1 **Mito-nuclear variation**
2 **in transgenerational and reproductive effects**
3 **of amino acid and lipid nutrition**
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Abstract

Animals vary genetically in responses to dietary change. Both mitochondrial and nuclear genomes contribute to this variation, but the role of combinatorial "mito-nuclear" genetic variation is understudied. We do not know whether specific nutrients modify patterns of mito-nuclear variation, nor whether putative epigenetic mechanisms play a role. Here, we show that enriching dietary essential amino acids or lipids modifies patterns of mito-nuclear variation in *Drosophila* life-history, including transgenerational effects of lipids. Systematically evaluating alternative statistical models revealed that diet-mito-nuclear interactions were a leading driver of phenotypic variation. Mito-nuclear genotype repeatably predicted phenotypic impacts of nutritional changes, but genotypes bearing naturally co-occurring pairs of mitochondria and nuclei did not necessarily outperform novel pairings, suggesting that nutrition-dependent phenotypes cannot easily be optimised by matching mitochondria to coincident nuclear genotypes. These results enhance understanding of how nutrition and genetics sculpt phenotype, with potential implications for human mitochondrial transfer therapies.

Introduction

Nutrients and a genetic code are the fundamentals of cellular life. Consequently, variation in nutrition and genotype are primary determinants of variation in health and biological fitness. Nutritional and genetic variation can also interact, meaning that different genotypes can respond distinctly to the same nutritional change (Dobson et al., 2015; Heymsfield and Wadden, 2017; Jumbo-Lucioni et al., 2010; Liao et al., 2009; Mulvey et al., 2017; Rikke et al., 2010). Biomedically, the interactive effects of diet and human genetic variation have led to interest in personalised nutrition (Ordovas et al., 2018), in which diets would be targeted individually, to treat maladies such as obesity and late-life morbidity (Fontana et al., 2010; Heymsfield and Wadden, 2017; Simpson and Raubenheimer, 2012). Biologically, unravelling genetic variation in how fitness is affected by nutrition will be a major part of bridging the genotype-phenotype gap.

Why do responses to diet vary? Ultimately, cellular metabolic function is the output of the coordinate activity of enzymes encoded by both the mitochondrial and nuclear genome. Both genomes exhibit sequence variation, yet the consequences of mitochondrial genome variation have often been overlooked. Effects of diet do indeed vary amongst mitochondrial haplotypes, altering metabolism and life-history (Aw et al., 2018; Bevers et al., 2019; Camus et al., 2020; Drummond et al., 2019; Mulvey et al., 2017; Nagarajan-Radha et al., 2019; Towarnicki and Ballard, 2018, 2017; Zhu et al., 2014). When both mitochondrial and nuclear genomes vary, we expect “mito-nuclear” variation, in which effects of variants in one can be either

buffered or augmented by variants in the other. In animals, this has been observed for fecundity (Mossman et al., 2016a), fertility (Camus et al., 2015), lifespan (Camus et al., 2015; Vaught et al., 2020), gene expression (Camus et al., 2015; Mossman et al., 2019, 2016b), and epigenomic modifications (Grunau et al., 2018). Novel mito-nuclear pairings can even change the course of nuclear genomic evolution (Healy and Burton, 2020), and lead to reproductive isolation (Hill, 2019; Ma et al., 2016). The intimacy of mito-nuclear coupling leads us to expect coadaptation (Dowling et al., 2008), and consequently to predict that pairs from the same population (sympatric) should function optimally. Some studies have supported this prediction (Baris et al., 2017; Chang et al., 2016; Ellison and Burton, 2006; Ma et al., 2016; Meiklejohn et al., 2013), but others have not (Dowling et al., 2010; Mossman et al., 2016b; Vaught et al., 2020). Despite genetic variation in responses to dietary variation, and the emerging importance of mito-nuclear interactions in overall genetic variation, there has been relatively little work to connect the three, by asking whether the phenotypic impacts of dietary alterations are subject to mito-nuclear interactions. Trailblazing work in *Drosophila melanogaster* has shown diet-mito-nuclear (DMN) variation in development time, lifespan and fecundity (Camus et al., 2020; Montooth et al., 2019; Mossman et al., 2016a; Rand et al., 2018; Zhu et al., 2014), but specific causal nutrients and cellular mechanisms remain to be established.

Perhaps diet's most immediate fitness effect is regulation of reproduction. Parents can gain fitness by increasing offspring quantity, or by promoting offspring quality (Lack, 1947; Smith et al., 1989; Stearns, 1992). Offspring quality and quantity will depend on fertility, fecundity, and development (viability) of those offspring, each of

which are influenced by diet. Equivalent changes in these traits can be elicited in distinct taxa by equivalent dietary changes: for example, in numerous studies of insects and mammals, amino acid nutrition has proven consistently important for fertility, fecundity and offspring development (Bong et al., 2014; Crean and Senior, 2019; Dong et al., 2016; Fanson et al., 2012; Grandison et al., 2009; Jensen et al., 2015; Lee et al., 2008; Leitão-Gonçalves et al., 2017; Liang and Zhang, 2006; Ma et al., 2020; Maklakov et al., 2008; McCracken et al., 2020; Mirth et al., 2018; Skorupa et al., 2008; Solon-Biet et al., 2015; Svajgr et al., 1972; Tufarelli et al., 2015; Winship et al., 2018; Wong et al., 2014a; Zanco et al., 2020); whereas high-lipid or high-sugar diets are obesogenic (Dobson et al., 2019; Hariri and Thibault, 2010; Jang et al., 2018; Rivera et al., 2015; Saud et al., 2015; Wong et al., 2014b; Woodcock et al., 2015; Zhao et al., 2020). However, importantly, reproduction is not only regulated by the individual's current diet: large nutritional fluctuations can induce enduring physiological and molecular changes, which can transmit across generations, influencing quality of offspring and even grand-offspring (Barker and Osmond, 1986; Barker and Thornburg, 2013; Deas et al., 2019; Duque-Guimarães and Ozanne, 2017; Holland et al., 2016; Li et al., 2018; Liang and Zhang, 2006; Öst et al., 2014; Rivera et al., 2015; Stefana et al., 2017; Wei et al., 2014; Winship et al., 2018). Thus, diet can influence reproduction via lasting, programmed effects on offspring quality, which point to epigenetic reprogramming, whilst acute effects of diet on both offspring quality and quantity point to metabolic changes. But do these effects vary amongst genotypes? If so, is variation shaped by mitochondrial genomes, nuclear genomes, or by mito-nuclear genotype?

Understanding health impacts of mito-nuclear interactions is not just a current topic in biology. It is also pertinent biomedically, because dietary intervention is a preeminent treatment for human mitochondrial disease (Gorman et al., 2016), and also following the advent of mitochondrial replacement therapies (Craven et al., 2010; Tachibana et al., 2018; Wolf et al., 2015). These procedures have been licensed in the United Kingdom and a few other countries (Cohen et al., 2020). They aim to treat mitochondrial diseases by placing embryonic nuclei into the cytoplasm, and therefore mitochondrial context, of a disease-free donor. Some argue that the conservation of mitochondrial function and the body of evidence for mito-nuclear interactions suggest that undesired consequences may ensue if the donor mitochondria function poorly with the nuclear genome (Dobler et al., 2018; Dunham-Snary and Ballinger, 2015; Reinhardt et al., 2013); although the UK Human Fertility and Embryology Authority concluded that risks remain “purely theoretical” (HFEA, 2016). To solve this debate it will be important to identify which traits to evaluate in patients, whether costs or benefits may be diet-dependent, and whether transgenerational variation may ensue.

