 [21] Patel, M. R., Miriyala, G. K., Littleton, A. J., Yang, H. K., Trinh, K., Young, J. M., Kennedy, S. R.,
615 Yamashita, Y. M., Pallanck, L. J. & Malik, H. S. 2016 A mitochondrial DNA hypomorph of cytochrome
616 oxidase specifically impairs male fertility in Drosophila melanogaster. *Elife* **5**. (DOI:ARTN e16923

617 10.7554/eLife.16923).
618 [22] Xu, H., DeLuca, S. Z. & O'Farrell, P. H. 2008 Manipulating the metazoan mitochondrial genome
619 with targeted restriction enzymes. *Science* **321**, 575-577. (DOI:10.1126/science.1160226).
620 [23] Smith, S., Turbill, C. & Suchentrunk, F. 2010 Introducing mother's curse: low male fertility
621 associated with an imported mtDNA haplotype in a captive colony of brown hares. *Mol Ecol* **19**, 36-
622 43. (DOI:10.1111/j.1365-294X.2009.04444.x).
623 [24] Drummond, E., Short, E. & Clancy, D. 2019 Mitonuclear gene X environment effects on lifespan
624 and health: How common, how big? *Mitochondrion* **49**, 12-18. (DOI:10.1016/j.mito.2019.06.009).
625 [25] Aw, W. C., Garvin, M. R., Melvin, R. G. & Ballard, J. W. O. 2017 Sex-specific influences of mtDNA
626 mitotype and diet on mitochondrial functions and physiological traits in Drosophila melanogaster.
627 *Plos One* **12**. (DOI:ARTN e0187554

628 10.1371/journal.pone.0187554).
629 [26] Wolff, J. N., Pichaud, N., Camus, M. F., Cote, G., Blier, P. U. & Dowling, D. K. 2016 Evolutionary
630 implications of mitochondrial genetic variation: mitochondrial genetic effects on OXPHOS respiration

