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

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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 which 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.

This article is part of the theme issue 'Linking the mitochondrial genotype to phenotype: a complex endeavour'.

1. 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 & Hurst [10], 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. Under this weak form, accumulation of 'male-harming, but female-benign' mutations would lead to a male-biased genetic load accumulating within the mitochondrial haplotypes of different populations. Furthermore, the identity, number and severity of the male-harming mutations would be expected to differ across haplotypes of different populations, given that each is evolving along its own independent trajectory. Thus, under the weak form of the hypothesis, it is predicted that the genetic variation which delineates distinct mitochondrial haplotypes (across the natural distribution of any given species of eukaryote) will confer greater effects on phenotypic expression in males than in females [8,12,13].

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

Ultimately, the evolutionary significance of the Mother's Curse hypothesis hinges on the capacity by which mutations, exhibiting sex-specific effects on the phenotype, can accrue within the mtDNA sequence. Several studies have now documented evidence for the presence of mutations conferring male-specific effects on components of adult life history—reproductive outcomes in vinegar flies (*Drosophila melanogaster*), mice (*Mus musculus*), chicken (*Gallus domesticus*), brown hares (*Lepus europaeus*) and humans [8,16–23], and longevity [12,24] 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, because it supports the contention that genetic variation which 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 intersexual 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.* [25] 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 13 mitochondrial haplotypes of *D. melanogaster*. In another study, Novicic *et al.* [45] reported that as much as 20% of the variation in whole-organism metabolic rate (measured as carbon dioxide (CO_2) production) across adult *Drosophila subobscura* could be mapped to genetic variation across three mtDNA haplotypes. However, 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 13 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*. [12] and reproductive fitness from Camus & Dowling [16] 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.

2. Methods

(a) 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 used a panel of 13 strains of *D. melanogaster*, each of which is characterized 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 backcrossing 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. Backcrosses were always conducted at low adult densities (five pairs), and only eggs produced by parents that were 4 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^{-1}) 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 v. 9.0.4 [49].

(b) 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 13 strains. The experiment was conducted over three temporally separated sampling blocks, each of which was separated by a single generation of fly propagation (14 days).

(i) Generating focal flies

All focal flies (i.e. those used in the experiment) were produced by parents and grandparents that were 4 days of adult age at the time of egg-laying. In the two generations leading up to the assay, all flies were reared under carefully controlled densities (10 pairs of adult flies per vial, and egg numbers per vial reduced to 80), at constant laboratory conditions (25°C). We ensured we had a steady daily supply of standard-aged focal flies for the metabolic rate ($\dot{V}CO_2$) measurements, by allowing the great-grandparents of the focal flies to lay eggs that produced the grandparental flies over several successive days (5 days in sampling blocks 1 and 2, and 8 days in block 3). Thus, although all focal flies had parents and grandparents of precisely standardized age, they had been produced by great-grandparents that differed in age by up to 7 days.

(c) Metabolic rate assay

The focal flies were collected under mild CO_2 anaesthesia within 6 h of their eclosion into adulthood, thus ensuring their virginity, and then housed in single-sex groups of 10 flies per vial. These flies remained in these vials for 4 days before measurement of their metabolic rate. For any given sampling day, we maintained one vial of 10 focal flies per strain duplicate per sex within each block. The use of virgin flies removed any physiological effects on metabolic rate caused by mating *per se* and post-mating intersexual harassment. Additionally, the 4 day recovery period following collection of the focal flies ensured that the impact of CO_2 anaesthesia on the metabolic rate had dissipated by the time of the assay [50].

A standard Sable Systems International (SSI, www.sablesys.com, Las Vegas, NV, USA) flow-through CO_2 respirometry system, connected to four LI-COR 7000 infrared CO_2/H_2O gas analysers (LICOR, Lincoln, NE, USA), was used to measure CO_2 production as a proxy of metabolic rate ($\dot{V}CO_2$) of adult flies. Two identical set-ups were created, each underpinned by two LI-COR 7000s (SSI, www.sablesys.com). 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^{-1} 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, MA, 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 manoeuvrable 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). Each chamber was measured once for

10 min, with a 2 min pause period between every measurement to allow time for the CO_2 readings to stabilize. The assaying chambers were flushed with a humidified airflow (80% relative humidity) 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, CT, USA) attached to a TC-2000 thermocouple meter (Sable Systems).