Here, we identify specific nutrients sufficient to drive diet-mito-nuclear variation in *Drosophila* life-history, and show that specific mito-nuclear genotypes can exhibit long-lasting, transgenerational responses to transient dietary changes. We bred a panel of flies with replicated and fully-factorial mito-nuclear variation, and show that essential amino acids and lipids can elicit novel patterns of variation in fecundity and offspring performance (development time). We then show for the first time that the capacity for effects of parental diet on offspring performance are genetically variable,

and mito-nuclear in this case. To elucidate overall variation in fitness, we integrate our analyses of offspring quality and quantity, showing mito-nuclear variation in how diet influences reproductive investment; and also that “mis-matched” pairs of mitochondrial and nuclear genomes do not necessarily perform worse than matched pairs. Finally, we evaluate performance of alternative models of our extensive datasets, in which interactions between diet, mitochondrial and nuclear haplotype were systematically included or excluded. We find that models which include mito-nuclear or diet-mito-nuclear interactions are favoured overwhelmingly over models which assume no interaction, suggesting that models which do not account for these terms are not adequate to explain variance in fitness and health.

Results

We used a set of *D. melanogaster* lines comprising replicated, fully-factorial combinations of varied mitochondrial and nuclear genomes (Figure 1a), derived from heterogeneous populations originally isolated in Australia and Benin (formerly Dahomey). For brevity, we term Australian and Beninese genotypes *A* and *B*, respectively. The populations were introgressed either reciprocally ($AB = A$ mitochondria / *B* nuclei; $BA = B$ mitochondria / *A* nuclei), or to themselves to control for introgression and drift (i.e. $AA = A$ mitochondria / *A* nuclei; $BB = B$ mitochondria / *B* nuclei). In each generation, a fixed number of females per line were mated to a fixed number of males from the ancestral population. Each combination was replicated in triplicate, generating twelve lines altogether (e.g. $AA1$, $AA2$, $AA3$, $BA1$, $BA2$, etc). The lines were created to retain equivalent standing nuclear variation from the ancestral populations in each of the two mitochondrial backgrounds. Introgressions had been iterated for 74 generations at the time of our first experiment, and so the percentage of nuclear genome that remained unreplaced by introgression in the AB and BA genotypes was expected to be negligibly small ($\sim 5.3e-21\%$). Ancestors of all lines were cleared of cytoplasmic *Wolbachia* endosymbionts long prior to these experiments (Vaught et al., 2020), and so we expect cytoplasmic genetic complements to comprise only mitochondria. The *A* and *B* nuclear genomes do not appear to select for differential mitochondrial haplotypes in this design (Vaught et al., 2020), and we expect the rigorous backcrossing regime to minimize differential segregation of nuclear genomic variants over *A* and *B*

171 mitochondria: this experimental design should therefore produce fully-factorial mito-
172 nuclear variation.

173

174

Figure 1

Figure 1. Study design. A. Genetically distinct and heterogeneous populations of flies were originally isolated from Benin (formerly Dahomey), and New South Wales, Australia. Mito-nuclear variation was generated by reciprocally introgressing each line either into its own respective cytoplasmic background, or into the other line's cytoplasmic background. Three biologically independent replicate lines were established per mito-nuclear combination. 74 iterations of introgression were completed before experiments. **B.** Fly lines were cultured on rearing diet and then fed experimental diets in adulthood. The heatmap shows estimated content of different macronutrients, bars at the top indicating caloric content.

EAA and lipid nutrition are sufficient to drive diet-mito-nuclear variation in life-history

To identify nutrients which could drive DMN interactions, we added nutrients to a sugar-yeast diet (Bass et al., 2007), as previously (Dobson et al., 2018; Emran et al., 2014; Grandison et al., 2009). To ensure that potential novelty effects were evenly distributed amongst conditions, the baseline diet was distinct from the medium on which the lines were maintained and developed. For experimental diets, we added one of two nutrients to the baseline, selected for their important and conserved health impacts. Essential amino acids (EAAs) are metabolised by mitochondria (Mariño et al., 2014), and their specific enrichment is sufficient to recapitulate fecundity effects of adding yeast (Emran et al., 2014; Grandison et al., 2009). We also manipulated dietary lipid, because mitochondria metabolise fatty acids, and high-fat animal diets can model Western human disease (Hariri and Thibault, 2010; Heymsfield and Wadden, 2017). Lipid source can have large physiological effects (Brankatschk et al., 2018), and flies are largely vegan in nature (Knittelfelder et al., 2020). Therefore, we supplied a plant-based lipid which set in agar (i.e. in contrast to oils), by adding margarine to the diet (15% w/v, after (Woodcock et al., 2015)). Whilst this design did not control calories, it enabled us to test whether specific, ecologically-relevant nutrients can cause diet-mito-nuclear variation. Estimated nutrient contents (Lesperance and Broderick, 2020) are presented in Figure 1b.

Previous work showed that manipulating complex nutrient sources elicits life-history variation in specific mito-nuclear genotypes, including in fecundity (egg laying), and

210 offspring development (Camus et al., 2020; Montooth et al., 2019; Mossman et al.,
211 2016a; Rand et al., 2018; Zhu et al., 2014). We tested whether EAAs and lipid were
212 sufficient to cause these effects. Starting three days after adult emergence, the
213 control, EAA-enriched and lipid-enriched foods were fed to flies of all lines for seven
214 days. We restricted treatment to adults, to avoid developmental variation (Figure 2a).
215 This experiment was repeated twice. In both experiments, EAAs increased fecundity,
216 and lipids repressed fecundity. Fecundity was correlated between the two
217 experiments, indicating technical repeatability (Figure 2b). Biologically, two important
218 trends were evident. First, genetic replicates consistently grouped together, with all
219 three replicates of each mito-nuclear genotype tending to sit within one standard
220 deviation of each other (Figure S1), despite having been separated for >70
221 generations of introgression. This suggested a highly stereotyped effect of mito-
222 nuclear genotype on fecundity, which overcame any genetic drift in the fly lines.
223 Therefore, for simplification, we replotted the data without distinguishing
224 experimental or genetic replicates (Figure 2c). This outlined the second important
225 trend, that mito-nuclear variation was shaped by diet, in agreement with a GLMM
226 analysis indicating a three-way interaction ($p=0.009$, Table S1). To visualise pairwise
227 statistical differences between every genotype, we used *post-hoc* estimated marginal
228 means (EMMs (Searle et al., 1980)) analysis. These EMMs represented the GLMM
229 output per experimental condition, and so their correlation to the raw data indicated a
230 good model fit (Figure 2c). Pairwise comparison of EMMs was consistent with our
231 interpretation that overall patterns of among-genotype variation in fecundity were
232 diet-dependent (Table S2). These results revealed distinct patterns of mito-nuclear
233 variation on different diets, and that impacts of consuming a certain diet depend on

234 mito-nuclear genotype.

