Mito-nuclear variation in transgenerational and reproductive effects 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 fundaments 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

64 buffered or augmented by variants in the other. In animals, this has been observed
65 for fecundity (Mossman et al., 2016a), fertility (Camus et al., 2015), lifespan (Camus
66 et al., 2015; Vaught et al., 2020), gene expression (Camus et al., 2015; Mossman et
67 al., 2019, 2016b), and epigenomic modifications (Grunau et al., 2018). Novel mito-
68 nuclear pairings can even change the course of nuclear genomic evolution (Healy
69 and Burton, 2020), and lead to reproductive isolation (Hill, 2019; Ma et al., 2016).
70 The intimacy of mito-nuclear coupling leads us to expect coadaptation (Dowling et
71 al., 2008), and consequently to predict that pairs from the same population
72 (sympatric) should function optimally. Some studies have supported this prediction
73 (Baris et al., 2017; Chang et al., 2016; Ellison and Burton, 2006; Ma et al., 2016;
74 Meiklejohn et al., 2013), but others have not (Dowling et al., 2010; Mossman et al.,
75 2016b; Vaught et al., 2020). Despite genetic variation in responses to dietary
76 variation, and the emerging importance of mito-nuclear interactions in overall genetic
77 variation, there has been relatively little work to connect the three, by asking whether
78 the phenotypic impacts of dietary alterations are subject to mito-nuclear interactions.
79 Trailblazing work in *Drosophila melanogaster* has shown diet-mito-nuclear (DMN)
80 variation in development time, lifespan and fecundity (Camus et al., 2020; Montooth
81 et al., 2019; Mossman et al., 2016a; Rand et al., 2018; Zhu et al., 2014), but specific
82 causal nutrients and cellular mechanisms remain to be established.
83
84 Perhaps diet's most immediate fitness effect is regulation of reproduction. Parents
85 can gain fitness by increasing offspring quantity, or by promoting offspring quality
86 (Lack, 1947; Smith et al., 1989; Stearns, 1992). Offspring quality and quantity will
87 depend on fertility, fecundity, and development (viability) of those offspring, each of

88 which are influenced by diet. Equivalent changes in these traits can be elicited in
89 distinct taxa by equivalent dietary changes: for example, in numerous studies of
90 insects and mammals, amino acid nutrition has proven consistently important for
91 fertility, fecundity and offspring development (Bong et al., 2014; Crean and Senior,
92 2019; Dong et al., 2016; Fanson et al., 2012; Grandison et al., 2009; Jensen et al.,
93 2015; Lee et al., 2008; Leitão-Gonçalves et al., 2017; Liang and Zhang, 2006; Ma et
94 al., 2020; Maklakov et al., 2008; McCracken et al., 2020; Mirth et al., 2018; Skorupa
95 et al., 2008; Solon-Biet et al., 2015; Svajgr et al., 1972; Tufarelli et al., 2015; Winship
96 et al., 2018; Wong et al., 2014a; Zanco et al., 2020); whereas high-lipid or high-sugar
97 diets are obesogenic (Dobson et al., 2019; Hariri and Thibault, 2010; Jang et al.,
98 2018; Rivera et al., 2015; Saud et al., 2015; Wong et al., 2014b; Woodcock et al.,
99 2015; Zhao et al., 2020). However, importantly, reproduction is not only regulated by
100 the individual's current diet: large nutritional fluctuations can induce enduring
101 physiological and molecular changes, which can transmit across generations,
102 influencing quality of offspring and even grand-offspring (Barker and Osmond, 1986;
103 Barker and Thornburg, 2013; Deas et al., 2019; Duque-Guimarães and Ozanne,
104 2017; Holland et al., 2016; Li et al., 2018; Liang and Zhang, 2006; Öst et al., 2014;
105 Rivera et al., 2015; Stefana et al., 2017; Wei et al., 2014; Winship et al., 2018). Thus,
106 diet can influence reproduction via lasting, programmed effects on offspring quality,
107 which point to epigenetic reprogramming, whilst acute effects of diet on both
108 offspring quality and quantity point to metabolic changes. But do these effects vary
109 amongst genotypes? If so, is variation shaped by mitochondrial genomes, nuclear
110 genomes, or by mito-nuclear genotype?
111

112 Understanding health impacts of mito-nuclear interactions is not just a current topic
113 in biology. It is also pertinent biomedically, because dietary intervention is a
114 preeminent treatment for human mitochondrial disease (Gorman et al., 2016), and
115 also following the advent of mitochondrial replacement therapies (Craven et al.,
116 2010; Tachibana et al., 2018; Wolf et al., 2015). These procedures have been
117 licensed in the United Kingdom and a few other countries (Cohen et al., 2020). They
118 aim to treat mitochondrial diseases by placing embryonic nuclei into the cytoplasm,
119 and therefore mitochondrial context, of a disease-free donor. Some argue that the
120 conservation of mitochondrial function and the body of evidence for mito-nuclear
121 interactions suggest that undesired consequences may ensue if the donor
122 mitochondria function poorly with the nuclear genome (Dobler et al., 2018; Dunham-
123 Snary and Ballinger, 2015; Reinhardt et al., 2013); although the UK Human Fertility
124 and Embryology Authority concluded that risks remain “purely theoretical” (HFEA,
125 2016). To solve this debate it will be important to identify which traits to evaluate in
126 patients, whether costs or benefits may be diet-dependent, and whether
127 transgenerational variation may ensue.
128
129 Here, we identify specific nutrients sufficient to drive diet-mito-nuclear variation in
130 *Drosophila* life-history, and show that specific mito-nuclear genotypes can exhibit
131 long-lasting, transgenerational responses to transient dietary changes. We bred a
132 panel of flies with replicated and fully-factorial mito-nuclear variation, and show that
133 essential amino acids and lipids can elicit novel patterns of variation in fecundity and
134 offspring performance (development time). We then show for the first time that the
135 capacity for effects of parental diet on offspring performance are genetically variable,

136 and mito-nuclear in this case. To elucidate overall variation in fitness, we integrate
137 our analyses of offspring quality and quantity, showing mito-nuclear variation in how
138 diet influences reproductive investment; and also that “mis-matched” pairs of
139 mitochondrial and nuclear genomes do not necessarily perform worse than matched
140 pairs. Finally, we evaluate performance of alternative models of our extensive
141 datasets, in which interactions between diet, mitochondrial and nuclear haplotype
142 were systematically included or excluded. We find that models which include mito-
143 nuclear or diet-mito-nuclear interactions are favoured overwhelmingly over models
144 which assume no interaction, suggesting that models which do not account for these
145 terms are not adequate to explain variance in fitness and health.
146
147

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 (~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*

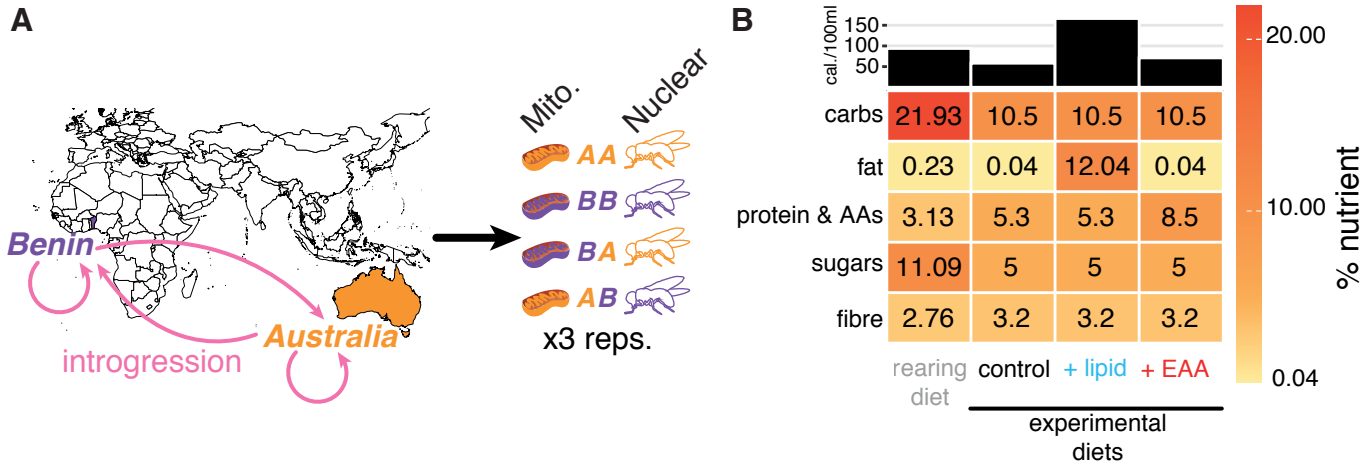
171 mitochondria: this experimental design should therefore produce fully-factorial mito-

172 nuclear variation.

