Diet's impact dictated by synonymous mitochondrial SNP interacting with nucleotype

Adam J. Dobson[1,2]*, Susanne Voigt[2], Luisa Kumpitsch[2], Lucas Langer[2], Emmely Voigt[2], Rita Ibrahim[1], Damian K. Dowling[3], Klaus Reinhardt[2]*

[1]Institute of Molecular Cell and Systems Biology, University of Glasgow, G12 8QQ, UK.

[2]Applied Zoology, Faculty of Biology, Technische Universität Dresden, 01069 Dresden, Germany.

[3]School of Biological Sciences, Monash University, Clayton, Victoria 3800, Australia

*Corresponding authors. Email: adam.dobson@glasgow.ac.uk, klaus.reinhardt@tu-dresden.de

ABSTRACT: Epistasis between mitochondrial and nuclear genomes can modulate fitness effects of nutrition. Are these "mitonuclear" effects deterministic with regard to optimal nutrition? Which nutrients and genetic loci participate? Here, in fruitflies, we show that mitonuclear epistasis repeatably dictates fitness effects of dietary lipids and amino acids, with a tripartite interaction emerging as an unprecedented source of quantitative variation. We also observed diet-dependent developmental lethality and parental effects, but only in specific mitonucleotypes. Associating phenotype to mtDNA variation implicated a non-coding RNA (*mt:lrRNA*), suggesting a novel mechanism, with epistasis between mitochondria-derived regulatory factors and the nuclear genome producing qualitative differences in how diet impacts fitness.

SUMMARY: Combined action of nuclear genotype and regulatory loci on mtDNA produces major differences in fitness impacts of nutrition.

Introduction

Nutrition and genotype are primary determinants of variation in health and biological fitness. They can also interact, resulting in different responses among genotypes to the same nutritional changes (*1*, *2*). In humans, there is interest in leveraging this variation to optimize nutrition, by personalizing diet to individual consumers' needs (*3*). To realize this ambition we must understand the genetic drivers of variation in response to nutrition, but this is challenging because independently segregating loci have non-additive, epistatic interactions (*4*), which may modulate response to nutrition, i.e. diet-by-genotype-by-genotype variation (*5*). Which genetic loci are involved? Mitochondria are critical metabolic hubs, with their own small genome, and variation in their function can contribute to variation in dietary optima. The mitochondrial genome segregates independently of the nuclear genome, and the combination of mitochondrial and nuclear variants can generate "mitonuclear" epistasis (*6*, *7*). This epistasis is thought to occur because mtDNA is transcribed, processed and translated by nuclear-encoded proteins, and mtDNA-encoded proteins function in pathways and complexes that include nuclear-encoded proteins (*8*). Reciprocally, outputs of genetic variation in the nucleus depend on how mitochondrial metabolites and signals feed into broader cellular networks. Mitonuclear epistasis has been reported for numerous traits and processes (*9*–*11*), but diet-by-mito-by-nuclear (DMN) interactions are less well-characterized, despite evidence for mitochondrial modulation of nutrient signaling (*12*). So far, DMN interactions have been shown for development time, lifespan, fecundity and gene expression in *Drosophila melanogaster* (*5*, *13*–*16*), but multiple important questions remain: (A) How much phenotypic variation do DMN interactions cause, relative to lower-order interactions (i.e. nuclear-diet, mitochondria-diet) and main effects (i.e. diet, nuclear, mitochondria)? (B) Is mitonuclear genotype deterministic for response to nutrition? In other words, when mitonuclear genotypes are replicated, are responses repeatable? (C) Parental nutrition can modulate offspring fitness, independent of offspring diet (*17*) - is this impact of nutritional variation subject to mitonuclear variation? (D) Which specific dietary nutrients cause DMN variation? Perhaps most importantly, (E) which mitochondrial polymorphisms underpin DMN interactions?

Here, in *Drosophila*, we study how variation among mitochondrial genotypes (mitotypes) modulates reproductive response to specific nutrients, in distinct populations of nuclear genotypes (nucleotypes),

using a panel of fully-factorial mitonuclear pairings (mitonucleotypes). We study reproductive traits because of their relevance to biological fitness, expanding on preceding studies (*5*, *13–16*) through multidimensional analysis of reproductive phenotype, and manipulating specific dietary nutrients (essential amino acids and lipid). We show that diets expected to promote fitness can in fact be lethal to specific mitonucleotypes. We also show that effects of parental nutrition on offspring performance are mitonucleotype-specific. This DMN variation was repeatable among independent genetic replicates. Effect sizes of DMN interactions were large for some traits, even exceeding those for diet:mitotype or diet:nucleotype interactions, implicating mitonuclear epistasis as a more important determinant of response to nutrition than variation in either genome alone, and showing that DMN variation can be a major source of phenotypic variation. Importantly, we observe DMN interactions among populations differentiated only by an mtDNA polymorphism was in a non-protein coding gene, long ribosomal RNA (*mt:lrRNA*), implicating a mitochondrial regulatory factor in DMN effects. Altogether these results suggest that mitonuclear epistasis can be a leading determinant of optimal diet, that this variation maps to putatively regulatory variants on mtDNA, and that the consequences can be a matter of life or death.

Results

We produced *D. melanogaster* populations (Figure S1A) comprising replicated and fully-factorial combinations of mitochondrial and nuclear genomes from Australia, Benin and Canada (*A*, *B*, *C*, respectively). The crossing scheme was designed to produce distinct mitochondrial backgrounds bearing equivalent pools of standing nuclear variation, by introgressing populations either reciprocally or to themselves. For brevity, we abbreviate population names, giving mitochondrial and then nuclear origin (e.g. *AB* = Australian mitochondria, Beninese nuclei). Each combination was triplicated (e.g. AB_1, AB_2, AB_3) at the beginning of the introgression and maintained in parallel for more than 160 introgressions, altogether generating 27 populations, comprising nine triplicated mitonucleotypes. We confirmed that this design (A) replaced original nucleotypes with donor nucleotypes (Figure S1B, Figure S1C), at frequencies from 94.4% to 99.9% (Figure S1F, Supplementary Text), and that (B) nucleotype replacement was equivalent in the presence of distinct mitotypes (Figure S1B, Supplementary Text). These analyses confirmed that the panel of populations was suitable for studying the behaviors of distinct mitotypes in populations with comparable nuclear genomic diversity. These analyses also

85 indicated that the majority of mitotype diversity was represented by populations bearing only Australian
86 or Beninese mitochondria (Figure S1B, Supplementary Text), and so populations with Canadian
87 ancestry were excluded from further study, to reduce the total number of populations for phenotyping
88 from 27 to 12 (Supplementary Text). We then examined the fitness response of these populations (*AA_{1-}*
89 *$_{3}$, AB_{1-3}, BA_{1-3} and BB_{1-3}*) to dietary manipulations, applying both an established manipulation that
90 promotes fecundity by enriching essential amino acids (EAA), and a novel manipulation that represses
91 fecundity by enriching plant-based lipids (Figure S2A, Supplementary Text). Feeding these EAA-
92 enriched and lipid-enriched diets to the focal panel of 12 populations (Figure S2C, Supplementary Text)
93 revealed mitonuclear variation in fecundity response (Figure S2D, Supplementary Text), which could
94 not be accounted for by caloric density (Figure S3B, Supplementary Text), revealing for the first time
95 specific nutrients that are sufficient to elicit DMN variation (Figure S3B, Supplementary Text). This
96 fecundity variation was both (A) technically repeatable among replicate experiments (Figure S3A,
97 Supplementary Text), suggesting that introgression maintained equivalent diversity across generations;
98 and (B) biologically repeatable among replicate lines (Figure S2E, Supplementary Text), suggesting
99 that the repeatable genetic variation in the lines led to predictable and repeatable phenotypic variation.
100

101 ***Large effects of DMN interactions on multiple fitness parameters***

102 Encouraged by initial fecundity results, we characterized a more extensive panel of fitness traits. In a
103 total of >25,000 individual flies we assayed fecundity, fertility and development time - traits that have
104 previously been shown to be sensitive to DMN variation (*13*, *16*) - as well as number of adult progeny
105 as a direct fitness measure. We varied feeding on experimental diets in two different ways (Figure 1A).
106 We hypothesized that there might be mitonucleotype-dependent variation in response to (A) chronic
107 dietary change for both parents and offspring, and (B) parental diet, which can influence offspring health
108 independent of offspring diet (*17*, *18*). Therefore, eggs were laid and developed either in a chronic
109 feeding paradigm, in which both parents and offspring were fed experimental diets; or a parental feeding
110 paradigm, in which diets were fed to parents before eggs were laid and developed on a standardized
111 medium, distinct from parental diet. In the latter context, DMN interactions can only result from a
112 parental effect. To ensure genetic consistency, the same parents were used in each paradigm, by laying
113 eggs for 24h after one week on experimental media (chronic paradigm), then switching to a universal

Figure 1

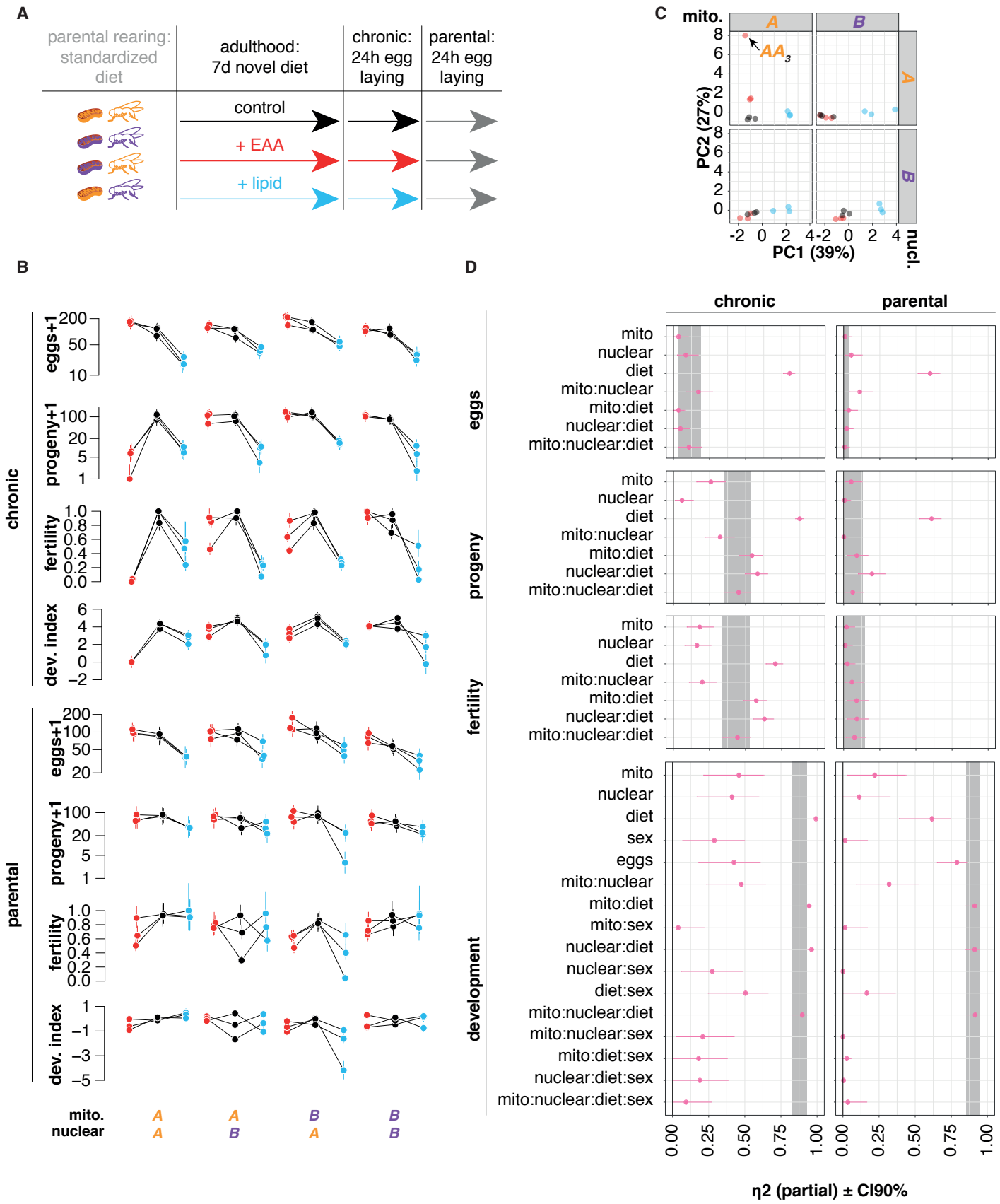
A
parental rearing: standardized diet
adulthood: 7d novel diet
chronic: 24h egg laying
parental: 24h egg laying
control
+ EAA
+ lipid
B
chronic
parental
eggs+1
progeny+1
fertility
dev. index
mito.
nuclear
C
mito.
nucl.
PC2 (27%)
PC1 (39%)
AA3
D
chronic
parental
eggs
progeny
fertility
development
mito
nuclear
diet
sex
eggs
mito:nuclear
mito:diet
mito:sex
nuclear:diet
nuclear:sex
diet:sex
mito:nuclear:diet
mito:nuclear:sex
mito:diet:sex
nuclear:diet:sex
mito:nuclear:diet:sex
η2 (partial) ± CI90%

Figure 1. Large and repeatable impacts of mitonucleotype on response to chronic and parental nutritional change. A. Key and experimental design. Flies 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, followed by a further 24h son standardized rearing medium. **B.** Repeatable mitonuclear variation in response to chronic and parental changes in nutrition. Panels show EMMs for trait indicated on y-axis. Feeding paradigm and mitonuclear variation are indicated at the top of the plot. Colors encode diet as per key. Development index shows EMMs for Cox mixed-effects models of proportion eclosed over time, excluding sex from plot. Development data are plotted in full as Kaplan-Meier plots in Figure S4. Note the exclusion of EMMs for development of genotype AA_3 in chronic feeding: EAA lethality prevented meaningful estimation. **C.** PCA shows ordination of populations according to mitotype, nucleotype, and diet. Results shown from one PCA of scaled and mean-centered EMMs, split by facets per genotype, with mitotype split by rows and nucleotype split by columns. **D.** Effect sizes for mitonuclear modification of responses to nutrition. Panels show effect size estimates (partial η^2) for traits indicated on left, in feeding paradigms indicated at top. Partial η^2 calculated from GLMMs (fecundity, progeny, fertility) or Cox Mixed Models (development). Grey bands indicate confidence interval (90%) for diet-mito-nuclear terms. For all traits in the chronic feeding paradigm, diet consistently had the largest effect size, but diet-mito-nuclear effect size was either greater than or equal to mito-diet and nuclear-diet terms, and also larger than mito or nuclear main effects. In the parental feeding paradigm, for fertility and development but not fecundity or progeny, diet-mito-nuclear effect size was either greater than or equal to other genetic modifiers of response to nutrition, and by ranking greater than main mito or nuclear effects.

114 standardized medium for another 24h of egg laying (parental paradigm). The parents developed on a
115 standardized medium that was distinct from all experimental media, to ensure that any novelty effects
116 were distributed evenly among experimental conditions.
117
118 To visualize patterns of DMN variation, and repeatability among replicate mitonucleotypes, we
119 calculated Estimated Marginal Means (EMMs (*19*), Figure 1B). DMN variation was visually apparent for
120 all traits (Figure 1B – noting exclusion of genotype AA_3 from development in chronic paradigm, see
121 Supplementary Text). Statistical models revealed DMN interactions for all traits in each feeding
122 paradigm (GLMMs for fecundity, progeny and fertility; Cox mixed-effects models for development time),
123 except for fecundity in the parental feeding paradigm (Table S5). No trait varied as a linear function of
124 caloric density (Figure S5), and so impact of diet was modelled as an unordered factor. For development
125 time models, we also included interactions with offspring sex, because of reports of sex-biased
126 mito:nuclear variation (*16*). However, sex did not modify genotype-by-diet interactions (all sex
127 interactions with diet, mitochondria, or nuclear background: $p>0.05$).
128
129 Chronic lipid feeding was deleterious for all traits, but mitonuclear genotype determined magnitude;
130 chronic EAA feeding promoted fecundity but, surprisingly, reduced fertility (Figure 1B). Consequently,
131 progeny counts on EAA did not exceed counts on control diet, suggesting that fitness was not enhanced
132 by EAAs. The magnitude of the fecundity benefits and fertility costs were again mitonucleotype-
133 dependent. In *AA* populations, uniquely, progeny count after chronic EAA feeding was even lower than
134 after chronic lipid enrichment, to near lethality in AA_3 (Figure 1B), but this sensitivity was rescued by
135 replacing either the mitochondria (*BA*) or nuclei (*AB*) (Figure 1B), confirming a mitonucleotype-specific
136 effect. Mitonuclear incompatibility is widely reported (*20*), but our data indicate that it can be diet-
137 dependent, under nutrient-enriched conditions that we had expected to promote fitness.
138
139 DMN variation was also apparent in the parental feeding paradigm, albeit less pronounced than after
140 chronic feeding. Lipid was less universally toxic. *AA* flies even exhibited a benefit of parental lipid
141 feeding, developing on average one day earlier (Figure S4), but again this was not evident in *BA* or *AB*
142 flies (Figure 1B), confirming another mitonucleotype-specific effect.

143

144 To estimate repeatability for all traits, we used the same approach as in our initial fecundity experiments
145 (Supplementary Text), and found among-replicate repeatability both within each diet and in response
146 to nutrient enrichment, for all traits (Figure S6).

147

148 To distill the complex patterns of variation in this multi-dimensional dataset, we performed PCA on
149 EMMs for all traits on all diets in all populations, for an integrative view of how mitonucleotype
150 determined response to diet (Figure 1C). Control or EAA-fed flies separated from lipid-fed flies on the
151 first principal component. However the second principal component revealed an orthogonal response
152 to EAAs in *AA* mitonucleotypes, particularly in line AA_3 (Figure 1C). This analysis was consistent with
153 an interpretation that DMN effects induce coordinate changes in multiple traits, leading to an altered
154 phenotype overall.