235

236 Animal fitness is a function of both quantity and quality of offspring, and DMN
237 variation in offspring development has already been documented. Having shown that
238 EAAs and lipid were sufficient to cause DMN variation in egg laying, we
239 hypothesised that variation in offspring quality may additionally manifest in fertility of
240 those eggs. To test this, after parents had been fed experimental diets for a week as
241 previously, we allowed eggs laid over 24h to develop in the same experimental
242 media. We scored time to adult and pupal emergence, along with sex in adults. We
243 take proportion of eggs emerging as a metric of parental reproductive success, and
244 time to development as a metric of relative offspring fitness. In every mito-nuclear
245 genotype, food enrichment with EAAs or lipid either reduced the proportion
246 developing, extended development time, or both. However, the magnitude of these
247 effects was highly genotype-dependent (Figure 2d). For explicit analysis, we fit Cox
248 mixed-effects (CME) survival models. The DMN interaction was statistically
249 significant for emergence of both males and females to adulthood (each sex
250 $p < 0.0005$, Table S3, Figure S2). We did not detect robust sex-specificity
251 (Supplementary Text, Figure S2), and results were broadly repeatable within the
252 triplicates of each fly genotype (Figure S2). To confirm genotype-specific effects, we
253 applied *post-hoc* EMM tests to the CME model. This analysis aggregated time to
254 development and proportion developing into a single quantitative output, because
255 the EMMs were a posterior view of our CME analysis, which integrates time to
256 development and proportion developing. In this framework, high EMMs correspond
257 to faster and/or more frequent development, and vice-versa (Figure 2e). These

EMMs confirmed that EAA or lipid enrichment disadvantaged specific genotypes (Figure 2e). The *AA* flies bore a particular cost of reduced survival on EAA food. The differential ranking of EMMs across genotypes on different diets indicated that the pattern of mito-nuclear genetic variation was dependent on EAA and lipid nutrition. Indeed, as with fecundity, there was little variation within each triplicate per mito-nuclear genotype (except that the detrimental effect of EAAs on development of *AA* flies was pushed to lethality in the *AA3* line, and *BB3* was somewhat more lipid-resistant than *BB1* and *BB2* (Figure S2)), suggesting that the developmental response to nutrition is highly stereotyped by mito-nuclear genotype. A parallel analysis of these same individuals' time to pupation revealed congruent patterns (Figure S3, Figure S4, Table S4). Altogether, these results show that either lipid or EAA enrichment are sufficient to potently modify developmental outputs of mito-nuclear genetic variation.

Figure 2

Figure 2. Diet-mito-nuclear interactions modulate *Drosophila* fecundity. A.

Experimental design. The 12 mito-nuclear lines were reared from egg to adult on rearing food, and allocated at random to experimental media 6-48h after eclosion, at a density of five of each sex per vial. After seven days, flies laid eggs on fresh food for 24 hours. **B.** Intra-line correlations in impact of diet-mito-nuclear variation on egg laying. Each point shows mean egg laying per line per diet in each of two replicate experiments, with the replicates of each haplotype grouped by dashed lines. Means were correlated between experiments (Pearson's $r = 0.87$, $p = 7.6e-12$). **C.** Impact of diet on fecundity is determined by mito-nuclear variation. Data are pooled across the experimental and genetic replicates presented in panel B. Boxplots show medians, first and third quartiles and 5th and 95th percentiles. Connected points to right of each box show *post-hoc* comparisons of means (by estimated marginal means with 95% confidence intervals, calculated from a generalised linear mixed effects model, showing exponent of EMMs in order to fit to original data scale). Translucent points to left of each box show raw data. Complementary statistical analysis presented in Table S1 and Table S2. **D.** Kaplan-Meier plots of development (egg-to-adult) after parents fed and offspring developed on experimental media. Mito-nuclear genotypes are indicated in top-left of each panel. **E.** Estimated marginal means (EMMs) with 95% confidence intervals summarising survival analysis (Cox mixed effects) of data from D. Higher values correspond to faster development and/or increased fertility, lower values correspond to slower development and/or reduced viability. Accompanying statistical analysis presented in Table S3.

Persistent life-history effects of dietary EAAs and lipid in specific mito-nuclear genotypes

DMN interactions could occur via numerous mechanisms, but no candidate has yet been confirmed. Dietary variation induces not only immediate changes to metabolism, but also long-lasting epigenetic changes (Barker and Osmond, 1986; Barker and Thornburg, 2013; Deas et al., 2019; Dobson et al., 2017; Duque-Guimarães and Ozanne, 2017; Holland et al., 2016; Li et al., 2018; Liang and Zhang, 2006; Öst et al., 2014; Rivera et al., 2015; Stefana et al., 2017; Wei et al., 2014; Winship et al., 2018). Mitochondria metabolise substrates for epigenetic modifications (Eisenberg et al., 2014; Mariño et al., 2014; Trefely et al., 2020), suggesting that nutritional regulation of epigenetically-encoded traits may vary amongst mito-nuclear genotypes. We asked whether EAAs and lipid might drive DMN interactions for life-history via long-term reprogramming. Whilst such effects are consistent with epigenetic modifications, we conservatively avoid that term for our data because we did not measure epigenetic marks directly. We tested for long-lasting effects of transient EAA and lipid enrichment, both in the generation that experienced the nutritional manipulation, and in their offspring.

We conducted a second fecundity experiment, with a week of adult-onset feeding on experimental diets as previously, but followed by a diet switch back to standardised rearing medium. Fecundity was measured at the end of experimental diet feeding, and 24h after switching back to standardised medium, yielding vial-matched egg counts pre- and post- switch (Figure 3a). The overall effect of experimental diets was

equivalent before and after the switch (EAAs elevated fecundity, lipid repressed fecundity), confirming that effects of experimental diets persisted for the 24h feeding on standardised diet (Figure 3b). Before the switch, on experimental diets, a DMN interaction was once again evident (Figure 3c, Table S5, Figure S5a), and pooling these data with preceding data (Figure 2) yielded the same qualitative result (Figure S6, Table S6), confirming that DMN effects were consistent across experiments.

A GLMM analysis confirmed that, after switching back to standardised diet, the main effects of prior EAA or lipid feeding persisted (Figure 3d, Table S7, Figure S5b). However, the DMN interaction did not persist (Figure 3d, Table S7, Figure S5b). These findings suggested that EAA and lipid modify the manifestation of mito-nuclear variation in fecundity via transient effects; on top of longer-term impacts which do not depend on mito-nuclear genotype. If so, recovery of fecundity after diet switch should exhibit a DMN interaction. We estimated recovery rate as the ratio of eggs laid per vial after/before the switch, with greater ratios indicating greater plasticity. Recovery from EAA or lipid feeding was indeed variable amongst mito-nuclear genotypes (Figure 3e; GLMM $p=2.91E-09$, Table S8; Figure S5c). Altogether, these analyses revealed two layers of variation, with genotype-independent main effects of dietary EAAs and lipid which persist after feeding, and additional DMN variation whilst feeding on the diets, but not after. This suggested that EAAs and lipid can have lasting fecundity effects which generalise amongst mito-nuclear genotypes, but did not exclude the possibility of longer-term, transgenerational diet-mito-nuclear impacts on offspring.

345 Diet can modulate fitness by effects that span one or more generations (Camilleri-
346 Carter et al., 2019; Deas et al., 2019; Öst et al., 2014; Rivera et al., 2015). We are
347 not aware of a prior demonstration of genetic variation in such transgenerational
348 reprogramming, nor of whether it is subject specifically to mito-nuclear variation.
349 Since we had already observed that feeding EAAs and lipid to both parents and
350 offspring modified the manifestation of mito-nuclear genetic variation in development,
351 we speculated that some of that variation could be due to differential legacies of
352 parental diet. If so, we expected that feeding EAAs or lipid to parents alone would be
353 sufficient to modify patterns of mito-nuclear variation in offspring development, even
354 when all offspring developed in a standardized medium.