173

174

Figure 1


A
Benin
Australia
introgression
Mito.
Nuclear
AA
BB
BA
AB
x3 reps.
B
cal./100ml
150
100
50
carbs 21.93 10.5 10.5 10.5
fat 0.23 0.04 12.04 0.04
protein & AAs 3.13 5.3 5.3 8.5
sugars 11.09 5 5 5
fibre 2.76 3.2 3.2 3.2
rearing diet
control
+ lipid
+ EAA
experimental diets
20.00
10.00
0.04
% nutrient

176 **Figure 1. Study design. A.** Genetically distinct and heterogeneous populations of
177 flies were originally isolated from from Benin (formerly Dahomey), and New South
178 Wales, Australia. Mito-nuclear variation was generated by reciprocally introgressing
179 each line either into its own respective cytoplasmic background, or into the other
180 line's cytoplasmic background. Three biologically independent replicate lines were
181 established per mito-nuclear combination. 74 iterations of introgression were
182 completed before experiments. **B.** Fly lines were cultured on rearing diet and then
183 fed experimental diets in adulthood. The heatmap shows estimated content of
184 different macronutrients, bars at the top indicating caloric content.
185

186 **EAA and lipid nutrition are sufficient to drive diet-mito-nuclear variation in life-**
187 **history**
188
189 To identify nutrients which could drive DMN interactions, we added nutrients to a
190 sugar-yeast diet (Bass et al., 2007), as previously (Dobson et al., 2018; Emran et al.,
191 2014; Grandison et al., 2009). To ensure that potential novelty effects were evenly
192 distributed amongst conditions, the baseline diet was distinct from the medium on
193 which the lines were maintained and developed. For experimental diets, we added
194 one of two nutrients to the baseline, selected for their important and conserved
195 health impacts. Essential amino acids (EAAs) are metabolised by mitochondria
196 (Mariño et al., 2014), and their specific enrichment is sufficient to recapitulate
197 fecundity effects of adding yeast (Emran et al., 2014; Grandison et al., 2009). We
198 also manipulated dietary lipid, because mitochondria metabolise fatty acids, and
199 high-fat animal diets can model Western human disease (Hariri and Thibault, 2010;
200 Heymsfield and Wadden, 2017). Lipid source can have large physiological effects
201 (Brankatschk et al., 2018), and flies are largely vegan in nature (Knittelfelder et al.,
202 2020). Therefore, we supplied a plant-based lipid which set in agar (i.e. in contrast to
203 oils), by adding margarine to the diet (15% w/v, after (Woodcock et al., 2015)). Whilst
204 this design did not control calories, it enabled us to test whether specific,
205 ecologically-relevant nutrients can cause diet-mito-nuclear variation. Estimated
206 nutrient contents (Lesperance and Broderick, 2020) are presented in Figure 1b.
207
208 Previous work showed that manipulating complex nutrient sources elicits life-history
209 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

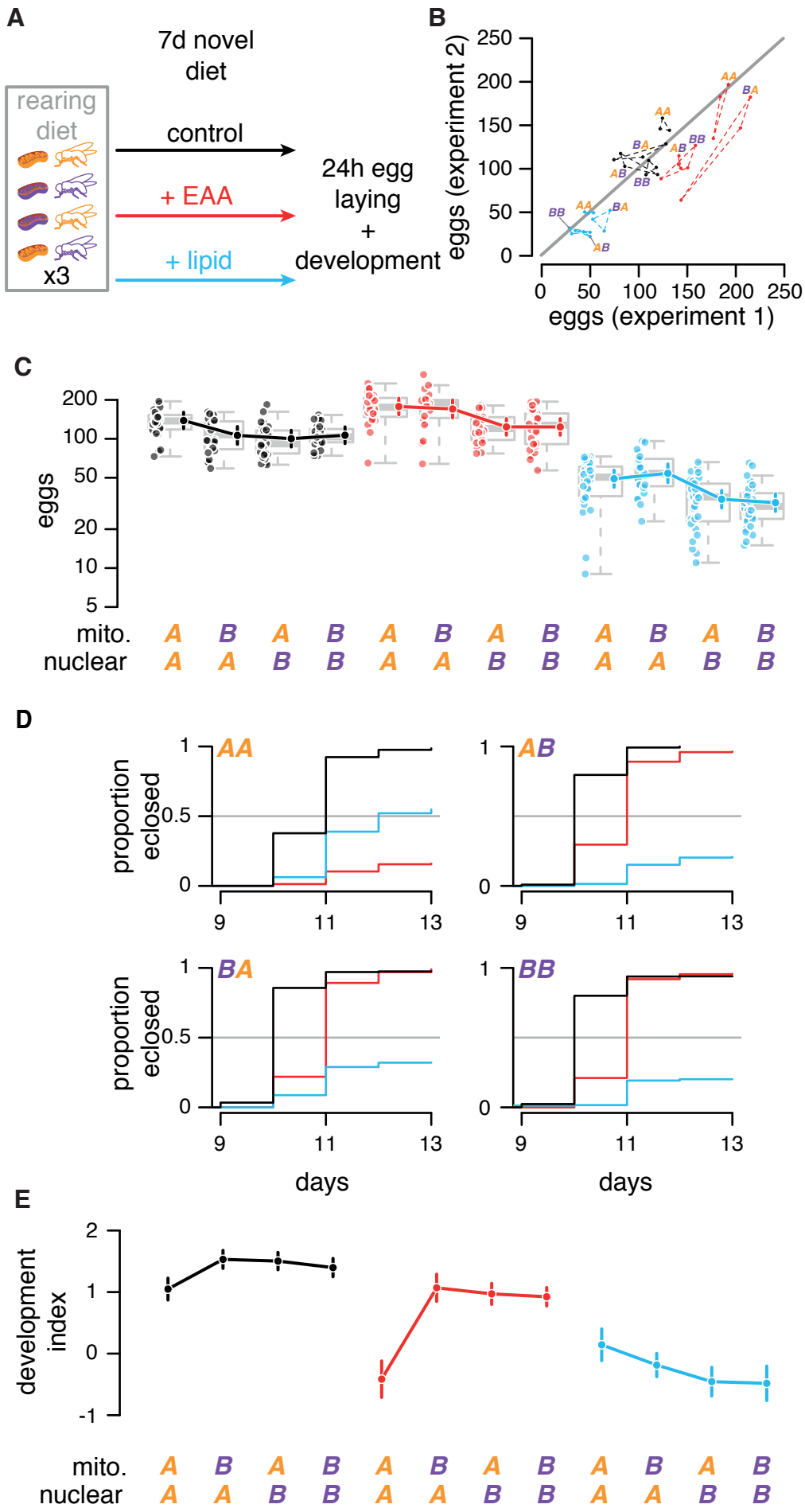
234 mito-nuclear genotype.

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

258 EMMs confirmed that EAA or lipid enrichment disadvantaged specific genotypes
259 (Figure 2e). The *AA* flies bore a particular cost of reduced survival on EAA food. The
260 differential ranking of EMMs across genotypes on different diets indicated that the
261 pattern of mito-nuclear genetic variation was dependent on EAA and lipid nutrition.
262 Indeed, as with fecundity, there was little variation within each triplicate per mito-
263 nuclear genotype (except that the detrimental effect of EAAs on development of *AA*
264 flies was pushed to lethality in the *AA3* line, and *BB3* was somewhat more lipid-
265 resistant than *BB1* and *BB2* (Figure S2)), suggesting that the developmental
266 response to nutrition is highly stereotyped by mito-nuclear genotype. A parallel
267 analysis of these same individuals' time to pupation revealed congruent patterns
268 (Figure S3, Figure S4, Table S4). Altogether, these results show that either lipid or
269 EAA enrichment are sufficient to potently modify developmental outputs of mito-
270 nuclear genetic variation.
271

Figure 2


A
rearing diet
x3
7d novel diet
control
+ EAA
+ lipid
24h egg laying + development
B
eggs (experiment 2)
eggs (experiment 1)
C
eggs
mito.
nuclear
D
proportion eclosed
days
E
development index
mito.
nuclear

274 **Figure 2. Diet-mito-nuclear interactions modulate *Drosophila* fecundity. A.**
275 Experimental design. The 12 mito-nuclear lines were reared from egg to adult on
276 rearing food, and allocated at random to experimental media 6-48h after eclosion, at
277 a density of five of each sex per vial. After seven days, flies laid eggs on fresh food
278 for 24 hours. **B.** Intra-line correlations in impact of diet-mito-nuclear variation on egg
279 laying. Each point shows mean egg laying per line per diet in each of two replicate
280 experiments, with the replicates of each haplotype grouped by dashed lines. Means
281 were correlated between experiments (Pearson's r = 0.87, p = 7.6e-12). **C.** Impact of
282 diet on fecundity is determined by mito-nuclear variation. Data are pooled across the
283 experimental and genetic replicates presented in panel B. Boxplots show medians,
284 first and third quartiles and 5th and 95th percentiles. Connected points to right of
285 each box show *post-hoc* comparisons of means (by estimated marginal means with
286 95% confidence intervals, calculated from a generalised linear mixed effects model,
287 showing exponent of EMMs in order to fit to original data scale). Translucent points
288 to left of each box show raw data. Complementary statistical analysis presented in
289 Table S1 and Table S2. **D.** Kaplan-Meier plots of development (egg-to-adult) after
290 parents fed and offspring developed on experimental media. Mito-nuclear genotypes
291 are indicated in top-left of each panel. **E.** Estimated marginal means (EMMs) with
292 95% confidence intervals summarising survival analysis (Cox mixed effects) of data
293 from D. Higher values correspond to faster development and/or increased fertility,
294 lower values correspond to slower development and/or reduced viability.
295 Accompanying statistical analysis presented in Table S3.
296

