Mitonuclear interactions and early-life diet shape adult nutritional behaviour

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Abstract

Mitochondrial function relies on close coordination between the mitochondrial and nuclear genomes. Disruption to this coordination—via mitonuclear mismatch—can impair metabolic efficiency, particularly under energetically demanding conditions such as during development. The nutritional environment further modulates mitochondrial demands, suggesting that mitonuclear genotype and diet may interact to shape life-history traits and behaviour. Here, we investigate how early-life diet and mitonuclear genotype jointly influence development time, adult body size, and nutritional preference in *Drosophila melanogaster*. Using a full-factorial panel of putatively matched and mismatched combinations (cybrids) of mitonuclear genotype derived from natural Australian populations, we reared flies on diets varying in their ratio of macronutrients and assessed how this influenced larval development and subsequent adult diet preference. Developmental rate was significantly influenced by mitonuclear coevolution and diet, with cybrids showing delayed development under all conditions, with dietary extremes exacerbating this effect. Despite this, egg-to-adult viability remained unaffected. Adult nutritional behaviour exhibited clear genotype- and diet-dependent effects. Flies reared on high-protein diets increased carbohydrate intake as adults, while those reared on high-carbohydrate diets increased protein intake, suggesting compensatory feeding responses. Mitonuclear mismatch further modulated nutrient consumption, particularly in females, whose carbohydrate intake was influenced by intergenomic compatibility and early-life dietary conditions. Males' protein consumption was also impacted by mitonuclear coevolution across all developmental diets. Finally, body size was also shaped by interactions between mitonuclear genotype and diet. Together, our findings demonstrate that mitonuclear compatibility and the composition of the early nutritional environment interact to shape developmental and behavioural phenotypes. These results support a role for mitonuclear coadaptation in mediating metabolic plasticity, highlighting the evolutionary and physiological significance of genotype-specific mitonuclear coordination.

Introduction

Developmental time is a core life-history trait with far-reaching implications for fitness (Metcalfe & Monaghan, 2001). It determines how rapidly organisms transition through life stages and affects their exposure to ecological pressures such as predation, competition, and seasonal constraints (Sniegula et al., 2024). In insects, selection on developmental timing can shape life cycle synchrony, dispersal potential, and reproductive output (Gershman et al., 2022). Since development is an energetically costly process, the pace at which it proceeds is tightly coupled to metabolic rate, which itself is governed by mitochondrial function (Brown et al., 2004, Norin & Metcalfe, 2019, Koch et al., 2021, Thoral et al., 2024).

Mitochondria, the cellular powerhouses, produce ATP via oxidative phosphorylation (OXPHOS), a process that depends on proteins encoded by both the mitochondrial (mtDNA) and nuclear (nuDNA) genomes (Gray et al., 1999, Martin & Koonin, 2006). This dual-genomic control renders mitochondrial function sensitive to mitonuclear compatibility (Burton et al., 2013, Rand, 2001). Mismatches between mtDNA and nuDNA—whether due to hybridisation, introgression, or experimental manipulation—can disrupt electron transport chain (ETC) function, leading to reduced metabolic efficiency and physiological performance (Hill et al., 2019). Increasingly, studies show that mitonuclear interactions shape diverse phenotypes including ageing, reproduction, locomotion, and metabolism (Ma et al., 2016, Meiklejohn et al., 2013, Rank et al., 2020, Latorre-Pellicer et al., 2016). Recent work has highlighted developmental time as another trait shaped by mitonuclear epistasis: Mossman et al. (2016) found that development rate in *Drosophila melanogaster* is influenced by nuclear and mitochondrial genotype, with dietary composition further modulating these effects (Mossman et al., 2016). Their results support the view that metabolic performance and developmental timing are not fixed traits, but dynamic outcomes of genome-by-genome-by-environment (G×G×E) interactions. Yet the precise metabolic pathways that underlie these effects—and how organisms compensate behaviourally—remain poorly understood.