155

156 We used these data to address one of our main questions: Is mitonuclear epistasis deterministic for the
157 impact of nutritional variation? We calculated a measure of effect size (partial η^2) for DMN interactions,
158 and compared to lower-order effects (Figure 1D), anticipating that diet would be the largest source of
159 variation for most traits, but that this this might be modified by mitonucleotype (i.e. large DMN terms).
160 However we were surprised to find that the magnitude of DMN effects approached or equaled diet's
161 effect for some traits, indicating a major source of variation. In the chronic feeding paradigm, DMN effect
162 sizes were greater than or equal to mito:diet and nuclear:diet effects for egg laying, progeny and fertility
163 (Figure 1D). For development in both paradigms, DMN effect sizes were large, on par with diet,
164 diet:mitotype, and diet:nucleotype; revealing DMN interactions as a major source of variation for
165 developmental impacts of chronic dietary change in these flies. Fertility effects were more pronounced
166 after chronic feeding than after parental feeding, but in both paradigms DMN effect sizes were ~75% of
167 diet's main effect, as were mito:diet and nuclear:diet terms, suggesting that these factors do not simply
168 modulate effect of diet, but their interaction with diet is a substantial source of variation outright. In fact,
169 in the parental nutrition paradigm, DMN effect sizes for fertility outranked the main effect of diet,
170 although with overlapping confidence intervals and modest effect size. However, for development,
171 effect sizes for DMN terms exceeded nearly all other terms (exception for the lower-order diet:mito and

172 diet:nuclear interactions), without overlapping confidence intervals (Figure 1D). The magnitude of effect
173 on this trait exceeded even the main effect of diet, suggesting that dietary regulation of this trait could
174 not be properly understood without accounting for mitonucleotype. We also validated our effect size
175 calculations orthogonally, by assessing how a range of alternative models described the data, and by
176 calculating variance explained (r^2) by each model, which gave congruent results (Supplementary Text).
177 These analyses suggest that mitonucleotype can modulate response to dietary variation, and the
178 emergent interaction can produce as much phenotypic variation as the main effect of diet.
179
180 ***Associating DMN interactions with mitochondrial polymorphisms implicates regulatory***
181 ***variation***
182 The second major question of our study was: what are the putative causes of DMN interactions?
183 Mitochondrial polymorphisms underlying DMN interactions are largely uncharacterized. We examined
184 our populations' mitochondrial SNPs in detail, to ask if phenotypic variance segregated according to
185 mitonucleotype. mtDNA SNP frequencies were stable over time (Supplementary Text, Table S8),
186 suggesting we could use these flies in the same way as established genetic mapping populations (*21*),
187 because introgression maintained variation and therefore phenotypic variation could be associated with
188 preceding sequence data. We found 27 SNPs, in both protein coding and non-coding regions of mtDNA
189 (Table S3), but only 2/27 SNPs were expected to change amino acid sequence (Table S3), suggesting
190 that synonymous and regulatory polymorphisms might underlie DMN interactions (Supplementary
191 Text). To reveal how major mtDNA alleles co-segregated, and how they intersected with nucleotypes,
192 we performed hierarchical clustering of major alleles, and examined how the clustering grouped
193 populations (Figure 2A). This separated *A* mitotypes from *B*, validating our initial approach of encoding
194 mitotype by geographic origin (Figures 2, 3). However, nested within this higher-level grouping, more
195 granular among-line differentiation was evident, with five distinct clusters of unique mitotypes. These
196 mitotypes were not nested within nucleotype, and some co-occurred with both *A* and *B* nucleotypes.
197 We confirmed this pattern of co-occurrence orthogonally, with network analysis (Supplementary Text).
198 These patterns of co-occurrence suggested that differential responses to diet could be associated to
199 the distinct mitotypes. To generate a final, sequence-informed mitonucleotype assignment, we
200 concatenated sequenced-based mitotype with nucleotype (i.e. A or B, since our sequencing data

Figure 2

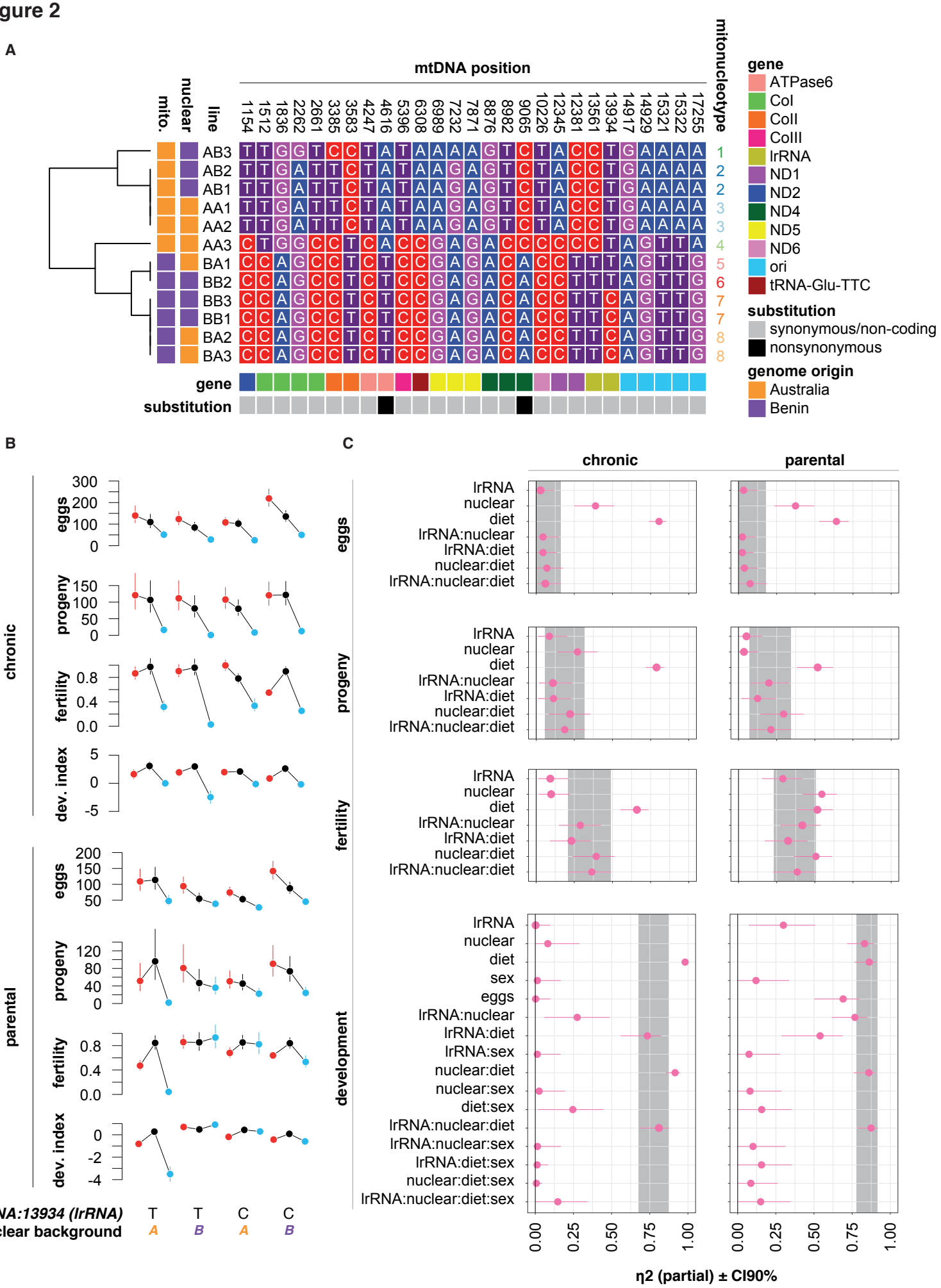
A
mtDNA position
mito.
nuclear
line
1154 1512 1836 2262 2661 3385 3583 4247 4616 5396 6308 6989 7232 7871 8876 8982 9065 10226 12345 12381 13561 13934 14917 14929 15321 15322 17255
mitonucleotype
AB3 T T G G T C C T A T A A A A G T C T A C C T G A A A A 1
AB2 T T G A T T C T A T A A G A G T C T A C C T G A A A A 2
AB1 T T G A T T C T A T A A G A G T C T A C C T G A A A A 2
AA1 T T G A T T C T A T A A G A G T C T A C C T G A A A A 3
AA2 T T G A T T C T A T A A G A G T C T A C C T G A A A A 3
AA3 C T G G C C T C A C C G A G A C C C C C C T A G T T A 4
BA1 C C A G C C T C T C C G A G A C A C C T T T A G T T G 5
BB2 C C A G C C T C T C C G A G A C A C C T T T A G T T G 6
BB3 C C A G C C T C T C C G A G A C A C C T T C A G T T G 7
BB1 C C A G C C T C T C C G A G A C A C C T T C A G T T G 7
BA2 C C A G C C T C T C C G A G A C A C C T T C A G T T G 8
BA3 C C A G C C T C T C C G A G A C A C C T T C A G T T G 8
gene
substitution
gene
ATPase6
CoI
CoII
CoIII
lrRNA
ND1
ND2
ND4
ND5
ND6
ori
tRNA-Glu-TTC
substitution
synonymous/non-coding
nonsynonymous
genome origin
Australia
Benin
B
chronic
parental
eggs
progeny
fertility
dev. index
mtDNA:13934 (lrRNA)
nuclear background
T T C C
A B A B
C
chronic
parental
lrRNA
nuclear
diet
lrRNA:nuclear
lrRNA:diet
nuclear:diet
lrRNA:nuclear:diet
eggs
progeny
fertility
development
sex
lrRNA:sex
nuclear:sex
diet:sex
lrRNA:nuclear:sex
lrRNA:diet:sex
nuclear:diet:sex
lrRNA:nuclear:diet:sex
0.00 0.25 0.50 0.75 1.00
η2 (partial) ± CI90%

Figure 2. A C/T polymorphism in mitochondrial *long ribosomal RNA* substantially alters nucleotype-specific response to dietary variation. A. Segregation of major alleles for significantly differentiated mitochondrial SNPs. Heatmap shows nucleotide identity at positions in mitochondrial genome indicated at top. Gene for each position and SNP classification (synonymous/non-synonymous) indicated by color bar at top, and geographic origin of mitochondrial and nuclear genomes indicated on right. Hierarchical clustering (dendrogram on left) shows separation of SNPs by geographic origin, with five constituent clusters. Concatenating SNP clusters with nucleotype reveals eight mitonucleotypes, indicated to right. **B.** Trait EMMs among subset of populations with mitochondria varying only by a C/T substitution in *mt:lrRNA* (position 13934). Significant SNP-by-nucleotype-by-diet effects were observed for progeny, fertility and fecundity (Table S10). Panels show EMMs for traits and feeding conditions indicated on left, with mitonucleotype indicated at bottom. EMMs calculated from GLMMs (fecundity, progeny, fertility) or Cox mixed models (development). Equivalent plot for all populations in the study shown in Figure S8. **C.** Effect sizes for interaction of mt::lrRNA (13934) C/T polymorphism, nucleotype and diet. Panels show effect size estimates (partial η^2) for traits indicated on left, in the two feeding paradigms indicated at the top. η^2 calculated from GLMMs (fecundity, progeny, fertility) or Cox Mixed Models (development). Grey bands indicate confidence interval (90%) for diet-mito-nuclear terms. Effect sizes suggest that, for progeny and fertility of these populations (mitonucleotypes 5-8), the variation resulting from the interaction of lrRNA polymorphism, nucleotype and diet is equivalent to standing genetic variation from nucleotype and lrRNA polymorphism; and also an equivalent determinant of response to diet.

201 suggested nuclear homogenization independent of mitotype (Figure S1)). This revealed eight distinct
202 mitonucleotypes. We re-analyzed our phenotype data with respect to these new mitonucleotype
203 assignments, fitting models that tested for interaction of mitonucleotype and diet, which confirmed
204 distinct responses to diet (Supplementary Text, Figure S8).
205
206 We were particularly interested by the paucity of nonsynonymous mtDNA polymorphisms, which
207 suggested roles for putatively regulatory variants. We noticed that a subset of populations
208 (mitonucleotypes 5, 6, 7, and 8) (Figure 2A), were distinguished by only one mitochondrial
209 polymorphism. This was a C/T polymorphism in *mt:lrRNA* (mtDNA position 13934), which occurred at
210 high frequencies (between 0.99 and 1, Table S3) in each nuclear background. The presence of only
211 one mtDNA SNP in these lines (contrasting other populations, which bore confounding variation at other
212 positions) suggested that any mitonuclear or DMN variation in this subset of lines was attributable to
213 the *mt:lrRNA* SNP. *mt:lrRNA* is not known to encode protein, so this SNP could provide an illustrative
214 example of the potential for mitochondrial regulatory variation to drive DMN variation in phenotype.
215
216 Plotting phenotype data from mitonucleotypes 5, 6, 7 and 8 (Figure 2B) revealed qualitative variation in
217 fertility. In populations with the *mt:lrRNA* T allele, chronic EAA feeding decreased fertility, in both
218 nucleotypes (mitonucleotypes 5 & 6). However, the *mt:lrRNA* C allele unleashed nucleotype-dependent
219 responses to diet: C allele populations with nucleotype A showed decreased fertility after EAA feeding
220 (mitonucleotype 8), but increased fertility with nucleotype *B* (mitonucleotype 7) (Figure 2B). Indeed,
221 mitonucleotype 7 was the only mitonucleotype that increased fertility upon EAA feeding.
222
223 In the parental feeding paradigm, nucleotypes *A* and *B* responded to diet equivalently in the presence
224 of the *mt:lrRNA C* allele (mitonucleotypes 7 & 8). However, nucleotype-specific responses to diet were
225 unleashed by the *mt:lrRNA* T allele: fertility was impaired by parental feeding on either EAA or lipid in
226 the presence of nucleotype *A* (mitonucleotype 5), but not in the presence of nucleotype *B*
227 (mitonucleotype 6). This altered fertility had apparent consequences for progeny count and
228 development index (Figure 2C). Statistical tests (Table S10) confirmed interactions of the *mt:lrRNA*
229 polymorphism, nucleotype, and diet, for all traits except egg laying. This exclusively post-embryonic

230 variation indicated impacts on offspring performance, but not parental reproductive effort. Effect size
231 calculations (Figure 2C) suggested that the lrRNA:nucleotype:diet interaction was an important source
232 of variation among these populations. For fertility during chronic feeding, lrRNA:nucleotype:diet effects
233 were bigger even than for nucleotype. Most strikingly, for development in both feeding paradigms, effect
234 size for the lrRNA:nucleotype:diet interaction was large, approaching or even equal to main effects of
235 diet. Altogether these results suggest that epistasis between nucleotype and a SNP in non-coding
236 mtDNA can dictate response to diet, which can produce more phenotypic variation than the main effects
237 of mitochondrial or nuclear genotype, and can equal the effect of diet.
238
239 **Discussion**
240 Predicting phenotype from genotype is a long-standing challenge. To this end, genome-wide
241 association studies (GWAS) have flourished. Two over-arching findings of the era of GWAS are that
242 non-coding variation is more important than previously expected; and that additive effects of
243 independently-segregating variants do not fully explain quantitative trait variation (*4*, *22*). This latter
244 finding implies "missing heritability", suggesting that additional processes are at work. Two hypothetical
245 explanations are that genotype-by-genotype epistasis (G*G) and genotype-by-environment (G*E)
246 interactions create non-additive effects. Speculation about epistasis has led to the "omnigenic model",
247 which posits that variation in a given trait is likely explained by G*G between a few "core genes", and
248 the sum effect of many (or all) small-effect variants throughout the rest of the genome (*4*, *22*).
249 Mitonuclear interactions may be a useful illustration of the omnigenic model, with epistasis between the
250 few genes on the mitochondrial genome and the sum of nuclear genomic variants producing substantial
251 phenotypic variation (*7*). We are not aware of any prior attempt to explicitly associate specific
252 mitochondrial SNPs with these interactions, and our results suggest that non-coding variants on mtDNA
253 can play a role. It remains to be seen if non-coding mtDNA variation is as important as non-coding
254 nDNA variation appears to be, though our effect size calculations (Figure 2C) indicate potentially large
255 roles. More generally, we have added to the growing body of evidence for diet-mito-nuclear interactions
256 (*5*, *13*, *16*), showing that outcomes of mitonuclear epistasis are environment-dependent. Thus,
257 altogether, DMN interactions show many of the hallmarks of a major source of phenotypic variation,
258 and we have demonstrated this for *Drosophila* fitness traits. The Darwinian view that reproduction

259 subjugates all other processes, and the central role of mitochondria in cellular function, suggest that
260 these interactions may be important but underappreciated sources of variation for many further traits,
261 and not just in flies.
262
263 While we partitioned phenotypic variance to SNPs, we have not attempted systematic GWAS. In
264 general, GWAS to test genome-wide epistasis are not tractable, due to the enormous sample that would
265 be required to maintain statistical power. For mitonuclear epistasis, testing consequences of
266 interactions between "only" every mtDNA variant and every nuclear variant would be simpler, but an
267 enormous sample would still be required. However, if the omnigenic hypothesis is correct (*4*), such a
268 systematic approach would fail to recognize the underlying biology, which is better modelled as
269 epistasis between a subset of core genes (i.e. mitochondrial genes) and nuclear genomic background
270 (e.g. represented by "background" as in our study; or alternatively dimension reduction, pedigrees,
271 marker loci, or pathway-level variation). If candidate mtDNA variants can be identified, methods to test
272 their role conclusively, by mtDNA editing, are on the horizon. mtDNA editing is in its infancy (*23*), but
273 would facilitate powerful tests of how mitochondria affect outputs of nuclear variation, including
274 response to diet. An additional question raised by our study is the mechanistic role of SNPs outside of
275 genic or protein-coding regions on mtDNA: are these variants regulatory? Our analyses indicate
276 statistical associations, which would also be testable by mtDNA editing. These tools would also enable
277 mechanistic investigation of how mtDNA variants impact mitochondrial function (e.g. respiration,
278 proteome), their consequences for cellular processes (e.g. metabolism, epigenome), and how their
279 impact on phenotype and response to diet varies among nuclear backgrounds. We do not dismiss the
280 importance of coding variation, but our data suggest that non-coding variation may yet prove important.
281
282 Diet is a major source of biological variation. But the importance of genotype-by-diet variation is
283 increasingly recognized, with genetic variation manifesting phenotypically only under certain dietary
284 conditions, and genotype-specific responses to diet (*1*, *24*). We manipulated two specific nutrient
285 classes (EAAs and lipid) normally derived from yeast in fly food, offering greater specificity than previous
286 DMN studies. The nutrients we identify are of particular interest, because EAAs regulate swathes of
287 life-history and health traits, while lipid consumption is associated with the pandemic of human

288 metabolic disease (*3*). We found that impacts of dietary lipid depend on mitonuclear genotype, which
289 may be relevant to understanding variation in impacts of high-fat human diets. The high-EAA diets that
290 we used have parallels to high-protein diets used to increase yields of livestock and human muscle
291 mass, and our finding that EAAs can decrease offspring quality may give pause for thought in use of
292 these diets. We were surprised that EAA enrichment did not enhance offspring development, because
293 we interpreted increased parental egg laying on this food to indicate parental preference, presumably
294 in anticipation of fitness benefits. However, the discrepancy with development and fertility may indicate
295 that EAAs function as signals of food quality as well as metabolites, which could drive deleterious
296 outcomes when EAA levels are not representative of the composition of food that would be found in the
297 yeasts which flies are thought to consume in nature.
298
299 An important finding of our study is that mitonucleotype can modify the qualitative output of dietary
300 variation, and can even result in lethality for some genotypes on EAA enriched-diet. For traits where
301 lethality was evident, effect size calculations suggested that impacts of DMN interactions were equal in
302 magnitude to the main effect of diet, suggesting not only that mitonucleotype modulates response to
303 diet, but that DMN interactions can be major sources of variation outright.
304
305 We have revealed a relationship between parental nutrition and mitonucleotype. Transient dietary
306 alterations and metabolic disease can drive persistent molecular and phenotypic change, within and
307 across generations(*17*, *25*). In our study, transient parental feeding on a high lipid diet even accelerated
308 offspring development in *AA* mitonucleotypes. A direct role for diet in selecting embryos can be
309 excluded because of the standardized post-embryonic environment, and so these effects are likely
310 explained by (A) mitonucleotype-specific parental allocation of development-accelerating factors, after
311 feeding on specific diets, or (B) mitonucleotype-specific selection on offspring from such factors. More
312 generally, after both chronic and parental feeding, effects manifested most strongly in post-embryonic
313 traits, i.e. for fertility, development time, and total progeny, and a dietary and nuclear interactions with
314 C/T polymorphism in *mt:lrRNA* is sufficient to cause these effects. Interestingly, in embryos, lrRNA has
315 been localized outside the mitochondria, in polar granules, suggesting functions in germline
316 determination (*26*), and highlighting this non-coding RNA as a potential mechanistic link to post-

317 embryonic variation. Additional or alternative candidate mechanisms to mediate mitonuclear variation
318 in parental effects include altered epigenetic marks, nutrient provision from mother to offspring, or
319 microbiota. It may be illuminating in the future to ask if effects of parental diet are modulated by parental
320 mitonucleotype (e.g. differential nutrient allocation to eggs, gamete epigenome), or mitonucleotype-
321 dependent processes in offspring (e.g. differential response to altered maternal nutrition). More
322 generally, our study suggests that regulatory variation in mitochondria may modify cellular function in
323 ways that are not yet understood, but appear to depend on dietary and nuclear genetic context. The
324 SNP in lrRNA may, for example, modify protein translation. Altered mitochondrial metabolism will alter
325 overall cellular metabolism, which can have myriad downstream consequences. Much further work is
326 now required to elucidate these mechanisms.
327
328 In summary, our study shows that (A) specific nutrients' fitness effects are determined by interplay of
329 mitochondrial and nuclear genetic variants, (B) DMN effects can produce unprecedented biological
330 variation, and (C) a single-nucleotide substitution in a mtDNA-encoded regulatory factor can underpin
331 these effects. This suggests that regulatory variation in mtDNA may be a hitherto unappreciated but
332 important factor that conspires with nuclear genome to dictate optimal nutrition.
333
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AUTHOR CONTRIBUTIONS

Conceptualization: AJD, KR

Methodology: AJD, SV, DKD, KR

421 Investigation: AJD, SV, LK, LL, EV, RI

422 Visualization: AJD

423 Funding acquisition: KR, AJD

424 Project administration: AJD

425 Supervision: AJD

426 Writing – original draft: AJD

427 Writing – review & editing: AJD, SV, DKD, KR

428

429 **COMPETING INTERESTS**

430 The authors declare no competing interests.

431

432 **DATA AND MATERIALS AVAILABILITY**

433 All data and statistical code will be made available at github.com/dobdobby. Sequence data are being

434 uploaded to NCBI (accession pending).