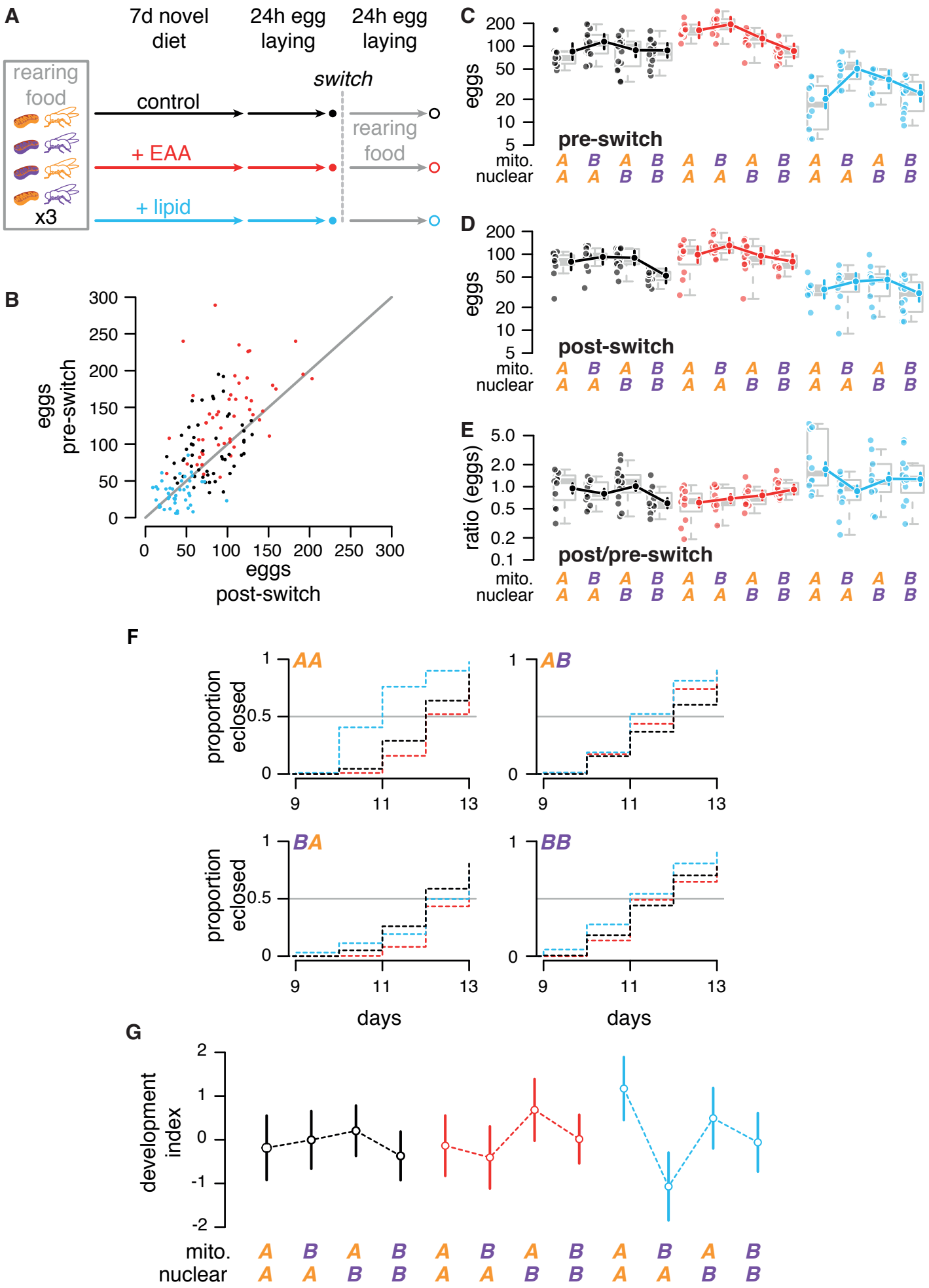
297 **Persistent life-history effects of dietary EAAs and lipid in specific mito-nuclear**
298 **genotypes**
299
300 DMN interactions could occur via numerous mechanisms, but no candidate has yet
301 been confirmed. Dietary variation induces not only immediate changes to
302 metabolism, but also long-lasting epigenetic changes (Barker and Osmond, 1986;
303 Barker and Thornburg, 2013; Deas et al., 2019; Dobson et al., 2017; Duque-
304 Guimarães and Ozanne, 2017; Holland et al., 2016; Li et al., 2018; Liang and Zhang,
305 2006; Öst et al., 2014; Rivera et al., 2015; Stefana et al., 2017; Wei et al., 2014;
306 Winship et al., 2018). Mitochondria metabolise substrates for epigenetic
307 modifications (Eisenberg et al., 2014; Mariño et al., 2014; Trefely et al., 2020),
308 suggesting that nutritional regulation of epigenetically-encoded traits may vary
309 amongst mito-nuclear genotypes. We asked whether EAAs and lipid might drive
310 DMN interactions for life-history via long-term reprogramming. Whilst such effects
311 are consistent with epigenetic modifications, we conservatively avoid that term for
312 our data because we did not measure epigenetic marks directly. We tested for long-
313 lasting effects of transient EAA and lipid enrichment, both in the generation that
314 experienced the nutritional manipulation, and in their offspring.
315
316 We conducted a second fecundity experiment, with a week of adult-onset feeding on
317 experimental diets as previously, but followed by a diet switch back to standardised
318 rearing medium. Fecundity was measured at the end of experimental diet feeding,
319 and 24h after switching back to standardised medium, yielding vial-matched egg
320 counts pre- and post- switch (Figure 3a). The overall effect of experimental diets was

321 equivalent before and after the switch (EAAs elevated fecundity, lipid repressed
322 fecundity), confirming that effects of experimental diets persisted for the 24h feeding
323 on standardised diet (Figure 3b). Before the switch, on experimental diets, a DMN
324 interaction was once again evident (Figure 3c, Table S5, Figure S5a), and pooling
325 these data with preceding data (Figure 2) yielded the same qualitative result (Figure
326 S6, Table S6), confirming that DMN effects were consistent across experiments.
327
328 A GLMM analysis confirmed that, after switching back to standardised diet, the main
329 effects of prior EAA or lipid feeding persisted (Figure 3d, Table S7, Figure S5b).
330 However, the DMN interaction did not persist (Figure 3d, Table S7, Figure S5b).
331 These findings suggested that EAA and lipid modify the manifestation of mito-
332 nuclear variation in fecundity via transient effects; on top of longer-term impacts
333 which do not depend on mito-nuclear genotype. If so, recovery of fecundity after diet
334 switch should exhibit a DMN interaction. We estimated recovery rate as the ratio of
335 eggs laid per vial after/before the switch, with greater ratios indicating greater
336 plasticity. Recovery from EAA or lipid feeding was indeed variable amongst mito-
337 nuclear genotypes (Figure 3e; GLMM p=2.91E-09, Table S8; Figure S5c).
338 Altogether, these analyses revealed two layers of variation, with genotype-
339 independent main effects of dietary EAAs and lipid which persist after feeding, and
340 additional DMN variation whilst feeding on the diets, but not after. This suggested
341 that EAAs and lipid can have lasting fecundity effects which generalise amongst
342 mito-nuclear genotypes, but did not exclude the possibility of longer-term,
343 transgenerational diet-mito-nuclear impacts on offspring.
344

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

369 lipid-enriched food (Figure 3f). A parallel analysis of these same individuals' time to
370 pupation revealed congruent patterns (Figure S3, Figure S4, Table S10). Whilst
371 some variation may be due to selection, our experimental design largely excludes
372 this possibility, because the only nutritional variation that individuals under study
373 experienced was *in utero* when mothers fed on experimental diets, before post-
374 embryonic development in standardised medium; and variation in rearing density
375 was also accounted for statistically. Furthermore, since the major effect we observe
376 is an acceleration of development in one condition (*AA* flies fed lipid), selection
377 would have to be otherwise ubiquitous but relaxed specifically for this condition. The
378 results therefore indicate that specific mito-nuclear genotypes are susceptible to
379 transgenerational effects of parental nutrition, in this case lipid.
380
381 Notably, the transgenerational effects we observed in development did not correlate
382 patterns when both parents and offspring were fed the diets. Our studies of
383 transgenerational and intergenerational effects of EAAs and lipid were conducted at
384 the same time (Figure 2d-e flies developed from eggs plotted in Figure 3c, Figure 3f-
385 g from eggs in Figure 3d), validating this comparison. Altogether, this discord
386 suggests that nutrients can induce transgenerational effects in specific mito-nuclear
387 genotypes, but this reprogramming in combination with ongoing nutrient enrichment
388 in offspring leads to additional changes to patterns of phenotypic variation.
389

Figure 3


A
7d novel diet
24h egg laying
24h egg laying
rearing food
x3
control
+ EAA
+ lipid
switch
rearing food
B
eggs pre-switch
eggs post-switch
C
eggs
pre-switch
mito.
nuclear
D
eggs
post-switch
E
ratio (eggs)
post/pre-switch
F
proportion eclosed
AA
AB
BA
BB
days
G
development index
mito.
nuclear

391 **Figure 3. Performance is determined by interplay of mito-nuclear genotype and**
392 **persistent intragenerational and transgenerational effects of diet. A.**
393 Experimental design. 12 Mito-nuclear lines were reared from egg to adult on rearing
394 food, and allocated at random to experimental media 6-48h after eclosion, at a
395 density of five of each sex per vial. After seven days, flies laid eggs on fresh food for
396 24 hours. Females were then reassigned back to standardised rearing medium, and
397 eggs were laid for a further 24h. **B.** Correspondence between egg laying rate per
398 vial, before and after switch from experimental diets. Egg laying rates before and
399 after switch across all diets were correlated (Pearson's product-moment correlation,
400 $r=0.65$, $p<2.2e-16$). Line of equivalence shown in grey. **C.** Egg laying rate before
401 dietary switch is determined by mito-nuclear variation (*vis* Fig 2C). Boxplots show
402 medians, first and third quartiles and 5th and 95th percentiles. Connected points to
403 right of boxes show *post-hoc* comparisons of means (by estimated marginal means
404 with 95% confidence intervals, calculated from a generalised linear mixed effects
405 model). Translucent points to left of each box show raw data. Complementary
406 statistical analysis presented in Table S5. **D.** Diet-mito-nuclear interactions do not
407 persist 24h after switch from experimental media. Statistical analysis presented in
408 Table S7. **E.** Mito-nuclear interactions govern recovery of fecundity after switch from
409 experimental media. Statistical analysis presented in Table S8. **F.** Kaplan-Meier plots
410 of development to adulthood of eggs laid on developmental media, after parents had
411 previously fed on experimental media. Mito-nuclear genotypes are indicated in top-
412 left of each panel. **G.** Estimated marginal means (EMMs) with 95% confidence
413 intervals summarising survival analysis (Cox mixed effects) of data from F. Higher
414 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