Diet is a central feature of the developmental environment, and its macronutrient composition exerts profound effects on mitochondrial metabolism (Rodriguez et al., 2021, Aw et al., 2018, Towarnicki & Ballard, 2017). Protein-rich diets often accelerate development by providing essential amino acids necessary for biosynthesis, yet the digestion of protein and the processing of nitrogenous waste are metabolically demanding, increasing energetic demands on mitochondrial function and oxidative stress (Chatterjee & Perrimon, 2021). Conversely, high-carbohydrate diets are more readily metabolised but can disrupt energy homeostasis by overwhelming glycolytic pathways, promoting lipid synthesis, and impairing mitochondrial

oxidative efficiency (Mattila & Hietakangas, 2017). Disruptions in these regulatory circuits can lead to metabolic imbalances, particularly in genotypes with compromised mitonuclear coordination where mitochondrial energy production is already suboptimal. Importantly, diet is not only an environmental input but also a behavioural choice, shaped by the organism's internal physiological state and energy demands (Corrales-Carvajal et al., 2016, Ribeiro & Dickson, 2010). Recent evidence suggests that nutritional preference itself is modulated by mitonuclear interactions. A study by Camus & Inwongwan (2023), leveraging a large panel of *Drosophila* genotypes, revealed that intergenomic epistasis shapes behavioural decisions regarding macronutrient intake, in a sex-specific manner (Camus & Inwongwan, 2023). These findings imply that mitochondrial efficiency shapes internal metabolic state, which in turn influences nutritional decision-making. However, it remains unclear how such behavioural flexibility emerges during development, or how developmental experience shapes later dietary choices.

Here, we extend this work by investigating how developmental diet and mitonuclear genotype jointly influence both developmental time and adult nutritional preference in *Drosophila melanogaster*. We used flies derived from subtropical (Townsville) and temperate (Melbourne) populations at opposite ends of the Australian east-coast cline (Hoffmann et al., 2002). From these, we created a full-factorial panel of mitonuclear genotypes comprising two putatively matched combinations and two putatively mismatched (cybrid) combinations. Each genotype was reared on diets differing in yeast-to-sugar ratio. We first measured time to adult emergence to assess whether mitonuclear compatibility buffers or exacerbates the effects of nutritional stress during development. We then asked whether the early dietary environment influences later feeding behaviour, using a binary macronutrient choice assay to quantify adult diet preference.

We hypothesise that: i) matched genotypes will develop more rapidly and consistently across diets, while mismatched genotypes will show developmental delays, particularly on high-protein or high-carbohydrate diets that challenge mitochondrial metabolism; ii) mitonuclear incompatibility will amplify the costs of stressful developmental environments, leading to more pronounced changes in diet preference in adulthood; and iii) flies reared on suboptimal diets may compensate behaviourally by shifting their adult feeding preferences in a genotype-dependent manner. By linking developmental environment, metabolic genotype, and adult nutritional behaviour, our study provides a valuable integrative perspective on how early-life conditions and genetic compatibility shape complex phenotypes. These findings have broader relevance for understanding the evolution of metabolic plasticity and the constraints imposed by mitonuclear coadaptation.

Materials and Methods

Fly Stocks and Mitonuclear Panel

We used a panel of *Drosophila melanogaster* lines derived from two natural populations collected from distinct latitudinal locations along the Australian east coast cline. The first population originated from Townsville (latitude −19.26°, longitude 146.81°), representing a northern subtropical environment, and the second from Melbourne (latitude −37.77°, longitude 144.99°), representing a temperate southern environment. These populations were collected in early 2021 from the wild and transferred to Monash University (Australia), where they were named according to their collection site: Townsville ('T') and Melbourne ('M'). Isofemale lines were initially established from wild-caught, inseminated females, and F2 progeny from each line were subsequently used to produce massbred populations. For Melbourne, 26 isofemale lines were established, each contributing 12 virgin males and 12 virgin females to the massbred pool. The Townsville population was established from 35 isofemale lines, with each contributing 10 virgin males and 10 virgin females. All isofemale lines were treated with tetracycline (0.3 mg/mL), to eliminate possible endosymbionts (like *Wolbachia*), and this was screened using PCR (ONeill et al., 1992). Each massbred population was maintained across two bottles at density of 400 flies per bottle under standard laboratory conditions (25°C, 50% relative humidity, 12:12 light:dark cycle) on a standard 1:1 yeast:sugar (SY) diet.