The respirometry assays were run over five consecutive days in blocks 1 and 2, and over eight consecutive days in block 3. We ran four 'experimental trials' per day at approximately 09.00, 11.30, 14.00 and 16.30. 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 owing 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).

(d) Statistical analyses

(i) Linear mixed-effect modelling of the global data

The data analyses were performed in the R statistical environment (v. 3.4 [59]), and graphs were plotted in *ggplot2* package [60] in R and GraphPad Prism software v. 8.1.0. 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 the $\dot{V}CO_2$ trace file) of each focal fly was modelled as the response variable, with mtDNA haplotype (13 levels), sex of the fly (two levels), time of day of the assay (four 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 (three levels), assay-day (eight 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 stepwise 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 the mean metabolic rate (referred to 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 the mean metabolic rate for body mass (electronic supplementary material, Manual correction of mean metabolic rate for body mass and figures S1 and 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 stepwise model reduction process and parameter estimation procedure as described above. Furthermore, we used these two models to estimate the marginal R^2 values (95% confidence intervals (CIs)) for each model using the 'nsj' method in the *r2glmm* package [64]; and further estimated the effect size attributed by the mtDNA haplotype on the 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).

(ii) Estimating intersexual 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 13 haplotypes to determine the magnitude and direction of intersexual correlation for metabolic rate. From this test, we estimated the Pearson's correlation coefficient and then 95% CIs 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 CIs of the correlation coefficient were estimated from the bias corrected and accelerated (BC_a) method in the *boot* package.

(iii) Estimating inter-trait correlations across haplotypes

The full panel of 13 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

Table 1. Results from the *lmer* model of the full dataset, which included metabolic rate of both sexes. (In this model, haplotype and sex were modelled as fixed effects, and centred body mass and ADS as fixed covariates. The final model was derived by sequentially eliminating non-significant higher-order interaction terms across both fixed and random effects using log-likelihood ratio tests, retaining only the final list of fixed and random effects, and any higher-order interactions that were statistically significant at $p < 0.05$. The random effects—trial[day(block)] denotes 'experimental trial nested within day nested within block' and day[block] denotes 'experimental day nested within block'.)

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)]	3.59×10^{-11}	1				
residual	0.133					

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 pairwise combinations of traits within and between the two sexes. We estimated the Pearson's correlation coefficient and 95% CIs 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 CIs of the correlation coefficient were estimated from the bias corrected and accelerated (BC_a) method in the *boot* package.

3. Results

(a) Mitochondrial genetic variation for metabolic rate is male-biased

A significant interaction between mtDNA haplotype and sex on metabolic rate was found (table 1). This indicates that the identity of the mtDNA haplotype affects metabolic rate, but that the pattern of effects across haplotypes differs across the sexes (figure 1*a–c*). Furthermore, analyses of the sex-specific datasets revealed the mtDNA haplotype effect on metabolic rate was significant only in males (table 2). A male-bias in the magnitude of the mtDNA haplotypic effect was further supported by the examination of standardized effect sizes (marginal R^2 values (95% CI)) estimated from the sex-specific models (R^2_{males} estimate ± 95% CIs = 0.236 [0.292, 0.202], $R^2_{\text{females}} = 0.167$ [0.223, 0.138] and Cohen's d ($d_{\text{males}} = 0.647$, $d_{\text{females}} = 0.461$).

(b) Metabolic rate of males is sensitive to circadian effects

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

(c) Intersexual correlation for metabolic rate, across haplotypes, is negative

We found a negative correlation between the sexes in metabolic rate across the 13 mtDNA haplotypes, controlling for body mass and activity (i.e. based on the emmeans of the final model in table 1, Pearson's correlation coefficient $r_p = -0.64$; bootstrapped 95% CIs = −0.84, −0.31). That is, haplotypes which conferred greater trait means in females conferred lower trait means in male flies (figure 1*d*).