355

356 We restricted diet manipulations to adults for one week, before egg-laying on
357 standardised developmental medium, and allowing those eggs to develop on that
358 standardised medium. This design differs from the preceding study of developmental
359 effects (Figure 2d-e), because diet was only manipulated in the parental generation.
360 Therefore any diet-mito-nuclear effect (or lower-order diet effect) detected in this
361 experiment is transgenerational. We scored time to adult emergence (along with sex
362 and pupation), and again, results were consistent within each triplicate fly genotype
363 (Figure S2), and so data were plotted after pooling all three replicates per genotype.
364 Plotting these data suggested that, indeed, parental nutrition had genotype-specific
365 effects on development (Figure 3f). This was confirmed by CME analysis (Table S9,
366 males $p=0.003$, female $p=0.03$), without strong evidence of sex bias (Supplementary
367 Text, Figure S2). EMM analysis confirmed genotype-specific impacts (Figure 3g):
368 Specifically, AA flies developed on average a full day faster when parents were fed

lipid-enriched food (Figure 3f). A parallel analysis of these same individuals' time to pupation revealed congruent patterns (Figure S3, Figure S4, Table S10). Whilst some variation may be due to selection, our experimental design largely excludes this possibility, because the only nutritional variation that individuals under study experienced was *in utero* when mothers fed on experimental diets, before post-embryonic development in standardised medium; and variation in rearing density was also accounted for statistically. Furthermore, since the major effect we observe is an acceleration of development in one condition (AA flies fed lipid), selection would have to be otherwise ubiquitous but relaxed specifically for this condition. The results therefore indicate that specific mito-nuclear genotypes are susceptible to transgenerational effects of parental nutrition, in this case lipid.

Notably, the transgenerational effects we observed in development did not correlate patterns when both parents and offspring were fed the diets. Our studies of transgenerational and intergenerational effects of EAAs and lipid were conducted at the same time (Figure 2d-e flies developed from eggs plotted in Figure 3c, Figure 3f-g from eggs in Figure 3d), validating this comparison. Altogether, this discord suggests that nutrients can induce transgenerational effects in specific mito-nuclear genotypes, but this reprogramming in combination with ongoing nutrient enrichment in offspring leads to additional changes to patterns of phenotypic variation.

Figure 3

Figure 3. Performance is determined by interplay of mito-nuclear genotype and persistent intragenerational and transgenerational effects of diet. A.

Experimental design. 12 Mito-nuclear lines were reared from egg to adult on rearing food, and allocated at random to experimental media 6-48h after eclosion, at a density of five of each sex per vial. After seven days, flies laid eggs on fresh food for 24 hours. Females were then reassigned back to standardised rearing medium, and eggs were laid for a further 24h. **B.** Correspondence between egg laying rate per vial, before and after switch from experimental diets. Egg laying rates before and after switch across all diets were correlated (Pearson's product-moment correlation, $r=0.65$, $p<2.2e-16$). Line of equivalence shown in grey. **C.** Egg laying rate before dietary switch is determined by mito-nuclear variation (*vis* Fig 2C). Boxplots show medians, first and third quartiles and 5th and 95th percentiles. Connected points to right of boxes show *post-hoc* comparisons of means (by estimated marginal means with 95% confidence intervals, calculated from a generalised linear mixed effects model). Translucent points to left of each box show raw data. Complementary statistical analysis presented in Table S5. **D.** Diet-mito-nuclear interactions do not persist 24h after switch from experimental media. Statistical analysis presented in Table S7. **E.** Mito-nuclear interactions govern recovery of fecundity after switch from experimental media. Statistical analysis presented in Table S8. **F.** Kaplan-Meier plots of development to adulthood of eggs laid on developmental media, after parents had previously fed on experimental media. Mito-nuclear genotypes are indicated in top-left of each panel. **G.** Estimated marginal means (EMMs) with 95% confidence intervals summarising survival analysis (Cox mixed effects) of data from F. Higher values correspond to faster development and/or increased fertility, lower values

415 correspond to slower development and/or reduced viability. Accompanying statistical
416 analysis presented in Table S9.
417

Mito-nuclear (mis-)matching does not predict differential life-history responses to nutrition

Together, offspring quantity and quality define fitness (Lack, 1947; Stearns, 1992). Our analyses suggested that both fecundity (quantity) and fertility (quality) are regulated by EAAs and lipid, but specific mito-nuclear genotypes are differentially receptive or resistant to these effects. To date, the widely-held prediction of a disadvantage to “mis-matched” mito-nuclear pairings, has received equivocal support (Baris et al., 2017; Chang et al., 2016; Ellison and Burton, 2006; Ma et al., 2016; Meiklejohn et al., 2013). Our datasets provided an opportunity to ask whether this prediction applied in terms of differential responses to specific nutrients; whilst taking an integrative view of fitness, encompassing both offspring quality and quantity, and putatively epigenetic effects. To this end, we integrated outputs of our previous analyses by plotted EMMs from against one another, indicating effects of diet and mito-nuclear genotype on offspring quality and quantity, and the relationship between them. We did this separately for data from parents and offspring both fed experimental diets (flies in Figure 2e, which developed from eggs in Figure 3c), and from parent-restricted feeding (flies in Figure 3g, which developed from eggs in Figure 3d).

First, we examined impacts of EAA feeding. Visual inspection indicated that EAA enrichment enhanced offspring quantity, but compromised offspring quality in flies of all genotypes except *BB* (Figure 4a). This was surprising, because we had assumed

that parents should discriminate diets that maximise their own fitness, and therefore that parental egg-laying would correlate offspring development indices. Instead, the apparent tradeoff indicates either that (1) EAAs signal diet quality to parents on normal diets when yeast is the source of most nutrients (including protein), but our manipulations rendered this quality signal misleading (Zanco et al., 2020); or that (2) diet is subject to parent-offspring conflict (Trivers, 1974), with parents maximising their fitness by producing more eggs on high EAA-diets, despite the cost to individual offspring.

Were costs of EAAs genotype-specific? The quality-quantity tradeoff induced by EAA feeding was exaggerated dramatically in *AA* flies, whose eggs developed exceptionally poorly on EAA-enriched food. However, this cost of EAAs was rescued in both *BA* and *AB* genotypes, indicating that neither *A* mitochondria nor *A* nuclei alone were sufficient for *AA*'s diet-dependent phenotype, and therefore that these effects are mito-nuclear (Figure 4a). This outlined a second surprising finding, in which a sympatric mito-nuclear pairing endured a higher cost of dietary EAAs than allopatric pairings (Figure 4a). Thus, unexpected, costly, and definitively mito-nuclear patterns of variation can emerge on specific diets. However, no clear effects of past EAA feeding on offspring quality or quantity were evident (Figure 4b). Together, these results indicated that EAAs interact with mito-nuclear genotype during feeding, but not thereafter. This suggests that the reproductive DMN variation we observed upon EAA enrichment is an acute effect of diet, and therefore likely metabolic and not epigenetic.

Next we examined the impact of lipid enrichment. Lipid mostly compromised both quality and quantity, but one genotype stood out: *AA* flies were most resistant to the reduction in offspring quality induced by high-lipid diet, and their development index on lipid-enriched food was in fact better than on EAA-enriched food (Figure 4a). By this metric, *AA* flies' performance was substantially better than the *AB* or *BA* flies, confirming that neither *A* mitochondria nor *A* nuclei alone were sufficient to explain *AA*'s response to diet. This again suggested that genotypes which exhibit notable responses to diet do not necessarily bear allopatric combinations of mitochondria and nuclear genomes. Furthermore, the finding that the *AA* genotype was beneficial in one condition (EAA) despite costs in another (lipid) reveals that it was not simply sick as would be expected with a poor-functioning mito-nuclear pairing, but instead exhibits its own genotype-specific pattern of response to diet.

Finally, we evaluated the impact on offspring of parent-restricted lipid enrichment. All genotypes bore costs, but impacts were highly genotype-specific. Again, the *AA* flies stood out. Specifically, *AA* flies bore a cost of parental lipid feeding on offspring quantity, but appeared to enjoy a compensatory benefit from enhanced offspring quality. In fact, these flies exhibited the highest offspring quality (development index) observed in any condition in our study. These benefits were not evident in either the *AB* or *BA* flies, confirming again that neither *A* mitochondria nor *A* nuclei alone are sufficient to explain diet-dependent phenotype: the observed effects are mito-nuclear.