To investigate the role of mitonuclear interactions, we established a full-factorial panel comprising four mitonuclear genotypes. Two of these retained their putatively matched combinations: Townsville mitochondrial DNA paired with Townsville nuclear DNA ("tT") and Melbourne mitochondrial DNA paired with Melbourne nuclear DNA ("mM"). We refer to the other as 'cytoplasmic hybrids' (cybrids) since they contain putatively mismatched genome combinations: Townsville mitochondrial DNA paired with Melbourne nuclear DNA ("tM") and Melbourne mitochondrial DNA paired with Townsville nuclear DNA ("mT"). Because mitochondrial haplotypes remain polymorphic in both Melbourne and Townsville locations, we refer to the most frequent mitonuclear combinations at each location as putatively matched, and the less frequent combinations as putatively mismatched. In this nomenclature, the lowercase letter refers to the mitochondrial origin and the uppercase letter to the nuclear background. The mitochondrial haplotypes correspond to those previously characterised along the Australian cline, with the Townsville haplotype ('t') aligning with haplotype A1 and the Melbourne haplotype ('m') with haplotype B1 (Camus et al., 2017, Lajbner et al., 2018). These haplotypes segregate latitudinally along the east coast of Australia, with 't' (A1) being more common at low latitudes and 'm' (B1) more common at higher latitudes. Specifically, Camus et al. (2017) reported that the frequencies of 't' versus 'm' in the Townsville population

were 55% and 27%, respectively, while in Melbourne they were 16% and 60% (Camus et al., 2017). For clarity and consistency, we use the two-letter genotype labels (tT, mT, mM, tM) introduced in (Bettinazzi et al., 2024).

All genotypes were generated in November 2021using a crossing scheme involving balancer chromosomes to replace the nuclear genome of a given population. We take advantage of the maternal inheritance of the mtDNA and couple each balancer chromosome to the desired nuclear genome. Consequently through 6 generations of crossings we are able to replace the nuclear genome, whilst keeping the mtDNA from the original population. For graphical representation of the fly crossing scheme see Supplementary figure 1 from (Bettinazzi et al., 2024). This crossing scheme was followed by ongoing backcrossing of the target nuclear genome, as one of the limitations of this scheme is that is does not consider the 4th (dot chromosome) or possible hyperrecombination due to balancer chromosomes. Regular PCR-based genotyping was conducted to verify mitochondrial haplotype integrity (Bettinazzi et al., 2024).

All mitonuclear fly populations are maintained under standard laboratory conditions, kept individually in 250mL bottles, with rough densities of approximately 250-400 flies in each bottle. These populations are maintained in discrete generations, with each generation being roughly every 2 weeks. To avoid nuclear adaptation to the fixed mitochondrial haplotype, nuclear heterogeneity was maintained through recurrent backcrossing. More specifically this was done using 100 virgin females of the each mitonuclear population with 100 male flies of the nuclear stock populations (tT and mT females with males of the T stock population, tM and mM females with males of the M stock population). To generate experimental flies for assays, bottled populations were placed on oviposition plates and given a fixed time to lay eggs. This fixed time was dependent on the experiment (see below for further detail). Following this lay, eggs clutches were randomly allocated into vials with egg densities being controlled (50-80 eggs per vial) to minimise environmental variance.

Diet Treatments

To manipulate developmental nutritional environments, we established three experimental diets by varying the yeast:sugar ratio of our standard sugar-yeast (SY) medium; with the aim of modulating the protein:carbohydrate (P:C) ratios of the diet. The protein source here is considered to be the brewer's yeast (50% protein, 12% carbohydrate), while the carbohydrate source is sucrose. While we acknowledge that yeast is not 100% protein, and contains both complex and simple carbohydrates, we are unable to perform these experiments using the *Drosophila* holidic diet since it does not sustain proper larval development (Piper et al., 2014).

The three diets represented a gradient of macronutrient balance: a high-carbohydrate diet (yeast:sugar 1:4), a standard control diet (yeast:sugar 1:1), and a high-protein diet (yeast:sugar 2:1), albeit they are not exactly isocaloric but close in amount of calories (Table S1). To achieve the desired diet ratios, yeast and sugar concentrations were adjusted proportionally while the concentrations of agar (gelling agent), nipagin, and propionic acid (preservatives) were kept constant across all diets (Table S1). These developmental diets were selected to impose nutritional challenges that are known to affect metabolic processes, growth rate, and adult physiology in *Drosophila* (Klepsatel et al., 2020).