435

436 **SUPPLEMENTARY MATERIALS**

437 Supplementary Text (including Materials and Methods)

438 Figs S1 to S8

439 Tables S1 to S10

440 Supplementary text references

441

442 **FIGURE LEGENDS**

443 **Figure 1. Large and repeatable impacts of mitonucleotype on response to chronic and parental**

444 **nutritional change. A.** Key and experimental design. Flies were reared from egg to adult on rearing

445 food, and allocated at random to experimental media 6-48h after eclosion, at a density of five of each

446 sex per vial. After seven days, flies laid eggs on fresh food for 24 hours, followed by a further 24h son

447 standardized rearing medium. **B.** Repeatable mitonuclear variation in response to chronic and parental

448 changes in nutrition. Panels show EMMs for trait indicated on y-axis. Feeding paradigm and mitonuclear

449 variation are indicated at the top of the plot. Colors encode diet as per key. Development index shows

450 EMMs for Cox mixed-effects models of proportion eclosed over time, excluding sex from plot.
451 Development data are plotted in full as Kaplan-Meier plots in Figure S4. Note the exclusion of EMMs
452 for development of genotype AA_3 in chronic feeding: EAA lethality prevented meaningful estimation. **C.**
453 PCA shows ordination of populations according to mitotype, nucleotype, and diet. Results shown from
454 one PCA of scaled and mean-centered EMMs, split by facets per genotype, with mitotype split by rows
455 and nucleotype split by columns. **D.** Effect sizes for mitonuclear modification of responses to nutrition.
456 Panels show effect size estimates (partial η^2) for traits indicated on left, in feeding paradigms indicated
457 at top. Partial η^2 calculated from GLMMs (fecundity, progeny, fertility) or Cox Mixed Models
458 (development). Grey bands indicate confidence interval (90%) for diet-mito-nuclear terms. For all traits
459 in the chronic feeding paradigm, diet consistently had the largest effect size, but diet-mito-nuclear effect
460 size was either greater than or equal to mito-diet and nuclear-diet terms, and also larger than mito or
461 nuclear main effects. In the parental feeding paradigm, for fertility and development but not fecundity
462 or progeny, diet-mito-nuclear effect size was either greater than or equal to other genetic modifiers of
463 response to nutrition, and by ranking greater than main mito or nuclear effects.
464
465 **Figure 2. A C/T polymorphism in mitochondrial *long ribosomal RNA* substantially alters**
466 **nucleotype-specific response to dietary variation. A.** Segregation of major alleles for significantly
467 differentiated mitochondrial SNPs. Heatmap shows nucleotide identity at positions in mitochondrial
468 genome indicated at top. Gene for each position and SNP classification (synonymous/non-
469 synonymous) indicated by color bar at top, and geographic origin of mitochondrial and nuclear genomes
470 indicated on right. Hierarchical clustering (dendrogram on left) shows separation of SNPs by geographic
471 origin, with five constituent clusters. Concatenating SNP clusters with nucleotype reveals eight
472 mitonucleotypes, indicated to right. **B.** Trait EMMs among subset of populations with mitochondria
473 varying only by a C/T substitution in *mt:lrRNA* (position 13934). Significant SNP-by-nucleotype-by-diet
474 effects were observed for progeny, fertility and fecundity (Table S10). Panels show EMMs for traits and
475 feeding conditions indicated on left, with mitonucleotype indicated at bottom. EMMs calculated from
476 GLMMs (fecundity, progeny, fertility) or Cox mixed models (development). Equivalent plot for all
477 populations in the study shown in Figure S8. **C.** Effect sizes for interaction of mt::lrRNA (13934) C/T
478 polymorphism, nucleotype and diet. Panels show effect size estimates (partial η^2) for traits indicated on

479 left, in the two feeding paradigms indicated at the top. η^2 calculated from GLMMs (fecundity, progeny,
480 fertility) or Cox Mixed Models (development). Grey bands indicate confidence interval (90%) for diet-
481 mito-nuclear terms. Effect sizes suggest that, for progeny and fertility of these populations
482 (mitonucleotypes 5-8), the variation resulting from the interaction of lrRNA polymorphism, nucleotype
483 and diet is equivalent to standing genetic variation from nucleotype and lrRNA polymorphism; and also
484 an equivalent determinant of response to diet.
485
486 **Figure S1. Mitonuclear diversity in a panel of *D. melanogaster*. A.** Fly populations from Australia,
487 Benin and Canada were introgressed in all possible combinations, generating novel combinations of
488 mitochondrial and nuclear genomes. Three biologically independent replicate populations were
489 established per introgression. **B.** Principal Components Analysis indicates nuclear replacement and
490 homogenization with nuclear genomes from donor populations. PCA was performed on per-population
491 allele frequencies, of all observed nuclear SNPs on the major chromosome arms (2L, 2R, 3L, 3R, and
492 X). Points representing diverse nucleotypes sit on top of one another, suggesting homogenized nuclear
493 genomes even in the presence of distinct mitochondria. **C.** Variance explained by PCA of nuclear SNPs.
494 Barplots show variance explained by each PC. **D.** PCA reveals population grouping by mitochondrial
495 allele frequency, suggesting that the dominant axis of variation (PC1) is driven by SNPs that differentiate
496 mitotypes B and C from mitotype A, with one line (AA_3) intermediate between the two major clusters.
497 PCA was performed on per-population allele frequencies of observed mitochondrial SNPs. **E.** Variance
498 explained by PCA of mitochondrial SNPs. Barplots show variance explained by each PC. **F.** Nuclear
499 and **G.** mitochondrial admixture proportions. Admixture proportions for each line were inferred by
500 model-based clustering with ConStruct (K=3). To minimize effects of linkage disequilibrium (LD), only
501 nuclear SNPs at least 1kb apart and outside regions of no recombination were considered for PCA and
502 admixture analyses.
503
504 **Figure S2. Enriching EAAs or lipid is sufficient to induce repeatable diet-mito-nuclear variation**
505 **in fecundity. A.** Reproductive manipulation by enriching fly medium with plant-based lipid. Egg laying
506 by wild-type *Dahomey* flies on control medium (10% yeast, 5% sugar) and control with 15% plant-based
507 lipid source (margarine) added. Boxplots show medians, first and third quartiles and 5th and 95th

508 percentiles. Two-sample t-test t = 1.98, df = 16, p = 0.03. Data shown per fly. **B.** Diet design: The
509 heatmap shows estimated macronutrient content of diets used in this study, bars at top indicate caloric
510 content. **C.** Key & experimental design. Flies were reared from egg to adult on rearing food, and
511 allocated at random to experimental media 6-48h after eclosion, at a density of five of each sex per vial.
512 After seven days, flies laid eggs on fresh food for 24 hours. **D.** Mitonuclear variation in fecundity
513 response to nutrient enrichment. Plot shows eggs laid in vial of five females and five males over 24h.
514 Boxplots show medians, first and third quartiles and 5th and 95th percentiles. Points to left of each box
515 show raw data. Connected points to right of each box show estimated marginal means (EMMs) with
516 90% confidence intervals. Data shown per vial (5 females + 5 males). **E.** Response to nutritional
517 variation is repeatable among mitonuclear genotypes. Bubble plot shows response index - signed,
518 logged, absolute fold-change in specified comparisons of EMMs - with point size scaled to indicate
519 probability of observed difference (-log10 FDR), and border opacity indicating threshold of statistical
520 significance (FDR≤0.05). Fold-change calculated for conditions on Y-axis relative to conditions on X-
521 axis, e.g. bottom-right cluster of points shows increase on EAA-enriched media relative to control.
522 Points along diagonal show comparisons within replicate genotypes on the same diet, with few
523 significant differences among replicate genotypes. In response to lipid enrichment, the same changes
524 were always evident among replicates of the same genotype, and in response to EAA enrichment
525 similar changes were evident in some replicates. Boxes indicate comparisons among replicates of the
526 same genotype on the same diet.
527
528 **Figure S3. Diet-mito-nuclear variation in fecundity: Technical repeatability and absence of linear**
529 **relationship to caloric content. A.** Intra-line correlations in impact of mitonuclear variation on egg
530 laying, and impact of diet on egg laying. Each point shows mean egg laying per line per diet in each of
531 two replicate experiments, with the replicates of each haplotype grouped by dashed lines. Means were
532 correlated between experiments (Pearson's r = 0.87, p = 7.6e-12). **B.** Fecundity does not correlate
533 caloric content of experimental media. Scatterplots show eggs at each caloric level, with facets per
534 each combination of mitochondrial (columns) and nuclear (rows) genotype. Diet indicated by color.
535 Lines show smoothed spline through points. Trait values do not linearly correlate with calories, therefore
536 caloric content is no more informative than modeling diet as an unordered factor.

537

538 **Figure S4. Kaplan-meier plots of development.** Plots show proportion eclosed over time. Note the
539 exclusion of EMMs for development of genotype AA_3 in chronic feeding: EAA lethality prevented
540 meaningful estimation.

541

542 **Figure S5. Trait values do not correlate with the caloric content of experimental media.** Trait and
543 feeding paradigm indicated above each panel of plots. Within each panel, scatterplots show trait values
544 at each caloric level, with facets per each combination of mitochondrial (columns) and nuclear (rows)
545 genotype. Diet indicated by color. Lines show smoothed spline through points. Trait values do not
546 linearly correlate calories.

547

548 **Figure S6. Structure of statistically significant differences among populations.** Bubble plot shows
549 response index - signed, logged, absolute fold-change in specified comparisons of EMMs - with point
550 size scaled to indicate probability of observed difference (-log10 FDR), and border opacity indicating
551 threshold of statistical significance (FDR≤0.05). Fold-change calculated for conditions on Y-axis relative
552 to conditions on X-axis, e.g. bottom-right cluster of points shows increase on EAA-enriched media
553 relative to control. Points along diagonal show comparisons within replicate genotypes on the same
554 diet, with few significant differences among replicate genotypes. In response to lipid enrichment, the
555 same changes were always evident in replicate genotypes, and in response to EAA enrichment similar
556 changes were evident in some replicates. Boxes indicate comparisons within replicate populations on
557 the same diet.

558

559 **Figure S7. Significantly differentiated loci between mtDNAs *A* and *B*.** In total, 146 SNPs were
560 observed within the 12 populations of which 27 were significantly differentiated between populations
561 with mtDNAs of different origins. Significant allele frequency differences were assessed by Fisher's
562 exact test (FDR<0.001). (A) Allele frequencies and (B) network analysis based on the major alleles in
563 each line at the 27 differentiated sites. Nonsynonymous changes (nonsyn) and nucleotide changes in
564 RNAs are in bold.

565

566 **Figure S8. SNP-driven segregation of populations reveals mitonucleotype-specific responses**
567 **to diet. A.** Panels show EMMs for traits indicated on y-axes, in feeding conditions indicated at top, with
568 mitonucleotype indicated at bottom. EMMs calculated from GLMMs (fecundity, progeny, fertility) or Cox
569 mixed models (development). Summary statistics beneath each panel were calculated from post-hoc
570 joint tests, showing the impact of diet in each genotype. Skull and crossbone motif indicate exclusion
571 of due to line-specific lethality. Genotype-specific F and P values indicate that non-coding variation in
572 the mitochondrial genome modulates nucleotype-specific response to diet. **B.** PCA shows ordination of
573 populations according to mitonucleotype and diet. PCA values as per Figure 1C, with facets showing
574 mitonucleotypes indicated at top of each panel.

Figure S1

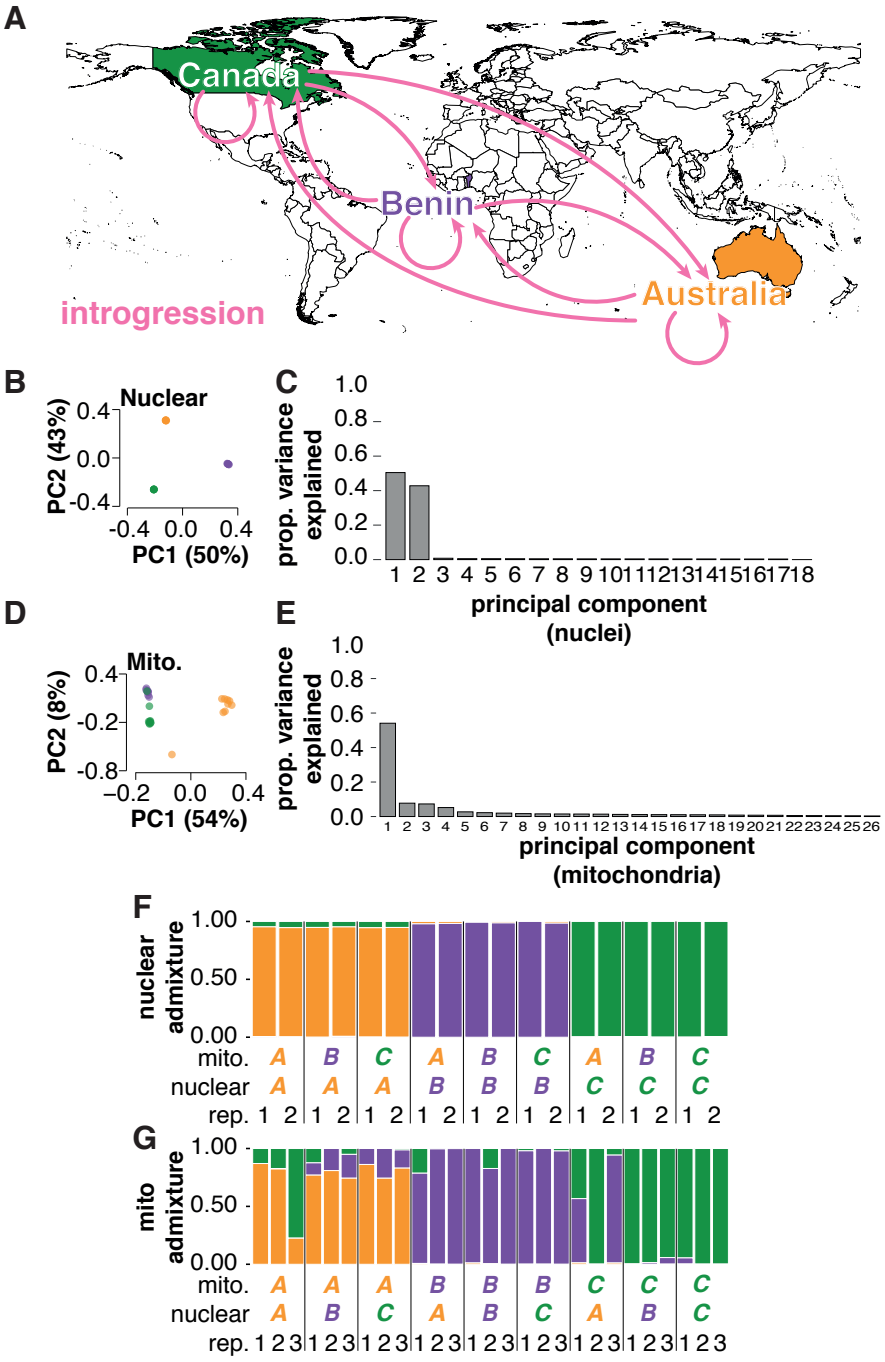

A
Canada
Benin
Australia
introgression
B
Nuclear
PC2 (43%)
PC1 (50%)
C
prop. variance explained
principal component (nuclei)
D
Mito.
PC2 (8%)
PC1 (54%)
E
prop. variance explained
principal component (mitochondria)
F
nuclear admixture
mito.
nuclear
rep.
G
mito admixture
mito.
nuclear
rep.

