

Interactions between mitochondrial haplotype and dietary macronutrient ratios confer sex-specific effects on longevity in *Drosophila melanogaster*

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Abstract

Recent studies have demonstrated that modifications to the ratio of dietary macronutrients affect longevity in a diverse range of species. However, the degree to which levels of natural genotypic variation shape these dietary effects on longevity remains unclear. The mitochondria have long been linked to the ageing process. The mitochondria possess their own genome, and previous studies have shown that mitochondrial genetic variation affects longevity in insects. Furthermore, the mitochondria are the sites in which dietary nutrients are oxidized to produce adenosine triphosphate, suggesting a capacity for dietary quality to mediate the link between mitochondrial genotype and longevity. Here, we measured longevity of male and female fruit flies, across a panel of genetic strains of *Drosophila melanogaster*, which vary only in their mitochondrial haplotype, when fed one of two isocaloric diets that differed in their protein-to-carbohydrate ratio. The mitochondrial haplotype affected the longevity of flies, but the pattern of these effects differed across the two diets in males, but not in females. We discuss the implications of these results in relation to an evolutionary theory linking maternal inheritance of mitochondria to the accumulation of male-harming mitochondrial mutations, and to the theory exploring the evolution of phenotypic plasticity to novel environments.

Keywords: gene-by-environment, Mother's Curse, nutrition, protein:carbohydrate ratio, sexual conflict

Introduction

Individual investment into the various components of life-history is expensive and relies on the acquisition of resources, including the regular uptake of dietary macronutrients (1). Indeed, one of the core facets of life-history is longevity; a trait whose expression is explicitly tied to variation in dietary quality and quantity (2). For many years, it was assumed that extensions in longevity could be achieved simply through reductions in total caloric intake (3). However, research advances over the past two decades have determined that variation in the balance of specific dietary macronutrients ingested, rather than total calories *per se*, may be the main contributor to longevity (1, 4). These insights came from studies that harnessed experimental designs able to decouple the influence of macronutrient ratios from the total caloric intake, which demonstrated that ratios of dietary protein-to-carbohydrate (P:C) are the primary contributors to longevity outcomes across crickets, *Drosophila* flies, and mice (1, 4). As such, the association between macronutrient ratios and longevity appears to be conserved across a range of bilaterian metazoans.

Recently, further progress has been made in understanding the link between macronutrient balance and longevity in metazoans. Firstly, several studies have demonstrated that the effects associated with modifying macronutrient ratios on longevity are largely consistent across the sexes, in insects and mice, albeit with minor differences in the optimal ratios of P:C in each sex (5, 6). Secondly, studies that have focused on the molecular pathways that mediate longevity responses to dietary intake have identified a key role for the Insulin-like Growth Factor 1 and mechanistic Target of Rapamycin (IGF-1/mTOR) network in the regulation of these effects (2). However, one area of research that remains less explored when it comes to the links between diet

and longevity, is the role that natural genetic variation plays in mediating the magnitude of longevity response to dietary manipulation. While it is clear that the phenotypic expression of longevity is underpinned by genetic variation within the nuclear (7, 8) and mitochondrial genomes (9, 10), little is known as to whether the expression of this genetic variation is sensitive to variation in the dietary environment, manifesting as macronutrient-mediated genotype-by-environment ($G \times E$) interactions for longevity. Temperature-mediated $G \times E$ interactions for longevity have been previously demonstrated in *D. melanogaster* (11), and given the widespread prevalence of $G \times E$ interactions underpinning the expression of quantitative traits (12), this would suggest considerable scope for genotype-specific responses in levels of macronutrient mediated plasticity for longevity. Furthermore, such $G \times E$ interactions could conceivably differ in their sensitivity across the sexes (13).

The mitochondria have long been at the forefront of hypotheses that seek to explain why ageing occurs (14). As the organelles that generate energy reserves used for organismal and somatic maintenance, their functionality is a key prerequisite for sustaining healthy life (15). Somewhat paradoxically, they generate highly Reactive Oxygen Species, as by-products, implicated in oxidative stress, and progressive physiological deterioration with advancing age (16, 17).

Furthermore, mitochondrial dysfunction is a known pathology of normal ageing, as well as late-onset diseases such as Alzheimer's and Parkinson's (18). From an evolutionary standpoint, the mitochondria are of particular interest to the ageing process given that they have retained their own genome, comprised of mitochondrial DNA (mtDNA), in which a set of 13 core protein-coding genes encode polypeptide subunits that interact intimately with nuclear-encoded subunits to assemble the five enzyme complexes that underpin oxidative phosphorylation (OXPHOS). As

such, interactions between genes encoded by the mtDNA and nuclear genes that contribute to the mitochondrial proteome underpin biological processes that are essential for eukaryotic life (19, 20).