All diets were prepared freshly, poured into standard 25 x 95 mm vials, and allowed to solidify. Throughout the experiments, flies were reared and maintained at 25°C on a 12:12 light:dark cycle at high humidity (>60%) to minimise food desiccation and evaporation from diet surfaces.

Developmental Time & Egg-to-Adult Viability Experiment

To assess the influence of mitonuclear genotype and developmental diet on developmental timing, ~300 5-day old adult flies from each mitonuclear genotype were placed on oviposition plates consisting of grape-agar media for a period of 2 hours. Following oviposition, 20 eggs were picked from each mitonuclear genotype combination and placed in individual vials containing one of the three developmental diet treatments. Development was monitored 3 times daily, and the time to adult eclosion was recorded. Eclosion checks were conducted at consistent time intervals across all treatment groups. Adult emergence was scored as the number of hours from egg lay to the appearance of fully eclosed flies. All emerging adults were removed promptly upon eclosion to prevent mating or crowding effects. Using this dataset we also examined egg-to-adult viability, which was calculated as the total number of flies that emerged from each vial of 20 eggs.

Diet Preference Experiment

For the nutritional preference experiment, ~200 4-day old adult flies from each mitonuclear genotype were allowed to lay eggs on petri dishes containing the appropriate treatment diet for a period of 2 hours. Following oviposition, sections of the food containing approximately 30–50 eggs were transferred to individual vials containing the same developmental diet. To synchronise developmental staging across treatments, we employed a staggered egg-lay design based on pilot data showing diet-specific effects on developmental rates. Specifically, flies destined for the high-carbohydrate (1:4) diet were from the first egg-lay, followed by those for the standard diet laid after 48 hours, and finally those for the high-protein (2:1) treatments were laid on the subsequent days. This strategy made sure that all focal adult flies emerged

roughly within a 24-hour window. We acknowledge that with our experimental design, the maternal age of the focal flies covaries with dietary treatment. However, we predict that small differences in maternal age should not have large effects on dietary preference of offspring or be involved in genotype by environment interactions for dietary preference.

We conducted a diet preference assay using a capillary feeding (CAFE) approach. Flies from each genotype/diet combination were placed in bottles containing the same diet treatment for 48-hours, giving them an opportunity to mate. After this mating period, cohorts of flies were split by sex and triplets of flies from each genotype/diet combination were placed in 1% agar vials. Agar vials provide no nutritional value, yet prevent flies from desiccation by providing a water source. Following an overnight starvation period on agar-only vials, two 5 µl microcapillary tubes were inserted into each vial: one filled with a carbohydrate solution and the other with a protein solution. We note that while the overnight starvation period is routinely used for these experiments, it is a significant amount of time for a fly to have no access to food. This could have greater repercussions on fly physiology/behaviour than we think, and should be further studied.

These diets were prepared following the established protocols, consisting of defined mixtures of amino acids (for the protein solution) and sucrose (for the carbohydrate solution) dissolved in a buffered aqueous solution (see Tables S2-S4 for recipes) (Camus et al., 2018). Capillary tubes were replaced every 24 hours over a three-day feeding period, and the volume of diet consumed from each tube was measured to the nearest 0.1 µl. Evaporation controls were included by placing identical capillaries into vials without flies and measuring changes in volume over the same time intervals. Food consumption for each day was corrected by subtracting evaporation losses.

Statistical Analyses

Data analysis was performed using the R software (R Core Team, 2021). We utilised several statistical packages including; 'ggplot2' (Wickham, 2016), 'lme4' (Bates et al., 2015), 'lmerTest' (Kuznetsova et al., 2017), 'car' (Fox & Weisberg, 2019), 'ggeffects' (Lüdecke, 2018), 'Rmisc', and 'emmeans' (Lenth, 2022).