Figure S2

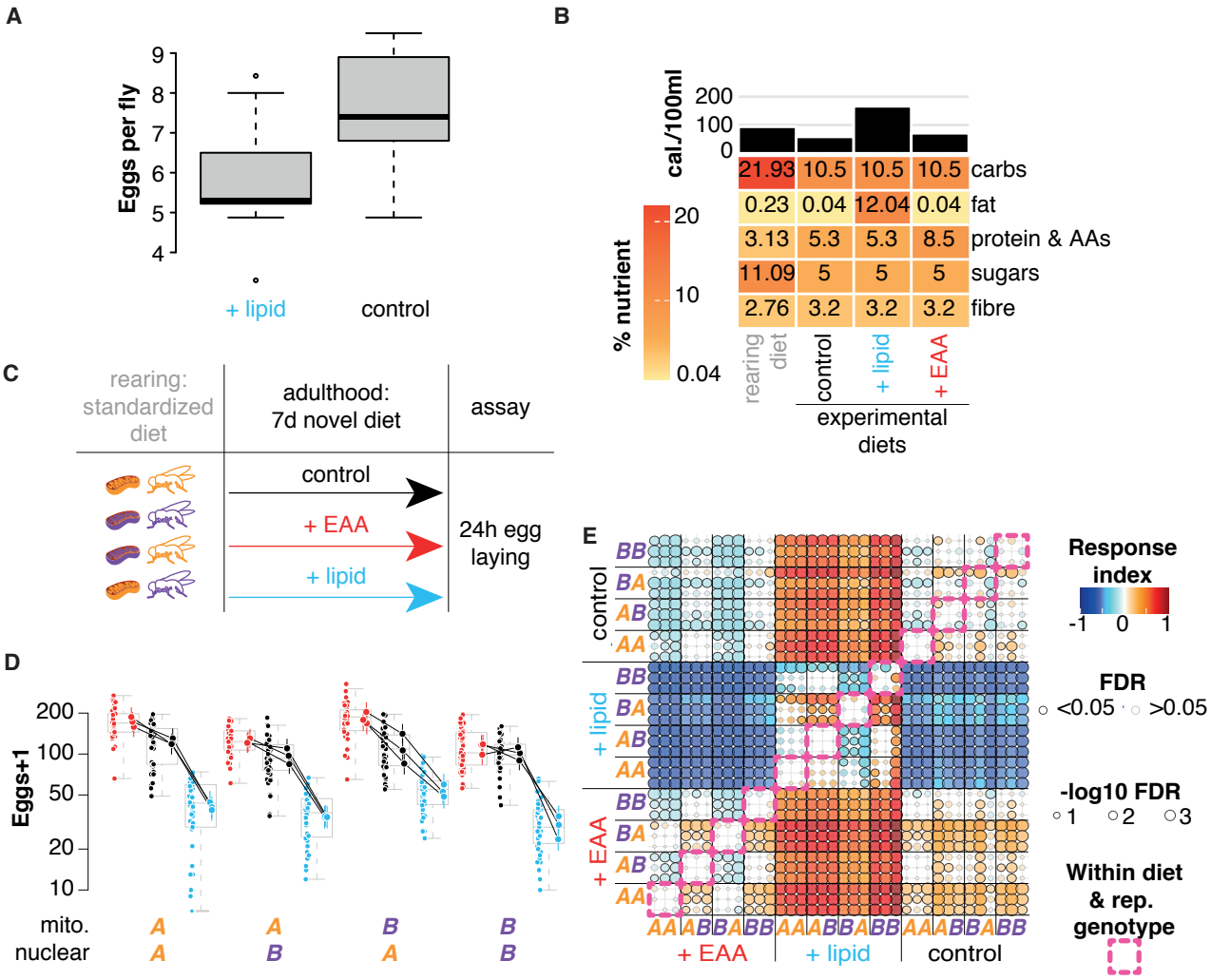

A
Eggs per fly
9
8
7
6
5
4
+ lipid
control
B
cal./100ml
200
100
0
% nutrient
20
10
0.04
21.93 10.5 10.5 10.5 carbs
0.23 0.04 12.04 0.04 fat
3.13 5.3 5.3 8.5 protein & AAs
11.09 5 5 5 sugars
2.76 3.2 3.2 3.2 fibre
rearing diet
control
+ lipid
+ EAA
experimental diets
C
rearing: standardized diet
adulthood: 7d novel diet
assay
control
+ EAA
+ lipid
24h egg laying
D
Eggs+1
200
100
50
20
10
mito.
nuclear
A A
A B
B A
B B
E
control
+ lipid
+ EAA
BB
BA
AB
AA
Response index
-1 0 1
FDR
<0.05 >0.05
-log10 FDR
1 2 3
Within diet & rep. genotype

Figure S3

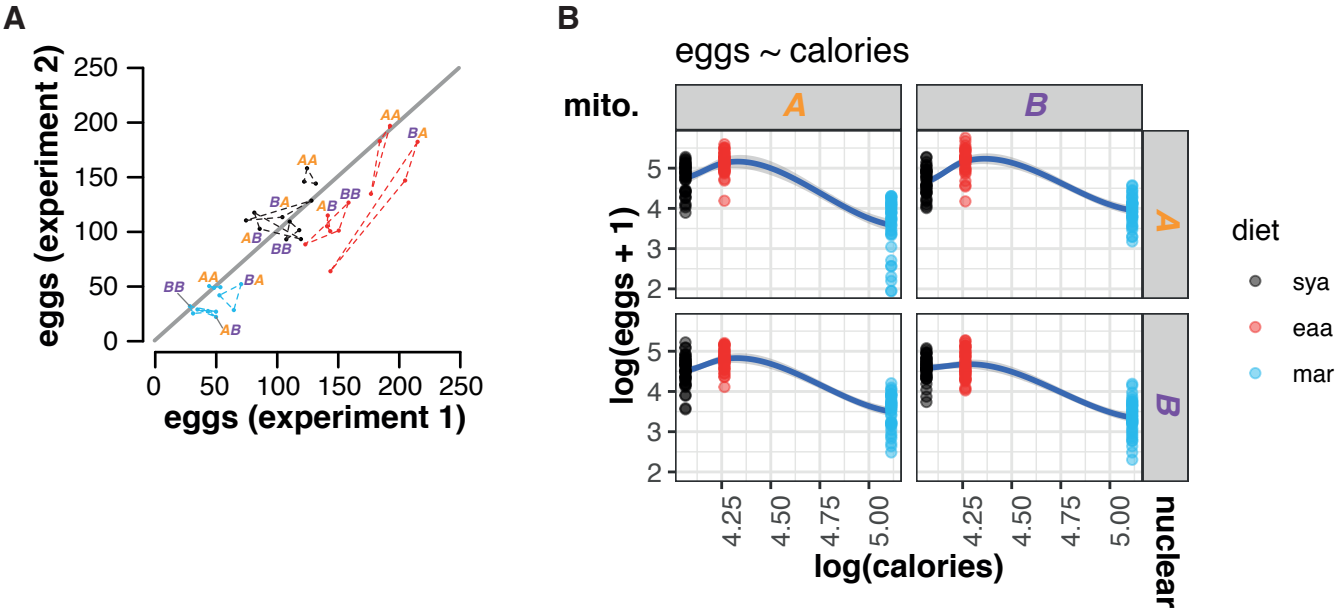

A
eggs (experiment 2)
eggs (experiment 1)
0
50
100
150
200
250
AA
BA
AB
BB
B
eggs ~ calories
mito.
A
B
nuclear
log(eggs + 1)
log(calories)
4.25
4.50
4.75
5.00
diet
sya
eaa
mar

Figure S4

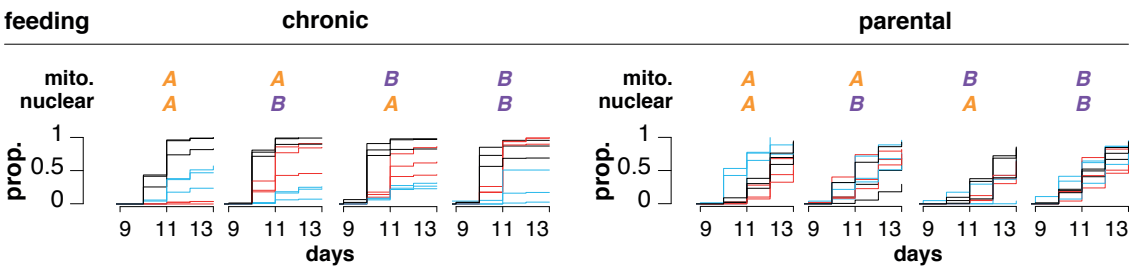

feeding
chronic
parental
mito.
nuclear
A
A
A
B
B
A
B
B
prop.
1
0.5
0
9 11 13
days

Figure S5

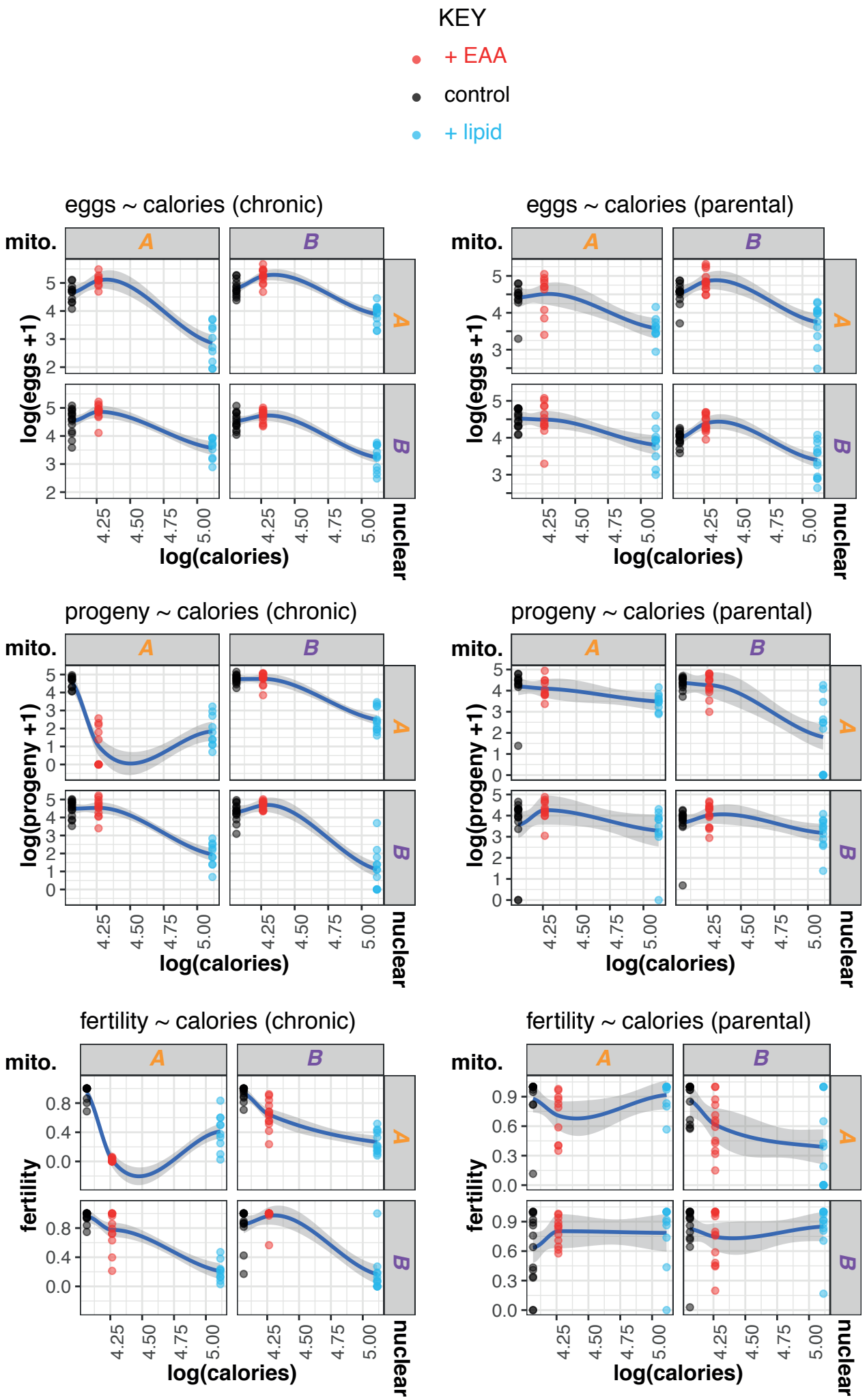

KEY
+ EAA
control
+ lipid
eggs ~ calories (chronic)
eggs ~ calories (parental)
progeny ~ calories (chronic)
progeny ~ calories (parental)
fertility ~ calories (chronic)
fertility ~ calories (parental)
mito.
A
B
nuclear
log(eggs +1)
log(progeny +1)
fertility
log(calories)
4.25
4.50
4.75
5.00

Figure S6

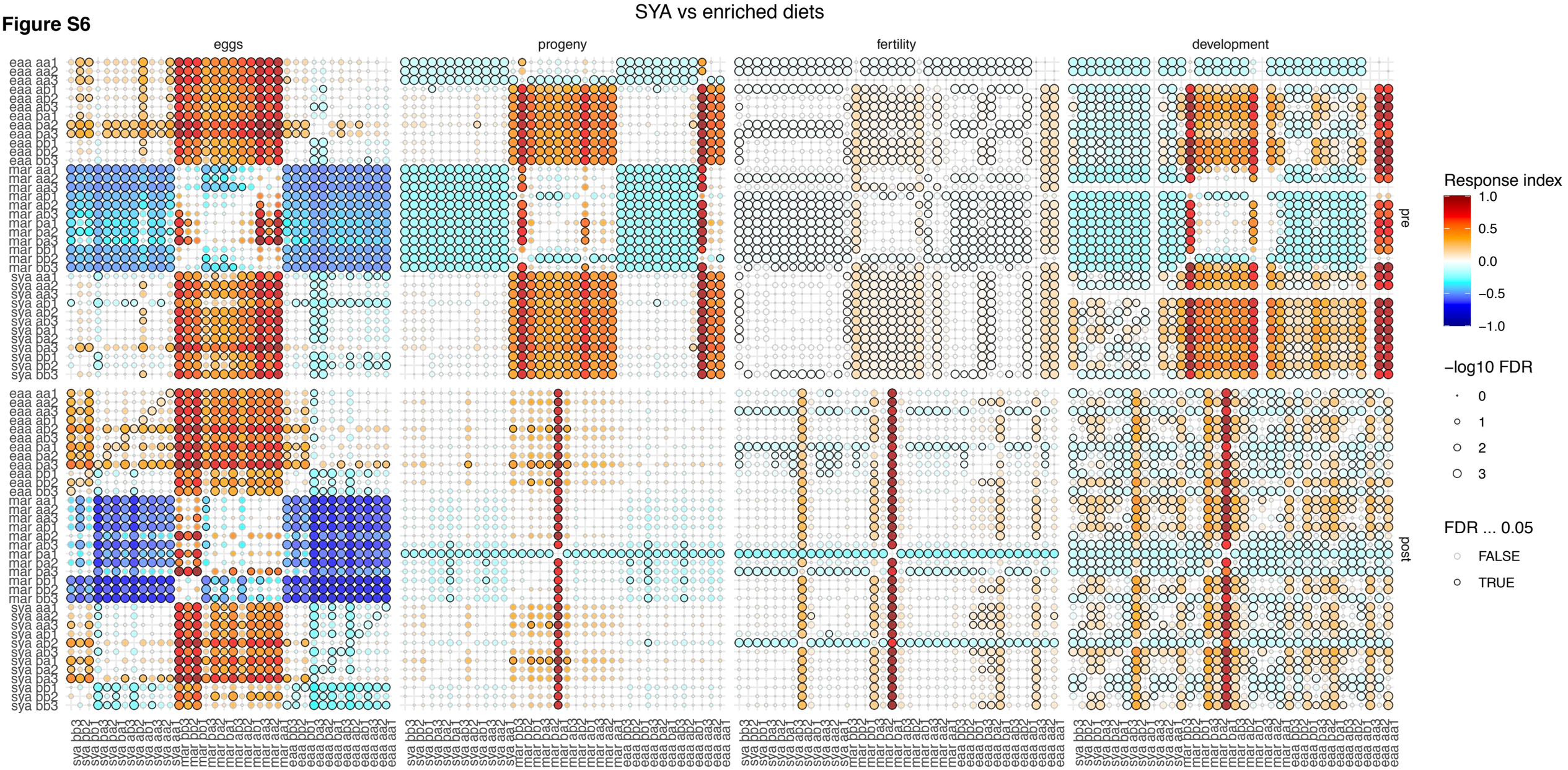
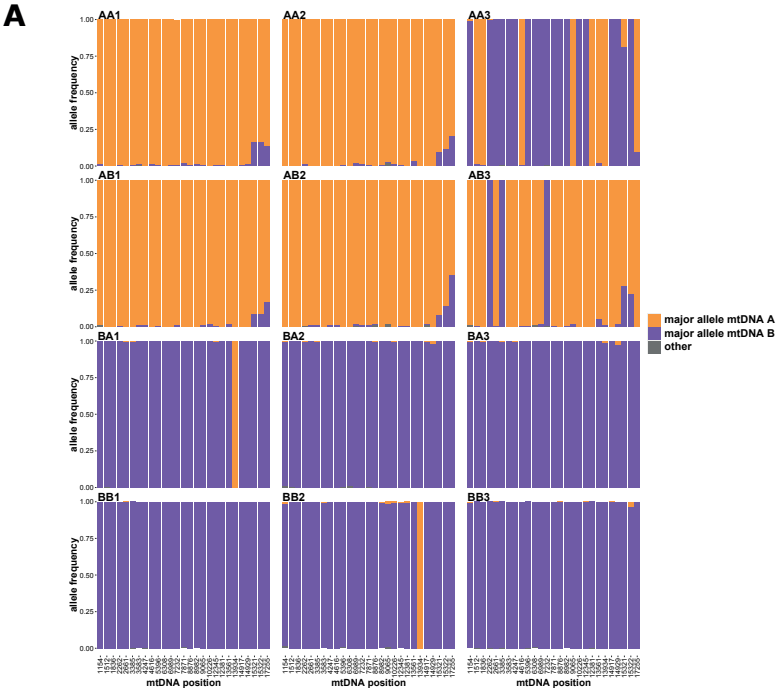

SYA vs enriched diets
eggs
progeny
fertility
development
pre
post
Response index
1.0
0.5
0.0
−0.5
−1.0
−log10 FDR
0
1
2
3
FDR ... 0.05
FALSE
TRUE

Figure S7


A
AA1
AA2
AA3
AB1
AB2
AB3
BA1
BA2
BA3
BB1
BB2
BB3
allele frequency
1.00
0.75
0.50
0.25
0.00
mtDNA position
major allele mtDNA A
major allele mtDNA B
other

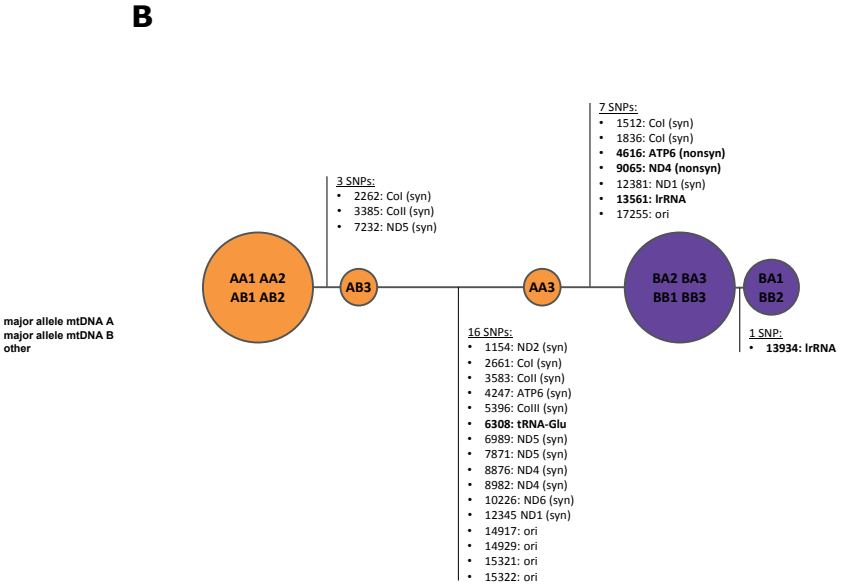

B
major allele mtDNA A
major allele mtDNA B
other
AA1 AA2
AB1 AB2
AB3
AA3
BA2 BA3
BB1 BB3
BA1
BB2
3 SNPs:
• 2262: CoI (syn)
• 3385: CoII (syn)
• 7232: ND5 (syn)
7 SNPs:
• 1512: CoI (syn)
• 1836: CoI (syn)
• 4616: ATP6 (nonsyn)
• 9065: ND4 (nonsyn)
• 12381: ND1 (syn)
• 13561: lrRNA
• 17255: ori
16 SNPs:
• 1154: ND2 (syn)
• 2661: CoI (syn)
• 3583: CoII (syn)
• 4247: ATP6 (syn)
• 5396: CoIII (syn)
• 6308: tRNA-Glu
• 6989: ND5 (syn)
• 7871: ND5 (syn)
• 8876: ND4 (syn)
• 8982: ND4 (syn)
• 10226: ND6 (syn)
• 12345 ND1 (syn)
• 14917: ori
• 14929: ori
• 15321: ori
• 15322: ori
1 SNP:
• 13934: lrRNA