489 Altogether, these results confirm diet-mito-nuclear shaping of fitness. They show that
490 diet can induce phenotypic change in specific mito-nuclear genotypes, with neither *A*
491 mitochondria nor *A* nuclei accounting for the *AA* flies' differential response to diet.
492 This suggests that mito-nuclear "matching" is not necessarily optimal. In wild
493 populations, the cost of EAA susceptibility may be balanced by lipid resistance, in
494 which case these flies were not sick in all circumstances, but rather exhibit a private
495 pattern of genotype-specific variation, which is evident only on specific diets.
496

Figure 4

Figure 4. Mito-nuclear interactions dictate dietary modulation of offspring quality versus quantity. Panels show relationships per diet and per genotype (data from Figures 2-3). **A** offspring development index and eggs laid, when both parents and offspring were fed experimental diets, and **B** development index before switch and release of egg laying after diet, when parents were switched from experimental to standard diets. Points show EMMs $\pm$ 95% confidence intervals, and grey lines indicate grand means.

Diet-mito-nuclear effects cause substantial biological variation

Finally, we sought to test the importance of the DMN interactions that we identified for overall variation in our dataset, for two reasons. First, because the DMN patterns that we identified may be of previously-unrecognised importance in nature. Second, because mitochondrial transfer therapy (Craven et al., 2010; Tachibana et al., 2018; Wolf et al., 2015) presents the possibility of a medical procedure giving patients novel combinations of mitochondrial and nuclear genomes, which our results suggest may lead to unpredictable responses to nutritional variation. Therefore, we sought effect sizes of DMN terms, complementing our preceding analyses by outlining whether DMN effects were substantial, or biologically trivial despite statistical significance.

Since *de facto* effect sizes for interaction terms are not easily interpreted, we used optimization procedures to compare simple models of the data against those including DMN interactions (Akaike, 1974). Calculating Akaike Weights reveals performance amongst a set of models, in terms of the relative probability of being the best description of the data (Wagenmakers and Farrell, 2004). We applied this procedure to all our previously-presented models (Figures 2-3, Figure S5), and simpler alternatives. Cases where this analysis favoured including DMN interactions argued for the importance of this novel tier of variation in health, which mitochondrial transfer will influence in ways that we cannot currently predict.

Akaike Weights suggested that models accounting for DMN interactions performed best described all datasets but one, and that purely additive models (assuming no interactions) were deficient by comparison (Figure 5).

For fecundity data (Figures 2-3), we fit a structured series of models, systematically including or eliminating all possible interactions amongst diet, mitochondrial and nuclear genotype. Akaike Weights favoured including DMN interactions (Figure 5a-d): for example, a model of data from Figure 2c including DMN interactions was 61.7 times more likely to be the best model than another with no interactions (Figure 5a); and approximately twice more likely than the next-best, which retained only the diet:nuclear interaction (Figure 5a). Models including DMN terms were also the best descriptor of fecundity before switching from experimental media (data from Figure 3c), and the release of fecundity after switching (data from Figure 3e). The exception to the emergent rule was the case of fecundity after switching from experimental media (Figure 3d), in which the favoured model included a mito-nuclear term, but not a DMN term. This exception demonstrated that the most complex models were not favoured automatically, but therefore encouraging confidence in other cases, suggesting that DMN interactions do indeed explain substantial variation.

For development data (Figures 2e, Figure 3f, Figure S4-S6), including terms for offspring sex added an additional order of complexity (i.e. a potential four-way interaction), rendering an assessment of all possible combinations hard to interpret. Therefore, we calculated Akaike Weights for models including either only first-degree terms (a purely additive model, without interaction terms), second-degree terms (all

pairwise combinations), third-degree terms (all three-way interactions), or fourth-degree terms (all four-way interactions, but only when sex was known, in models of adult emergence). We did this both for development to pupa (Figure 5e-f) and to adult (Figure 5g-h), and when diet was manipulated either for both parents and offspring (Figures 5e and 5g), or solely for parents (Figures 5f and 5h). In each case, models including all possible interaction terms performed best, often by several orders of magnitude (note logarithmic scale on figures). This analysis indicated that DMN interactions exert a substantial influence on developmental variation.

In summary, our modelling indicates that key health and fitness metrics - fecundity and development - are subject to significant interactions amongst mito-nuclear genotype and diet, and that those interactions account for significant variation in our data, which reflects the real-world mosaic of diet-mito-nuclear variation.

Figure 5

Figure 5. Models including interactions amongst mitochondrial and nuclear genotypes and diet treatment are favoured over alternatives. Barplots indicate Akaike weights analyses of sets of alternative models of data from Figures 2-3. Columns to left of each panel indicate terms in each model. Higher values indicate a better-supported model. The probability that one describes data better than another can be calculated by the ratio of their respective Akaike Weights. Plots compare models of data of fecundity of varied mito-nuclear genotypes fed experimental diets (**A**, data from Figure 2; and **B**, data from Figure 3c), after feeding on experimental diets (**C**, data from Figure 3d), and the ratio of fecundity before and after switching from experimental to standardised diets (**D**, data from Figure 3e). Plots E-H compare models of development time and rate, when models included either no interactions between predictive terms, or various levels of interactions as indicated, for development to pupa when experimental diets were fed to either parents and offspring (**E**, data from Figure S3) or parents alone (**F**, data from Figure S3), and development to adult when experimental diets were fed to either parents and offspring (**G**, data from Figure 2) or parents alone (**H**, data from Figure 3).

586

587

Discussion

588

589 Diet is a major source of biological variation. Evidence is now mounting that mito-
590 nuclear interactions are also a substantial source of variation, with implications in
591 areas as diverse as mitochondrial disease, ageing, evolution of sex, and speciation
592 (Ballard and Melvin, 2010; Đorđević et al., 2017; Gemmell et al., 2004; Gershoni et
593 al., 2009; Havird et al., 2015; Hill, 2017; Innocenti et al., 2011; Latorre-Pellicer et al.,
594 2019; Milot et al., 2017; Reinhardt et al., 2013; Wallace and Chalkia, 2013). Our
595 work complements previous studies showing that diet and mito-nuclear variation
596 interact to produce complex phenotypes, with diet enhancing or reducing the
597 consequences of mito-nuclear interactions. We now reveal specific nutrients which
598 are sufficient to drive these effects. The nutrients we identify are of particular
599 interest, because EAAs are currently attracting a great deal of attention for their
600 extensive regulation of swathes of life-history and health traits, whilst lipid
601 consumption is associated with the pandemic of metabolic disease in humans
602 (Simpson and Raubenheimer, 2012). We now show that responses these nutrients
603 are exaggerated in some mito-nuclear genotypes, and buffered in others. An
604 additional, and perhaps more important novel finding, is that transgenerational
605 effects of these nutrients can emerge in specific mito-nuclear genotypes. These
606 interactions appear to explain substantial variance in reproductive traits and life-
607 history. The Darwinian view that reproduction subjugates all other processes
608 suggests that many further traits may be similarly regulated.

609

610 Transient dietary alterations and metabolic disease can drive persistent molecular
611 and phenotypic change, within and across generations (Dobson et al., 2017; Duque-
612 Guimarães and Ozanne, 2017; Holland et al., 2016; Stefana et al., 2017). Our study
613 now shows that specific mito-nuclear genotypes are susceptible to a
614 transgenerational effect of lipid nutrition. To our knowledge, this is the first
615 demonstration of any form of genetic variation in transgenerational effects of diet.
616 Currently, we do not know how this transgenerational effect of lipid is transmitted, but
617 it may be due to altered epigenetic marks, nutrient provision from mother to
618 offspring, or microbiota. It will be interesting in future work to ask whether mito-
619 nuclear genotype shapes further aspects of non-genetic inheritance, and identify
620 mechanisms. Epigenomic profiling will likely prove insightful. Lipids and mito-nuclear
621 genotype may interact via mitochondrial metabolic processes such as beta-oxidation,
622 or by affecting rates of mitochondrial fusion and fission (Senyilmaz et al., 2015). It
623 will also be interesting to ask whether the transgenerational effects we have
624 identified in F₁ descendants extend further, to subsequent generations. These
625 findings indicate that detailed pedigrees containing both mitochondrial and nuclear
626 information may be required to predict transgenerational effects of nutrition.