(d) Intrasexual correlations involving metabolic and life-history traits

We observed a negative correlation between emmeans metabolic rate and longevity across the 13 haplotypes in males (Pearson's correlation coefficient $r_p = -0.63$; bootstrapped 95% CIs = −0.88, −0.021), but not in females ($r_p = -0.38$; 95% CIs = −0.78, 0.26) (figure 3). The correlation between body mass and longevity was positive in males ($r_p = 0.63$, 95% CIs = −0.03, 0.84) but negative in females ($r_p = -0.70$, 95% CIs = −0.98, 0.09; electronic supplementary material, figure S3), but the CIs of each overlapped with zero. We did not observe any signatures of pleiotropy between the metabolic rate or body mass and components of fertility outcomes, in either of the sexes (electronic supplementary material, figure S4).

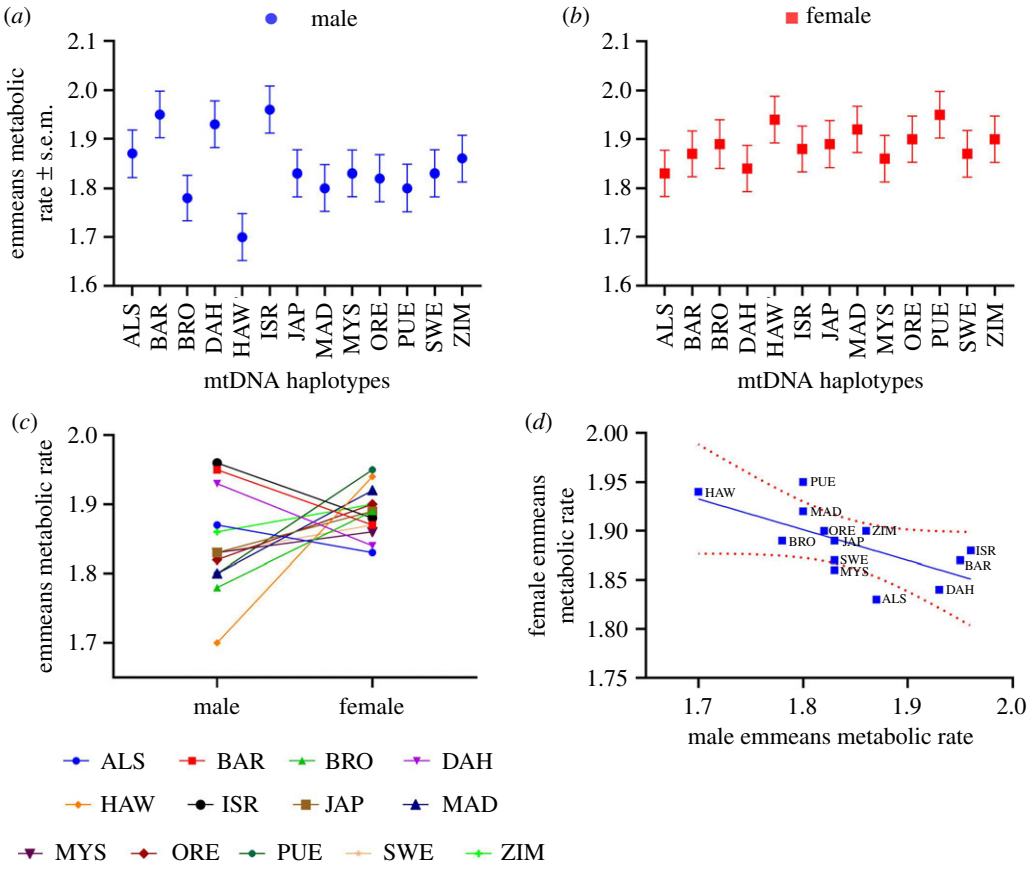

Figure 1. Effects of mtDNA haplotype on the metabolic rate of (*a*) male and (*b*) female flies. In (*a,b*), the estimated marginal means (emmeans) ± 1 s.e. of metabolic rate for each mtDNA haplotype-by-sex combination were derived from the final model built on global data, using the *emmeans* package in R. The emmeans accounted for variation attributable to body mass and ADS in the final global model. (*c*) Interaction plot showing variation in emmeans metabolic rate between the sexes, across the 13 mtDNA haplotypes. (*d*) Negative intersexual mitochondrial correlation for emmeans metabolic rate (Pearson's correlation coefficient $r_p = -0.64$, bootstrapped 95% CIs = −0.84, −0.31). The scales in both axes are adjusted across the panels to elucidate the magnitude of variation and relationship between the trait means. For annotations of the mtDNA haplotypes, refer to the Methods section. (Online version in colour.)