631 and mitochondrial quantity change with age and sex in fruit flies. *J Evol Biol* **29**, 736-747.
632 (DOI:10.1111/jeb.12822).
633 [27] Dordevic, M., Stojkovic, B., Savkovic, U., Immonen, E., Tucic, N., Lazarevic, J. & Arnqvist, G. 2017
634 Sex-specific mitonuclear epistasis and the evolution of mitochondrial bioenergetics, ageing, and life
635 history in seed beetles. *Evolution* **71**, 274-288. (DOI:10.1111/evo.13109).
636 [28] Mossman, J. A., Tross, J. G., Jourjine, N. A., Li, N., Wu, Z. J. & Rand, D. M. 2017 Mitonuclear
637 Interactions Mediate Transcriptional Responses to Hypoxia in Drosophila. *Molecular Biology and*
638 *Evolution* **34**, 447-466. (DOI:10.1093/molbev/msw246).
639 [29] Mossman, J. A., Tross, J. G., Li, N., Wu, Z. J. & Rand, D. M. 2016 Mitochondrial-Nuclear
640 Interactions Mediate Sex-Specific Transcriptional Profiles in Drosophila. *Genetics* **204**, 613-630.
641 (DOI:10.1534/genetics.116.192328).
642 [30] Hoekstra, L. A., Julick, C. R., Mika, K. M. & Montooth, K. L. 2018 Energy demand and the context-
643 dependent effects of genetic interactions underlying metabolism. *Evol Lett* **2**, 102-113.
644 (DOI:10.1002/evl3.47).
645 [31] Mossman, J. A., Biancani, L. M., Zhu, C. T. & Rand, D. M. 2016 Mitonuclear Epistasis for
646 Development Time and Its Modification by Diet in Drosophila. *Genetics* **203**, 463-+.
647 (DOI:10.1534/genetics.116.187286).
648 [32] Nagarajan-Radha, V., Rapkin, J., Hunt, J. & Dowling, D. K. 2019 Interactions between
649 mitochondrial haplotype and dietary macronutrient ratios confer sex-specific effects on longevity in
650 Drosophila melanogaster. *The Journals of Gerontology: Series A*. (DOI:10.1093/gerona/glz104).
651 [33] Dowling, D. K. 2014 Evolutionary perspectives on the links between mitochondrial genotype and
652 disease phenotype. *Bba-Gen Subjects* **1840**, 1393-1403. (DOI:10.1016/j.bbagen.2013.11.013).
653 [34] Camus, M. F., Wolf, J. B. W., Morrow, E. H. & Dowling, D. K. 2015 Single Nucleotides in the
654 mtDNA Sequence Modify Mitochondrial Molecular Function and Are Associated with Sex-Specific
655 Effects on Fertility and Aging. *Current Biology* **25**, 2717-2722. (DOI:10.1016/j.cub.2015.09.012).
656 [35] Camus, M. F., Wolff, J. N., Sgro, C. M. & Dowling, D. K. 2017 Experimental Support That Natural
657 Selection Has Shaped the Latitudinal Distribution of Mitochondrial Haplotypes in Australian
658 Drosophila melanogaster. *Molecular Biology and Evolution* **34**, 2600-2612.
659 (DOI:10.1093/molbev/msx184).
660 [36] Mossman, J. A., Biancani, L. M. & Rand, D. M. 2019 Mitochondrial genomic variation drives
661 differential nuclear gene expression in discrete regions of Drosophila gene and protein interaction
662 networks. *Bmc Genomics* **20**, 691. (DOI:10.1186/s12864-019-6061-y).
663 [37] Hulbert, A. J., Pamplona, R., Buffenstein, R. & Buttemer, W. A. 2007 Life and death: Metabolic
664 rate, membrane composition, and life span of animals. *Physiological Reviews* **87**, 1175-1213.
665 (DOI:10.1152/physrev.00047.2006).
666 [38] Zera, A. J. & Harshman, L. G. 2001 The physiology of life history trade-offs in animals. *Annual*
667 *Review of Ecology and Systematics* **32**, 95-126. (DOI:DOI
668 10.1146/annurev.ecolsys.32.081501.114006).
669 [39] Arnqvist, G., Dowling, D. K., Eady, P., Gay, L., Tregenza, T., Tuda, M. & Hosken, D. J. 2010 Genetic
670 architecture of metabolic rate: environment specific epistasis between mitochondrial and nuclear
671 genes in an insect. *Evolution* **64**, 3354-3363. (DOI:10.1111/j.1558-5646.2010.01135.x).
672 [40] Correa, C. C., Aw, W., Melvin, R. G., Pichaud, N. & Ballard, J. W. O. 2012 Mitochondrial DNA
673 variants influence mitochondrial bioenergetics in Drosophila melanogaster. *Mitochondrion* **12**, 459-
674 464. (DOI:10.1016/j.mito.2012.06.005).
675 [41] Pichaud, N., Ballard, J. W. O., Tanguay, R. M. & Blier, P. U. 2012 Naturally Occurring
676 Mitochondrial DNA Haplotypes Exhibit Metabolic Differences: Insight into Functional Properties of
677 Mitochondria. *Evolution* **66**, 3189-3197. (DOI:10.1111/j.1558-5646.2012.01683.x).
678 [42] Matoo, O. B., Julick, C. R. & Montooth, K. L. 2019 Genetic Variation for Ontogenetic Shifts in
679 Metabolism Underlies Physiological Homeostasis in Drosophila. *Genetics* **212**, 537-552.
680 (DOI:10.1534/genetics.119.302052).