418

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

421

422 Together, offspring quantity and quality define fitness (Lack, 1947; Stearns, 1992).
423 Our analyses suggested that both fecundity (quantity) and fertility (quality) are
424 regulated by EAAs and lipid, but specific mito-nuclear genotypes are differentially
425 receptive or resistant to these effects. To date, the widely-held prediction of a
426 disadvantage to "mis-matched" mito-nuclear pairings, has received equivocal
427 support (Baris et al., 2017; Chang et al., 2016; Ellison and Burton, 2006; Ma et al.,
428 2016; Meiklejohn et al., 2013). Our datasets provided an opportunity to ask whether
429 this prediction applied in terms of differential responses to specific nutrients; whilst
430 taking an integrative view of fitness, encompassing both offspring quality and
431 quantity, and putatively epigenetic effects. To this end, we integrated outputs of our
432 previous analyses by plotted EMMs from against one another, indicating effects of
433 diet and mito-nuclear genotype on offspring quality and quantity, and the relationship
434 between them. We did this separately for data from parents and offspring both fed
435 experimental diets (flies in Figure 2e, which developed from eggs in Figure 3c), and
436 from parent-restricted feeding (flies in Figure 3g, which developed from eggs in
437 Figure 3d).

438

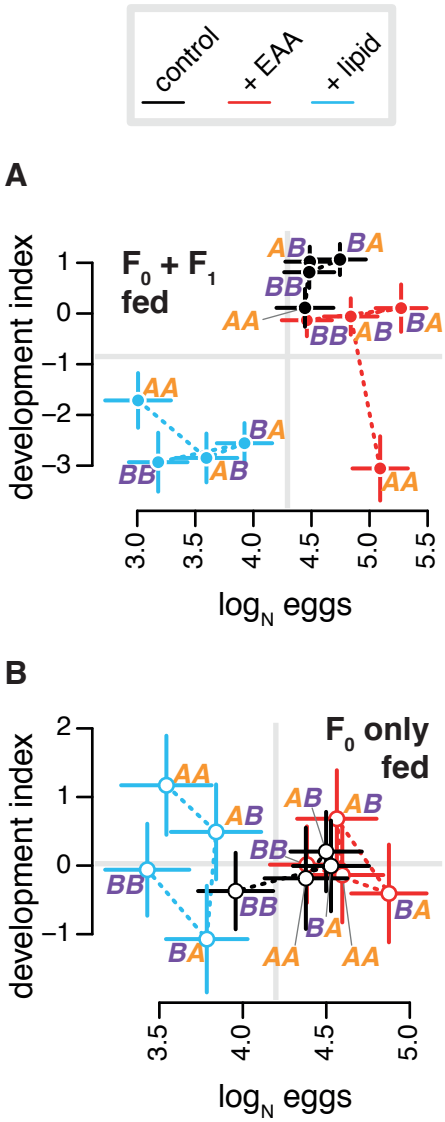
439 First, we examined impacts of EAA feeding. Visual inspection indicated that EAA
440 enrichment enhanced offspring quantity, but compromised offspring quality in flies of
441 all genotypes except *BB* (Figure 4a). This was surprising, because we had assumed

442 that parents should discriminate diets that maximise their own fitness, and therefore
443 that parental egg-laying would correlate offspring development indices. Instead, the
444 apparent tradeoff indicates either that (1) EAAs signal diet quality to parents on
445 normal diets when yeast is the source of most nutrients (including protein), but our
446 manipulations rendered this quality signal misleading (Zanco et al., 2020); or that (2)
447 diet is subject to parent-offspring conflict (Trivers, 1974), with parents maximising
448 their fitness by producing more eggs on high EAA-diets, despite the cost to individual
449 offspring.
450
451 Were costs of EAAs genotype-specific? The quality-quantity tradeoff induced by EAA
452 feeding was exaggerated dramatically in *AA* flies, whose eggs developed
453 exceptionally poorly on EAA-enriched food. However, this cost of EAAs was rescued
454 in both *BA* and *AB* genotypes, indicating that neither *A* mitochondria nor *A* nuclei
455 alone were sufficient for *AA*'s diet-dependent phenotype, and therefore that these
456 effects are mito-nuclear (Figure 4a). This outlined a second surprising finding, in
457 which a sympatric mito-nuclear pairing endured a higher cost of dietary EAAs than
458 allopatric pairings (Figure 4a). Thus, unexpected, costly, and definitively mito-nuclear
459 patterns of variation can emerge on specific diets. However, no clear effects of past
460 EAA feeding on offspring quality or quantity were evident (Figure 4b). Together,
461 these results indicated that EAAs interact with mito-nuclear genotype during feeding,
462 but not thereafter. This suggests that the reproductive DMN variation we observed
463 upon EAA enrichment is an acute effect of diet, and therefore likely metabolic and
464 not epigenetic.
465

466 Next we examined the impact of lipid enrichment. Lipid mostly compromised both
467 quality and quantity, but one genotype stood out: *AA* flies were most resistant to the
468 reduction in offspring quality induced by high-lipid diet, and their development index
469 on lipid-enriched food was in fact better than on EAA-enriched food (Figure 4a). By
470 this metric, *AA* flies' performance was substantially better than the *AB* or *BA* flies,
471 confirming that neither *A* mitochondria nor *A* nuclei alone were sufficient to explain
472 *AA*'s response to diet. This again suggested that genotypes which exhibit notable
473 responses to diet do not necessarily bear allopatric combinations of mitochondria
474 and nuclear genomes. Furthermore, the finding that the *AA* genotype was beneficial
475 in one condition (EAA) despite costs in another (lipid) reveals that it was not simply
476 sick as would be expected with a poor-functioning mito-nuclear pairing, but instead
477 exhibits its own genotype-specific pattern of response to diet.
478
479 Finally, we evaluated the impact on offspring of parent-restricted lipid enrichment. All
480 genotypes bore costs, but impacts were highly genotype-specific. Again, the *AA* flies
481 stood out. Specifically, *AA* flies bore a cost of parental lipid feeding on offspring
482 quantity, but appeared to enjoy a compensatory benefit from enhanced offspring
483 quality. In fact, these flies exhibited the highest offspring quality (development index)
484 observed in any condition in our study. These benefits were not evident in either the
485 *AB* or *BA* flies, confirming again that neither *A* mitochondria nor *A* nuclei alone are
486 sufficient to explain diet-dependent phenotype: the observed effects are mito-
487 nuclear.
488

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


control
+ EAA
+ lipid
A
F0 + F1
fed
development index
log N eggs
B
F0 only
fed
development index
log N eggs

498 **Figure 4. Mito-nuclear interactions dictate dietary modulation of offspring**
499 **quality versus quantity.** Panels show relationships per diet and per genotype (data
500 from Figures 2-3). **A** offspring development index and eggs laid, when both parents
501 and offspring were fed experimental diets, and **B** development index before switch
502 and release of egg laying after diet, when parents were switched from experimental
503 to standard diets. Points show EMMs±95% confidence intervals, and grey lines
504 indicate grand means.
505

506

507 **Diet-mito-nuclear effects cause substantial biological variation**

508

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

519

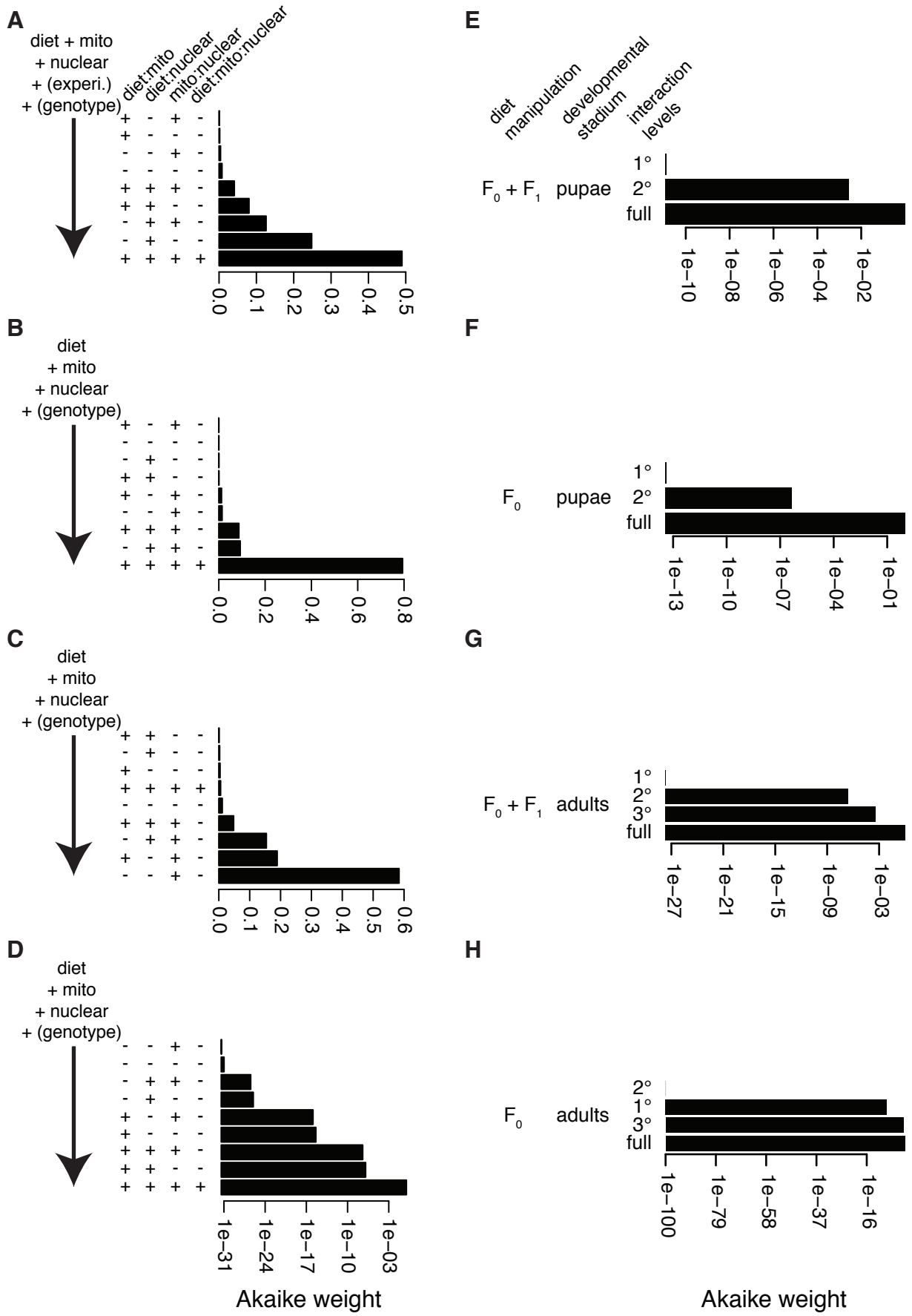
520 Since *de facto* effect sizes for interaction terms are not easily interpreted, we used
521 optimization procedures to compare simple models of the data against those
522 including DMN interactions (Akaike, 1974). Calculating Akaike Weights reveals
523 performance amongst a set of models, in terms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