4. Discussion

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

(a) Sex-specific effects of mtDNA haplotype on metabolic rate

Not all studies that have tested for mtDNA haplotype effects on phenotypic expression have uncovered evidence for a male-bias in the magnitude of effects [27–31]. However, studies that leveraged this same panel of 13 mtDNA haplotypes, or subsets of haplotypes from the panel, have reported male-biases in the magnitude of haplotype effects on traits such as longevity and survival, patterns of nuclear gene expression, early-life mitochondrial 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 which affect metabolic rate can consistently confer effects that are larger in males than in females.

(b) 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

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

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)]	4.93×10^{-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					

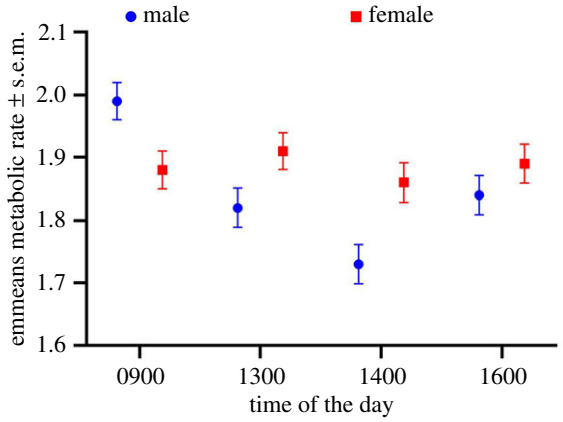

Figure 2. Circadian effects on the metabolic rate of each sex. The emmeans metabolic rate (adjusted for body mass and ADS) for each sex-by-time of the day combination was estimated from the model in table 1 using the *emmeans* package in R. (Online version in colour.)

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 & Dowling [16] 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 & Dowling [16] do not correspond with those conferring relatively higher and lower metabolic rates in our study (electronic supplementary material, figure S4). Furthermore, another difference between the two studies is that Camus & Dowling [16] 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, as 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 4 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 1*b* is suggestive of a