[43] Hoekstra, L. A., Siddiq, M. A. & Montooth, K. L. 2013 Pleiotropic effects of a mitochondrial-nuclear incompatibility depend upon the accelerating effect of temperature in Drosophila. *Genetics* **195**, 1129-1139. (DOI:10.1534/genetics.113.154914).
[44] Immonen, E., Ronn, J., Watson, C., Berger, D. & Arnqvist, G. 2016 Complex mitonuclear interactions and metabolic costs of mating in male seed beetles. *Journal of Evolutionary Biology* **29**, 360-370. (DOI:10.1111/jeb.12789).
[45] Novicic, Z. K., Immonen, E., Jelic, M., Andelkovic, M., Stamenkovic-Radak, M. & Arnqvist, G. 2015 Within-population genetic effects of mtDNA on metabolic rate in Drosophila subobscura. *Journal of Evolutionary Biology* **28**, 338-346. (DOI:10.1111/jeb.12565).
[46] Lighton, J. R. B. 2018 Measuring Metabolic Rates: A Manual for Scientists. *Oxford University Press* **Second Edition**.
[47] Clancy, D. J. 2008 Variation in mitochondrial genotype has substantial lifespan effects which may be modulated by nuclear background. *Aging Cell* **7**, 795-804. (DOI:DOI 10.1111/j.1474-9726.2008.00428.x).
[48] Wolff, J. N., Camus, M. F., Clancy, D. J. & Dowling, D. K. 2016 Complete mitochondrial genome sequences of thirteen globally sourced strains of fruit fly (Drosophila melanogaster) form a powerful model for mitochondrial research. *Mitochondrial DNA Part A* **27**, 4672-4674. (DOI:10.3109/19401736.2015.1106496).
[49] Kearse, M., Moir, R., Wilson, A., Stones-Havas, S., Cheung, M., Sturrock, S., Buxton, S., Cooper, A., Markowitz, S., Duran, C., et al. 2012 Geneious Basic: An integrated and extendable desktop software platform for the organization and analysis of sequence data. *Bioinformatics* **28**, 1647-1649. (DOI:10.1093/bioinformatics/bts199).
[50] Colinet, H. & Renault, D. 2012 Metabolic effects of CO2 anaesthesia in Drosophila melanogaster. *Biol Letters* **8**, 1050-1054. (DOI:10.1098/rsbl.2012.0601).
[51] Rezende, E. L., Chappell, M. A., Gomes, F. R., Malisch, J. L. & Garland, T. 2005 Maximal metabolic rates during voluntary exercise, forced exercise, and cold exposure in house mice selectively bred for high wheel-running. *J Exp Biol* **208**, 2447-2458. (DOI:10.1242/jeb.01631).
[52] Jensen, P., Overgaard, J., Loeschcke, V., Schou, M. F., Malte, H. & Kristensen, T. N. 2014 Inbreeding effects on standard metabolic rate investigated at cold, benign and hot temperatures in Drosophila melanogaster. *Journal of Insect Physiology* **62**, 11-20. (DOI:10.1016/j.jinsphys.2014.01.003).
[53] Lighton, J. R. B. & Turner, R. J. 2004 Thermolimit respirometry: an objective assessment of critical thermal maxima in two sympatric desert harvester ants, Pogonomyrmex rugosus and P-californicus. *J Exp Biol* **207**, 1903-1913. (DOI:10.1242/jeb.00970).
[54] Stevens, M. M., Jackson, S., Bester, S. A., Terblanche, J. S. & Chown, S. L. 2010 Oxygen limitation and thermal tolerance in two terrestrial arthropod species. *J Exp Biol* **213**, 2209-2218. (DOI:10.1242/jeb.040170).
[55] DeVries, Z. C., Kells, S. A. & Appel, A. G. 2016 Estimating the critical thermal maximum (CTmax) of bed bugs, Cimex lectularius: Comparing thermolimit respirometry with traditional visual methods. *Comparative Biochemistry and Physiology a-Molecular & Integrative Physiology* **197**, 52-57. (DOI:10.1016/j.cbpa.2016.03.003).
[56] Klok, C. J., Kaiser, A., Lighton, J. R. B. & Harrison, J. F. 2010 Critical oxygen partial pressures and maximal tracheal conductances for Drosophila melanogaster reared for multiple generations in hypoxia or hyperoxia. *Journal of Insect Physiology* **56**, 461-469. (DOI:10.1016/j.jinsphys.2009.08.004).
[57] Lighton, J. R. B., Weier, J. A. & Feener, D. H. 1993 The Energetics of Locomotion and Load Carriage in the Desert Harvester Ant Pogonomyrmex-Rugosus. *J Exp Biol* **181**, 49-61.
[58] Vorhees, A. S. & Bradley, T. J. 2012 Differences in critical thermal maxima and mortality across life stages of the mealworm beetle Tenebrio molitor. *J Exp Biol* **215**, 2319-2326. (DOI:10.1242/jeb.070342).