Figure S8

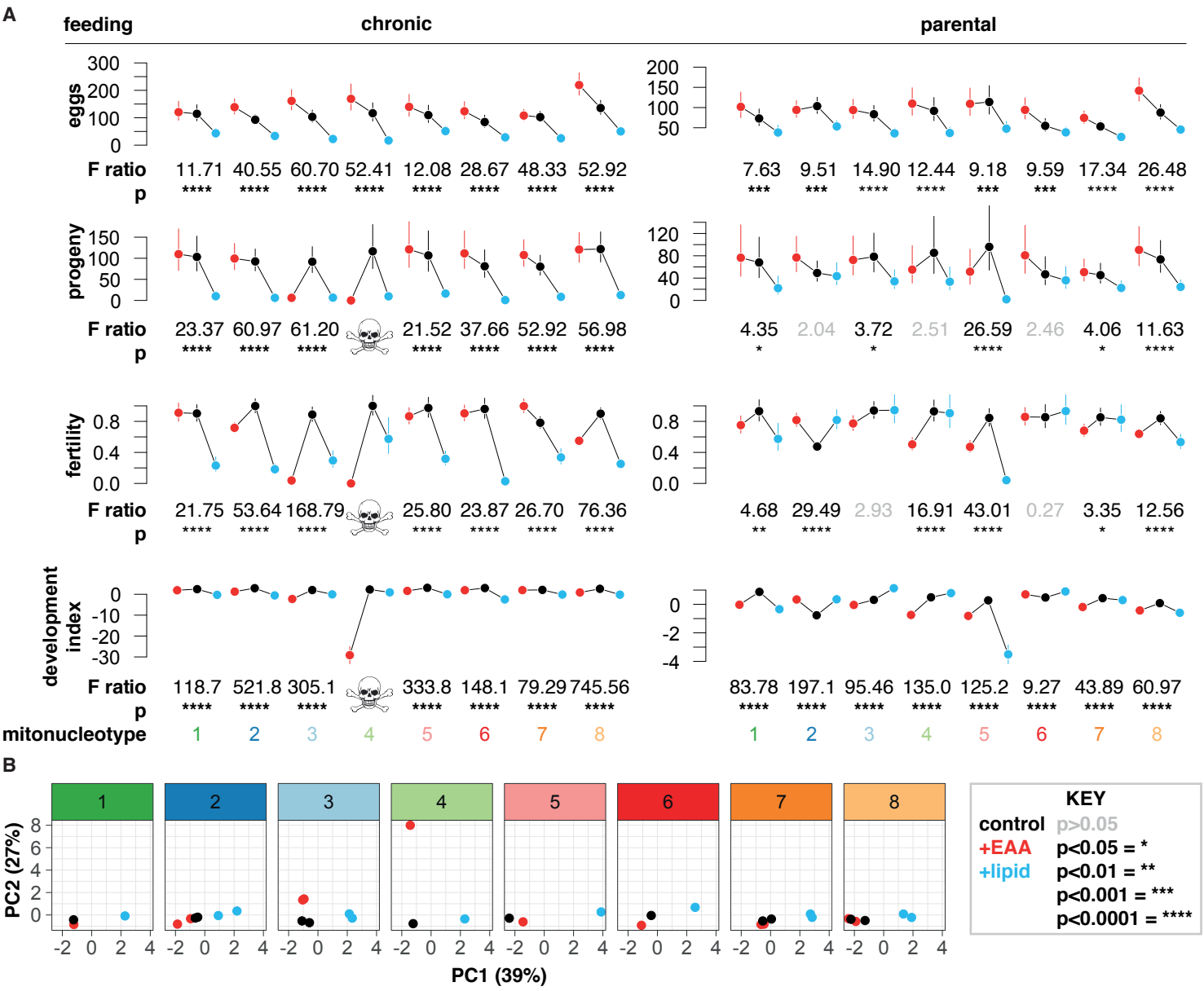

A
feeding
chronic
parental
eggs
F ratio
p
11.71 40.55 60.70 52.41 12.08 28.67 48.33 52.92
7.63 9.51 14.90 12.44 9.18 9.59 17.34 26.48
progeny
23.37 60.97 61.20 21.52 37.66 52.92 56.98
4.35 2.04 3.72 2.51 26.59 2.46 4.06 11.63
fertility
21.75 53.64 168.79 25.80 23.87 26.70 76.36
4.68 29.49 2.93 16.91 43.01 0.27 3.35 12.56
development index
118.7 521.8 305.1 333.8 148.1 79.29 745.56
83.78 197.1 95.46 135.0 125.2 9.27 43.89 60.97
mitonucleotype
1 2 3 4 5 6 7 8
B
PC2 (27%)
PC1 (39%)
KEY
control p>0.05
+EAA p<0.05 = *
+lipid p<0.01 = **
p<0.001 = ***
p<0.0001 = ****

Table S1. Rounds of introgression at different phases of the study

Experiment	N. rounds introgression
Mitochondrial genome sequencing	89
Nuclear & mitochondrial genome sequencing	117 + 120
First fecundity experiment (Fig 2)	158
Second fecundity experiment (Fig 2)	160
Fitness indices experiment (Fig 3)	161

Table S2. Percentage nuclear admixture (most prevalant background) per population

Line	% admixture
AA1	94.9
AA2	94.4
AB1	97.9
AB2	98.3
AC1	99.9
AC3	99.7
BA2	94.8
BA3	94.5
BB1	99.2
BB3	98.7
BC1	99.6
BC2	99.9
CA2	94.5
CA3	94.8
CB2	99.9
CB3	98.5
CC1	99.9
CC2	99.6

Table S3. Significantly differentiated SNPs between mtDNAs A and B. SNPs are ranked according to FST. Pairwise FST per SNP were calculated between mitonuclear genotypes (replicates were pooled) based on (64) using Popoolation2 (65). Means of pairwise FST between genotypes with mtDNA A and those with mtDNA B are given. Mitochondrial (mt) positions are according to Flybase Release 6 (66).

				mtDNA A		mtDNA B		major allele frequency (major allele)											
mt position	F_{ST}	gene	substitution	major allele	amino acid	major allele	amino acid	AA1	AA2	AA3	AB1	AB2	AB3	BA1	BA2	BA3	BB1	BB2	BB3
1,154	0.74	*ND2*	synonymous	T	Asn	C	Asn	0.99 (T)	1.00 (T)	0.99 (C)	0.99 (T)	1.00 (T)	0.99 (T)	1.00 (T)	0.98 (C)	0.99 (C)	1.00 (C)	0.98 (C)	0.99 (C)
1,512	0.99	*CoI*	synonymous	T	Ile	C	Ile	1.00 (T)	1.00 (T)	0.99 (T)	1.00 (T)	1.00 (T)	1.00 (T)	0.99 (C)	0.99 (C)	1.00 (C)	1.00 (C)	1.00 (C)	1.00 (C)
1,836	1	*CoI*	synonymous	G	Gly	A	Gly	1.00 (G)	1.00 (G)	1.00 (G)	1.00 (G)	1.00 (G)	1.00 (G)	1.00 (A)	1.00 (A)	0.99 (A)	1.00 (A)	1.00 (A)	1.00 (A)
2,262	0.47	*CoI*	synonymous	A	Lys	G	Lys	1.00 (A)	0.99 (A)	1.00 (G)	1.00 (A)	0.99 (A)	1.00 (G)	1.00 (G)	1.00 (G)	1.00 (G)	1.00 (G)	1.00 (G)	1.00 (G)
2,661	0.76	*CoI*	synonymous	T	Pro	C	Pro	1.00 (T)	1.00 (T)	1.00 (C)	1.00 (T)	0.99 (T)	1.00 (T)	0.99 (C)	1.00 (C)	1.00 (C)	1.00 (C)	1.00 (C)	1.00 (C)
3,385	0.48	*CoII*	synonymous	T	Gly	C	Gly	1.00 (T)	1.00 (T)	1.00 (C)	1.00 (T)	0.99 (T)	1.00 (C)	0.99 (C)	1.00 (C)	1.00 (C)	1.00 (C)	1.00 (C)	1.00 (C)
3,583	0.76	*CoII*	synonymous	C	Ala	T	Ala	0.99 (C)	1.00 (C)	1.00 (T)	0.99 (C)	1.00 (C)	1.00 (C)	1.00 (T)	1.00 (T)	1.00 (T)	1.00 (T)	0.99 (T)	1.00 (T)
4,247	0.77	*ATPase6*	synonymous	T	Gly	C	Gly	1.00 (T)	1.00 (T)	1.00 (C)	0.99 (T)	0.99 (T)	1.00 (T)	1.00 (C)	1.00 (C)	1.00 (C)	1.00 (C)	1.00 (C)	1.00 (C)
4,616	0.99	*ATPase6*	nonsynonymous	A	Met	T	Ile	0.99 (A)	1.00 (A)	1.00 (A)	1.00 (A)	0.99 (A)	1.00 (A)	1.00 (T)	1.00 (T)	1.00 (T)	1.00 (T)	1.00 (T)	1.00 (T)
5,396	0.74	*CoIII*	synonymous	T	Leu	C	Leu	0.99 (T)	1.00 (T)	1.00 (C)	0.99 (T)	1.00 (T)	0.99 (T)	1.00 (C)	0.99 (C)	1.00 (C)	1.00 (C)	0.99 (C)	1.00 (C)
6,308	0.71	*tRNA-Glu-TTC*	-	A	-	C	-	1.00 (A)	1.00 (A)	1.00 (C)	1.00 (A)	1.00 (A)	0.99 (A)	1.00 (C)	0.99 (C)	1.00 (C)	1.00 (C)	1.00 (C)	0.99 (C)
6,989	0.72	*ND5*	synonymous	A	Tyr	G	Tyr	1.00 (A)	0.98 (A)	1.00 (G)	1.00 (A)	0.98 (A)	0.99 (A)	1.00 (G)	1.00 (G)	1.00 (G)	1.00 (G)	1.00 (G)	1.00 (G)
7,232	0.47	*ND5*	synonymous	G	Thr	A	Thr	1.00 (G)	0.99 (G)	1.00 (A)	0.99 (G)	0.99 (G)	1.00 (A)	1.00 (A)	1.00 (A)	1.00 (A)	1.00 (A)	1.00 (A)	1.00 (A)
7,871	0.73	*ND5*	synonymous	A	Phe	G	Phe	0.98 (A)	0.99 (A)	1.00 (G)	1.00 (A)	0.99 (A)	0.99 (A)	1.00 (G)	0.99 (G)	1.00 (G)	1.00 (G)	1.00 (G)	1.00 (G)
8,876	0.74	*ND4*	synonymous	G	Leu	A	Leu	1.00 (G)	1.00 (G)	1.00 (A)	1.00 (G)	0.98 (G)	1.00 (G)	1.00 (A)	1.00 (A)	1.00 (A)	1.00 (A)	1.00 (A)	1.00 (A)
8,982	0.75	*ND4*	synonymous	T	Leu	C	Leu	0.99 (T)	0.99 (T)	1.00 (C)	1.00 (T)	1.00 (T)	0.99 (T)	1.00 (C)	1.00 (C)	1.00 (C)	1.00 (C)	0.99 (C)	0.99 (C)
9,065	0.97	*ND4*	nonsynonymous	C	Val	A	Leu	0.99 (C)	0.98 (C)	1.00 (C)	0.99 (C)	0.98 (C)	0.98 (C)	1.00 (A)	1.00 (A)	1.00 (A)	1.00 (A)	0.98 (A)	1.00 (A)
10,226	0.75	*ND6*	synonymous	T	Leu	C	Leu	1.00 (T)	0.99 (T)	1.00 (C)	0.98 (T)	1.00 (T)	1.00 (T)	1.00 (C)	0.99 (C)	1.00 (C)	1.00 (C)	0.99 (C)	1.00 (C)
12,345	0.76	*ND1*	synonymous	A	Gly	C	Gly	1.00 (A)	1.00 (A)	1.00 (C)	1.00 (A)	0.99 (A)	1.00 (A)	0.99 (C)	1.00 (C)	1.00 (C)	1.00 (C)	0.99 (C)	1.00 (C)
12,381	0.99	*ND1*	synonymous	C	Met	T	Met	1.00 (C)	1.00 (C)	1.00 (C)	1.00 (C)	0.99 (C)	1.00 (C)	1.00 (T)	1.00 (T)	1.00 (T)	1.00 (T)	0.99 (T)	1.00 (T)
13,561	0.96	*lrRNA*	-	C	-	T	-	1.00 (C)	0.97 (C)	0.98 (C)	0.98 (C)	1.00 (C)	0.95 (C)	1.00 (T)	1.00 (T)	1.00 (T)	1.00 (T)	1.00 (T)	1.00 (T)
13,934	0.66	*lrRNA*	-	T	-	A	-	1.00 (T)	1.00 (T)	1.00 (T)	1.00 (T)	1.00 (T)	0.99 (T)	1.00 (T)	1.00 (C)	0.99 (C)	1.00 (C)	1.00 (T)	1.00 (C)
14,917	0.77	*ori*	-	G	-	A	-	0.99 (G)	0.98 (G)	1.00 (A)	1.00 (G)	0.97 (G)	0.98 (G)	1.00 (A)	1.00 (A)	1.00 (A)	1.00 (A)	1.00 (A)	1.00 (A)
14,929	0.74	*ori*	-	A	-	G	-	0.99 (A)	1.00 (A)	1.00 (G)	1.00 (A)	1.00 (A)	0.98 (A)	1.00 (G)	0.98 (G)	0.97 (G)	1.00 (G)	1.00 (G)	1.00 (G)
15,321	0.61	*ori*	-	A	-	T	-	0.84 (A)	0.91 (A)	0.81 (T)	0.92 (A)	0.92 (A)	0.72 (A)	1.00 (T)	1.00 (T)	1.00 (T)	1.00 (T)	1.00 (T)	1.00 (T)
15,322	0.56	*ori*	-	A	-	T	-	0.84 (A)	0.89 (A)	1.00 (T)	0.91 (A)	0.86 (A)	0.78 (A)	1.00 (T)	1.00 (T)	1.00 (T)	1.00 (T)	1.00 (T)	0.97 (T)
17,255	0.71	*ori*	-	A	-	G	-	0.87 (A)	0.80 (A)	0.91 (A)	0.83 (A)	0.65 (A)	1.00 (A)	1.00 (G)	1.00 (G)	1.00 (G)	1.00 (G)	1.00 (G)	1.00 (G)

Table S4. GLMM of fecundity, analysis of deviance table (Type III Wald chi-square tests)

Term	Chisq	Df	Pr(>Chisq)
(Intercept)	13063.3133	1	<2.20E-16
mito	0.0003	1	0.9854094
nuclear	69.1324	1	<2.20E-16
diet	1297.4867	2	<2.20E-16
experi	33.8005	1	6.11E-09
mito:nuclear	3.9543	1	0.0467519
mito:diet	1.5993	2	0.4494799
nuclear:diet	13.9395	2	0.0009399
mito:nuclear:diet	13.8816	2	0.0009675

Table S5. GLMM and CoxME analyses of reproductive traits, analysis of deviance tables (Type III Wald chi-square tests)

Feeding paradigm	Trait	Term	Chisq	Df	Pr(>Chisq)
Chronic	Fecundity	(Intercept)	24252.889	1	<2.20E-16
		mito	4.8822	1	0.0271354
		nuclear	12.3859	1	0.0004326
		diet	543.1248	2	<2.20E-16
		mito:nuclear	27.6546	1	1.45E-07
		mito:diet	4.8455	2	0.0886776
		nuclear:diet	6.7757	2	0.033781
		mito:nuclear:diet	15.9878	2	0.0003375
Chronic	Progeny	(Intercept)	5433.0112	1	<2.20E-16
		mito	46.1956	1	1.07E-11
		nuclear	8.3393	1	0.00388
		diet	912.305	2	<2.20E-16
		mito:nuclear	63.0979	1	1.97E-15
		mito:diet	157.546	2	<2.20E-16
		nuclear:diet	184.3683	2	<2.20E-16
		mito:nuclear:diet	108.5569	2	<2.20E-16
Chronic	Fertility	(Intercept)	421.339	1	<2.20E-16
		mito	29.542	1	5.47E-08
		nuclear	25.809	1	3.77E-07
		diet	317.228	2	<2.20E-16
		mito:nuclear	33.173	1	8.43E-09
		mito:diet	178.054	2	<2.20E-16
		nuclear:diet	225.867	2	<2.20E-16
		mito:nuclear:diet	105.408	2	<2.20E-16
Chronic	Development	mito	21.1991	1	4.14E-06
		nuclear	17.5192	1	2.84E-05
		diet	3088.2725	2	<2.20E-16
		sex	10.1661	1	0.00143
		eggs	18.4549	1	1.74E-05
		mito:nuclear	22.6814	1	1.91E-06
		mito:diet	435.358	2	<2.20E-16
		nuclear:diet	630.2221	2	<2.20E-16
		mito:sex	1.0107	1	0.314724
		nuclear:sex	9.4998	1	0.002055
		diet:sex	25.5007	2	2.90E-06
		mito:nuclear:diet	218.9798	2	<2.20E-16
		mito:nuclear:sex	6.59	1	0.010256
		mito:diet:sex	5.486	2	0.064376
		nuclear:diet:sex	5.8227	2	0.054403
		mito:nuclear:diet:sex	2.5633	2	0.277582
Parental	Fecundity	(Intercept)	13933.3199	1	<2.20E-16
		mito	1.279	1	0.25809
		nuclear	7.3251	1	0.0068
		diet	196.5383	2	<2.20E-16
		mito:nuclear	16.5213	1	4.81E-05
		mito:diet	4.9494	2	0.08419
		nuclear:diet	2.7931	2	0.24745
		mito:nuclear:diet	0.954	2	0.62063
Parental	Progeny	(Intercept)	9319.8784	1	<2.20E-16
		mito	7.2333	1	0.007156
		nuclear	0.7827	1	0.376327
		diet	205.1253	2	<2.20E-16
		mito:nuclear	0.1328	1	0.715592
		mito:diet	13.1708	2	0.00138
		nuclear:diet	32.1407	2	1.05E-07
		mito:nuclear:diet	8.8102	2	0.012215
Parental	Fertility	(Intercept)	45.4784	1	1.54E-11
		mito	2.5667	1	0.109138
		nuclear	1.4556	1	0.227634
		diet	3.4451	2	0.178611
		mito:nuclear	8.0326	1	0.004594
		mito:diet	12.9935	2	0.001508
		nuclear:diet	13.2591	2	0.001321
		mito:nuclear:diet	10.6506	2	0.004867
Parental	Development	mito	7.0451	1	0.007948
		nuclear	3.1984	1	0.073708
		diet	40.1913	2	1.87E-09
		sex	0.387	1	0.533892
		eggs	93.3797	1	<2.20E-16
		mito:nuclear	11.739	1	0.000612
		mito:diet	257.9248	2	<2.20E-16
		nuclear:diet	259.2143	2	<2.20E-16
		mito:sex	0.3823	1	0.536368
		nuclear:sex	0.0033	1	0.954262
		diet:sex	4.9558	2	0.08392
		mito:nuclear:diet	269.7261	2	<2.20E-16
		mito:nuclear:sex	0.0024	1	0.961141
		mito:diet:sex	0.6849	2	0.710015
		nuclear:diet:sex	0.1064	2	0.948203
		mito:nuclear:diet:sex	0.879	2	0.644372

Table S6. GLMMs of reproductive traits. Model optimisation favors retention of DMN terms. Model terms were systematically included or eliminated, and AICc, delta AICc, Akaike weights and variance explained (r^2) were calculated for each model.