Recent studies investigating the role of genetic variation in dietary-mediated effects on longevity have focused explicitly on the role of genetic variation across mitochondrial haplotypes, and epistatic combinations of mitochondrial-nuclear (mito-nuclear) genotype. For example, Zhu *et al.* (2014) examined effects of caloric and macronutrient modification on the longevity of female flies harbouring different combinations of mito-nuclear genotype (two mtDNA haplotypes from *D. melanogaster* and two from *D. simulans*, placed against two different isogenic nuclear backgrounds from *D. melanogaster* = eight mito-nuclear combinations). They reported evidence that longevity was affected by interactions between mtDNA haplotype, the nuclear background and the dietary regime, thus highlighting a prominent role for mitochondrial genetics in regulating the longevity response to the dietary modification. Mito-nuclear effects on ageing were moderated both by changes in the caloric content of the diet, and the macronutrient content (21). While Aw *et al.* (2017) examined sex-specific effects on a range of mitochondrial biochemical, physiological and life-history traits including survival, associated with interactions between two mtDNA haplotypes and variation in macronutrient ratios, in *D. melanogaster*. They reported the effects of the mitochondrial haplotype, and interactions between the mitochondrial haplotype and diet, on survival in males, but not females (22).

The findings of male-specific mitochondrial genotype by dietary environment interactions for survival, reported by Aw *et al.* (2017), are interesting in light of an evolutionary hypothesis

known as Mother's Curse (23), which predicts that mitochondrial genetic effects on the phenotype will be male-biased in magnitude. This prediction is based on the premise that maternal inheritance of mitochondria will facilitate the accumulation of mutations within the mitochondrial genome that exert male-biases in effects on the expression of life-history and physiological traits (23). Maternal inheritance means that the evolutionary fate of any mutation in the mtDNA sequence will be determined by its fitness effects in females. If a mutation arises in the mitochondrial genome that is benign or positive in its effects on female fitness, this mutation can accumulate and even fix within the mtDNA sequence, even if the same mutation incurs significant costs to male fitness (24). In theory, this will lead to the accumulation of mutation loads in the mitochondrial genome that exert male-biases in their associated phenotypic effects. A key prediction of the hypothesis, therefore, is that the genetic variation that delineates different mtDNA haplotypes will exert larger effects on the expression of phenotypes in males than females. That is, levels of mitochondrial genetic variation underpinning phenotypic trait expression should be male-biased. This prediction has received some empirical support from studies of genetic strains of *D. melanogaster*, which differ only in their mtDNA sequence. These studies have revealed male-biases in levels of mitochondrial genetic variation underpinning several traits, such as longevity and ageing rates (9), as well as nuclear genome-wide patterns of gene expression (25). These studies have been supported by other studies reporting specific mtDNA mutations, or haplotypes, associated with negative phenotypic outcomes in males, but not females (26-28)

In this study, we aimed to extend on previous studies that have linked mitochondrial genetic variation to longevity (29-31), and that have examined the role of mitochondrial genotype-by-

diet interactions for longevity (21, 22). We explored three questions. Firstly, whether the genetic variation that occurs across a panel of 13 mtDNA haplotypes, sourced from distinct global localities, affects longevity, and whether any such mitochondrial haplotypes effects are male-biased in magnitude, as was previously observed by Camus *et al.* (2012). Secondly, whether this variation is sensitive to macronutrient-mediated mitochondrial $G \times E$ interactions, and finally, whether these $G \times E$ interactions are male-biased in their manifestation as previously indicated by Aw *et al.* (2017) in their study of two haplotypes. To address these questions, we utilized the same panel of haplotypes as previously used by Camus *et al.* (2012), who uncovered clear male-biases for longevity and ageing rates across the 13 haplotypes. This panel also includes the two haplotypes used by Aw *et al.* (2017). Specifically, we measured longevity of replicated cohorts of male and female flies that were maintained on one of two isocaloric diets that differed only in their levels of protein and carbohydrate content but not total nutritional content (32). We also investigated whether mitochondrial haplotype effects on longevity, measured on artificial diets used in our study, matched those reported by Camus *et al.* (2012), who measured longevity of flies kept on standard yeast-based diets.

Methods

Mitochondrial panel

We measured the longevity of male and female flies, across a panel of 13 genetic strains of *D. melanogaster*, on each of two different diets. Each strain is characterised by a distinct and naturally occurring mtDNA haplotype in an otherwise isogenic nuclear background, derived from the w^{1118} line (33). These strains are annotated based on the geographical location from which they were originally sourced, thus - **Alstonville**, Australia; **Barcelona**, Spain;

Brownsville, USA; **Dahomey**, Benin, **Madang**, Papua New Guinea; **Mysore**, India; **Hawai'i**, USA, **Israel**; **Japan**; **Oregon**, USA; **Puerto Montt**, Chile; **Sweden** and **Zimbabwe** (9). The breeding scheme used to create the mitochondrial strains is outlined in Clancy (2008). The mitochondrial strains were obtained in 2007 from Dr David J Clancy and immediately divided into two biological duplicates, which have since been independently maintained through more than 100 generations of backcrossing of virgin females of each strain to males of the isogenic w^{1118} line. Additionally, the w^{1118} line has been propagated each generation via a single full-sibling pair to maintain isogenicity throughout the nuclear genome. By backcrossing the strain duplicates into the w^{1118} nuclear background over ~100 successive generations, we ensured that the nuclear backgrounds of each of the mitochondrial strains were truly isogenic and devoid of any cryptic nuclear genetic variation that may have accumulated over multiple generations of laboratory maintenance.