[59] R Development Core Team. 2013 R: A language and environment for statistical computing. (R Foundation for Statistical Computing.
[60] Wickham, H. 2016 ggplot2: Elegant Graphics for Data Analysis. *Springer-Verlag New York*, https://ggplot2.tidyverse.org.
[61] Bates, D., Machler, M., Bolker, B. M. & Walker, S. C. 2015 Fitting Linear Mixed-Effects Models Using lme4. *Journal of Statistical Software* **67**, 1-48.
[62] Kuznetsova, A., Brockhoff, P. B. & Christensen, R. H. B. 2017 lmerTest Package: Tests in Linear Mixed Effects Models. *Journal of Statistical Software; Vol 1, Issue 13 (2017)*. (DOI:10.18637/jss.v082.i13).
[63] Searle, S. R., Speed, F. M. & Milliken, G. A. 1980 Population Marginal Means in the Linear-Model - an Alternative to Least-Squares Means. *Am Stat* **34**, 216-221. (DOI:Doi 10.2307/2684063).
[64] Nakagawa, S. & Schielzeth, H. 2013 A general and simple method for obtaining R2 from generalized linear mixed-effects models. *Methods Ecol Evol* **4**, 133-142. (DOI:10.1111/j.2041-210x.2012.00261.x).
[65] Davison, A. C., Hinkley D.V. 1997 Bootstrap Methods and Their Applications. *Cambridge University Press, Cambridge*.
[66] Rand, D. M., Clark, A. G. & Kann, L. M. 2001 Sexually antagonistic cytonuclear fitness interactions in Drosophila melanogaster. *Genetics* **159**, 173-187.
[67] Careau, V., Thomas, D., Humphries, M. M. & Reale, D. 2008 Energy metabolism and animal personality. *Oikos* **117**, 641-653. (DOI:10.1111/j.0030-1299.2008.16513.x).
[68] Dammhahn, M., Dingemanse, N. J., Niemela, P. T. & Reale, D. 2018 Pace-of-life syndromes: a framework for the adaptive integration of behaviour, physiology and life history. *Behav Ecol Sociobiol* **72**. (DOI:UNSP 62

10.1007/s00265-018-2473-y).
[69] Reale, D., Garant, D., Humphries, M. M., Bergeron, P., Careau, V. & Montiglio, P. O. 2010 Personality and the emergence of the pace-of-life syndrome concept at the population level. *Philos Trans R Soc Lond B Biol Sci* **365**, 4051-4063. (DOI:10.1098/rstb.2010.0208).
[70] Auer, S. K., Dick, C. A., Metcalfe, N. B. & Reznick, D. N. 2018 Metabolic rate evolves rapidly and in parallel with the pace of life history. *Nature Communications* **9**. (DOI:ARTN 14

10.1038/s41467-017-02514-z).
[71] Deutsch, C. A., Tewksbury, J. J., Tigchelaar, M., Battisti, D. S., Merrill, S. C., Huey, R. B. & Naylor, R. L. 2018 Increase in crop losses to insect pests in a warming climate. *Science* **361**, 916-919. (DOI:10.1126/science.aat3466).
[72] Roeder, K. A. & Behmer, S. T. 2014 Lifetime consequences of food protein-carbohydrate content for an insect herbivore. *Funct Ecol* **28**, 1135-1143. (DOI:10.1111/1365-2435.12262).
[73] Sheldon, B. C. & Verhulst, S. 1996 Ecological immunology: costly parasite defences and trade-offs in evolutionary ecology. *Trends Ecol Evol* **11**, 317-321.
[74] Stearns, S. C. 1989 Trade-Offs in Life-History Evolution. *Funct Ecol* **3**, 259-268. (DOI:Doi 10.2307/2389364).
[75] Rand, D. M. 1994 Thermal Habit, Metabolic-Rate and the Evolution of Mitochondrial-DNA. *Trends Ecol Evol* **9**, 125-131. (DOI:Doi 10.1016/0169-5347(94)90176-7).
[76] Arnqvist, G., Stojkovic, B., Ronn, J. L. & Immonen, E. 2017 The pace-of-life: A sex-specific link between metabolic rate and life history in bean beetles. *Funct Ecol* **31**, 2299-2309. (DOI:10.1111/1365-2435.12927).
[77] Dunham-Snary, K. J., Sandel, M. W., Sammy, M. J., Westbrook, D. G., Xiao, R., McMonigle, R. J., Ratcliffe, W. F., Penn, A., Young, M. E. & Ballinger, S. W. 2018 Mitochondrial - nuclear genetic interaction modulates whole body metabolism, adiposity and gene expression in vivo. *EBioMedicine* **36**, 316-328. (DOI:10.1016/j.ebiom.2018.08.036).