529

530 Akaike Weights suggested that models accounting for DMN interactions performed
531 best described all datasets but one, and that purely additive models (assuming no
532 interactions) were deficient by comparison (Figure 5).
533
534 For fecundity data (Figures 2-3), we fit a structured series of models, systematically
535 including or eliminating all possible interactions amongst diet, mitochondrial and
536 nuclear genotype. Akaike Weights favoured including DMN interactions (Figure 5a-
537 d): for example, a model of data from Figure 2c including DMN interactions was 61.7
538 times more likely to be the best model than another with no interactions (Figure 5a);
539 and approximately twice more likely than the next-best, which retained only the
540 diet:nuclear interaction (Figure 5a). Models including DMN terms were also the best
541 descriptor of fecundity before switching from experimental media (data from Figure
542 3c), and the release of fecundity after switching (data from Figure 3e). The exception
543 to the emergent rule was the case of fecundity after switching from experimental
544 media (Figure 3d), in which the favoured model included a mito-nuclear term, but not
545 a DMN term. This exception demonstrated that the most complex models were not
546 favoured automatically, but therefore encouraging confidence in other cases,
547 suggesting that DMN interactions do indeed explain substantial variation.
548
549 For development data (Figures 2e, Figure 3f, Figure S4-S6), including terms for
550 offspring sex added an additional order of complexity (i.e. a potential four-way
551 interaction), rendering an assessment of all possible combinations hard to interpret.
552 Therefore, we calculated Akaike Weights for models including either only first-degree
553 terms (a purely additive model, without interaction terms), second-degree terms (all

554 pairwise combinations), third-degree terms (all three-way interactions), or fourth-
555 degree terms (all four-way interactions, but only when sex was known, in models of
556 adult emergence). We did this both for development to pupa (Figure 5e-f) and to
557 adult (Figure 5g-h), and when diet was manipulated either for both parents and
558 offspring (Figures 5e and 5g), or solely for parents (Figures 5f and 5h). In each case,
559 models including all possible interaction terms performed best, often by several
560 orders of magnitude (note logarithmic scale on figures). This analysis indicated that
561 DMN interactions exert a substantial influence on developmental variation.
562
563 In summary, our modelling indicates that key health and fitness metrics - fecundity
564 and development - are subject to significant interactions amongst mito-nuclear
565 genotype and diet, and that those interactions account for significant variation in our
566 data, which reflects the real-world mosaic of diet-mito-nuclear variation.
567

Figure 5


A
diet + mito
+ nuclear
+ (experi.)
+ (genotype)
diet:mito
diet:nuclear
mito:nuclear
diet:mito:nuclear
0.0
0.1
0.2
0.3
0.4
0.5
B
diet
+ mito
+ nuclear
+ (genotype)
0.0
0.2
0.4
0.6
0.8
C
diet
+ mito
+ nuclear
+ (genotype)
0.0
0.1
0.2
0.3
0.4
0.5
0.6
D
diet
+ mito
+ nuclear
+ (genotype)
1e−31
1e−24
1e−17
1e−10
1e−03
Akaike weight
E
diet
manipulation
developmental
stadium
interaction
levels
F0 + F1
pupae
1°
2°
full
1e−10
1e−08
1e−06
1e−04
1e−02
F
F0
pupae
1°
2°
full
1e−13
1e−10
1e−07
1e−04
1e−01
G
F0 + F1
adults
1°
2°
3°
full
1e−27
1e−21
1e−15
1e−09
1e−03
H
F0
adults
2°
1°
3°
full
1e−100
1e−79
1e−58
1e−37
1e−16
Akaike weight

569 **Figure 5. Models including interactions amongst mitochondrial and nuclear**
570 **genotypes and diet treatment are favoured over alternatives.** Barplots indicate
571 Akaike weights analyses of sets of alternative models of data from Figures 2-3.
572 Columns to left of each panel indicate terms in each model. Higher values indicate a
573 better-supported model. The probability that one describes data better than another
574 can be calculated by the ratio of their respective Akaike Weights. Plots compare
575 models of data of fecundity of varied mito-nuclear genotypes fed experimental diets
576 (**A,** data from Figure 2; and **B**, data from Figure 3c), after feeding on experimental
577 diets (**C,** data from Figure 3d), and the ratio of fecundity before and after switching
578 from experimental to standardised diets (**D**, data from Figure 3e). Plots E-H compare
579 models of development time and rate, when models included either no interactions
580 between predictive terms, or various levels of interactions as indicated, for
581 development to pupa when experimental diets were fed to either parents and
582 offspring (**E**, data from Figure S3) or parents alone (**F**, data from Figure S3), and
583 development to adult when experimental diets were fed to either parents and
584 offspring (**G**, data from Figure 2) or parents alone (**H**, data from Figure 3).
585

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 F_1 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

634 could drive deleterious outcomes when EAA levels are not representative of the
635 composition of food that would be found in the yeasts which flies are thought to
636 consume in nature. Alternatively, the discrepancy may prove an example of parent-
637 offspring conflict (Trivers, 1974), in which EAAs promote parental fecundity, despite
638 the detriment of reduced investment into individual offspring. How parent-offspring
639 conflict is regulated at the interface of diet and genetic variation in general is not
640 well-studied.
641
642 To our knowledge, our study is the first investigation of diet-mito-nuclear interactions
643 to manipulate specific nutrient classes, rather than a complex ingredient such as
644 yeast. Our finding that impacts of dietary lipid depend on mito-nuclear genotype may
645 be relevant to understanding variation in impacts of high-fat human diets. The high-
646 EAA diets that we used have parallels to high-protein anabolic diets used to increase
647 yields of livestock and human muscle mass. Our findings that EAAs generally
648 decrease offspring quality may give pause for thought in use of these diets.
649 Furthermore, the finding of mito-nuclear variation in the response to EAAs
650 enrichment demonstrates that certain individuals may bear particularly strong costs
651 of eating such diets. At present, we do not know the mechanistic basis of the diet-
652 mito-nuclear interactions that we have uncovered, but the transgenerational effects
653 hint at epigenetics, consistent with preceding findings that epigenetic marks respond
654 to genetic variation in the cytoplasm (Bellizzi et al., 2012; Grunau et al., 2018; Vivian
655 et al., 2017; Yan et al., 2011).
656

657 Our findings have implications for understanding variation that could emerge when
658 novel mito-nuclear pairings come to be, such as after mitochondrial replacement
659 therapy. Our results do not necessarily support the proposal to “match” mito-nuclear
660 pairs as a positive predictor of therapeutic success (Dowling et al., 2010; Mossman
661 et al., 2016a; Vaught et al., 2020), because a naturally co-occurring mito-nuclear pair
662 (*AA*) responded uniquely to diet, suggesting that costs and benefits are not
663 necessarily straightforward functions of mito-nuclear matching or mis-matching.
664 Thus, comparing performance of matched versus unmatched groups may not be
665 sufficient to evaluate risks of mitochondrial transfer therapy. Our analysis suggests
666 that mitochondrial transfer may be variously deleterious, beneficial, or have
667 unforeseen costs and benefits. Dietary recommendations may promote patient
668 health. Our data also lend kudos to the suggestion of transgenerational effects of
669 mitochondrial replacement therapy, especially when diet varies. A change of
670 nomenclature may ultimately be called for, since “matched” and “mis-matched” carry
671 intrinsic value judgments, but it seems that whether mitochondria and nuclei are of
672 shared origin does not necessarily predict cost or benefit. We suggest that deep
673 knowledge of patient and donor nuclear and mitochondrial genotype will likely be
674 required to predict a healthy match.
675
676 Our results have wider implications for the conduct of nutritional and genetic
677 research. Fly nutrition studies have drawn scrutiny for dietary variation between
678 studies, attributed to methodological reporting (Lesperance and Broderick, 2020) and
679 stochastic differences in ingredients (principally yeast) over time (Piper et al., 2014),
680 which may limit repeatability. Similar issues can ensue after inconsistent