The strains were cleared of infection from the bacterial symbiont *Wolbachia* via antibiotic (tetracycline hydrochloride) treatment (33). Before the start of our experiment, the absence of *Wolbachia* among the mitochondrial strains was confirmed via a diagnostic PCR, using whole genomic DNA isolated from two female flies per strain duplicate. This PCR amplified the *Wolbachia* Cytochrome Oxidase subunit I (*CoxA*) gene (34). We used a separate true-positive control (DNA sample extracted from *Wolbachia*-positive wild-type strain) and a true-negative control (DNA sample obtained from a tetracycline treated laboratory strain) in our diagnostic PCRs to confirm the *Wolbachia* status of the mitochondrial strain duplicates. Using a secondary analysis, we further confirmed the absence of *Wolbachia* in the mitochondrial strains by

screening for the presence of *Wolbachia* gene sequences in the pool of Illumina paired-end reads obtained from the mitochondrial panel NGS data (35).

In summary, this panel of mitochondrial strains serves as a valuable genetic resource to explore mitochondrial genetic effects on the phenotype, because the 13 haplotypes of the panel provide a broad representation of the total levels of mitochondrial genetic variation found across the globe for this species (35). Furthermore, because each of the haplotypes persists across two independent biological duplicates, mitochondrial genetic effects on longevity can be statistically decoupled from confounding sources of environmental variance, as well as from possible effects mediated by residual nuclear variation that might have accumulated across the strains despite our best efforts to maintain these as isogenic.

Generating experimental flies

The flies of each strain duplicate were subjected to a stringent breeding scheme for three generations leading up to the generation of the focal flies, to control for potential sources of environmental variance (such as parental and grandparental age effects, and density-dependent effects). In the first generation, for each strain duplicate, 15 pairs of flies were housed in vials with access to standard laboratory food (see **supplementary material**) until two days of adult age. The protein:carbohydrate ratio of the lab food is estimated to be 1:2.5 (that is, P = 22.5% and C = 57.4%). The mating pairs were then transferred to a second vial for 24 h, during which time females deposited eggs onto the surface of the food. These egg densities were reduced to 80 eggs per vial, by removing excess eggs with a clean spatula. This process was continued in the subsequent generation, with flies collected within 24 h of eclosion used to propagate the next

generation. In the second generation, 15 pairs of flies per vial were stored until four days of adult age, then transferred to fresh vials with access to fresh lab food supplemented with *ad libitum* dry yeast. The females were allowed to oviposit for 24 h, and egg density again trimmed to 80 eggs. The same procedure was followed for a third generation, which produced the parents to our “focal” flies used in our experiments.

In the fourth generation, the focal flies were collected as virgins, within 6 h of eclosion into adulthood, and then housed separately by sex, across two vials (10 flies per vial) per strain duplicate, for 24 h. This 24-h period enabled us to confirm the virginity of the female flies (as gauged by the absence of viable eggs over this time-period). When the focal flies were two days of adult-age, we added a group of 10 two-day-old “tester” flies of the opposite sex, collected from the *w¹¹¹⁸* line, to each vial. The flies were allowed to cohabit for 24 h, during which time the flies will have mated (9), and then the tester flies removed from these vials under light CO₂ anaesthesia. Focal flies were transferred to fresh vials with standard laboratory food in their respective same-sex cohorts for 24 h. This 24 h resting period allowed focal flies to recover from post-mating stress and CO₂ anaesthesia.

Isocaloric diets

When five days of age, each cohort of focal flies was placed onto one of two isocaloric, solid diets that varied only in the ratio of protein and carbohydrate, but otherwise had standardised quantities of Wesson’s salts, ascorbic acid, cholesterol and vitamin mix (32) and the same total nutritional content (i.e. diets were isocaloric). One of the diets was protein biased (2P:1C), while the other was carbohydrate biased (1P:8C). The protein used in these diets consisted of a 3:1:1

mixture of casein, peptone and albumen, whereas the carbohydrate was a 1:1 mixture of sucrose and dextrin. The constituents of the solid diets were added to warm distilled water that was boiled with 1% agar. This boiled food mixture was allowed to cool down to room temperature, before propionic acid and 10% nipagin were added. Two millilitres of food was dispensed into individual vials using a peristaltic pump (Watson Marlow Limited, UK), allowed to solidify overnight, before use in the longevity assay. Fresh food was prepared *ad hoc*, and the excess food was stored in the refrigerator for no longer than four days, before deployment into the longevity experiment. Refer to **supplementary material** for full methods on the preparation of the isocaloric diets.

Longevity assay

Each cohort of focal flies remained on the diet to which it had been assigned for the duration of the longevity experiment. Flies were transferred to fresh vials, every 48 h. This method of transferring flies to vials with fresh food ensured the availability of fresh food to the adult flies, free of fungal or bacterial growth and free from advanced stages of larval activity; and controlled for accidental deaths caused by flies getting stuck to the old food, which becomes stickier with age (9). We housed each of these technical replicates in separate trays, and the trays were kept in separate parts of the temperature-controlled cabinet (maintained at 25°C and ~30% relative humidity, Panasonic MLR-352H-PE environmental growth cabinet) to ensure that micro-environmental variation across the vials did not confound our capacity to accurately home in on mitochondrial genetic effects on longevity. When transferring the flies between vials, every 48 h, we recorded the number of dead flies in each vial. The longevity assay was run over four independent ‘experimental blocks’ that were temporally separated from each other by one

generation time (14 days). The experimental units (combinations of mitochondrial strain duplicate \times sex \times diet) were balanced in their representation across all the blocks, with each unit represented twice per block. That is, across all the experimental blocks, we maintained two independent copies of each strain duplicate-by-sex combination in separate vials for the two isocaloric diets. These vial replicates were denoted as the “technical replicates” in the statistical analysis.