779 [78] Arking, R., Buck, S., Wells, R. A. & Pretzlaff, R. 1988 Metabolic Rates in Genetically Based Long
780 Lived Strains of Drosophila. *Experimental Gerontology* **23**, 59-76. (DOI:Doi 10.1016/0531-
781 5565(88)90020-4).
782 [79] Johnston, S. E., Gratten, J., Berenos, C., Pilkington, J. G., Clutton-Brock, T. H., Pemberton, J. M. &
783 Slate, J. 2013 Life history trade-offs at a single locus maintain sexually selected genetic variation.
784 *Nature* **502**, 93-+. (DOI:10.1038/nature12489).
785 [80] Khazaeli, A. A., Van Voorhies, W. & Curtsinger, J. W. 2005 Longevity and metabolism in
786 Drosophila melanogaster: Genetic correlations between life span and age-specific metabolic rate in
787 populations artificially selected for long life. *Genetics* **169**, 231-242.
788 (DOI:10.1534/genetics.104.030403).
789 [81] Pearl, R. 1928 The Rate of Living. *Knopf, New York*.
790 [82] Rubner, M. 1908 Das Problem det Lebensdaur und seiner beziehunger zum Wachstum und
791 Ernarnhung. *Munich: Oldenberg*.
792 [83] Bronikowski, A. & Vleck, D. 2010 Metabolism, body size and life span: a case study in
793 evolutionarily divergent populations of the garter snake (Thamnophis elegans). *Integr Comp Biol* **50**,
794 880-887. (DOI:10.1093/icb/icq132).
795 [84] Burggren, W., Souder, B. M. & Ho, D. H. 2017 Metabolic rate and hypoxia tolerance are affected
796 by group interactions and sex in the fruit fly (Drosophila melanogaster): new data and a literature
797 survey. *Biol Open* **6**, 471-480. (DOI:10.1242/bio.023994).
798 [85] Van Voorhies, W. A., Khazaeli, A. A. & Curtsinger, J. W. 2004 Testing the "rate of living" model:
799 further evidence that longevity and metabolic rate are not inversely correlated in Drosophila
800 melanogaster. *J Appl Physiol* **97**, 1915-1922. (DOI:10.1152/japplphysiol.00505.2004).
801 [86] Groot, A. T. 2014 Circadian rhythms of sexual activities in moths: a review. *Frontiers in Ecology
802 and Evolution* **2**. (DOI:10.3389/fevo.2014.00043).
803 [87] Partridge, L., Hoffmann, A. & Jones, J. S. 1987 Male Size and Mating Success in Drosophila-
804 Melanogaster and Drosophila-Pseudoobscura under Field Conditions. *Animal Behaviour* **35**, 468-476.
805 (DOI:Doi 10.1016/S0003-3472(87)80272-5).
806 [88] Sakai, T. & Ishida, N. 2001 Circadian rhythms of female mating activity governed by clock genes
807 in Drosophila. *P Natl Acad Sci USA* **98**, 9221-9225. (DOI:DOI 10.1073/pnas.151443298).
808 [89] Rand, D. M., Haney, R. A. & Fry, A. J. 2004 Cytonuclear coevolution: the genomics of
809 cooperation. *Trends Ecol Evol* **19**, 645-653. (DOI:10.1016/j.tree.2004.10.003).
810 [90] Wolff, J. N., Ladoukakis, E. D., Enriquez, J. A. & Dowling, D. K. 2014 Mitonuclear interactions:
811 evolutionary consequences over multiple biological scales. *Philos T R Soc B* **369**. (DOI:Artn 20130443
812 Doi 10.1098/Rstb.2013.0443).