681 standardisation of genetic background (Burnett et al., 2011). The mito-nuclear field
682 as a whole suggests that different outcomes are to be expected when mitochondrial
683 and nuclear haplotypes are not carefully controlled by backcrossing, and our results
684 indicate that variation between studies may be amplified when diet is manipulated.
685 Even stochastic variation in diet may interact with mito-nuclear variation, making the
686 case for careful reporting, and perhaps the use of chemically-defined media (Piper et
687 al., 2014). Accounting for these sources of variation will likely improve repeatability.
688
689 In summary, we have shown that diet, mitochondrial and nuclear haplotype have
690 complex interactive effects, each capable of modifying the impact of change in
691 another. Dietary lipid and EAAs are both implicated separately in these interactions,
692 with relevance not just for proximate reproductive output, but also for lasting
693 transgenerational effects of diet which impact relative offspring fitness. These
694 varying, mito-nuclear impacts of diet on offspring quantity and quality appear to be
695 an important determinant of individual fitness and health.
696

697 **Materials & methods**

698

699 **Diets**

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

703

704 Experimental media build on published protocols (Bass et al., 2007; Dobson et al.,
705 2018; Emran et al., 2014). These media contained a final concentration of 10%
706 brewer's yeast, 5% sucrose, 1.5% agar (w/v), 3% nipagin and 0.3% propionic acid
707 (v/v). EAAs were purchased as powder (Sigma), and supplemented by dissolving
708 into a 6.66x solution in ddH_20 pH 4.5 (final media concentrations: L-arginine 0.43 g/l,
709 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-
710 methionine 0.1 g/l, L-phenylalanine 0.26 g/l, L-threonine 0.37 g/l, L-tryptophan 0.09
711 g/l, L-valine 0.4 g/l). Margarine (*Ja! Pflanzenmargarine* from Rewe Supermarkets,
712 Germany; 720 kcal / 100g; 80/100g fat from 23/100g saturated fatty acids, 40/100g
713 monounsaturated fatty acids, 17/100g polyunsaturated fatty acids) was briefly melted
714 then mixed thoroughly into the food (15% w/v). Final nutrient contents of rearing and
715 control media were estimated using the *Drosophila* diet content calculator
716 (Lesperance and Broderick, 2020), with additional protein, lipid and caloric content
717 after nutrient supplements calculated according to margarine nutrient content report,
718 and by assuming caloric equity between EAAs and protein at a caloric value of 4
719 calories/g (USDA). Vials contained ~5ml of food, and were stored at 4°C for up to 1
720 week before use.

721

722 **Flies**

723 *D. melanogaster* fly lines were established as described in Figure 1 and maintained
724 at 25°C on development medium throughout their history prior to our experiments.
725 The ancestral Australian population was isolated in Coffs Harbour, NSW, Australia
726 (Dowling et al., 2014). The Benin population is the widely-used *Dahomey* population,
727 isolated in the 1970s in Dahomey (now Benin). Ancestral lines had been cured of the
728 cytoplasmic endosymbiont *Wolbachia* 66 generations previously by tetracycline
729 treatment prior to the experiment. For each fly line, 45 females of the desired
730 mitochondrial background were crossed to 45 males of the desired nuclear
731 background per generation. Iterating this process over many generations led to
732 introgression of the desired nuclear background (from males) into each mitochondrial
733 background. For experiments, flies were collected upon eclosion to adulthood and
734 fed fresh developmental medium, before being pooled, split and assigned at random
735 to experimental medium in groups of 5 males and 5 females. Experimental flies were
736 maintained at 25°C, and transferred to fresh media every 48-72h for one week. Flies
737 were transferred to fresh medium 24h before egg laying experiments. For
738 development experiments, eggs were incubated at 25°C and pupation and eclosion
739 were scored daily. Eclosing adults were lightly CO_2-anaesthetised before counting
740 and sexing.

741

742

743 **Analysis**

744 Data were analysed in R 3.6.1. Markdowns of all analyses are provided as
745 supplementary material. Fit of fecundity data to a negative binomial distribution was
746 determined with firdistrplus::descdist and firdistrplus::fitdist. Generalised linear mixed
747 models of the form
748

749 $$y \sim diet * mitochondria * nuclear + (genotype)$$

750

751 were fit with lme4::glmer.nb (egg counts, negative binomial distribution) or
752 lme4::glmer (binomial of egg counts before and after diet switch); in which *diet*
753 (control/EAA/lipid), *mitochondria* (A/B) and *nuclear* (A/B) were fixed factors,
754 genotype was a random factor denoting the full replicated genotype (e.g. *AA1, AA2,*
755 *AA3, AB1, AB2, BA1*, etc). Where relevant (Figure 2), experimental replicate was
756 also included as an additional random factor. Dispersion was assessed with
757 blmeco::dispersion_glmer, and model singularity was tested with lme4::isSingular.
758 Anova tests (type-3) were conducted with car::Anova, and *post-hoc* analyses were
759 applied with the functions emmeans::joint_tests, emmeans::pairs, emmeans::emmip
760 (Searle et al., 1980). AIC was determined with stats::AIC, Akaike weights with
761 MuMIn::dredge. Four vials were excluded from the diet-switching experiment to
762 enable model fitting, on the basis of extreme values (minimum and maximum
763 observations in the experiment) or deviation from a QQ plot before diet switch.
764 These data are retained, with note, in the data associated to this paper.
765

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

768 greater than eggs in some vials, due to measurement error). A 50:50 sex ratio was
769 assumed amongst censors. Development to adult was modelled by fitting Cox mixed
770 effects models of the form
771

772 $$y \sim diet * mitocondria * nuclear * sex + eggs + (\frac{genotype}{vial})$$

773

774 with coxme::coxme. *Diet, mitochondria, nuclear* and *genotype* terms were as in
775 models of egg laying, and *vial* was an additional random effect nested in *genotype*.
776 *Eggs* coded number of eggs laid in the vial in which the individual developed, to
777 account for variation in rearing density. Pupal sex was unknown, so the sex term
778 was excluded from models of pupal development. Anova and *post-hoc* EMM tests
779 were conducted as per fecundity analyses, with additional stratification by sex when
780 applying emmeans::joint_tests.
781
782 Figures were assembled in Adobe Illustrator. Mitochondria graphics were recoloured
783 from files freely distributed under an open commons licence. The heatmap of nutrient
784 content was plotted in R with the superheat library.
785
786
787
788

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Declaration of interests

The authors declare no competing interests.

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1188

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
	mito	1	9.262	0.0023
	nuclear	1	1.331	0.2486
	diet	2	0.396	0.6731
male	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

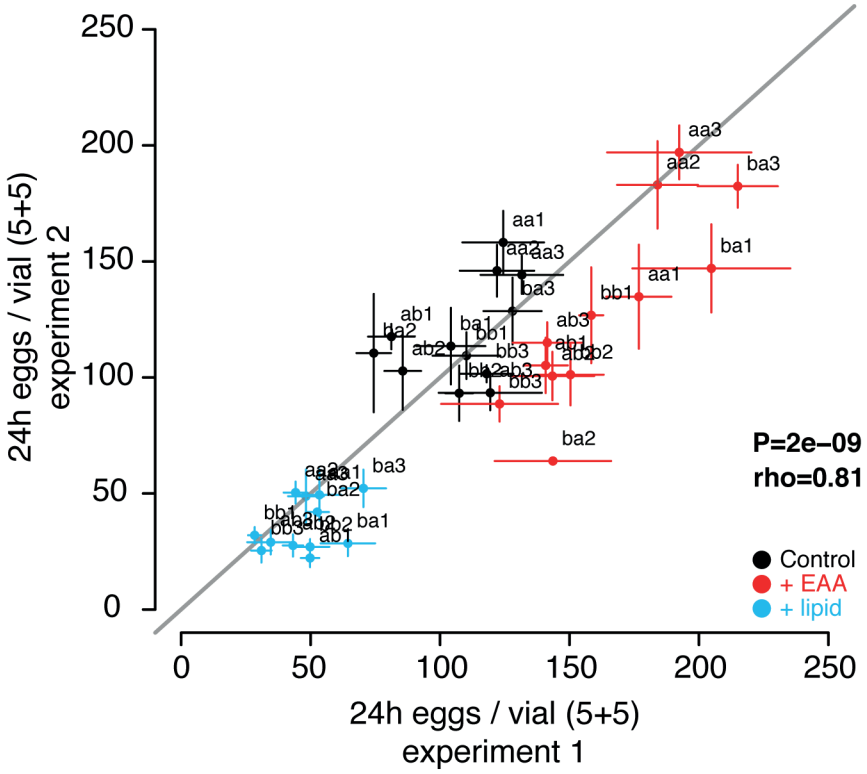
250
200
150
100
50
0
24h eggs / vial (5+5)
experiment 2
0
50
100
150
200
250
24h eggs / vial (5+5)
experiment 1
aa3
aa2
ba3
aa1
aa2
aa3
ba3
ba1
aa1
bb1
ab1
ba2
ba1
bb1
ab3
ab2
bb3
ab1
ab2
bb2
bb2
ab3
bb3
ba2
ba3
ba2
bb1
ba1
ab1
P=2e−09
rho=0.81
Control
+ EAA
+ lipid