Statistical analyses

We constructed a linear mixed effects model using the *lme4* package in R (36) in R v3.4 to analyse sources of variation affecting longevity. Here, we treated the mtDNA haplotype as a fixed effect in the models, because we were interested in characterising the nature of the mitochondrial genotype \times dietary environment (G \times E) interactions for longevity, rather than estimating sex-specific levels of mitochondrial genetic variance across diets. Thus, mtDNA haplotype (13 levels), sex (2 levels), diet (2 levels), and the higher-order interactions among these factors were included as fixed effects. In the same model, strain duplicates (26 levels), technical replicates (104 levels), experimental blocks (4 levels), and higher-order interactions between these factors were included as random effects. We included all possible interactions (up to second order) between fixed and random factors as random effects in the full model.

We derived a reduced model by progressively eliminating higher-order random effects whose removal did not explain a significant ($p < 0.05$) amount of change in the model (estimated using log-likelihood tests, and maximum likelihood estimation), commencing first with the highest-order interactions between random effects. We used this step-wise elimination approach to first

simplify the random effects component of the model, before proceeding to the fixed effects component of the model. Once we had derived our final model, we estimated the variance attributable to each random effect using restricted maximum likelihood (REML) estimation. We then used the *Anova* function of the *car* package (37) to estimate the significance of the fixed effects in the final model, using a Type III model and Chi-square distribution.

In the above analysis of the full dataset, we found that the second-order interaction term mtDNA haplotype \times sex \times diet explained statistically significant variation in longevity (log-likelihood ratio test, $\chi^2 = 61.114$, $p < 0.0001$). To further probe this three-way interaction, we divided the dataset into two and analysed the male and female data separately. In this second step of the analysis, we built separate *lmer* models for each sex-specific dataset with mtDNA haplotype (13 levels), dietary P:C ratio (2 levels) and the first-order interaction between these two variables as fixed effects. In each sex-specific model, we included strain duplicates (26 levels), experimental blocks (4 levels), technical replicates (52 levels), higher-order interactions between random effects, and higher-order interactions between fixed and random effects as random effects. We took the above described step-wise elimination approach to retain or reduce random effects from the model and derived a final reduced model using the log-likelihood ratio tests in R. The standard deviation attributable to each random effect and their higher-order interactions were estimated using the restricted maximum likelihood method from the final reduced model. The significance of the fixed effects was estimated from the Type III Wald's Chi-squared test using the maximum likelihood estimation approach in the *car* package.

In a third analysis, we estimated levels of mitochondrial genetic variance for longevity across haplotypes separately for each sex and diet combination. In this analysis, for each combination of sex and diet, we built a random effect *lmer* model with longevity as the response variable, and mtDNA haplotype, strain duplicate, technical replicate, and first-order interactions between mtDNA haplotype and block, and strain duplicate and block as the random effects in the models. We estimated the variance attributable to each random effect from each of the sex and diet random effects models, using the restricted maximum likelihood (REML) estimation. The proportion of variance attributable to the mtDNA haplotypes was estimated from the variance of each random effect using a formula = $(\text{Var}(X_1)/(\text{Var}(X_1) + \text{Var}(X_2) + \dots + \text{Var}(X_n))) * 100$, where X_n is the random effect and ‘Var’ is the variance of each random effect estimated from the *lmer* model. In addition, we estimated the 95% confidence intervals for the variance of each random effect from the same model using a parametric bootstrapping approach in *confint.lmerMod* function in R.

The panel of 13 mitochondrial haplotypes used in this study was also used in an earlier study that examined the effects of mitochondrial haplotype on male and female longevity on a standard yeast-based diet (9). We obtained the mean longevity of all possible combinations of mtDNA haplotype \times sex from Camus *et al.* (2012) and estimated the mitochondrial genetic correlations for mean longevity assayed on the isocaloric diets (used in this study) and yeast-based diet (used by Camus *et al.* 2012). We ran these correlation tests in *boot* package in R (38). For all possible pair-wise comparisons between trait means, we estimated the Pearson’s correlation coefficients (r_p), and the 95% confidence intervals for the r_p . 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 method in *boot* package (38).

Results

Sources of variance affecting longevity

Both the mtDNA haplotype and dietary treatment affected the longevity of flies (*lmer* analysis, mtDNA haplotype: $\chi^2 = 116.115$, $p < 0.0001$, diet: $\chi^2 = 199.907$, $p < 0.0001$, Table 1). Moreover, longevity was affected by interactions between the mtDNA haplotype, sex and diet ($\chi^2 = 61.524$, $p < 0.0001$, Table 1). To further probe this interaction, we analysed the data separately for each sex and observed that the interaction between mtDNA haplotype and dietary P:C ratio was statistically significant only in males (*lmer* analysis, $\chi^2 = 41.038$, $p < 0.0001$, Table 2). Thus, in males, but not in females (*lmer* analysis, mtDNA haplotype \times diet: $\chi^2 = 12.089$, $p = 0.4386$, Table 2), the magnitude of the dietary-mediated longevity response was affected by the mtDNA haplotype (Figure 1A and 1B).