Development time (hours) was modelled with mitochondrial haplotype, nuclear genotype, developmental treatment and sex (plus their interaction) as fixed effects. For this model, we also included the term "vial" as a random effect, representing the group of flies sharing the same developmental environment. To further test if diets influenced the degree of mitonuclear mismatch effects, we generated a subsequent model with diet, mitonuclear match (coevolved

or mismatch), and their interaction as fixed effect. Vial and mitonuclear genotype ("strain") were also included as random effects. This was followed by a *post-hoc* test examining if mitonuclear coevolution had an effect within each diet, and we calculated the effect size for these comparisons. For the egg-to-adult viability dataset, we used a binomial generalized linear mixed model with developed offspring and flies that failed to develop (deaths) as a response variable. We used mitochondrial haplotype, nuclear genotype and developmental treatment (plus their interaction) as fixed effects, with vial as a random effect. In this case we did not include sex as a fixed factor because it assumes that the clutch of eggs has an equal starting number of male and female eggs. We added an observation-level random effect to the mixed model to deal with overdispersion of the data.

For the dietary preference we ran two analyses. We first analysed the data using multivariate analyses of variance (MANOVA), whereby the response variable was the joint consumption of carbohydrate and protein with the interactive effect of mitochondrial haplotype, nuclear background, sex and diet treatment as fixed effects. This experiment was performed across two consecutive blocks, thus we included this as a factor in our analyses. Given one cannot add random effects to MANOVA analyses, we decided to include it as a fixed effect in these models. We then ran these same analyses on each sex separately, to probe any sex-specific patterns more deeply. The second type of analyses we ran on this data were separate linear mixed models for each response variable separately (protein consumption and carbohydrate consumption). Here we modelled either the total consumption of carbohydrate or protein with the effects of mitochondrial haplotype, nuclear background, diet treatment and sex (and their interactions) as fixed effects. Block was modelled here as a random effect. We repeated these analyses separately for each sex.

We also tested if disrupting putatively coevolved mitonuclear genotypes had an effect on diet choice. For this we generated secondary models, where we had coevolutionary status (coevolved vs mismatched), diet, sex and their interactive effects as fixed effects. Depending on the models, block and mitonuclear genotype were added as a fixed (MANOVA) or random (LMM) effects. All statistical models are reported in Tables 1, S2-S12, and supplementary code file.

Results

Development time

Development time was significantly influenced by diet, with flies developing more slowly on the high-carbohydrate diet and more quickly on the high-protein diet ($F_{2,56.11}$ = 9916.2, p < 0.001; Figure 1A; Table S5). Across all diets, females tended to develop more quickly than males ($F_{1,56.12}$ = 13.42, p = 0.001), although the magnitude of this sex difference varied with diet. While neither mitochondrial ($F_{1,56.12}$ = 0.13, p = 0.718) nor nuclear genotype ($F_{1,56.12}$ = 0.06, p = 0.811) alone had a significant main effect, their interaction was highly significant ($F_{1,56.12}$ = 205.36, p < 0.001), indicating strong mitonuclear epistasis. This interaction was evident across all dietary conditions and sexes, where the two mismatched genotypes (mT and tM) exhibited divergent developmental profiles (Figure 1A). We also detected significant interactions between mitochondrial genotype and sex ($F_{1,56.12}$ = 5.8, p = 0.019), as well as nuclear genotype and sex ($F_{1,56.12}$ = 11.31, p = 0.001), suggesting sex-specific sensitivity to genetic background. Additionally, a diet × sex interaction ($F_{2,56.11}$ = 15.50, p < 0.001) revealed that females were particularly delayed on the high-carbohydrate diet relative to males. Three-way interactions between mitochondrial and nuclear genotypes and diet ($F_{2,56.11}$ = 6.04, p = 0.004), as well as between mitochondrial and nuclear genotypes and sex ($F_{1,56.12}$ = 5.64, p = 0.021) further showed that the developmental effects of mitonuclear genotype depended on the nutritional environment or sex (Table S5). For example, the tT genotype consistently exhibited the fastest development across diets and sexes, whereas both cybrid lines (mT and tM) showed slower and more variable development, particularly under nutritional stress. This is further supported by complementary analyses which revealed a significant interaction between coevolution status (i.e. fly lines grouped as either “coevolved” or “mismatched”) and diet ($F_{2,121.65}$ = 4.10, p = 0.002) (Table 1A). *Post-hoc* analysis revealed that mismatched lines (mT plus tM) developed more slower than coevolved lines (tT plus mM), across all three diets (ST: t = -5.526, p < 0.0001; HC: t = -5.702, p < 0.0001; HP: t = -9.079, p < 0.0001), with estimates and effect sizes suggesting stronger mitonuclear effect under both extreme diets (Tables 1B,C, Supplementary code section 2.2).