813

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

Fixed effects	*Sum sq*	*Mean sq*	*NumDF*	*DenDF*	*F value*	*P - value*
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

Random effects	*Variance*	*p-value*
Strain duplicate	0	1
Day[Block]	0.0035	<0.0001
Trial[Day[Block]]	3.59e-11	1
Residual	0.133	

824

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***

Fixed effects	*Sum sq*	*Mean sq*	*NumDF*	*DenDF*	*F value*	*P - value*
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

Random effects	*Variance*
Day[Block]	0.0073

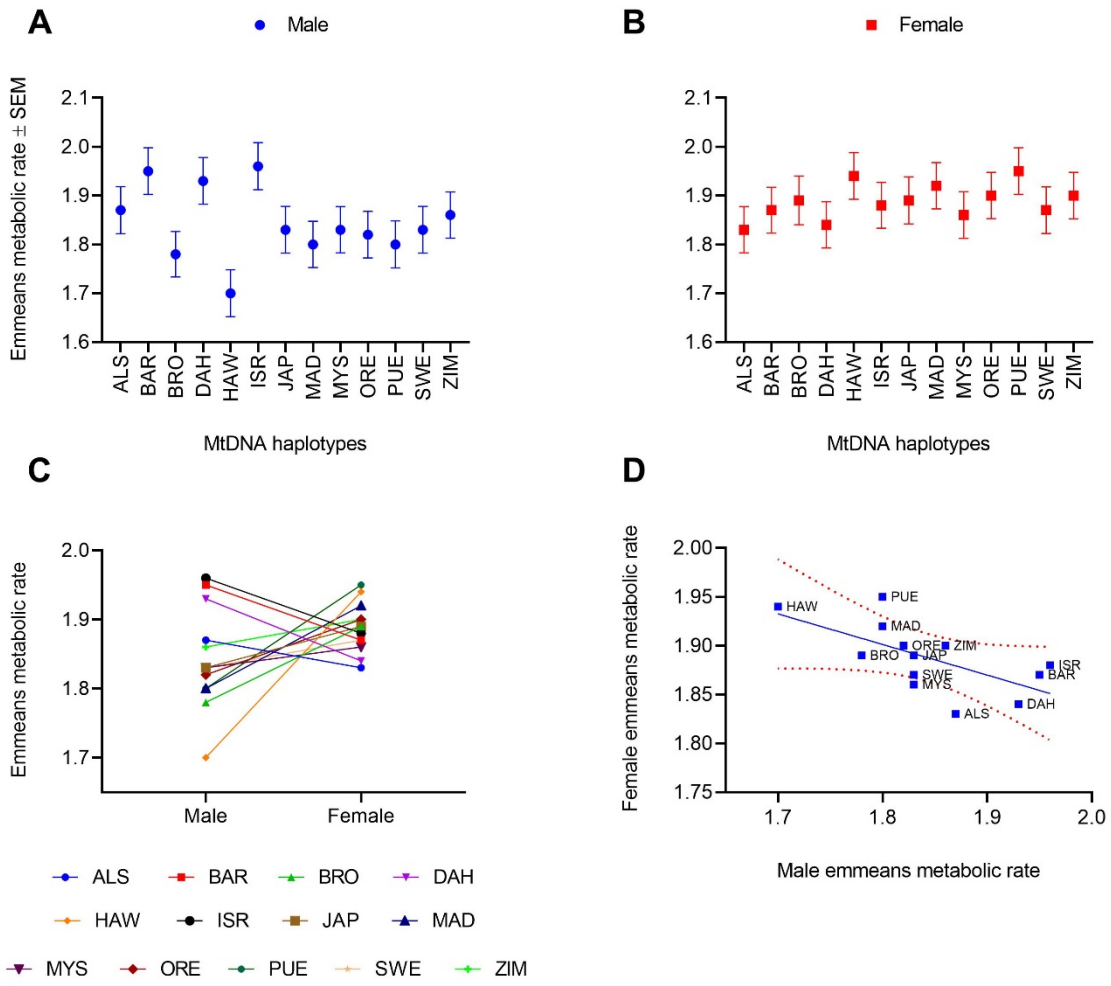
Trial[Day[Block]]	4.93e-09
Residual	0.168

Female-specific lmer model

Fixed effects	*Sum sq*	*Mean sq*	*NumDF*	*DenDF*	*F value*	*P - value*
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