response variable										AIC				r2		top ranking	
trait	feeding paradigm	diet	mito	nuclear	diet:mito	diet:nuclear	mito:nuclear	diet:mito:nuclear	df	logLik	AICc	delta AICc	Akaike weight	marginal	conditional	r2	AIC
		+	+	+	+	+	+	+	14	-684.30	1399.81	0.00	0.983780819	0.8270	0.8280	*	*
		+	+	+	+	+	+		12	-692.32	1411.00	11.19	0.003662494	0.8190	0.8190		
		+	+	+	+	+			11	-698.70	1421.36	21.55	2.06E-05	0.7858	0.8161		
		+	+	+	+		+		w	-694.86	1411.36	11.55	0.003056125	0.8181	0.8181		
		+	+	+	+				9	-701.28	1421.87	22.07	1.59E-05	0.7849	0.8150		
		+	+	+		+	+		10	-694.48	1410.59	10.78	0.004478885	0.8169	0.8169		
		+	+	+		+			9	-701.08	1421.48	21.67	1.94E-05	0.7836	0.8136		
		+	+	+			+		8	-696.69	1410.43	10.62	0.004863981	0.8163	0.8163		
fecundity	chronic	+	+	+					7	-703.22	1421.26	21.45	2.16E-05	0.7836	0.8129		
		+	+		+				8	-703.84	1424.72	24.91	3.83E-06	0.7623	0.8175		
		+	+						6	-705.99	1424.58	24.77	4.12E-06	0.7591	0.8152		
		+		+		+			8	-701.77	1420.59	20.79	3.02E-05	0.7784	0.8132		
		+		+					6	-703.86	1420.33	20.52	3.44E-05	0.7788	0.8126		
		+							5	-706.44	1423.31	23.50	7.76E-06	0.7535	0.8151		
			+	+			+		6	-933.16	1878.93	479.12	8.99E-105	0.2082	0.2405		
			+	+					5	-936.31	1883.06	483.25	1.14E-105	0.1334	0.2393		
			+						4	-939.60	1887.49	487.68	1.24E-106	0.0078	0.2374		
				+					4	-936.54	1881.36	481.55	2.67E-105	0.1268	0.2379		
		+	+	+	+	+	+	+	14	-667.00	1365.21	0.00	1	0.9396	0.9463	*	*
		+	+	+	+	+	+		12	-725.36	1477.06	111.86	5.14E-25	0.9287	0.9402		
		+	+	+	+	+			11	-734.41	1492.79	127.58	1.97E-28	0.8667	0.9408		
		+	+	+	+		+		10	-792.55	1606.73	241.52	3.59E-53	0.9160	0.9312		
		+	+	+	+				9	-799.54	1618.41	253.20	1.04E-55	0.8666	0.9315		
		+	+	+		+	+		10	-780.53	1582.70	217.49	5.93E-48	0.9261	0.9349		
		+	+	+		+			9	-789.03	1597.39	232.18	3.82E-51	0.8767	0.9353		
		+	+	+			+		8	-824.59	1666.24	301.03	4.28E-66	0.9164	0.9282		
progeny	chronic	+	+	+					7	-830.42	1675.66	310.45	3.85E-68	0.8843	0.9284		
		+	+		+				8	-799.96	1616.98	251.77	2.13E-55	0.8614	0.9312		
		+	+						6	-830.58	1673.77	308.57	9.90E-68	0.8830	0.9283		
		+		+		+			8	-792.00	1601.06	235.85	6.10E-52	0.8376	0.9360		
		+		+					6	-833.27	1679.15	313.94	6.74E-69	0.8555	0.9290		
		+							5	-833.37	1677.16	311.96	1.82E-68	0.8542	0.9288		
			+	+			+		6	-1314.55	2641.71	1276.51	6.46E-278	0.4099	0.4648		
			+	+					5	-1321.53	2653.49	1288.28	1.79E-280	0.2058	0.4854		
			+						4	-1322.22	2652.73	1287.53	2.61E-280	0.1700	0.4865		
				+					4	-1324.11	2656.50	1291.29	3.97E-281	0.0392	0.4867		
		+	+	+	+	+	+	+	13	-590.81	1210.37	0.00	1	0.9697	0.9813	*	*
		+	+	+	+	+	+		11	-779.24	1582.46	372.09	1.59E-81	0.9484	0.9682		
		+	+	+	+	+			10	-787.77	1597.16	386.79	1.02E-84	0.8759	0.9682		
		+	+	+	+		+		9	-1029.80	2078.93	868.56	2.48E-189	0.9290	0.9568		
		+	+	+	+				8	-1035.23	2087.52	877.14	3.40E-191	0.8814	0.9569		
		+	+	+		+	+		9	-969.22	1957.76	747.38	5.10E-163	0.9367	0.9607		
		+	+	+		+			8	-975.56	1968.17	757.80	2.79E-165	0.8844	0.9608		
		+	+	+			+		7	-1133.60	2282.01	1071.64	1.98E-233	0.9258	0.9546		
fertility	chronic	+	+	+					6	-1138.47	2289.54	1079.17	4.58E-235	0.8839	0.9548		
		+	+		+				7	-1040.55	2095.92	885.55	5.08E-193	0.7636	0.9562		
		+	+						5	-1143.83	2298.09	1087.72	6.38E-237	0.7713	0.9542		
		+		+		+			7	-979.66	1974.14	763.76	1.42E-166	0.8089	0.9614		
		+		+					5	-1142.87	2296.16	1085.79	1.68E-236	0.8060	0.9556		
		+							4	-1146.03	2300.34	1089.96	2.08E-237	0.6927	0.9547		
			+	+			+		5	-1488.18	2986.78	1776.41	0	0.7260	0.8138		
			+	+					4	-1492.86	2994.01	1783.63	0	0.6121	0.8216		
			+						3	-1499.07	3004.30	1793.93	0	0.2120	0.8253		
				+					3	-1496.86	2999.89	1789.52	0	0.4066	0.8245		
		+	+	+	+	+	+	+	14	-665.58	1362.36	5.99	0.019410256	0.6390	0.6615	*	
		+	+	+	+	+	+		12	-666.05	1358.45	2.08	0.13682632	0.6376	0.6600		
		+	+	+	+	+			11	-671.37	1366.71	10.34	0.002201296	0.5654	0.6560		
		+	+	+	+		+		10	-667.37	1356.37	0.00	0.387806634	0.6350	0.6594		*
		+	+	+	+				9	-672.53	1364.38	8.01	0.007069829	0.5631	0.6555		
		+	+	+		+	+		10	-668.65	1358.93	2.56	0.107592684	0.6339	0.6562		
		+	+	+		+			9	-674.03	1367.38	11.01	0.00157598	0.5602	0.6524		
		+	+	+			+		8	-669.90	1356.86	0.49	0.303511343	0.6314	0.6559		
fecundity	parental	+	+	+					7	-675.14	1365.09	8.72	0.004949029	0.5578	0.6521		
		+	+		+				8	-674.23	1365.52	9.15	0.003996452	0.5280	0.6591		
		+	+						6	-676.77	1366.15	9.78	0.002912181	0.5238	0.6556		
		+		+		+			8	-674.31	1365.67	9.30	0.003701858	0.5538	0.6527		
		+		+					6	-675.42	1363.44	7.07	0.011309499	0.5514	0.6523		
		+							5	-676.96	1364.36	7.99	0.007136639	0.5182	0.6554		
			+	+			+		6	-764.51	1541.63	185.26	2.29E-41	0.2494	0.2905		
			+	+					5	-770.08	1550.58	194.21	2.61E-43	0.0909	0.2776		
			+						4	-771.80	1551.87	195.51	1.36E-43	0.0089	0.2778		
				+					4	-770.31	1548.91	192.54	6.01E-43	0.0814	0.2791		
		+	+	+	+	+	+	+	14	-782.00	1595.20	0.00	0.659299911	0.6693	0.6989	*	*
		+	+	+	+	+	+		12	-786.40	1599.15	3.95	0.09161432	0.6577	0.6885		
		+	+	+	+	+			11	-786.63	1597.23	2.02	0.239762563	0.6564	0.6892		
		+	+	+	+		+		10	-802.55	1626.74	31.54	9.36E-08	0.6245	0.6566		
		+	+	+	+				9	-802.77	1624.87	29.67	2.38E-07	0.6230	0.6571		
		+	+	+		+	+		10	-792.50	1606.62	11.42	0.002181207	0.6403	0.6685		
		+	+	+		+			9	-792.72	1604.76	9.56	0.005548254	0.6393	0.6692		
		+	+	+			+		8	-807.87	1632.79	37.59	4.54E-09	0.6071	0.6374		
		+	+	+					7	-808.17	1631.15	35.95	1.03E-08	0.6047	0.6375		
progeny	parental	+	+		+				8	-803.90	1624.85	29.65	2.40E-07	0.6152	0.6614		
		+	+						6	-809.58	1631.76	36.55	7.61E-09	0.5936	0.6421		
		+		+		+			8	-795.10	1607.25	12.05	0.001593141	0.6098	0.6677		
		+		+					6	-810.17	1632.94	37.73	4.22E-09	0.5786	0.6359		
		+							5	-811.18	1632.79	37.59	4.53E-09	0.5677	0.6398		
			+	+			+		6	-901.89	1816.38	221.18	6.18E-49	0.1143	0.1562		
			+	+					5	-903.00	1816.43	221.22	6.04E-49	0.0892	0.1509		
			+						4	-905.16	1818.61	223.40	2.03E-49	0.0332	0.1508		
				+					4	-904.41	1817.09	221.89	4.32E-49	0.0553	0.1503		
		+	+	+	+	+	+	+	13	-804.95	1638.67	0.00	0.999999284	0.6520	0.7660	*	*
		+	+	+	+	+	+		11	-821.73	1667.43	28.76	5.68E-07	0.5582	0.7092		
		+	+	+	+	+			10	-824.24	1670.11	31.44	1.49E-07	0.4580	0.7053		
		+	+	+	+		+		9	-854.33	1727.99	89.33	4.01E-20	0.4844	0.6426		
		+	+	+	+				8	-857.44	1731.92	93.26	5.62E-21	0.3509	0.6410		
		+	+	+		+	+		9	-841.88	1703.09	64.43	1.02E-14	0.4680	0.6288		
		+	+	+		+			8	-844.46	1705.97	67.30	2.43E-15	0.3562	0.6251		
		+	+	+			+		7	-879.77	1774.35	135.68	3.45E-30	0.2891	0.4735		
fertility	parental	+	+	+					6	-883.15	1778.90	140.24	3.53E-31	0.1118	0.4739		
		+	+		+				7	-857.63	1730.07	91.40	1.42E-20	0.3399	0.6405		
		+	+						5	-883.30	1777.03	138.37	9.00E-31	0.0990	0.4725		
		+		+		+			7	-844.75	1704.31	65.65	5.56E-15	0.3415	0.6248		
		+		+					5	-883.36	1777.14	138.47	8.52E-31	0.0950	0.4720		
		+							4	-883.51	1775.30	136.64	2.14E-30	0.0819	0.4709		
			+	+			+		5	-889.07	1788.56	149.89	2.82E-33	0.2214	0.4237		
			+	+					4	-892.33	1792.94	154.27	3.17E-34	0.0334	0.4226		
			+						3	-892.46	1791.09	152.43	7.96E-34	0.0226	0.4220		
				+					3	-892.61	1791.39	152.73	6.85E-34	0.0109	0.4213		

Table S7. CoxME of development indices. Model optimisation favors retention of DMN terms. Model terms were systematically included or eliminated, and AICc, delta AICc and Akaike weights were calculated for each model.

feeding paradigm	Terms																AIC				top ranking
	diet	mito	nuclear	sex	diet:mito	diet:nuclear	diet:sex	mito:nuclear	mito:sex	nuclear:sex	diet:mito:nuclear	diet:mito:sex	diet:nuclear:sex	mito:nuclear:sex	diet:mito:nuclear:sex	df	logLik	AICc	delta	weight	
	+	+	+	+	+	+	+	+	+	+	+			+		25	-86048.18256	172148.0489	0	0.220363148	*
	+	+	+	+	+	+	+	+	+	+	+		+	+		27	-86046.4059	172148.5081	0.459206251	0.175155427	
	+	+	+	+	+	+	+	+	+	+	+	+	+	+		29	-86044.68608	172149.0858	1.036927457	0.131211842	
	+	+	+	+	+	+	+	+	+	+	+					24	-86049.80176	172149.2773	1.22847662	0.11922811	
	+	+	+	+	+	+	+	+	+	+	+	+		+		27	-86047.11245	172149.925	1.876157262	0.08624553	
	+	+	+	+	+	+	+	+		+	+					23	-86051.14066	172149.9465	1.89762164	0.085324876	
	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	31	-86043.36605	172150.462	2.413095999	0.06593892	
	+	+	+	+	+	+	+	+	+	+	+		+			26	-86048.89514	172151.4767	3.427827596	0.039700471	
	+	+	+	+	+	+	+	+	+	+	+	+				26	-86048.9144	172151.5179	3.469073521	0.038890115	
	+	+	+	+	+	+	+	+		+	+		+			25	-86050.47126	172152.6197	4.570847771	0.022417748	
	+	+	+	+	+	+	+	+	+	+	+	+	+			28	-86048.21081	172154.1241	6.075201176	0.01056637	
	+	+	+	+	+	+	+	+			+					22	-86055.53253	172156.7254	8.676574104	0.002877696	
	+	+	+	+	+	+	+	+	+		+					23	-86055.21762	172158.1031	10.05427986	0.00144504	
	+	+	+	+	+	+	+	+	+		+	+				25	-86054.04154	172159.7656	11.71670551	0.000629345	
	+	+	+	+	+	+		+		+	+					21	-86063.62516	172170.904	22.85512437	2.40E-06	
	+	+	+	+	+	+		+	+	+	+					22	-86063.08344	172171.8283	23.77943459	1.51E-06	
	+	+	+	+	+	+		+	+	+	+			+		23	-86062.20492	172172.0799	24.03107736	1.33E-06	
	+	+	+	+	+	+		+			+					20	-86068.02035	172177.6898	29.64096131	8.07E-08	
	+	+	+	+	+	+		+	+		+					21	-86067.96579	172179.587	31.53814651	3.12E-08	
	+	+	+		+	+		+			+					19	-86071.62588	172182.8912	34.8423025	5.99E-09	
	+	+	+	+	+	+	+	+	+	+				+		23	-86236.13251	172519.8843	371.8354173	3.98E-82	
	+	+	+	+	+	+	+	+	+	+						22	-86237.75845	172521.127	373.0781103	2.14E-82	
	+	+	+	+	+	+	+	+	+	+			+	+		25	-86234.77623	172521.1832	373.1343639	2.08E-82	
	+	+	+	+	+	+	+		+	+						22	-86237.73618	172521.402	373.3531667	1.86E-82	
	+	+	+	+	+	+	+	+		+						21	-86239.35262	172522.3072	374.2583042	1.19E-82	
	+	+	+	+	+	+	+			+						21	-86239.33073	172522.5847	374.5358002	1.03E-82	
	+	+	+	+	+	+	+	+	+	+		+		+		25	-86235.78521	172523.2042	375.1553316	7.57E-83	
	+	+	+	+	+	+	+	+	+	+			+			24	-86236.9079	172523.4374	375.3885664	6.74E-83	
	+	+	+	+	+	+	+		+	+			+			24	-86236.88544	172523.7139	375.6650011	5.87E-83	
	+	+	+	+	+	+	+	+	+	+		+				24	-86237.43912	172524.5021	376.4532613	3.96E-83	
	+	+	+	+	+	+	+	+	+	+		+	+	+		27	-86234.5437	172524.7332	376.6843106	3.53E-83	
	+	+	+	+	+	+	+		+	+		+				24	-86237.41683	172524.7767	376.727876	3.45E-83	
	+	+	+	+	+	+	+	+		+			+			23	-86238.63526	172524.8834	376.8345207	3.27E-83	
	+	+	+	+	+	+	+			+			+			23	-86238.61314	172525.1623	377.1133972	2.84E-83	
	+	+	+	+	+	+	+	+	+	+		+	+			26	-86236.87976	172527.3957	379.3468579	9.31E-84	
	+	+	+	+	+	+	+		+	+		+	+			26	-86236.85729	172527.6721	379.6232197	8.11E-84	
	+	+	+	+	+	+	+	+								20	-86243.79215	172529.1827	381.1338513	3.81E-84	
	+	+	+	+	+	+	+									20	-86243.7703	172529.4577	381.4088389	3.32E-84	
	+	+	+	+	+	+	+	+	+							21	-86243.35887	172530.3232	382.2743423	2.15E-84	
	+	+	+	+	+	+	+		+							21	-86243.33698	172530.5969	382.5480534	1.88E-84	
	+	+	+	+	+	+	+	+	+			+				23	-86242.82525	172533.2693	385.2204534	4.94E-85	
	+	+	+	+	+	+	+		+			+				23	-86242.80335	172533.5425	385.4936278	4.31E-85	
	+	+	+	+	+	+		+		+						19	-86252.55924	172544.7099	396.6610339	1.62E-87	
	+	+	+	+	+	+				+						19	-86252.53671	172544.9852	396.9363055	1.41E-87	
	+	+	+	+	+	+		+	+	+				+		21	-86250.81894	172545.2446	397.1957171	1.24E-87	
	+	+	+	+	+	+		+	+	+						20	-86251.82752	172545.2537	397.2048164	1.23E-87	
	+	+	+	+	+	+			+	+						20	-86251.80458	172545.5268	397.4779173	1.08E-87	
	+	+	+	+	+	+		+								18	-86257.07973	172551.7473	403.6984194	4.80E-89	
	+	+	+	+	+	+										18	-86257.05734	172552.0208	403.9719312	4.19E-89	
	+	+	+	+	+	+		+	+							19	-86256.96301	172553.5198	405.4709515	1.98E-89	
	+	+	+	+	+	+			+							19	-86256.94046	172553.7924	405.7435399	1.73E-89	
	+	+	+		+	+		+								17	-86260.30201	172556.1819	408.1329855	5.23E-90	
	+	+	+		+	+										17	-86260.27955	172556.4603	408.4113963	4.55E-90	
	+	+	+	+		+	+	+	+	+				+		21	-86572.94078	173189.5235	1041.47461	1.55E-227	
	+	+	+	+		+	+	+		+						19	-86575.89635	173191.4206	1043.371686	6.00E-228	
	+	+	+	+		+	+	+	+	+						20	-86574.89621	173191.4273	1043.37845	5.98E-228	
	+	+	+	+		+	+			+						19	-86575.89302	173191.6596	1043.610756	5.32E-228	
	+	+	+	+		+	+	+	+	+			+	+		23	-86572.00348	173191.6606	1043.611699	5.32E-228	
	+	+	+	+		+	+		+	+						20	-86574.89286	173191.6653	1043.616413	5.31E-228	
	+		+	+		+	+			+						19	-86575.91411	173191.7617	1043.712874	5.06E-228	
	+	+	+	+		+	+	+	+	+			+			22	-86574.0829	173193.8118	1045.762919	1.81E-228	
	+	+	+	+		+	+	+		+			+			21	-86575.2028	173194.044	1045.995164	1.62E-228	
	+	+	+	+		+	+		+	+			+			22	-86574.07939	173194.0505	1046.001636	1.61E-228	
	+	+	+	+		+	+			+			+			21	-86575.19935	173194.2839	1046.235049	1.43E-228	
	+		+	+		+	+			+			+			21	-86575.22038	173194.386	1046.337107	1.36E-228	
	+	+	+	+		+	+	+								18	-86581.74001	173201.1051	1053.056202	4.73E-230	
	+	+	+	+		+	+									18	-86581.73657	173201.3411	1053.292218	4.20E-230	
	+		+	+		+	+									18	-86581.7573	173201.4424	1053.393502	4.00E-230	
	+	+	+	+		+	+	+	+							19	-86581.55336	173202.7378	1054.688912	2.09E-230	
	+	+	+	+		+	+		+							19	-86581.54979	173202.9729	1054.924001	1.86E-230	
	+	+	+	+		+		+		+						17	-86589.76091	173215.139	1067.090143	4.24E-233	
	+	+	+	+		+				+						17	-86589.75741	173215.3781	1067.329186	3.76E-233	
	+	+	+	+		+		+	+	+				+		19	-86587.90196	173215.4335	1067.384608	3.66E-233	
	+		+	+		+				+						17	-86589.77866	173215.4803	1067.431438	3.58E-233	
	+	+	+	+		+		+	+	+						18	-86589.05101	173215.7253	1067.676424	3.16E-233	
	+	+	+	+		+			+	+						18	-86589.04748	173215.9636	1067.914716	2.81E-233	