Egg-to-adult viability

In contrast to development time, egg-to-adult viability was not significantly affected by any main effects or interactions in our model. There were no significant effects of nuclear genotype (χ^2 = 0.0001, df = 1, p = 0.993), mitochondrial genotype (χ^2 = 0.139, df = 1, p = 0.709), or developmental diet (χ^2 = 0.149, df = 2, p = 0.928) on viability (Figure 1B). Likewise, none of the interaction terms—including the mitonuclear interaction (χ^2 = 0.002, df = 1, p = 0.966) or

any genotype × diet interactions—approached significance ($p > 0.82$ for all; see Table S6 and Supplementary code section 3.1).

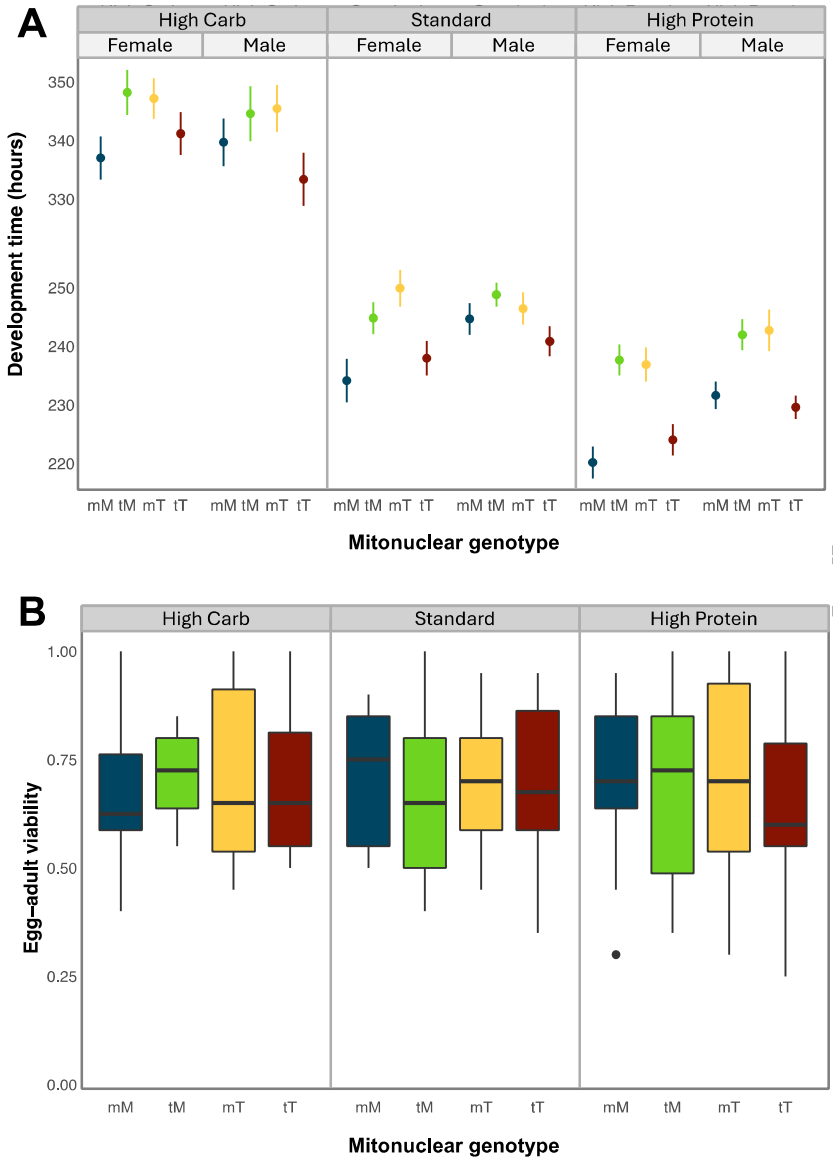

Figure 1: (A) Development time (hours from egg to adult emergence) for females and males across three developmental diets varying in yeast:sugar ratio. **(B) Egg-to-adult viability** (proportion of eggs developing into viable adults) under the same dietary conditions. Colours represent the four mitonuclear genotypes: *mM* (Melbourne mtDNA × Melbourne nuclear background), *mT* (Melbourne mtDNA × Townsville nuclear background), *tM* (Townsville mtDNA × Melbourne nuclear background), and *tT* (Townsville mtDNA × Townsville nuclear background). Error bars in panel A indicate ± SEM; boxes in panel B show the interquartile

range (IQR), with horizontal lines indicating the median and whiskers indicating the range of the data within 1.5× IQR.