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

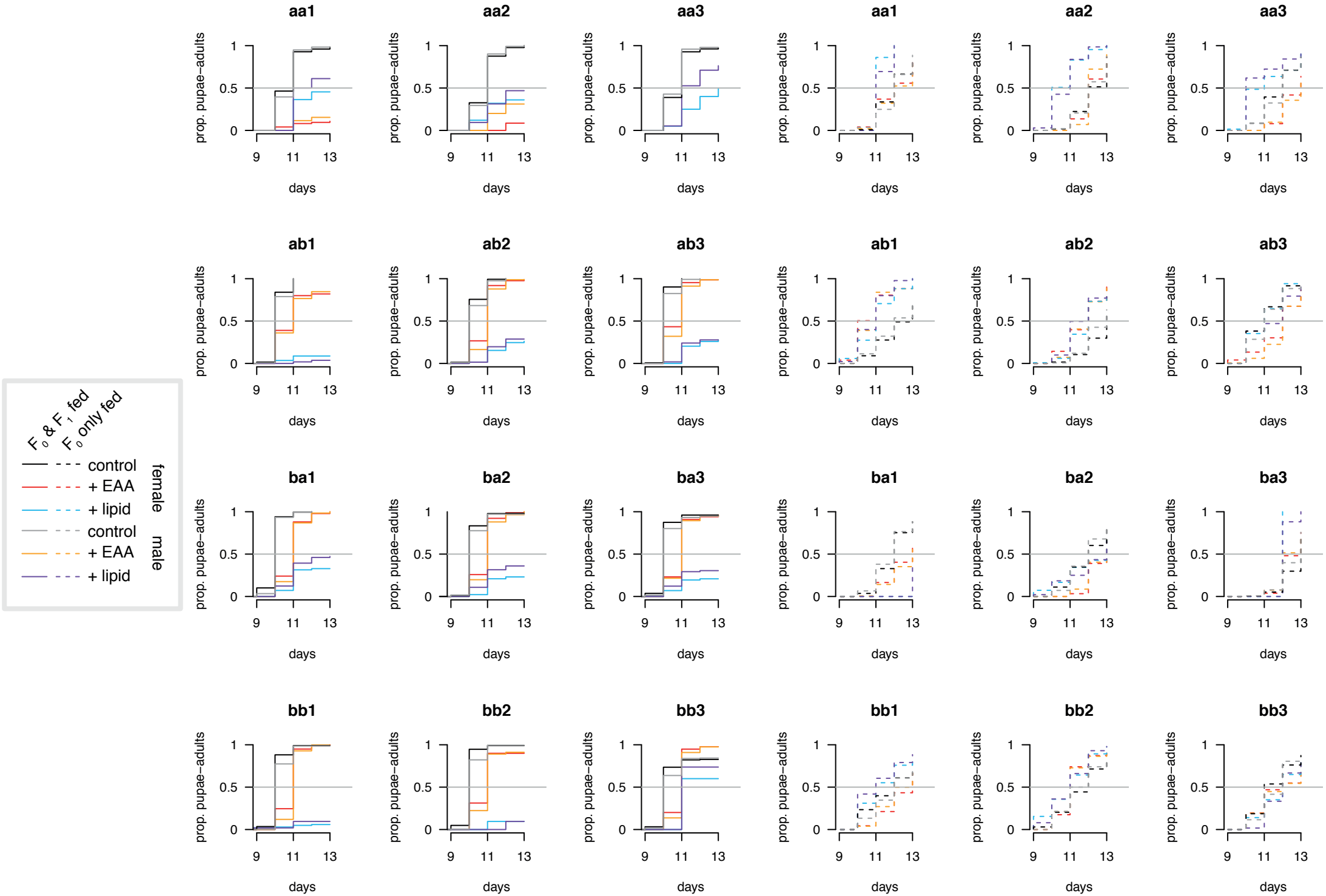
aa1
aa2
aa3
ab1
ab2
ab3
ba1
ba2
ba3
bb1
bb2
bb3
prop. pupae–adults
days
0
0.5
1
9
11
13
F0 & F1 fed
F0 only fed
control
+ EAA
+ lipid
control
+ EAA
+ lipid
female
male

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

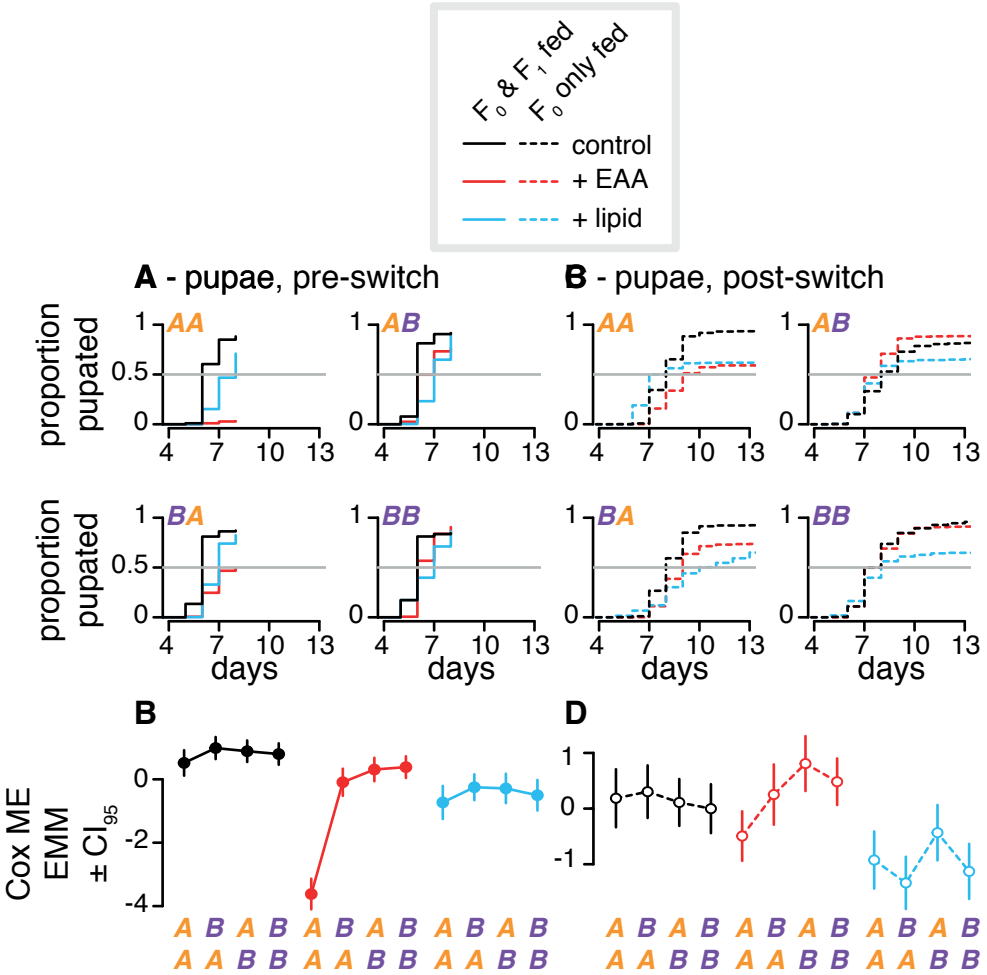
F0 & F1 fed
F0 only fed
control
+ EAA
+ lipid
A - pupae, pre-switch
B - pupae, post-switch
proportion pupated
AA
AB
BA
BB
days
B
D
Cox ME EMM ± CI95
A B A B A B A B A B A B
A A B B A A B B A A B B

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

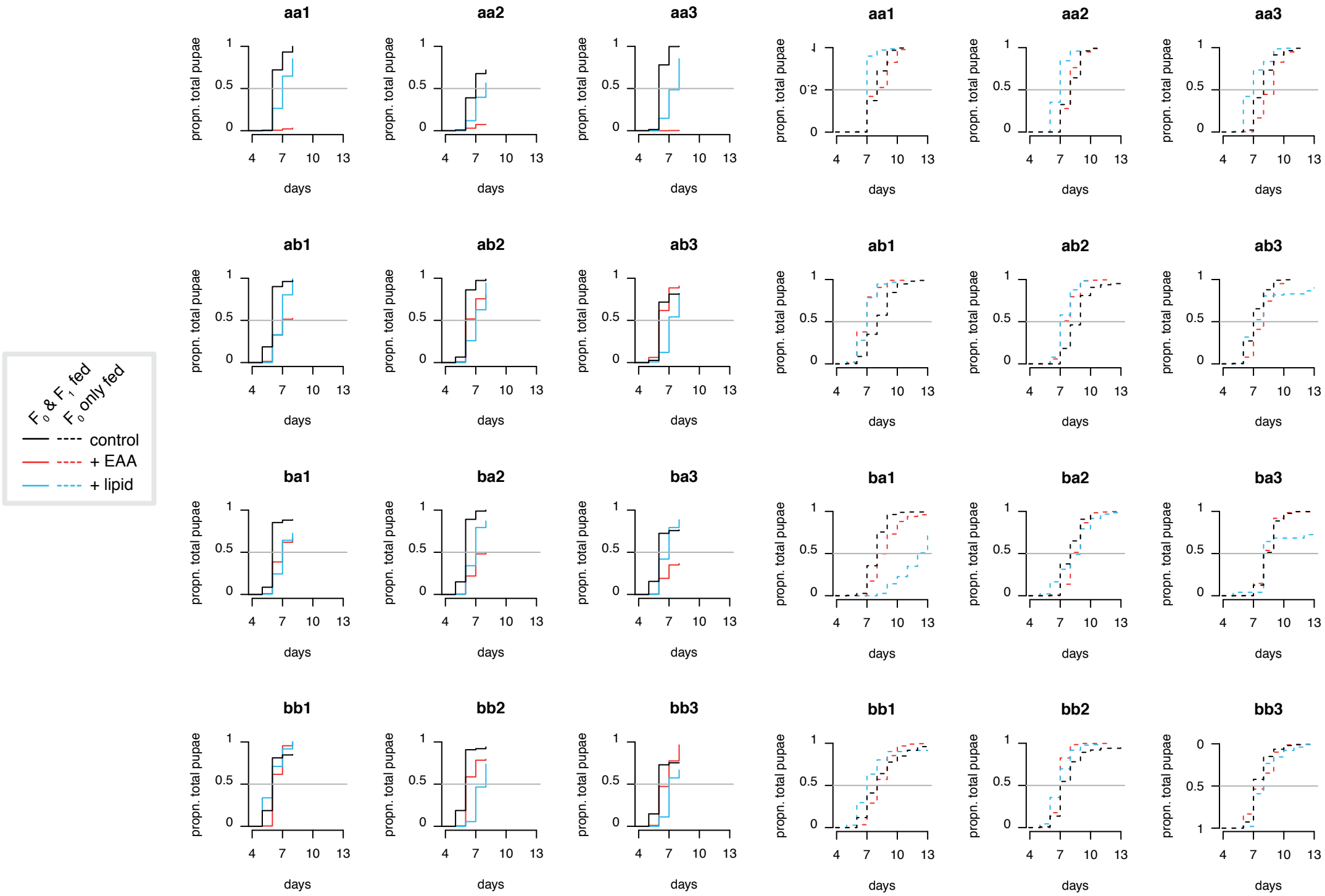
aa1
aa2
aa3
ab1
ab2
ab3
ba1
ba2
ba3
bb1
bb2
bb3
propn. total pupae
days
F0 & F1 fed
F0 only fed
control
+ EAA
+ lipid