Random effects	*Variance*
Day[Block]	0.0045
Trial[Day[Block]]	0.0011
Residual	0.093

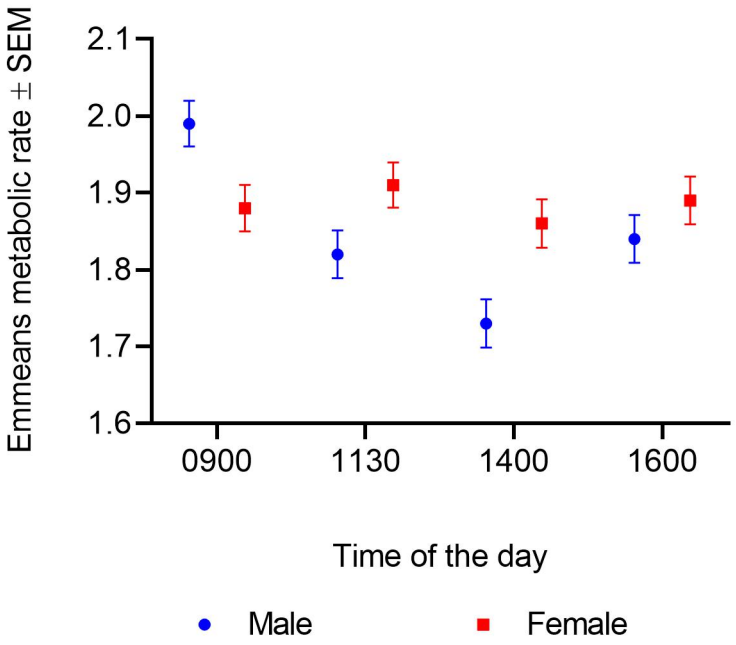
Figures



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) ± 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.

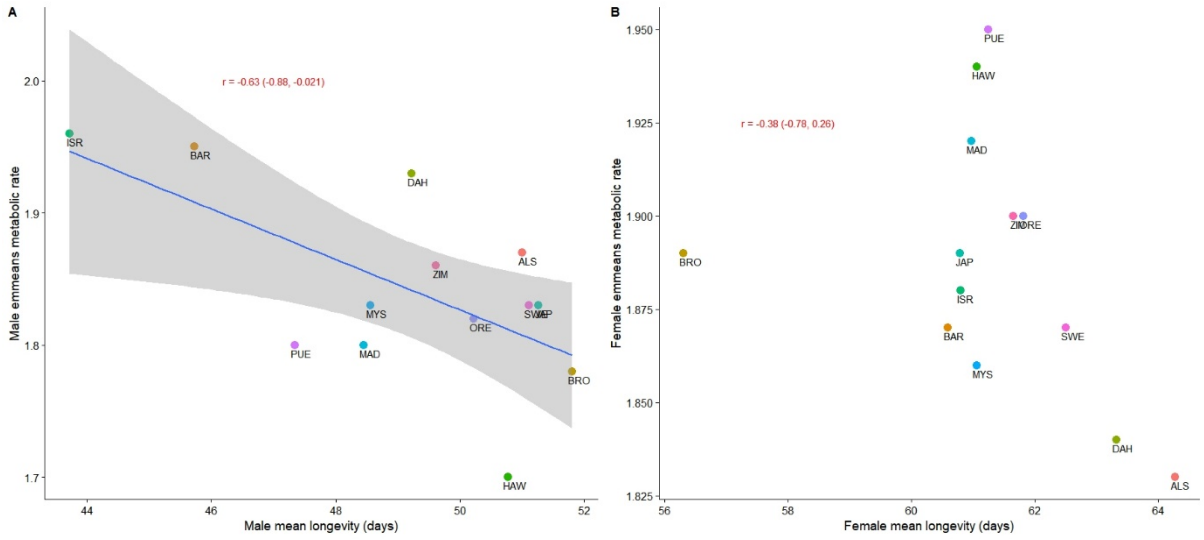
845



846

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

850



851

852 **Figure 3**. Intra-sexual mitochondrial correlation between longevity and emmeans metabolic
853 rate in A) males and B) females. The scales in both axes are adjusted to show the direction of
854 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).