	+	+	+	+		+		+							16	-86595.62807	173224.8705	1076.82164	3.27E-235
	+	+	+	+		+									16	-86595.62447	173225.107	1077.058119	2.90E-235
	+		+	+		+									16	-86595.64532	173225.2084	1077.159497	2.76E-235
	+	+	+	+		+		+	+						17	-86595.56091	173226.7413	1078.692469	1.28E-235
	+	+	+	+		+			+						17	-86595.55723	173226.9773	1078.928478	1.14E-235
	+	+	+			+		+							15	-86598.91873	173229.4429	1081.394031	3.32E-236
	+	+	+			+									15	-86598.91546	173229.6845	1081.635588	2.94E-236
Chronic	+		+			+									15	-86598.93636	173229.7859	1081.737011	2.80E-236
	+	+	+	+	+		+	+	+	+				+	21	-86790.73508	173624.8502	1476.801323	4.59481050632359e-322
	+	+	+	+	+		+	+	+	+					20	-86792.17619	173625.7241	1477.675221	2.96439387504748e-322
	+	+	+	+	+		+		+	+					20	-86792.19316	173626.2491	1478.200196	2.27270197086973e-322
	+	+	+	+	+		+	+		+					19	-86794.25741	173627.8772	1479.828339	9.88131291682493e-323
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Parental	+	+	+	+		+	+	+	+	+			+	+		23	-70740.95088	141529.5028	493.1090916	2.62E-108
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	+	+	+	+	+		+	+	+							19	-70747.53978	141534.6757	498.2819984	1.98E-109
	+	+		+	+		+		+							19	-70747.56504	141534.7701	498.3764007	1.89E-109
	+	+	+	+	+		+		+							19	-70747.55763	141534.7773	498.3835246	1.88E-109
	+	+	+	+	+		+	+		+						19	-70747.82402	141535.2439	498.8501574	1.49E-109
	+	+	+	+	+		+			+						19	-70747.84191	141535.346	498.9522673	1.41E-109
	+	+	+	+	+			+	+							17	-70750.03773	141535.657	499.2632616	1.21E-109
	+	+		+	+				+							17	-70750.06321	141535.7525	499.3587241	1.15E-109
	+	+	+	+	+				+							17	-70750.05579	141535.7596	499.3658097	1.15E-109
	+	+	+	+	+			+		+						17	-70750.23921	141536.0597	499.6659623	9.89E-110
	+	+	+	+	+					+						17	-70750.2573	141536.1627	499.7689918	9.40E-110
	+	+	+	+	+		+	+	+	+						20	-70747.50355	141536.6108	500.2170152	7.51E-110
	+	+	+	+	+		+		+	+						20	-70747.5214	141536.7123	500.3185666	7.14E-110
	+	+	+	+	+			+	+	+						18	-70750.03199	141537.6522	501.2584986	4.46E-110
	+	+	+	+	+				+	+						18	-70750.05005	141537.7548	501.3610683	4.24E-110
	+	+	+	+	+		+	+	+			+				21	-70747.19197	141537.9952	501.6014714	3.76E-110
	+	+		+	+		+		+			+				21	-70747.21726	141538.0893	501.6955728	3.59E-110
	+	+	+	+	+		+		+			+				21	-70747.20984	141538.0965	501.7027434	3.57E-110
	+	+	+	+	+		+	+	+	+					+	21	-70747.47032	141538.5522	502.1584278	2.84E-110
	+	+	+	+	+			+	+	+					+	19	-70750.0317	141539.6587	503.2649973	1.64E-110
	+	+	+	+	+		+	+	+	+		+				22	-70747.13781	141539.8951	503.5013553	1.45E-110
	+	+	+	+	+		+		+	+		+				22	-70747.15571	141539.9964	503.6027023	1.38E-110
	+	+	+	+	+		+	+	+	+		+			+	23	-70747.08305	141541.7942	505.4004576	5.62E-111
	+	+	+					+								13	-70885.57793	141798.7222	762.3284225	9.10E-167
	+															13	-70885.59616	141798.7845	762.3907354	8.82E-167
	+		+													13	-70885.59291	141798.7953	762.4015107	8.77E-167
	+	+														13	-70885.59884	141798.8005	762.4067863	8.75E-167
	+	+	+													13	-70885.59255	141798.8078	762.4140606	8.72E-167
	+	+	+	+				+								14	-70885.53042	141800.6327	764.2389071	3.50E-167
	+			+												14	-70885.54862	141800.6947	764.3009426	3.39E-167
	+		+	+												14	-70885.54539	141800.7055	764.3117908	3.37E-167
	+	+		+												14	-70885.55129	141800.7107	764.316916	3.37E-167
	+	+	+	+			+	+								16	-70883.56564	141800.7153	764.321562	3.36E-167
	+	+	+	+												14	-70885.54501	141800.718	764.3242563	3.35E-167
	+			+			+									16	-70883.58363	141800.7767	764.382908	3.26E-167
	+		+	+			+									16	-70883.58042	141800.7876	764.393811	3.24E-167
	+	+		+			+									16	-70883.58629	141800.7924	764.3986933	3.23E-167
	+	+	+	+			+									16	-70883.58002	141800.7998	764.4060694	3.22E-167
	+	+	+	+			+	+	+							17	-70883.17281	141801.9363	765.5425965	1.82E-167
	+	+		+			+		+							17	-70883.19344	141802.0128	765.6190688	1.76E-167
	+	+	+	+			+		+							17	-70883.18718	141802.0203	765.6265477	1.75E-167
	+	+	+	+				+	+							15	-70885.2251	141802.0279	765.6341735	1.74E-167
	+	+		+					+							15	-70885.2459	141802.1053	765.7115479	1.68E-167
	+	+	+	+					+							15	-70885.23962	141802.1127	765.7189954	1.67E-167
	+	+	+	+				+		+						15	-70885.52725	141802.632	766.2382104	1.29E-167
	+	+	+	+			+	+		+						17	-70883.54722	141802.6849	766.2911238	1.25E-167
	+		+	+						+						15	-70885.54222	141802.7048	766.3110826	1.24E-167
	+	+	+	+						+						15	-70885.54183	141802.7173	766.3235542	1.23E-167
	+		+	+			+			+						17	-70883.562	141802.7571	766.36334	1.21E-167
	+	+	+	+			+			+						17	-70883.5616	141802.7694	766.3756079	1.20E-167
	+	+	+	+			+	+	+	+						18	-70883.16451	141803.9265	767.5327203	6.74E-168
	+	+	+	+			+		+	+						18	-70883.17887	141804.0104	767.6166894	6.46E-168
	+	+	+	+				+	+	+						16	-70885.22494	141804.0336	767.6398514	6.39E-168
	+	+	+	+					+	+						16	-70885.2395	141804.1185	767.7247708	6.12E-168
	+	+	+	+			+	+	+	+					+	19	-70883.14012	141805.8848	769.4910967	2.53E-168
	+	+	+	+				+	+	+					+	17	-70885.22481	141806.0397	769.6459454	2.34E-168
		+	+					+								11	-70915.39382	141854.3519	817.9581087	7.57E-179
																11	-70915.40534	141854.4459	818.0521379	7.22E-179
		+														11	-70915.40486	141854.4489	818.0551644	7.21E-179
		+	+													11	-70915.40092	141854.4493	818.0555302	7.21E-179
			+													11	-70915.40451	141854.451	818.0572347	7.20E-179
		+	+	+				+								12	-70915.38091	141856.3307	819.9369475	2.81E-179
				+												12	-70915.39241	141856.4246	820.0308722	2.68E-179
		+		+												12	-70915.39191	141856.4276	820.0338628	2.68E-179
		+	+	+												12	-70915.38798	141856.428	820.0342519	2.68E-179
			+	+												12	-70915.39159	141856.4297	820.0359932	2.68E-179
		+	+	+				+	+							13	-70915.15819	141857.8904	821.4966711	1.29E-179
		+		+					+							13	-70915.16917	141857.9871	821.5933352	1.23E-179
		+	+	+					+							13	-70915.16524	141857.9875	821.5937636	1.23E-179
		+	+	+				+		+						13	-70915.37677	141858.3273	821.9335928	1.04E-179
		+	+	+						+						13	-70915.38383	141858.4246	822.0308813	9.88E-180
			+	+						+						13	-70915.38744	141858.4264	822.0326215	9.87E-180
		+	+	+				+	+	+						14	-70915.15753	141859.8944	823.5006387	4.74E-180
		+	+	+					+	+						14	-70915.16458	141859.9915	823.5977386	4.51E-180
		+	+	+				+	+	+					+	15	-70915.15473	141861.8944	825.5006913	1.74E-180

Table S8. mtDNA major allele frequency does not vary substantially over course of introgression. Table shows major allele frequency for indicated mitonucleotypes at indicated times in introgression. Sequence data from 2016 were published by Vaught et al (22). mtDNA from 2018 was collected alongside samples for nuclear DNA sequencing. Allele frequencies for each mitonuclear genotype are based on the two replicates for which sequence data was available in both datasets. Minus (-) correspond to sites with insufficient sequencing depth. Mitochondrial (mt) positions are according to Flybase Release 6.

mito-nuclear combination	AA		AB		BA		BB	
year	**2016**	**2018**	**2016**	**2018**	**2016**	**2018**	**2016**	**2018**
mt position								
1154	0.995	1	0.992	1	0.985	0.936	0.993	0.978
1154	1	1	1	0.993	0.996	0.941	0.999	0.992
1154	1	1	1	0.997	0.996	0.922	1	0.994
2262	0.994	1	0.995	0.992	0.998	0.99	0.999	1
2661	1	1	0.997	0.986	1	0.913	0.998	0.985
3385	0.998	1	0.997	0.995	0.995	1	0.997	0.986
3583	0.994	0.98	0.996	0.997	1	0.906	0.999	0.981
4247	1	1	0.99	0.995	0.998	0.912	0.999	1
4616	0.993	1	0.997	1	1	0.952	1	1
5396	0.994	1	0.997	0.996	0.996	0.922	0.999	0.99
6308	1	1	1	0.996	0.996	0.898	0.997	1
6989	0.992	1	0.991	0.995	1	0.844	1	1
7232	0.993	1	0.99	0.989	1	0.984	1	0.957
7871	0.987	1	0.995	0.992	0.997	0.872	0.998	0.971
8876	0.998	1	0.994	0.996	0.998	0.8	0.997	1
8982	0.99	1	1	1	1	0.885	0.995	1
9065	0.987	1	0.987	1	1	0.885	1	1
10226	0.997	1	0.988	0.99	0.996	0.965	1	0.987
12345	0.997	1	0.995	0.982	1	0.886	0.999	1
12381	0.997	1	0.998	0.997	1	0.87	0.999	0.977
13561	0.987	-	0.987	1	1	0.881	1	0.958
13934	1	1	1	0.995	0.994	0.948	1	0.989
14917	0.986	1	0.984	1	1	0.913	1	1
14929	0.991	1	1	1	0.976	0.914	1	1
15321	0.887	-	0.919	1	1	0.963	1	1
15322	0.875	-	0.892	0.964	1	0.938	0.979	1
17255	0.844	-	0.724	-	1	-	1	-

Table S9. GLM and CoxPH analyses of reproductive traits, including sequence-driven mitonucleotype, analysis of deviance tables (Type III Wald chi-square tests)

Feeding paradigm	Trait	Term	Chisq	Df	Pr(>Chisq)
Chronic	Fecundity	diet	464.94	2	<2.20E-16
		mitoNuclear	64.53	7	1.87E-11
		diet:mitoNuclear	38.52	14	0.0004326
Chronic	Progeny	diet	465.93	2	<2.20E-16
		mitoNuclear	157.37	7	<2.20E-16
		diet:mitoNuclear	230.07	14	<2.20E-16
Chronic	Fertility	diet	1005.33	2	<2.20E-16
		mitoNuclear	291.03	7	<2.20E-16
		diet:mitoNuclear	1205.51	14	<2.20E-16
Chronic	Development	mitoNuclear	695.4	7	<2.20E-16
		diet	5480.1	2	<2.20E-16
		sex	0	1	1
		eggs	23.1	1	1.52E-06
		mitoNuclear:diet	2035.6	14	<2.20E-16
		mitoNuclear:sex	9.2	7	0.24108
		diet:sex	6.3	2	0.04274
		mitoNuclear:diet:sex	15.5	14	0.34802
Parental	Fecundity	diet	166.224	2	<2.20E-16
		mitoNuclear	51.815	7	6.35E-09
		diet:mitoNuclear	16.571	14	0.2797
Parental	Progeny	diet	77.94	2	<2.20E-16
		mitoNuclear	23.689	7	0.001292
		diet:mitoNuclear	45.666	14	3.18E-05
Parental	Fertility	diet	67.008	2	2.81E-15
		mitoNuclear	187.741	7	<2.20E-16
		diet:mitoNuclear	278.077	14	<2.20E-16
Parental	Development	mitoNuclear	762.38	7	<2.20E-16
		diet	240.77	2	<2.20E-16
		sex	2.91	1	0.087805
		eggsD2	149.48	1	<2.20E-16
		mitoNuclear:diet	1523.17	14	<2.20E-16
		mitoNuclear:sex	7.42	7	0.386128
		diet:sex	9.66	2	0.007998
		mitoNuclear:diet:sex	19.14	14	0.159726

Table S10. GLM and CoxPH analyses of reproductive traits, in flies where only known mtDNA SNP is in mt:lrRNA. Analysis of deviance tables (Type III Wald chi-square tests)

Feeding paradigm	Trait	Term	Chisq	Df	Pr(>Chisq)
Chronic	Fecundity	lnRNA	2.202	1	0.13784
		nuclear	47.952	1	4.37E-12
		diet	278.969	2	<2.20E-16
		lnRNA:nuclear	3.407	1	0.06491
		lnRNA:diet	3.285	2	0.19348
		nuclear:diet	5.142	2	0.07646
		lnRNA:nuclear:diet	4.204	2	0.12221
Chronic	Progeny	lnRNA	7.665	1	0.0056297
		nuclear	31.93	1	1.60E-08
		diet	315.665	2	<2.20E-16
		lnRNA:nuclear	9.743	1	0.0018005
		lnRNA:diet	10.301	2	0.0057951
		nuclear:diet	23.378	2	8.38E-06
		lnRNA:nuclear:diet	18.214	2	0.0001109
Chronic	Fertility	lnRNA	10.09	1	0.001492
		nuclear	10.71	1	0.001066
		diet	332.84	2	<2.20E-16
		lnRNA:nuclear	45.24	1	1.74E-11
		lnRNA:diet	32.64	2	8.16E-08
		nuclear:diet	53.92	2	1.95E-12
		lnRNA:nuclear:diet	47.77	2	4.24E-11
Chronic	Development	lnRNA	0.0531	1	0.8177
		nuclear	2.2179	1	0.13641
		diet	1322.4979	2	<2.20E-16
		sex	0.337	1	0.56159
		eggs	0.0539	1	0.81649
		lnRNA:nuclear	9.4512	1	0.00211
		lnRNA:diet	69.0919	2	9.93E-16
		nuclear:diet	271.5175	2	<2.20E-16
		lnRNA:sex	0.3288	1	0.56637
		nuclear:sex	0.6378	1	0.4245
		diet:sex	8.1407	2	0.01707
		lnRNA:nuclear:diet	106.7222	2	<2.20E-16
		lnRNA:nuclear:sex	0.3517	1	0.55316
		lnRNA:diet:sex	0.3005	2	0.8605
		nuclear:diet:sex	0.1746	2	0.9164
		lnRNA:nuclear:diet:sex	4.3173	2	0.11548
Parental	Fecundity	lnRNA	2.61	1	0.10622
		nuclear	44.878	1	2.10E-11
		diet	126.125	2	<2.20E-16
		lnRNA:nuclear	1.871	1	0.17133
		lnRNA:diet	1.738	2	0.41935
		nuclear:diet	2.708	2	0.25827
		lnRNA:nuclear:diet	5.411	2	0.06683
Parental	Progeny	lnRNA	4.139	1	0.0419077
		nuclear	2.779	1	0.0955088
		diet	72.689	2	<2.20E-16
		lnRNA:nuclear	17.838	1	2.41E-05
		lnRNA:diet	10.171	2	0.0061867
		nuclear:diet	29.181	2	4.61E-07
		lnRNA:nuclear:diet	18.184	2	0.0001125
Parental	Fertility	lnRNA	40.296	1	2.18E-10
		nuclear	131.243	1	<2.20E-16
		diet	101.565	2	<2.20E-16
		lnRNA:nuclear	70.774	1	<2.20E-16
		lnRNA:diet	47.812	2	4.15E-11
		nuclear:diet	102.671	2	<2.20E-16
		lnRNA:nuclear:diet	55.579	2	8.53E-13
Parental	Development	lnRNA	10.6929	1	0.001075
		nuclear	123.1882	1	<2.20E-16
		diet	155.9845	2	<2.20E-16
		sex	3.4132	1	0.064678
		eggs	55.8584	1	7.79E-14
		lnRNA:nuclear	82.8245	1	<2.20E-16
		lnRNA:diet	29.3376	2	4.26E-07
		nuclear:diet	152.6736	2	<2.20E-16
		lnRNA:sex	2.0061	1	0.156664
		nuclear:sex	2.2006	1	0.137961
		diet:sex	4.6641	2	0.097097
		lnRNA:nuclear:diet	173.1133	2	<2.20E-16
		lnRNA:nuclear:sex	2.8005	1	0.094237
		lnRNA:diet:sex	4.6327	2	0.098633
		nuclear:diet:sex	2.3554	2	0.307986
		lnRNA:nuclear:diet:sex	4.431	2	0.1091

Supplementary text

We produced *D. melanogaster* populations (Figure S1A) comprising replicated and fully-factorial combinations of mitochondrial and nuclear genomes from Australia, Benin and Canada (*A*, *B*, *C*, respectively). The crossing scheme was designed to produce distinct mitochondrial backgrounds bearing equivalent pools of standing nuclear variation, by introgressing populations either reciprocally or to themselves. For brevity, we abbreviate population names, giving mitochondrial and then nuclear origin (e.g. *AB* = Australian mitochondria, Beninese nuclei). Each combination was triplicated (e.g. AB_1, AB_2, AB_3) at the beginning of the introgression and maintained in parallel throughout more than 160 introgressions, altogether generating 27 populations, comprising nine triplicated mitonucleotypes.

After >100 introgressions (Table S1), assuming no mitochondrial incompatibility, we expected nucleotypes to be replaced, and that variation among population with co-originating nuclear backgrounds would be indistinguishable, regardless of mitotype. For a proof of principle, single nucleotide polymorphisms (SNPs) in the nuclear genome were identified by Pool-seq, sampling two populations per mitonucleotype (i.e. 18/27 total). Within each nucleotype, principal components analysis (PCA) suggested negligible differentiation by mitotype, with all samples in each nucleotype sitting indistinguishably on top of one another in an ordination plot (Figure S1B), on axes that explained 93% total variance (Figure S1F). Admixture analysis found between 94.4% and 99.9% replacement of nuclear genomes (Figure S1F, Table S2). These analyses confirmed that the panel of populations was suitable for studying the behaviors of distinct mitotypes in populations with comparable nuclear genomic diversity.