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

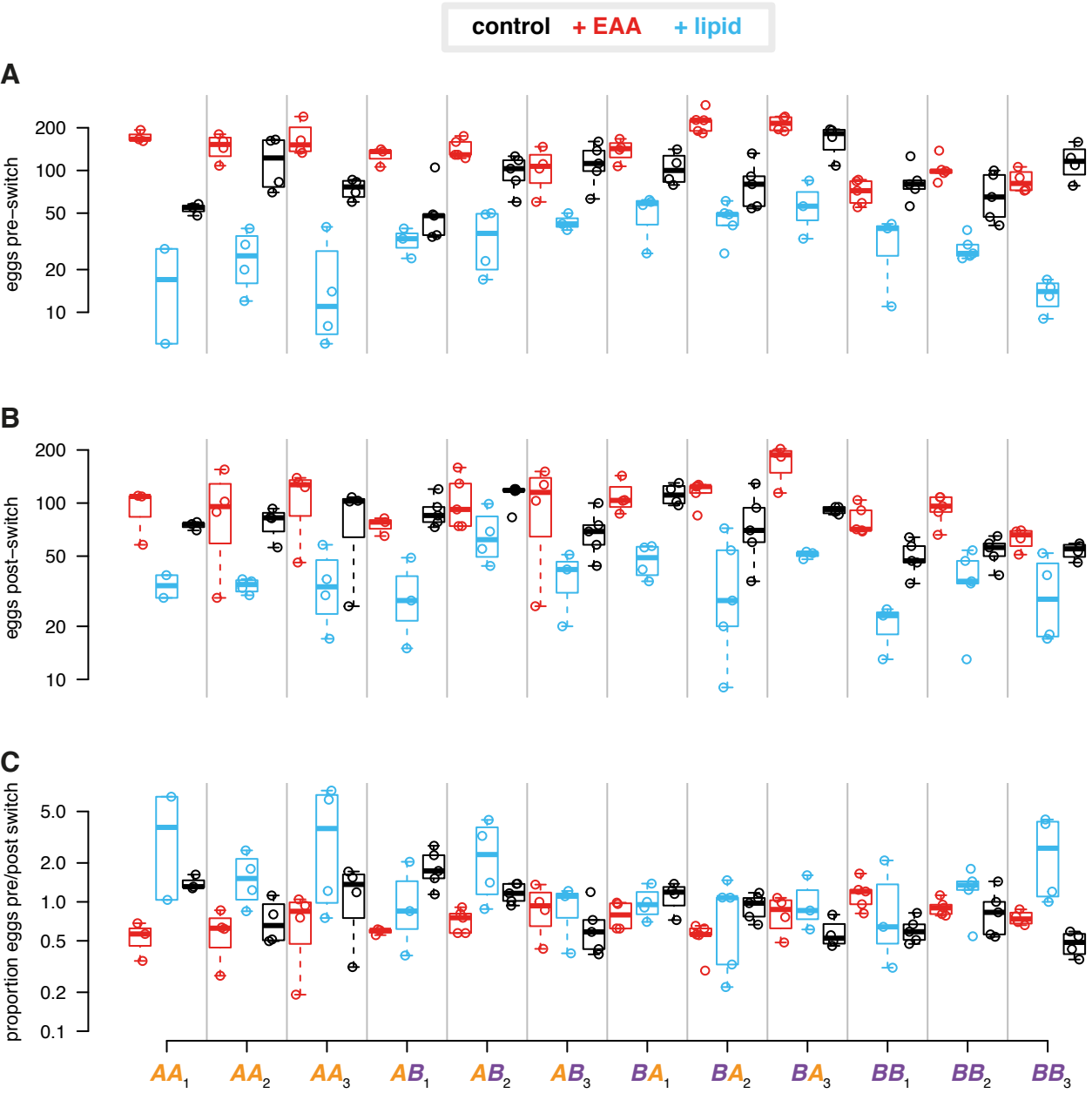
control
+ EAA
+ lipid
A
eggs pre–switch
200
100
50
20
10
B
eggs post–switch
200
100
50
20
10
C
proportion eggs pre/post switch
5.0
2.0
1.0
0.5
0.2
0.1
AA1
AA2
AA3
AB1
AB2
AB3
BA1
BA2
BA3
BB1
BB2
BB3

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

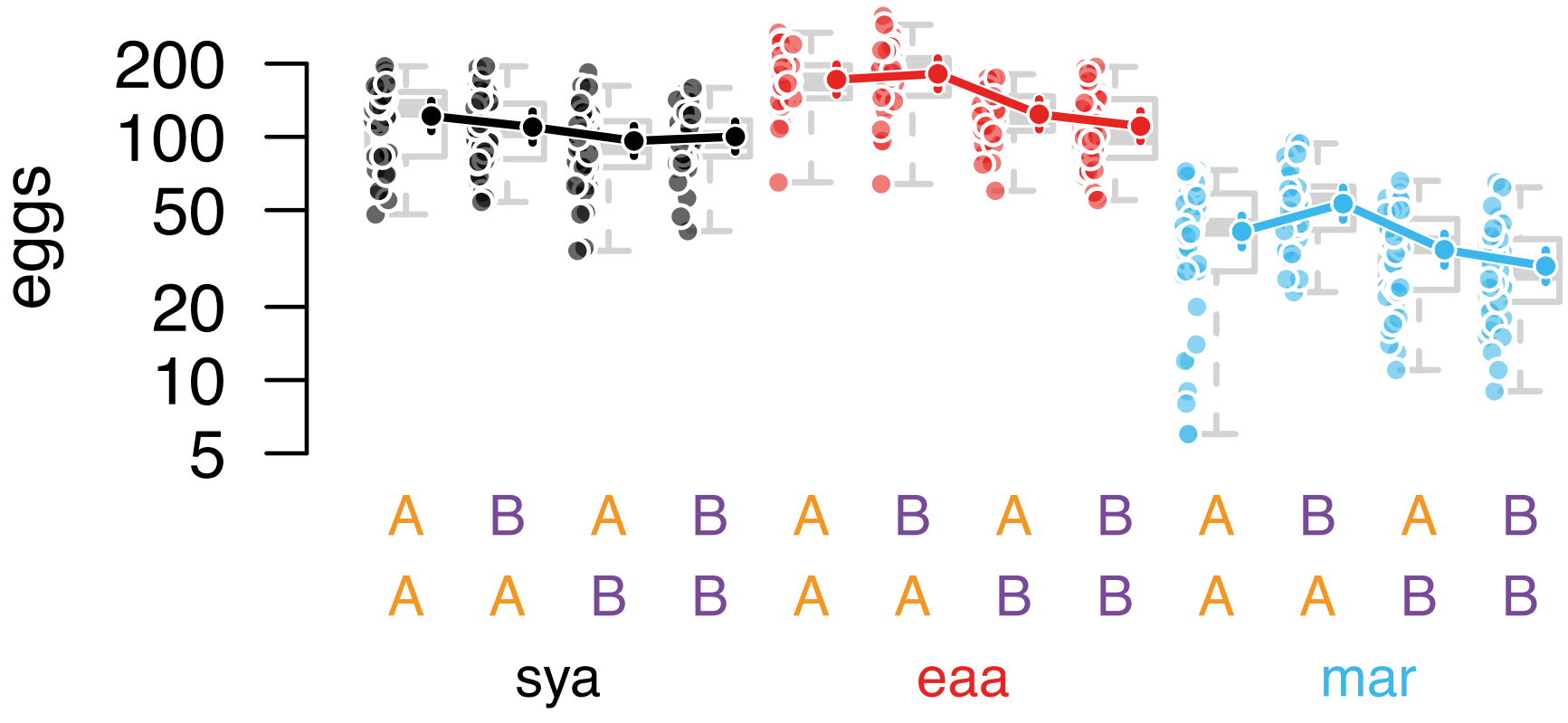
eggs
200
100
50
20
10
5
A B A B A B A B A B A B
A A B B A A B B A A B B
sya
eaa
mar

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.