Our analyses of levels of mitochondrial haplotype variation for longevity, per sex-by-diet combination, showed substantial mitochondrial variation underpinning longevity in females across both diets, but lower levels of mitochondrial variation in males (Table 3). These sex differences in mitochondrial variation for longevity appear to be primarily attributable to low female trait values of two haplotypes (Brownsville and Oregon) relative to the other haplotypes on both of the diets, and a low female trait value of the Hawai'i haplotype on the high P:C diet (Figure 1C and 1D).

Generally, changes in dietary macronutrient balance affected the longevity of flies. In general, the diet with high P:C ratio caused early death in flies, with the longevity of both sexes reduced by approximately 35% across the mtDNA haplotypes, compared to the low P:C diet (Figure 1C and 1D). Moreover, levels of sexual dimorphism in longevity were larger on the low P:C diet, with the level of dimorphism, eroded on the high P:C diet (Figure 1C and 1D).

Mitochondrial genetic correlations for longevity, across diets

Generally, intra- and inter-sexual mitochondrial genetic correlations for longevity were positive across the two isocaloric diets used in our study (Figure 2). This means that haplotypes that conferred high longevity on the high P:C diet generally conferred high longevity on the low P:C diet relative to other haplotypes, with the rank order of haplotypes generally consistent across the sexes. In contrast, mitochondrial genetic correlations were weak to absent, when comparing longevity on the isocaloric diets, in which the protein content was determined primarily by the casein content, to longevity on the yeast-based diets of Camus *et al.* (2012). However, there was a signature of a negative mitochondrial genetic correlation involving male longevity when assayed on the high P:C casein diet and male longevity when assayed on the yeast-based diet (Figure 2). Furthermore, there were signatures of negative correlations, across haplotypes, for female longevity when sampled on the isocaloric diets and male longevity when sampled on the standard yeast-based diet.

Discussion

Here, we examined the contribution of mitochondrial genetic variation to longevity in male and female fruit flies subjected to diets differing only in macronutrient balance. We had two aims.

First, to determine whether previously observed male-biases in the magnitude of mitochondrial genetic variance for longevity, measured using the same panel of haplotypes, would be replicable across novel dietary contexts that differed in their ratios of proteins and carbohydrates, but not calorie content. Second, to test whether longevity was affected by $G \times E$ interactions involving the mitochondrial haplotype and dietary P:C ratio, and to test whether any such interactions were male-biased in their manifestation, as reported by Aw *et al.* (2017) who studied mtDNA haplotype-by-diet interactions across two of the haplotypes used in our current study. While we found evidence that mitochondrial genetic variation for longevity was sex-specific in its pattern, unexpectedly this pattern was seemingly attributable to lower levels of mitochondrial genetic variance for longevity in males maintained on the high P:C diet relative to males or females maintained on the other diets. This decrease in mitochondrial genetic variance in males on high P:C diets, resulted in a $G \times E$ effect that was apparent only in males, consistent with the results of Aw *et al.* (2017) over an extended mitochondrial panel that included eleven additional haplotypes than used by Aw and colleagues. We discuss the implications of our findings, in light of previous research into the Mother's Curse hypothesis, and recent developments in the study of phenotypic plasticity, and outline suggested avenues of future research enquiry.

A key prediction to arise from the Mother's Curse hypothesis is that the genetic variation that delineates the naturally-occurring mtDNA haplotypes within any given species will confer male-biases in the magnitude of its effects on phenotypic expression (25). Of those studies that have sought to test this prediction, the evidence for male-biases in levels of mitochondrial genetic effects has been mixed (9, 25, 39-41). Notwithstanding, in many studies, the number of haplotypes surveyed has been too few to accurately home in on true levels of intra-specific

mitochondrial variance underpinning the focal traits, whilst overcoming effects of sampling error (22, 42, 43). While in other cases, inferences have been deduced following inter-specific crosses which placed mtDNA haplotypes of one species alongside the nuclear background of a congeneric species with which the haplotypes have no recent evolutionary exposure (21, 40), potentially unmasking cryptic mitochondrial genetic variation (44), and complicating inferences.

In particular, few studies have screened for sex differences in levels of mitochondrial genetic variation for longevity, or the capacity for sex differences in patterns of $G \times E$ interactions, between mtDNA haplotypes and different dietary contexts, to affect longevity. In 2012, Camus *et al.* screened the same panel of 13 haplotypes in *D. melanogaster* used in our study and reported that the effects of mitochondrial haplotype variation on longevity were specific to males. While, Aw *et al.* (2017) utilised two of the haplotypes used in our study, to reveal mitochondrial genotype-by-diet interactions for longevity in males but not females, across four diets differing in the ratios of protein to carbohydrates. Inspired by these two studies, we set out to screen for sex-biases in effects of mitochondrial haplotype variation for longevity across two isocaloric diets that differed only in their P:C ratios. Contrary to previously reported findings by Camus *et al.* (2012), we did not detect a general male-bias in the magnitude of mitochondrial genetic variation for longevity in our study. Instead, levels of mitochondrial genetic variation were specific to particular combinations of diet in each sex, and in particular, were lower for males maintained on the diet with high P:C ratio. We contend that the discrepancy in our results relative to those of Camus *et al.* (2012) may stem directly from the novel protein sources used in our study (casein, peptone, albumen). Fruit flies of the species *D. melanogaster* have evolved over long timescales to derive their protein from the yeast of fermenting fruits, which is the

protein source used in our standard laboratory food and that used by Camus *et al.* (2012). In contrast, the casein-based protein provided to flies in our current study is derived from bovine milk, and likely to represent a novel protein source, whose constitution and relative contributions of essential amino acids, as well as vitamins and minerals, is potentially mismatched to that found in yeast on which flies have evolved. This point is particularly pertinent in light of recent research that has demonstrated that optimal life-history trait expression in fruit flies and mice can be achieved on diets in which the amino-acid constitution is matched to the relative representation of the amino acids found within a species' exome; a paradigm known as "exome-matching" (45).