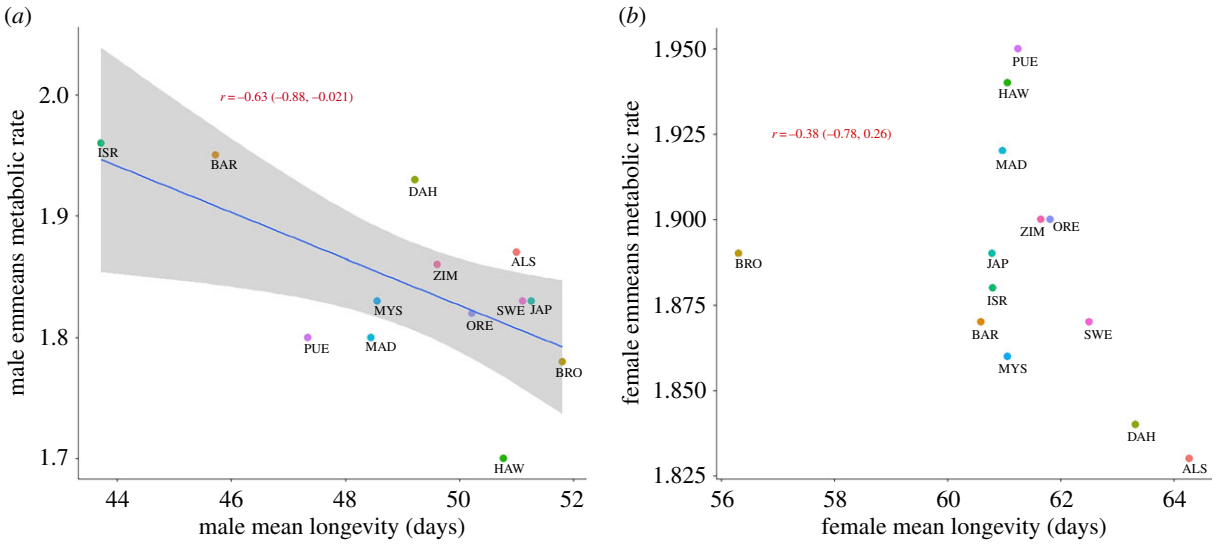

Figure 3. Intrasexual mitochondrial correlation between longevity and emmeans metabolic rate in (*a*) males and (*b*) females. The scales in both axes are adjusted to show the direction of the relationship between the traits in each sex. For annotations of the mtDNA haplotypes, refer to the Methods section. The mean longevity scores for each sex-by-haplotype combination were sourced from Camus *et al*. [12]. (Online version in colour.)

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 standardized effect size calculations, presented in the results, suggest the haplotypic effect in females is likely to be smaller than males, but greater than zero. However, 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], and the importance of even small variation in metabolic rates or other traits over extended periods (e.g. [71,72]). Ultimately, further work is required to test whether the intersexual correlations for metabolic rate, observed here, are genetic or phenotypic in origin.

(c) Negative correlation between metabolic rate and lifespan, across haplotypes, is limited to males

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

Here, we identified a negative correlation across haplotypes for metabolic rate and longevity in males, which suggests a possible male-specific genetic trade-off between the optimal expression of these traits. Indeed, a negative correlation between these two traits is the key prediction of the 'rate of living' hypothesis [78–82], which contends that organisms exhibiting high metabolic rates should have shorter lifespans. Despite its widespread appeal, little evidence exists for this prediction at either an interspecific or intraspecific scale [76,80,83]. Our observation of a male-specific negative mitochondrial correlation between metabolic rate and longevity is striking because it suggests that genetic variation might accumulate within the mitochondrial genome in a manner consistent with the rate of living hypothesis, albeit with a twist. Maternal inheritance of the mitochondrial genome will, in theory, render selection efficient at removing the pool of mutations that reduce female metabolic rate and longevity, but less efficient in removing the pool of mutations that exert male-specific effects on each of these two traits. The male-specificity of the mitochondrial correlation between metabolic rate and longevity would, therefore, suggest that any mtDNA mutations which underpin this correlation are likely to be non-adaptive, accumulating under a selection shadow, and therefore, not associated with fitness benefits to males. This contention is supported by the lack of a positive genetic correlation, across haplotypes, between metabolic rate and reproductive fitness in males (electronic supplementary material, figure S4).

(d) Sex and circadian contexts of metabolic rate expression in vinegar flies

Our study also provided new insights into the magnitude and context-dependency of sexual dimorphism in the metabolic rate of *D. melanogaster*. The mean mass- and activity-adjusted metabolic rate (i.e. emmeans of the final model in table 1) across the haplotypes was generally higher in females than in males (females: mean = 1.89, s.d. = 0.04; males: mean = 1.84, s.d. = 0.07). However, the magnitude of the sex difference in metabolic rate changed across the day owing to high levels of time-mediated plasticity in metabolic rate in males. The existing literature confirms that sexual dimorphism in the expression of metabolic rate in vinegar flies is context-dependent [84,85]. In this regard, the genotype of flies, number of flies assayed

in the respirometer (single fly versus group of flies), type of respirometry set-up (open versus flow-through), mating status of the focal flies (virgins versus mated), age of the focal fly (young versus old) and the type of assaying area (confined versus unconfined) have all been shown to influence patterns of sexual dimorphism in metabolic rate of vinegar flies [84]. Notwithstanding, while 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.

(e) 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 13 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]. However, 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.

5. Conclusion

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.

Data accessibility. The analyses were carried out by running generic codes explained in the R documentation. Raw data and electronic supplementary material are available through the Monash Figshare repository; https://doi.org/10.26180/5dae68bb85221.

Authors' contributions. D.K.D. conceived the experiment; V.N.-R., D.K.D., S.L.C., I.A. and D.J.C. designed the experiment; V.N.-R. and I.A. conducted the metabolic rate measurements; V.N.-R. conducted data analyses; D.K.D. advised on data analyses; V.N.-R. and D.K.D. wrote the first draft of the manuscript; S.L.C., I.A. and D.J.C. contributed to subsequent drafts and the final version of the manuscript.

Competing interests. We declare we have no competing interests.

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