We reanalyzed previously-reported mitochondrial sequences for the populations (*1*), finding 28 significantly differentiated SNPs (Fisher's exact test, FDR<0.001). PCA (Figure S1D) revealed consistent within-mitotype clustering, except population AA_3, which was distinct from other *A* mitotypes. Little differentiation was apparent between mitotypes *B* and *C* on the PCA, and only one SNP was significantly differentiated between these mitotypes. These findings suggested that phenotyping both *B* and *C* mitochondria in the same experiment would likely prove redundant. By contrast, 27 of the 28

SNPs were significantly differentiated between mitotypes *A* and *B* (Table S3), i.e. the majority of mitochondrial diversity in the full panel was represented by these two mitotypes. Therefore, we chose to work with only one mitochondrial background, retaining Beninese mitochondria because of the widespread use of this background in fly research, and excluding Canadian mitochondria (i.e. studying only *AA, AB, BA* and *BB*). We also excluded Canadian nucleotypes (*AC, BC*) because of lack of coevolutionary history with *A* or *B* mitotypes. This reduced number of populations for phenotyping from 27 to 12.

Novel high-lipid diet represses fecundity

Previous studies showed mitonuclear modulation of effects of diluting dietary yeast (*2–6*), which is a source of multiple nutrients. We studied effects of specific nutrients, normally derived from yeast, by specifically enriching either lipids or essential amino acids (EAAs). EAA enrichment increases fly fecundity ~20% (*7–9*). We were interested in lipids because of relevance to Western human diets (*10*, *11*), and so we developed a new high-lipid diet (see materials & methods). Lipid decreased wild-type fecundity by 20% (Figure S2A), confirming that this diet modulates reproduction, but inversely mirroring EAA's effect. Figure S2B shows approximate nutrient contents of control, EAA-enriched and lipid-enriched diets. Flies were maintained on a distinct "development" medium prior to experiments, to distribute any novelty effects of switch to experimental food evenly among conditions.

Specific nutrients sufficient for DMN variation

After ≥158 introgressions of the flies, EAA and lipid were each sufficient to cause DMN variation in reproduction (Figure S2B, Figure S2C). Repeating the same assay across multiple generations of introgression gave the same results, without differences that would indicate intergenerational drift (Figure S3A). Fecundity did not correlate with the increased caloric density in the EAA and lipid enriched diets (Figure S3B), as shown previously (*12*, *13*): fecundity therefore cannot be explained as a simple linear effect of calories. Diet was therefore modelled as an unordered factor. A DMN interaction was visually apparent (Figure S2D). A statistical model (GLMM, Table S4), which explained the majority of variance (conditional R^2=0.768), confirmed the DMN interaction (p=0.001).

59 To assess repeatability among genetic replicates, and to identify differential effects of EAA versus lipid
60 enrichment, we calculated estimated marginal means (EMMs) (*14*) for each line on each diet. EMMs
61 were visually correlated among genetic replicates (Figure S2D). To assess repeatability, we calculated
62 an index of differences between conditions (Supplementary text) and p-value for each pairwise diet:line
63 comparison (Figure S2E). First, we used these values to assess consistency among the genetic
64 replicates of each mitonucleotype (e.g. AA_1, AA_2, AA_1) which would be refuted by frequent among-
65 replicate differences on the same diet. Of the 36 total comparisons (3 diets, 12 populations), only four
66 were significantly different, indicating that mitonuclear replication led to repeatable fecundity. Second,
67 we assessed whether genetic replicates responded equivalently to dietary change. Adding lipid
68 consistently repressed fecundity, but the magnitude varied repeatably by mitonucleotype (Figure S2D,
69 FIgure S2E). Responses to EAA enrichment were more nuanced but were still overall repeatable
70 among replicates, with most populations increasing egg laying, to the greatest extent in *BA* populations
71 but more modestly in *AA*; and fewer populations responding to EAAs in *B* nucleotypes independent of
72 mitochondria (Figure S2D, Figure S2E). These analyses confirmed that (A) fecundity across varied
73 diets was repeatable among independently replicated mitonucleotypes, and (B) dietary EAAs and lipid
74 can cause this variation
75
76 **AIC and r^2 calculations**
77 For orthogonal tests of the importance of DMN terms, we asked if alternative models, excluding DMN
78 terms, were better descriptors of the data. We fit a structured series of models for each trait,
79 systematically including or eliminating diet, mitotype and nucleotype, and their interactions. Interactions
80 with offspring sex were also included for development models. We calculated Akaike Weights (Table
81 S6, Table S7), which evaluate the relative performance of a set of models. For all traits but one
82 (fecundity, in parental feeding paradigm), Akaike Weights showed that the best-performing models
83 included DMN interaction terms. For development time in each feeding paradigm, higher interactions
84 between sex and diet, mitotype and nucleotype were generally not favored, consistent with the
85 suggestion from effect size calculations that offspring sex was not a major modulator of genetic and
86 dietary effects in these flies.
87

88 We also calculated variance explained (r^2) by GLMMs (fecundity, progeny, fertility: r^2 cannot be
89 calculated for Cox models of development time), to evaluate each model's capacity to statistically
90 predict phenotype. GLMMs favored by Akaike Weights explained between 65% and 95% of variance
91 (Table S6), suggesting that most sources of variation in our flies were accounted for by our models.
92

93 **Mitochondrial SNP analysis**

94 All 27 SNPs in *A* and *B* populations were biallelic (Figure S7A). 70% of major alleles were fixed, and
95 89% were at frequency ≥0.99 (Figure S7A, Table S3). We mapped the mitochondrial SNPs to genes
96 and regulatory sequences. 19/27 mitochondrial SNPs were in protein-coding regions, in genes
97 encoding subunits of electron transport chain complexes and *ATPase subunit 6,* but only 2/19 were
98 predicted to be nonsynonymous (in *ATPase subunit 6* and *ND4*). However, 8/27 SNPs were in non-
99 coding regions (in the origin of replication, a tRNA, and *long ribosomal RNA*). Thus, the majority of
100 significantly differentiated SNPs in our populations do not appear to change amino acid sequence.
101 Resequencing at a two-year interval indicated that these frequencies were stable over time and unlikely
102 to have changed substantially between the time of genotyping and phenotyping (Table S8), suggesting
103 it was valid to associate this mitochondrial genetic variation with phenotype.
104

105 We examined among-line distribution of major mitochondrial alleles. Network analysis (Figure S7B)
106 revealed a punctuated continuum of among-line variation, independent of nucleotype, including some
107 populations with unique mitotypes (AB_3, AA_3), but others with identical mitotypes, even over distinct
108 nucleotypes (AA_1, AA_2, AB_2, AB_2; and BA_2, BA_3, BB_1, BB_3; and BA_1, BB_2). This analysis, focussed just
109 on populations phenotyped in this study, reinforced findings of our previous network analysis of the full
110 set of populations (shown in Figure S1) (*1*).
111

112 **Associations between mito-nucleotype and differential responses to diet**

113 We studied the interaction between diet and sequence-driven mitonucleotype. We used mitonucleotype
114 rather than SNPs because (A) tests of linked SNPs would have been redundant; and (B) AA_3's mitotype
115 did not occur over the *B* nucleotype, so its interaction with nucleotype could not be assessed.
116

117 Re-calculating and plotting EMMs per mitonucleotype indicated considerable variation in response to
118 diet (Figure 2B). We excluded mitonucleotype 4 (line AA_3) from statistical analysis because its extreme
119 trait values complicated modelling. We do not discard this line as an outlier: rather we highlight its
120 extreme and unique response to diet, which is self-evident without statistical modelling. Among other
121 populations, ANOVA tests revealed significant mitonucleotype:diet interactions (Table S9). To estimate
122 variability in response to dietary change, we calculated F-ratios and P-values for effect of diet per
123 mitonucleotype. F-ratios varied up to 10-fold, depending on trait. Diet effects were significant for all
124 mitonucleotypes in the chronic feeding paradigm ($p<0.001$ in all cases), but not in the parental feeding
125 paradigm. These analyses suggest that variance in response to diet can be partitioned by sequence-
126 based mitonucleotype.
127

128 **Supplementary text - Materials & methods**

129

130 **Diets**

131 Development medium contained 1.4% agar and 4.5% brewer's yeast (both Gewürzmühle Brecht,
132 Germany), 10% cornmeal and 11.1% sucrose (both Mühle Milzitz, Germany) (all w/v), 0.45% propionic
133 acid and 3% nipagin (v/v). Experimental media built on published protocols (*8*, *9*, *15*). These media
134 contained a final concentration of 10% brewer's yeast, 5% sucrose, 1.5% agar (w/v), 3% nipagin and
135 0.3% propionic acid (v/v). EAAs were purchased as powder (Sigma), and supplemented by dissolving
136 into a 6.66x solution in ddH_20 pH 4.5 (final media concentrations: L-arginine 0.43 g/l, L-histidine 0.21
137 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
138 g/l, L-threonine 0.37 g/l, L-tryptophan 0.09 g/l, L-valine 0.4 g/l). We added margarine (15% w/v, after
139 (*16*)) to ensure that lipid supply was plant-based, because wild fly physiology is likely influenced by their
140 consumption of what appears to be a vegan diet (*17*, *18*), and because margarine sets in agar (in
141 contrast to oils). Margarine (*Ja! Pflanzenmargarine* from Rewe Supermarkets, Germany;
142 manufacturer's analysis 720 kcal / 100g; 80/100g fat from 23/100g saturated fatty acids, 40/100g
143 monounsaturated fatty acids, 17/100g polyunsaturated fatty acids) was briefly melted then mixed
144 thoroughly into the food (15% w/v). Final nutrient contents of rearing and control media were estimated
145 using the *Drosophila* diet content calculator (*19*), with additional protein, lipid and caloric content after
146 nutrient supplements calculated according to margarine nutrient content report, and by assuming caloric
147 equity between EAAs and protein at a caloric value of 4 calories/g (USDA). Vials contained ~5ml of
148 food, and were stored at 4^oC for up to 1 week before use.

149

150 **Flies**

151 *D. melanogaster* fly lines were established as described in Figure S1 and maintained on development
152 medium throughout their history prior to our experiments. The ancestral Australian population was
153 isolated in Coffs Harbour, NSW, Australia (*20*). The Benin population is the widely-used *Dahomey*
154 population, isolated in the 1970s in Dahomey (now Benin). The cytoplasmic endosymbiont *Wolbachia*
155 was cleared by tetracycline treatment 66 generations prior to experiments. For each line, 45 females of
156 the desired mitochondrial background were crossed to 45 males of the desired nuclear background per

157 generation. Iterating this process over many generations led to introgression of the desired nuclear
158 background (from males) into each mitochondrial background. Fly lines were maintained at 25°C on
159 development medium throughout their history prior to experimentation. For experiments, flies were
160 collected upon eclosion to adulthood and fed fresh developmental medium, before being pooled, split
161 and assigned at random to experimental medium in groups of 5 males and 5 females. Experimental
162 flies were maintained at 25°C, and transferred to fresh media every 48-72h for one week. Flies were
163 transferred to fresh medium 24h before egg laying experiments. For development experiments, eggs
164 were incubated at 25°C and pupation and eclosion were scored daily. Eclosing adults were lightly CO_2-
165 anaesthetised before counting and sexing.
166
167 **Genome sequencing**
168 Input DNA controls from an unpublished ChIP-Seq experiment were used for whole genome sequence
169 analysis. Genomes of two replicates for each mitonuclear combination were sequenced. Pools of 50
170 adult flies were subjected to a standard native ChIP protocol as described in Cosseau *et al.* 2009. The
171 protocol included an MNase digestion step of six minutes at 37°C using 15U of the enzyme (Thermo
172 Fisher Scientific) per sample which yielded fragments between 284 and 300bp in length. DNA was
173 extracted with the QIAquick PCR purification kit (Qiagen) and 100ng genomic DNA of the unChIPped
174 input/negative controls were used for library preparations with NEB Next Ultra DNA lib Prep kit for
175 Illumina. Over 50 million 2x75bp (PE) reads per sample were sequenced on an Illumina Nextseq 500
176 platform in High Output (150 cycles) mode.
177
178 **Nuclear genome analysis**
179 Raw FASTQ reads were trimmed and filtered to remove low-quality reads (minimum base PHRED
180 quality of 18 and minimum read length of 50bp) prior to mapping using *cutadapt* (version 2.4; (*21*)).
181 Reads were mapped to the reference genome of *D. melanogaster* (Flybase Release 6.28) with *bwa aln*
182 (version 0.7.12; (*22*)) using parameters optimized for Pool-seq data (*23*). Mapped reads were filtered
183 for proper pairing and a mapping quality of at least 20 using *samtools* (version 1.9; (*24*)). Duplicates
184 were removed with *Picard* (version 2.18.11; http://broadinstitute.github.io/picard/) and sequences
185 flanking indels were re-aligned with *GATK* (version 3.8.1.0; (*25*)). Sequencing depth was assessed

186 using *Qualimap* (version 2.2.1; (*26*)) and ranged from 46-58x for autosomes and 22-28x for X
187 chromosomes. Individual *bam* files from all samples were then combined into a single *mpileup* file using
188 *samtools* (version 1.9; (*24*)). SNPs were called with the *PoolSNP* pipeline (version 1.05;
189 https://github.com/capoony/PoolSNP) from the DrosEU project (*27*) which was specifically developed
190 for SNP detection in Poolseq data. Parameters for SNP calling were as those used and optimized by
191 the DrosEU project (except minimum count was set to 10) as their dataset closely resembled the
192 present one. The resulting *vcf* file was converted into a *sync* file using the python script *VCF2sync.py*
193 from the DrosEU pipeline.
194
195 General genetic differentiation among nuclear genomes was assessed by principal component analysis
196 (PCA) using the R package *LEA* (version 3.4.0; (*28*)) and by estimating admixture proportions with the
197 R package *ConStruct* (version 1.0.4; (*29*)). Both approaches were based on major allele frequencies of
198 nuclear SNPs on all major chromosome arms (2L, 2R, 3L, 3R, and X). In order to minimize the effects
199 of linkage disequilibrium (LD), only SNPs at least 1kb apart and outside regions of no recombination
200 (*30*) were considered. Major allele frequencies were calculated with the python script *sync2AF.py* from
201 the DrosEU pipeline. Admixture proportions (K=3) for each line were inferred by non-spatial modelling
202 with three MCMC chains per run and 10,000 iterations.
203
204 **Mitochondrial genome analysis**
205 Sequence data of mitochondrial genomes were retrieved from data of (*1*) who had sequenced all 27
206 mitonuclear lines in pools of 150 flies including a prior mitochondrial enrichment protocol. These data
207 were reanalysed with more stringent sequencing depth criteria. *Bam* files from this previous study were
208 combined into a single *mpileup* file using *samtools* (version 1.9; (*24*)), which was then converted into a
209 *sync* file with *Popoolation2* (*31*). As for nuclear SNPs, a PCA using the R package *LEA* (version 3.4.0;
210 (*28*)) was performed and admixture proportions with the R package *ConStruct* (version 1.0.4; (*29*)) were
211 estimated based on the major allele frequencies of all mitochondrial SNPs. Major allele frequencies
212 were calculated with the python script *sync2AF.py* from the DrosEU pipeline. Admixture proportions
213 (K=3) for each line were inferred by non-spatial modelling with three MCMC chains per run and 10,000
214 iterations. Genetic differentiation for each mitochondrial SNP was assessed by estimating F_{ST} according

215 to (*32*) and Fisher's exact tests to estimate the significance of the allele frequency differences. Pairwise
216 F_{ST} and Fisher's exact tests per SNP were calculated between mitonuclear genotypes (replicates were
217 pooled) with *Popoolation2* (*31*). Resulting *P*-values from the Fisher's exact tests were corrected for
218 multiple testing using FDR correction (*33*) and SNPs significant at an FDR<0.001 were considered as
219 significantly differentiated between mitonuclear genotypes.
220
221 **Quantitative trait analysis**
222 Phenotype data were analysed in R 3.6.1. Fit of fecundity data to a negative binomial distribution was
223 determined with firdistrplus::descdist and firdistrplus::fitdist. Generalised linear mixed models of the
224 form
225
226 $$y \sim diet * mitochondria * nuclear + (genotype)$$
227
228 were fit with lme4::glmer.nb (egg counts & progeny, negative binomial distribution) or lme4::glmer
229 (fertility, binomial of progeny and egg counts); in which *diet* (control/EAA/lipid), *mitochondria* (A/B) and
230 *nuclear* (A/B) were fixed factors, genotype was a random factor denoting fly line (e.g. *AA1, AA2, AA3,*
231 *AB1, AB2, BA1*, etc). Where relevant (Figure S2), experimental replicate was also included as an
232 additional random factor. An observation-level random effect was also included for fertility under
233 chronic feeding to ameliorate overdispersion. Anova tests (type-3) were conducted with car::Anova,
234 and *post-hoc* analyses were applied with the functions emmeans::joint_tests, emmeans::pairs,
235 emmeans::emmip (*14*). Options for contrasts were set to orthogonal polynomials and sum-to-zero
236 contrasts.
237
238 Development to adult was modelled by fitting Cox mixed effects models of the form
239
240 $$y \sim diet * mitocondria * nuclear * sex + eggs + (genotype)$$
241

with coxme::coxme. *Diet, mitochondria, nuclear* and *genotype* terms were as in models of egg laying. *Eggs* coded number of eggs laid in the vial in which the individual developed, to account for variation in rearing density. Anova and and *post-hoc* EMM tests were conducted as per fecundity analyses.

PCA of phenotype data was conducted with prcomp on scaled EMMs, for each trait on each diet.

r^2 was calculated with MuMIn::r.squaredGLMM. Akaike weights were calculated with MuMIn::dredge.

To overcome challenges in calculating effect sizes for three-way DMN interactions, for each trait we used EMMs and joint tests to calculate F ratios, from which a measure of effect size within the sample population (partial η^2) can be estimated (*14*, *34*, *35*). Effect sizes were calculated with custom functions built around effectsize::F_to_eta2. Application of this function was necessarily specific to the type of model in question. In all cases, F statistics were taken from ANOVA tables returned by emmeans::joint_tests. For GLMMs and Cox mixed-effect models (Figure 4), degrees of freedom were taken from ANOVA tables returned by emmeans::joint_tests; with residual degrees of freedom calculated by df.residual for GLMMs, or taken from model output for Cox mixed-effect models. For GLMs (Figure 4), degrees of freedom and residual degrees of freedom were taken from stats::anova.

EMMs superimposed on plots were calculated by fitting a GLMs of the form

$$y \sim diet * line$$

and calculating EMM per fly line per diet. A distinct model was used for EMM calculation because models used to calculate statistical effects did not return main effects for each line (e.g. coefficient for mitotype A, nucleotype A, control diet) and therefore did not show among-mitonucleotype replication (i.e. coefficients for each of lines AA_1, AA_2, AA_3 on control diet). Confidence interval of development time EMM for line AA_3 on +EAA diet was too large to plot owing to lethality in that condition, therefore this line was excluded from the plot (see Figure 3 legend). Values were returned to original scale by exponentiation when emmeans returned logged values.

Difference indices were calculated from EMMs per line per diet described above. For each pairwise comparison, fold-change was calculated as

$$\frac{(Y - X)}{X}$$

Where Y represented post-treatment value and X represented starting value. Absolutes of these values were then logged, re-signed, and scaled to a -1:1 scale. P-values for difference in EMM was calculated for each pairwise comparison using emmeans::pairs, from which FDR was returned with stats::p.adjust. Bubble plots were produced using ggplot2.

Figures were assembled in Adobe Illustrator. Mitochondria graphics were recoloured from files freely distributed under an open commons license. Skull and crossbone graphics were sourced from all-free-download.com. Heatmap of nutrient content was plotted in R with superheat.

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