Theory proposes that exposure to stressful, previously-unencountered environments can disrupt adaptive responses in plasticity, unmask cryptic genetic variation, and take individuals away from their fitness optima (44, 46). This may then account for the failure of our current study to replicate previously observed male biases in the effects of mitochondrial haplotype variation on longevity. Mother's Curse theory proposes that maternal inheritance will remove mtDNA mutations that harm female fitness but fail to screen the set of male expression-specific mutations and that this will lead to male biases in levels of mitochondrial genetic variation underpinning the expression of life history traits (23). Notwithstanding, the environmental arena in which selection acts on the mtDNA sequence should be key to this process. Novel environmental conditions, such as those provided by the isocaloric diets used here, will plausibly unmask cryptic genetic variation in the mtDNA sequence, which while benign or adaptive to females on the yeast-based diets in which the flies have evolved, may incur fitness costs on a novel diet. This contention is supported by our finding of positive correlations, across mtDNA

haplotypes, for longevity when measured across the two isocaloric diets used here (both for intra- and inter-sexual mitochondrial genetic correlations), but an absence of mitochondrial genetic correlations when comparing longevity on the isocaloric diets to longevity on the yeast-based diet of Camus *et al.* (2012). That is, the mitochondrial polymorphisms that affect the longevity of females reared on isocaloric diets do not affect the longevity of females when reared on yeast-based diets. Accordingly, we propose that the mitochondrial genetic variation affecting female and male longevity on the isocaloric diets is likely to be non-adaptive, given the lack of prior exposure of fruit flies to these isocaloric diets means that these environments will plausibly represent extreme environments (44). A priority for future research will be to test this idea by measuring levels of sex-specific mitochondrial haplotype variation across diets that differ in their macronutrient ratios, using yeast-based diets that are more closely aligned to the protein and carbohydrate sources that the flies have evolved to feed on in the wild.

As has been shown previously in a number of invertebrate and vertebrate species (5, 6, 47-49), diets of high P:C ratio conferred striking reductions in longevity; an effect that was upheld across each of the sexes in our study. However, the genetic architecture of such longevity responses to dietary modifications was generally thought to be strictly associated with genetic variation in the nuclear genome of fruit flies, nematodes and mice (50-52). Here, we have contributed to emerging studies that suggest that mitochondrial genetic variation plays an important role in mediating the link between dietary quality and longevity (21, 22). Finally, we point out while the strength of our experimental design is that we have been able to assess sex-specific patterns of phenotypic plasticity in longevity responses to dietary quality across a large panel of mitochondrial haplotypes, a limitation of the design is that these effects have all been assessed

within the one nuclear genetic background. Mitochondrial functionality hinges on interactions between polypeptides encoded by nuclear and mitochondrial genomes, and this point alone suggests that mito-nuclear interactions will be important in regulating the longevity phenotype (19, 20). Indeed, several studies have provided evidence to support this contention (21, 30). The next frontier will, therefore, be to explore whether previously reported evidence for Mother's Curse effects (9), and male-biases in levels of mitochondrial gene-by-environment interactions (22), are upheld or change when surveyed across multiple nuclear genetic backgrounds. Such research could reveal hidden complexity in the evolutionary trajectories of life-history traits if it confirms that the expression of traits such as longevity is routinely underpinned by epistatic interactions spanning two genomes, whose outcomes are moderated across environmental contexts.

Funding

This research was funded by the Australian Research Council (FT160100022 and DP170100165) to DKD. VNR was supported by a Monash Graduate Scholarship; DKD by the Australian Research Council (FT160100022), JH by the Natural Environment Research Council (NE/G00949X/1) and Australian Research Council (DP180101708).

Acknowledgements

We thank Brooke Zanco and Violeta Lopez for their help during the experiment. DKD conceived the experiment and sourced the funding; VNR, DKD and JH designed the experiment; JH designed and advised on the isocaloric diets; JR prepared the isocaloric diets; VNR conducted

the experiment and analysed the data. DKD advised on data analysis. VNR & DKD wrote the manuscript, JH & JR contributed to drafting and final preparation of the manuscript.

Conflict of interest: None.