627

628 A significant outcome of our study is that impacts of diet on offspring quality and
629 quantity do not necessarily correlate, and their relationship is subject to mito-nuclear
630 variation. We were surprised that EAA enrichment did not enhance offspring
631 development, because parental preference for laying eggs on this food led us to
632 expect that EAAs would promote offspring anabolism. This discrepancy may indicate
633 that EAAs function as signals of food quality as well as metabolites. This dual role

could drive deleterious outcomes when EAA levels are not representative of the composition of food that would be found in the yeasts which flies are thought to consume in nature. Alternatively, the discrepancy may prove an example of parent-offspring conflict (Trivers, 1974), in which EAAs promote parental fecundity, despite the detriment of reduced investment into individual offspring. How parent-offspring conflict is regulated at the interface of diet and genetic variation in general is not well-studied.

To our knowledge, our study is the first investigation of diet-mito-nuclear interactions to manipulate specific nutrient classes, rather than a complex ingredient such as yeast. Our finding that impacts of dietary lipid depend on mito-nuclear genotype may be relevant to understanding variation in impacts of high-fat human diets. The high-EAA diets that we used have parallels to high-protein anabolic diets used to increase yields of livestock and human muscle mass. Our findings that EAAs generally decrease offspring quality may give pause for thought in use of these diets. Furthermore, the finding of mito-nuclear variation in the response to EAAs enrichment demonstrates that certain individuals may bear particularly strong costs of eating such diets. At present, we do not know the mechanistic basis of the diet-mito-nuclear interactions that we have uncovered, but the transgenerational effects hint at epigenetics, consistent with preceding findings that epigenetic marks respond to genetic variation in the cytoplasm (Bellizzi et al., 2012; Grunau et al., 2018; Vivian et al., 2017; Yan et al., 2011).

Our findings have implications for understanding variation that could emerge when novel mito-nuclear pairings come to be, such as after mitochondrial replacement therapy. Our results do not necessarily support the proposal to “match” mito-nuclear pairs as a positive predictor of therapeutic success (Dowling et al., 2010; Mossman et al., 2016a; Vaught et al., 2020), because a naturally co-occurring mito-nuclear pair (AA) responded uniquely to diet, suggesting that costs and benefits are not necessarily straightforward functions of mito-nuclear matching or mis-matching. Thus, comparing performance of matched versus unmatched groups may not be sufficient to evaluate risks of mitochondrial transfer therapy. Our analysis suggests that mitochondrial transfer may be variously deleterious, beneficial, or have unforeseen costs and benefits. Dietary recommendations may promote patient health. Our data also lend kudos to the suggestion of transgenerational effects of mitochondrial replacement therapy, especially when diet varies. A change of nomenclature may ultimately be called for, since “matched” and “mis-matched” carry intrinsic value judgments, but it seems that whether mitochondria and nuclei are of shared origin does not necessarily predict cost or benefit. We suggest that deep knowledge of patient and donor nuclear and mitochondrial genotype will likely be required to predict a healthy match.

Our results have wider implications for the conduct of nutritional and genetic research. Fly nutrition studies have drawn scrutiny for dietary variation between studies, attributed to methodological reporting (Lesperance and Broderick, 2020) and stochastic differences in ingredients (principally yeast) over time (Piper et al., 2014), which may limit repeatability. Similar issues can ensue after inconsistent

standardisation of genetic background (Burnett et al., 2011). The mito-nuclear field as a whole suggests that different outcomes are to be expected when mitochondrial and nuclear haplotypes are not carefully controlled by backcrossing, and our results indicate that variation between studies may be amplified when diet is manipulated. Even stochastic variation in diet may interact with mito-nuclear variation, making the case for careful reporting, and perhaps the use of chemically-defined media (Piper et al., 2014). Accounting for these sources of variation will likely improve repeatability.

In summary, we have shown that diet, mitochondrial and nuclear haplotype have complex interactive effects, each capable of modifying the impact of change in another. Dietary lipid and EAAs are both implicated separately in these interactions, with relevance not just for proximate reproductive output, but also for lasting transgenerational effects of diet which impact relative offspring fitness. These varying, mito-nuclear impacts of diet on offspring quantity and quality appear to be an important determinant of individual fitness and health.

Materials & methods

Diets

Development medium contained 1.4% agar and 4.5% brewer's yeast (both Gewürzmühle Brecht, Germany), 10% cornmeal and 11.1% sucrose (both Mühle Milzitz, Germany) (all w/v), 0.45% propionic acid and 3% nipagin (v/v).

Experimental media build on published protocols (Bass et al., 2007; Dobson et al., 2018; Emran et al., 2014). These media contained a final concentration of 10% brewer's yeast, 5% sucrose, 1.5% agar (w/v), 3% nipagin and 0.3% propionic acid (v/v). EAAs were purchased as powder (Sigma), and supplemented by dissolving into a 6.66x solution in ddH₂O pH 4.5 (final media concentrations: L-arginine 0.43 g/l, L-histidine 0.21 g/l, L-isoleucine 0.34 g/l, L-leucine 0.48 g/l, L-lysine 0.52 g/l, L-methionine 0.1 g/l, L-phenylalanine 0.26 g/l, L-threonine 0.37 g/l, L-tryptophan 0.09 g/l, L-valine 0.4 g/l). Margarine (*Ja! Pflanzenmargarine* from Rewe Supermarkets, Germany; 720 kcal / 100g; 80/100g fat from 23/100g saturated fatty acids, 40/100g monounsaturated fatty acids, 17/100g polyunsaturated fatty acids) was briefly melted then mixed thoroughly into the food (15% w/v). Final nutrient contents of rearing and control media were estimated using the *Drosophila* diet content calculator (Lesperance and Broderick, 2020), with additional protein, lipid and caloric content after nutrient supplements calculated according to margarine nutrient content report, and by assuming caloric equity between EAAs and protein at a caloric value of 4 calories/g (USDA). Vials contained ~5ml of food, and were stored at 4°C for up to 1 week before use.

Flies

D. melanogaster fly lines were established as described in Figure 1 and maintained at 25°C on development medium throughout their history prior to our experiments. The ancestral Australian population was isolated in Coffs Harbour, NSW, Australia (Dowling et al., 2014). The Benin population is the widely-used *Dahomey* population, isolated in the 1970s in Dahomey (now Benin). Ancestral lines had been cured of the cytoplasmic endosymbiont *Wolbachia* 66 generations previously by tetracycline treatment prior to the experiment. For each fly line, 45 females of the desired mitochondrial background were crossed to 45 males of the desired nuclear background per generation. Iterating this process over many generations led to introgression of the desired nuclear background (from males) into each mitochondrial background. For experiments, flies were collected upon eclosion to adulthood and fed fresh developmental medium, before being pooled, split and assigned at random to experimental medium in groups of 5 males and 5 females. Experimental flies were maintained at 25°C, and transferred to fresh media every 48-72h for one week. Flies were transferred to fresh medium 24h before egg laying experiments. For development experiments, eggs were incubated at 25°C and pupation and eclosion were scored daily. Eclosing adults were lightly CO₂-anaesthetised before counting and sexing.