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Tables

Table 1. Results from the final *lmer* model of the full dataset. The final model included only the significant fixed and random effects including their significant higher-order interactions. We used the Type III Wald's Chi-squared test to estimate the significance of each fixed effect and higher-order interactions in the final model. Furthermore, we used the restricted maximum likelihood method to estimate the variance attributable to each random effect in the final model.

| <i>Fixed effects</i> | <i>d.f.</i> | <i>Chi.sq</i> | <i>P - value</i> |
|------------------------|-------------|---------------|------------------|
| Intercept | 1 | 1200.023 | <0.0001 |
| mtDNA haplotype | 12 | 116.115 | <0.0001 |
| Sex | 1 | 63.698 | <0.0001 |
| Diet | 1 | 199.907 | <0.0001 |
| mtDNA haplotype × sex | 12 | 74.732 | <0.0001 |
| Sex × diet | 1 | 9.127 | <0.005 |
| mtDNA haplotype × diet | 12 | 15.664 | 0.207 |
| mtDNA × sex × diet | 12 | 61.524 | <0.0001 |

| <i>Random effects</i> | <i>Variance</i> |
|--------------------------|-----------------|
| Block | 2.5347 |
| Strain duplicate | 0 |
| Strain duplicate × diet | 1.0653 |
| Strain duplicate × block | 0.7578 |
| Technical replicate | 2.1564 |
| mtDNA haplotype × block | 1.2199 |
| Residual | 78.5497 |

Table 2. Results from the final sex-specific *lmer* models. The final reduced model for each sex was derived separately using log-likelihood ratio test in R. The models included only the significant fixed and random effects, along with their second-order interactions within and between fixed and random effects. We used the Type III Wald's Chi-squared test in *car* package to estimate the significance of fixed effects and their higher-order interactions from the final reduced model. The restricted maximum likelihood method was used to estimate the variance attributable to each random effect in the final reduced model.

a) Male longevity

| <i>Fixed effects</i> | <i>d.f.</i> | <i>Chi.sq</i> | <i>P - value</i> |
|--------------------------|-----------------|---------------|------------------|
| (Intercept) | 1 | 631.772 | <0.0001 |
| mtDNA haplotype | 12 | 45.289 | <0.0001 |
| Diet | 1 | 107.83 | <0.0001 |
| mtDNA haplotype × diet | 12 | 41.038 | <0.0001 |
| <i>Random effects</i> | <i>Variance</i> | | |
| Block | 4.069 | | |
| Strain duplicate | 0 | | |
| Technical replicate | 1.763 | | |
| MtDNA haplotype × block | 0.69 | | |
| Strain duplicate × diet | 1.661 | | |
| Strain duplicate × block | 2.220 | | |
| Residual | 81.729 | | |

b) Female longevity

| <i>Fixed effects</i> | <i>d.f.</i> | <i>Chi.sq</i> | <i>P - value</i> |
|------------------------|-------------|---------------|------------------|
| (Intercept) | 1 | 889.794 | <0.0001 |
| mtDNA haplotype | 12 | 82.801 | <0.0001 |
| Diet | 1 | 163.063 | <0.0001 |
| mtDNA haplotype × diet | 12 | 12.089 | 0.4386 |

| <i>Random effects</i> | <i>Variance</i> |
|--------------------------|-----------------|
| Block | 2.574 |
| Strain duplicate | 0 |
| Technical replicate | 2.288 |
| mtDNA haplotype × block | 2.753 |
| Strain duplicate × diet | 2.208 |
| Strain duplicate × block | 1.670 |
| Residual | 70.954 |

Table 3. Results from the random effects model analysed for each combination of sex and diet datasets. The proportion of variance was estimated only for the mtDNA haplotype in each model.

a) Male longevity on low P:C diet

| Random effect | Variance | 95% Confidence interval | Proportion of variance of mtDNA haplotype |
|------------------|-----------|-------------------------|---|
| mtDNA haplotype | 3.001e-13 | (-5.53, 0) | 0% |
| Strain duplicate | 0 | (-5.22, 0) | |

| | | |
|--------------------------|-------|---------------|
| Technical replicate | 5.23 | (2.39, 11.79) |
| mtDNA haplotype × block | 14.26 | (7.17, 31.62) |
| Strain duplicate × block | 11.55 | (5.96, 21.11) |
| Residual | 88.44 | (82.5, 93.59) |

b) Male longevity on high P:C diet

| Random effect | Variance | 95% Confidence interval | Proportion of variance of mtDNA haplotype |
|--------------------------|----------|-------------------------|---|
| mtDNA haplotype | 0.09 | (-0.47, 0.34) | 0.14% |
| Strain duplicate | 0 | (-1.49, 0) | |
| Technical replicate | 1.06 | (0.36, 4.23) | |
| mtDNA haplotype × block | 2.19 | (0.38, 7.84) | |
| Strain duplicate × block | 2.39 | (0.85, 6.22) | |
| Residual | 57.80 | (54.94, 62.04) | |

c) Female longevity on low P:C diet

| Random effect | Variance | 95% Confidence interval | Proportion of variance of mtDNA haplotype |
|-------------------------|----------|-------------------------|---|
| mtDNA haplotype | 10.51 | (1.62, 42.05) | 9.2% |
| Strain duplicate | 8.27e-12 | (-8.56, 0.3e-10) | |
| Technical replicate | 6.46 | (3.51, 14.1) | |
| mtDNA haplotype × block | 9.76 | (5.07, 21.16) | |

| | | |
|--------------------------|------|----------------|
| Strain duplicate × block | 5.29 | (1.78, 11.9) |
| Residual | 82.2 | (77.58, 87.28) |

d) Female longevity on high P:C diet

| Random effect | Variance | 95% Confidence interval | Proportion of variance of mtDNA haplotype |
|--------------------------|----------|-------------------------|---|
| mtDNA haplotype | 12.78 | (2.75, 29.11) | 17.18% |
| Strain duplicate | 0 | (-4.98, 0) | |
| Technical replicate | 3.28 | (1.72, 6.44) | |
| mtDNA haplotype × block | 2.85 | (0.49, 10.23) | |
| Strain duplicate × block | 5.43 | (2.89, 11.78) | |
| Residual | 50.07 | (47.18, 53.46) | |

Figure legends

Figure 1. The effects of interactions between mtDNA haplotype and dietary P:C ratios for longevity are shown as reaction norms for A) male and B) female longevity on low P:C (1:8) and high P:C (2:1) diets. Mean longevity \pm Standard Error for each combination of mtDNA haplotype and sex on C) the low P:C diet and D) the high P:C diet. The scales of Y-axis are adjusted for each diet in panels C and D to highlight the variation in longevity within each diet. In legend: low P:C indicates an isocaloric diet with P:C ratio = 1:8, high P:C is an isocaloric diet with P:C ratio = 2:1.