Analysis

Data were analysed in R 3.6.1. Markdowns of all analyses are provided as supplementary material. Fit of fecundity data to a negative binomial distribution was determined with `firdistrplus::descdist` and `firdistrplus::fitdist`. Generalised linear mixed models of the form

$$y \sim \text{diet} * \text{mitochondria} * \text{nuclear} + (\text{genotype})$$

were fit with `lme4::glmer.nb` (egg counts, negative binomial distribution) or `lme4::glmer` (binomial of egg counts before and after diet switch); in which *diet* (control/EAA/lipid), *mitochondria* (A/B) and *nuclear* (A/B) were fixed factors, genotype was a random factor denoting the full replicated genotype (e.g. AA1, AA2, AA3, AB1, AB2, BA1, etc). Where relevant (Figure 2), experimental replicate was also included as an additional random factor. Dispersion was assessed with `blme4::dispersion_glmer`, and model singularity was tested with `lme4::isSingular`. Anova tests (type-3) were conducted with `car::Anova`, and *post-hoc* analyses were applied with the functions `emmeans::joint_tests`, `emmeans::pairs`, `emmeans::emmip` (Searle et al., 1980). AIC was determined with `stats::AIC`, Akaike weights with `MuMIn::dredge`. Four vials were excluded from the diet-switching experiment to enable model fitting, on the basis of extreme values (minimum and maximum observations in the experiment) or deviation from a QQ plot before diet switch. These data are retained, with note, in the data associated to this paper.

Censoring (i.e. failure to develop) was inferred per vial when n. emerging adults was less than n. eggs (noting assignment of zero censors when adult counts were

greater than eggs in some vials, due to measurement error). A 50:50 sex ratio was assumed amongst censors. Development to adult was modelled by fitting Cox mixed effects models of the form

$$y \sim \text{diet} * \text{mitochondria} * \text{nuclear} * \text{sex} + \text{eggs} + \left(\frac{\text{genotype}}{\text{vial}} \right)$$

with `coxme::coxme`. *Diet*, *mitochondria*, *nuclear* and *genotype* terms were as in models of egg laying, and *vial* was an additional random effect nested in *genotype*. *Eggs* coded number of eggs laid in the vial in which the individual developed, to account for variation in rearing density. Pupal sex was unknown, so the sex term was excluded from models of pupal development. Anova and *post-hoc* EMM tests were conducted as per fecundity analyses, with additional stratification by sex when applying `emmeans::joint_tests`.

Figures were assembled in Adobe Illustrator. Mitochondria graphics were recoloured from files freely distributed under an open commons licence. The heatmap of nutrient content was plotted in R with the `superheat` library.

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790

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Declaration of interests

801

802 The authors declare no competing interests.

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Table S1. GLMM of fecundity across diets and mito-nuclear haplotypes

Term	Chisq	Df	Pr(> χ^2)
(Intercept)	4342.751	1	<2.20E-16
mito	8.3384	1	0.0039
nuclear	13.6946	1	0.0002
diet	314.9242	2	<2.20E-16
mito:nuclear	6.5883	1	0.0103
mito:diet	10.2151	2	0.0061
nuclear:diet	0.2024	2	0.9036
mito:nuclear:diet	9.3347	2	0.0094

Table S2. Pairwise comparisons of fecundity across diets and mito-nuclear haplotypes

diet	contrast	estimate	SE	Z ratio	P value
sya	AA vs BA	0.264859	0.0917	2.888	0.0203
	AA vs AB	0.32267	0.0872	3.701	0.0012
	AA vs BB	0.262022	0.0871	3.008	0.014
	BA vs AB	0.057811	0.0922	0.627	0.9233
	BA vs BB	-0.002838	0.092	-0.031	1
	AB vs BB	-0.060649	0.0876	-0.692	0.9001
EAA	AA vs BA	0.04355	0.0918	0.474	0.9648
	AA vs AB	0.362769	0.0865	4.193	0.0002
	AA vs BB	0.363417	0.0866	4.199	0.0002

	BA vs AB	0.319219	0.0922	3.464	0.003
	BA vs BB	0.319866	0.092	3.475	0.0029
	AB vs BB	0.000647	0.087	0.007	1
mar	AA vs BA	-0.09705	0.097	-1.001	0.7491
	AA vs AB	0.365225	0.0932	3.918	0.0005
	AA vs BB	0.42652	0.0935	4.56	<.0001
	BA vs AB	0.462275	0.0982	4.706	<.0001
	BA vs BB	0.523571	0.0986	5.309	<.0001
	AB vs BB	0.061296	0.0951	0.645	0.9174

Table S3. Development on experimental diets: Cox Mixed-Effects model of adult emergence, sex-stratified posterior analysis by Estimated Marginal Means

Sex	Term	Df	F ratio	P
female	mito	1	15.517	0.0001
	nuclear	1	13.541	0.0002
	diet	2	322.17	<.0001
	mito:nuclear	1	20.686	<.0001
	mito:diet	2	25.789	<.0001
	nuclear:diet	2	16.855	<.0001
	mito:nuclear:diet	2	25.733	<.0001
male	mito	1	10.307	0.0013
	nuclear	1	0.223	0.6371

diet	2	226.121	<.0001
mito:nuclear	1	16.511	<.0001
mito:diet	2	8.529	0.0002
nuclear:diet	2	15.201	<.0001
mito:nuclear:diet	2	7.778	0.0004

Table S4. Development on experimental diets: Cox Mixed-Effects model of egg-pupa survival

Term	Chisq	Df	Pr(> χ^2)
mito	1	129.732	<2.20E-16
nuclear	1	148.282	<2.20E-16
diet	2	146.228	<2.20E-16
eggs	1	12.377	0.0004
mito:nuclear	1	66.987	2.73E-16
mito:diet	2	74.55	<2.20E-16
nuclear:diet	2	90.713	<2.20E-16
mito:nuclear:diet	2	35.714	1.76E-08

Table S5. Fecundity before switch from experimental diets: GLMM of egg laying across mito-nuclear haplotypes, after feeding on experimental diets

Term	Chisq	Df	Pr(> χ^2)
(Intercept)	1748.5684	1	<2.20E-16
mito	1.1888	1	0.2756
nuclear	2.2325	1	0.1351

diet	127.1386	2	<2.20E-16
mito:nuclear	5.845	1	0.0156
mito:diet	9.7233	2	0.0077
nuclear:diet	10.8029	2	0.0045
mito:nuclear:diet	9.1987	2	0.0101

Table S6. GLMM of fecundity across diets and mito-nuclear haplotypes – data pooled from Figure 2 and Figure 3c

Term	χ^2	Df	Pr(> χ^2)
(Intercept)	4338.6592	1	<2.20E-16
mito	1.7911	1	0.1808
nuclear	9.8394	1	0.0017
diet	500.2988	2	<2.20E-16
mito:nuclear	1.8161	1	0.1779
mito:diet	14.5664	2	0.0006
nuclear:diet	2.829	2	0.2430
mito:nuclear:diet	17.8267	2	0.0001

Table S7. Fecundity after switch from experimental diets: GLMM of egg laying across mito-nuclear haplotypes, after feeding on experimental diets

Term	χ^2	Df	Pr(> χ^2)
(Intercept)	1341.3688	1	<2.20E-16
mito	2.6895	1	0.1010
nuclear	0.0347	1	0.8522

diet	36.59	2	1.13E-08
mito:nuclear	3.7278	1	0.0535
mito:diet	0.3195	2	0.8523
nuclear:diet	1.7084	2	0.4256
mito:nuclear:diet	0.5948	2	0.7427

Table S8. Rescue of fecundity after switch from experimental diets: GLMM of egg laying across mito-nuclear haplotypes: after feeding, relative to during feeding (binomial)

Term	χ^2	Df	Pr(> χ^2)
(Intercept)	44.7143	1	2.28E-11
mito	1.4802	1	0.2237
nuclear	4.4348	1	0.0352
diet	139.1165	2	<2.20E-16
mito:nuclear	0.1714	1	0.6788
mito:diet	51.4892	2	6.60E-12
nuclear:diet	17.9358	2	0.0001
mito:nuclear:diet	39.3128	2	2.91E-09

Table S9. Fertility & development after parental feeding on experimental diets: sex-stratified posterior (EMM) analysis of adult emergence

Sex	Term	Df	F ratio	P
female	mito	1	10.243	0.0014
	nuclear	1	1.313	0.2518

	diet	2	0.345	0.7083
	mito:nuclear	1	0.218	0.6407
	mito:diet	2	4.961	0.007
	nuclear:diet	2	0.777	0.4599
	mito:nuclear:diet	2	3.477	0.0309
male	mito	1	9.262	0.0023
	nuclear	1	1.331	0.2486
	diet	2	0.396	0.6731
	mito:nuclear	1	0.07	0.7913
	mito:diet	2	3.454	0.0316
	nuclear:diet	2	1.545	0.2133
	mito:nuclear:diet	2	5.862	0.0028

Table S10. Development after parental feeding on experimental diets: Cox Mixed-Effects model of egg-pupa survival

Term	Chisq	Df	Pr(> χ^2)
mito	1	3.8079	0.0510
nuclear	1	13.3143	0.0003
diet	2	10.8094	0.0045
eggs	1	31.5515	1.94E-08
mito:nuclear	1	3.8928	0.0485

mito:diet	2	5.5291	0.0630
nuclear:diet	2	8.4317	0.0148
mito:nuclear:diet	2	1.717	0.4238

Table S11. Fertility & development on experimental diets: Cox Mixed-Effects model of adult emergence

Term	Chisq	Df	Pr(> χ^2)
mito	1	75.1042	<2.20E-16
nuclear	1	65.8859	4.78E-16
diet	2	86.1542	<2.20E-16
sex	1	12.9677	0.0003
eggs	1	1.7022	0.1920
mito:nuclear	1	55.7929	8.05E-14
mito:diet	2	86.1953	<2.20E-16
nuclear:diet	2	68.6659	1.23E-15
mito:sex	1	14.1785	0.0002
nuclear:sex	1	14.9357	0.0001
diet:sex	2	12.8281	0.0016
mito:nuclear:diet	2	51.4656	6.67E-12
mito:nuclear:sex	1	13.8949	0.0002
mito:diet:sex	2	25.9358	2.33E-06
nuclear:diet:sex	2	11.5055	0.0032
mito:nuclear:diet:sex	2	16.6248	0.0002

Table S12. Fertility & development after parental feeding on experimental diets: Cox Mixed-Effects model of adult emergence

Term	Chisq	Df	Pr(>χ^2)
mito	1	0.3711	0.5423
nuclear	1	1.9887	0.1585
diet	2	10.3938	0.0055
sex	1	0.1665	0.6832
eggs	1	7.2316	0.0072
mito:nuclear	1	0.2117	0.6454
mito:diet	2	14.3708	0.0008
nuclear:diet	2	6.0394	0.0488
mito:sex	1	1.517	0.2181
nuclear:sex	1	1.4268	0.2323
diet:sex	2	2.9565	0.2280
mito:nuclear:diet	2	6.9531	0.0309
mito:nuclear:sex	1	0.565	0.4522
mito:diet:sex	2	0.2748	0.8716
nuclear:diet:sex	2	1.0484	0.5920
mito:nuclear:diet:sex	2	6.6992	0.0351

Figure S1

Figure S1. Diet-mito-nuclear interactions modulate *Drosophila* fecundity.

Genetic correlations in impact of mito-nuclear variation on egg laying, and impact of diet on egg laying. Each point shows mean egg laying per line per diet in each of two replicate experiments. Bars show standard error. Accompanying statistical analysis presented in Table S1 and Table S2.

Figure S2

Figure S2. Mito-nuclear variation in dietary regulation of adult development: impacts per genetic replicate. Kaplan-Meier plots of development after feeding on experimental diets as specified in key. Mito-nuclear genotypes are indicated per panel. Data are presented pooled across each replicate genotype in Figure 4. Accompanying statistical analysis presented in Table S3.

Figure S3. Mito-nuclear variation in dietary regulation of pupation. (A) Kaplan-Meier plots of pupation after feeding on experimental diets as specified in key. Mito-nuclear genotypes are indicated in top-left of each panel. (B). Estimated marginal means (EMMs) with 95% confidence intervals summarising survival analysis (Cox mixed effects) of data from A. Higher values correspond to faster development and/or increased fertility, lower values correspond to slower development and/or reduced viability. Data are presented per genetic replicate in Figure S4. Accompanying statistical analysis presented in Table S4.

Figure S4

Figure S4. Mito-nuclear variation in dietary regulation of pupation: impacts per genetic replicate. Kaplan-Meier plots of pupation after feeding on experimental diets as specified in key. Mito-nuclear genotypes are indicated per panel. Data are presented pooled across each replicate genotype in Figure S5. Accompanying statistical analysis presented in Table S4 and Table S10

Figure S5

Figure S5. Mito-nuclear variation in fecundity response to switching from experimental diets to a standardised diet: impacts per genetic replicate.

Fecundity measures are shown per genotype as specified on bottom x axis. Boxplots show medians, first and third quartiles and 5th and 95th percentiles. Points show individual data. **A.** Fecundity before diet switch. **B.** Fecundity after diet switch. **C.** Ratio of fecundity before and after diet switch. Data are presented pooled across each replicate genotype in Figure 3. Accompanying statistical analysis presented in Tables S5, S7 and S8.

Figure S6

Figure S6. Diet-mito-nuclear interactions modulate *Drosophila* fecundity - emergent pattern from three replicate experiments. Fecundity is shown per genotype as specified on bottom x axis. Boxplots show medians, first and third quartiles and 5th and 95th percentiles. Connected points to right of each box show *post-hoc* comparisons of means (by estimated marginal means with 95% confidence intervals, calculated from a generalised linear mixed effects model, showing exponent of EMMs in order to fit to original data scale). Translucent points to left of each box show raw data. Accompanying statistical analysis presented in Table S6.

Supplementary Text

Much recent research in *Drosophila* and other organisms has been directed to characterising the consequences transmission of mitochondria from mothers only, which restricts mito-nuclear coevolution to females (Gemmell et al., 2004; Maklakov and Lummaa, 2013; Montooth et al., 2019; Nagarajan-Radha et al., 2020). This is thought to cause a “mother’s curse” (Gemmell et al., 2004), in which males bear their mothers' mitochondria despite potential mismatch to variants inherited from fathers. In our study of development we manipulated diet and mito-nuclear genotype, and recorded sex of emerging adult offspring, giving four potential predictive factors. Our main analyses are presented stratified by sex. ANOVA tests of the complex diet:mito:nuclear:sex interaction were nominally supportive of sex biases in DMN interactions, but did not point solely to males. We detected significant sex:diet:mito:nuclear interactions, but even with our large sample sizes we are cautious in interpreting such complex interaction terms. F-ratios for DMN interactions were greater in males when only parents were fed experimental diets (Table S12), but were greater in females when both F0+F1 were fed experimental diets (Table S11), suggesting that sex dimorphisms manifest as different magnitudes of the same interaction. Visual inspection of the data led to a similar conclusion (Figure S2). We therefore interpret that any statistical differences reflected relatively modest differences in the present data, suggesting that mothers curse may exert a relatively benign influence in this case.