Figure 2. Intra- and inter-sexual mitochondrial genetic correlations for longevity across the different diets used in our study, and those of Camus *et al.* (2012) are shown here. Sex-specific longevity means for each mtDNA haplotype on isocaloric diets and yeast-based lab diet was estimated separately. Pearson's correlation tests were performed on all combinations of pairwise comparison between longevity means. In the horizontal axis, M refers to male, F is female, std – refers to standard laboratory food (yeast-based) used in Camus *et al.* (2012), carb refers to the low protein high carbohydrate diet (P:C = 1:8) and prot is the high protein low carbohydrate diet (P:C = 2:1) used in this study.

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Figure 1

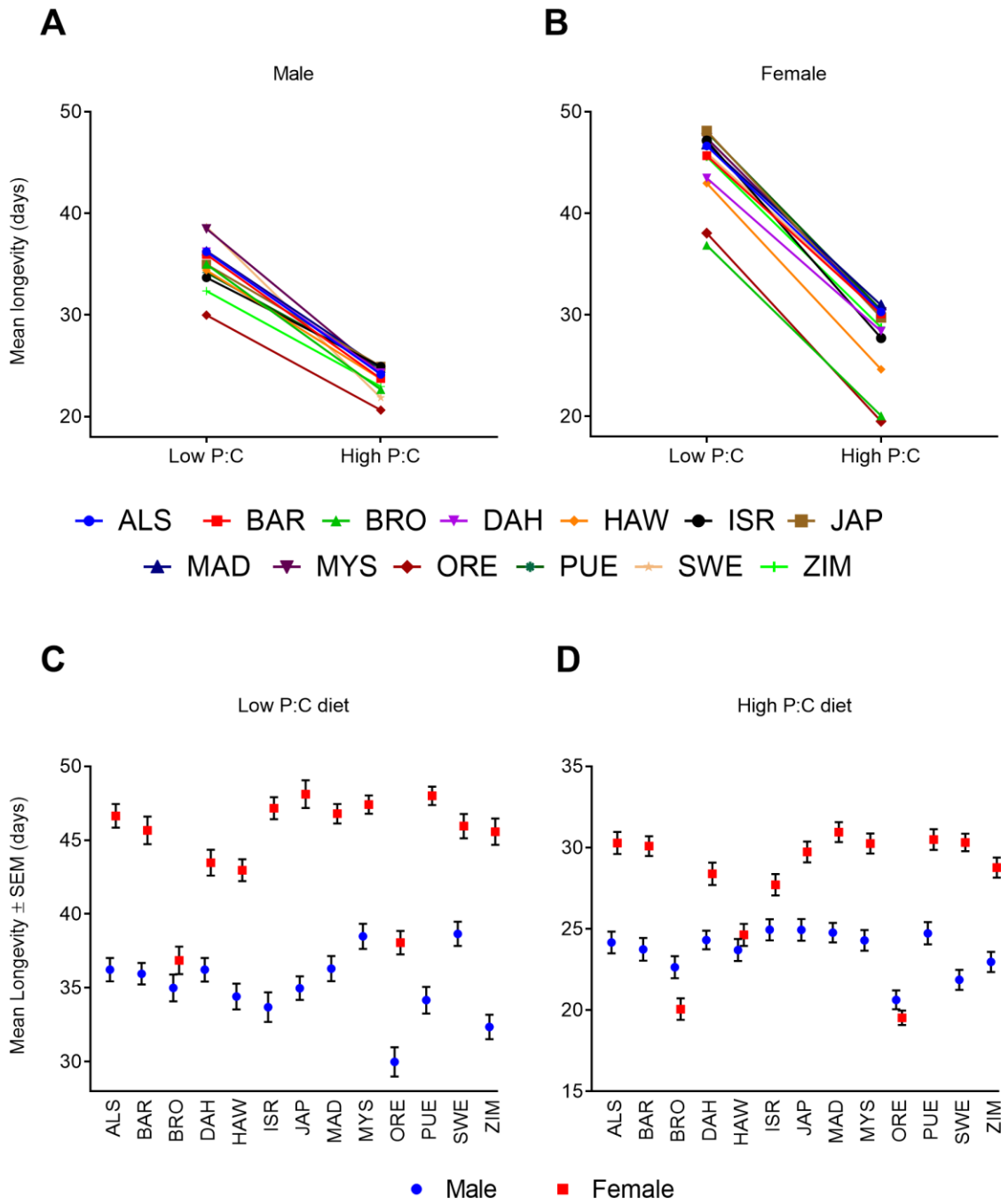


Figure 2