Table 1: Effect of mitonuclear coevolution and dietary treatment on developmental time. **(A)** Linear mixed model testing the interaction effect between mitonuclear coevolution (matched vs mismatched) and early-life dietary treatment on developmental time. **(B)** *Post-hoc* comparisons of mitonuclear matched and mismatched lines for development time across three dietary treatments. **(C)** Effect sizes associated with the *post hoc* analysis. We investigated whether more stressful nutritional environments exacerbated mitonuclear effects. Using linear mixed models (See supplementary code section 2.2), we find evidence for greater effects in the diet treatments compared to control diet. Note: because decreased development time is better, we observe negative estimates when comparing coevolved vs mismatch. Fixed effects: 'coev': matched (mM, tT) vs mismatched (tM, mT) lines; 'diet': HC (high-carbohydrates diet - 1:4 yeast:sugar), ST (standard diet - 1:1 yeast:sugar), HP (high-protein diet – 2:1 yeast:sugar).

A	Sum Sq	Mean Sq	NumDF	DenDF	F value	Pr(>F)
coev	31074	31074	1	121.64	137.9075	< 0.001
diet	3035867	1517933	2	121.65	6736.5539	< 0.001
coev × diet	1851	925	2	121.65	4.1065	0.002

	Coevolved - mismatch				
B	estimate	SE	df	t-ratio	p-value
High Carbohydrate	*-8.36*	1.47	15.8	-5.702	<.0001
Standard	*-8.13*	*1.47*	16	-5.526	<.0001
High Protein	*-13.41*	*1.48*	16.1	-9.079	<.0001

	Coevolved - mismatch	
C	effect size	SE
High Carbohydrate	-0.557	0.0976
Standard	-0.542	0.0980
High Protein	-0.893	0.0984

Nutritional preference

Nutritional preference was strongly sexually dimorphic, with females consuming significantly more protein and carbohydrate than males across all treatments (Pillai's trace = 0.479, $F_{2,367}$ = 168.72, p < 0.001). Genotypes and developmental diet also significantly influenced feeding behaviour (mito: Pillai's trace = 0.022, $F_{2,367}$ = 4.12, p = 0.017; nuc: Pillai's trace = 0.039, $F_{2,367}$ = 7.53, p < 0.001; diet: Pillai's trace = 0.059, $F_{4,736}$ = 5.56, p < 0.001), along with significant interactions between sex and mitochondrial genotype (sex × mito: Pillai's trace = 0.023, $F_{2,367}$ = 4.27, p = 0.015) and between sex and diet (Pillai's trace = 0.036, $F_{4,736}$ = 3.39, p = 0.009), indicating sex-specific response to both dietary treatment and haplotype presence (Table S7). When carried on each sex separately, multivariate analysis revealed a strong effects of both genotype and developmental diet in females (mito × nuc × diet: Pillai's trace = 0.057, $F_{4,418}$ = 3.10, p = 0.016) (Table S8). Univariate analysis on females showed that protein intake was significantly affected by mitochondrial ($F_{1,209}$ = 9.82, p = 0.002) and nuclear genotype ($F_{1,209}$ = 12.98, p < 0.001), as well as developmental diet ($F_{2,209}$ = 6.74, p = 0.001). Carbohydrate intake was also influenced by developmental diet ($F_{2,209}$ = 4.85, p = 0.009), though not by any of the genotypes alone. Importantly, a significant three-way interaction between mitochondrial genotype, nuclear genotype, and diet was detected for carbohydrate intake ($F_{2,209}$ = 4.88, p = 0.008), highlighting that female nutrient preferences are shaped by complex gene-by-gene-by-environment interactions (Figure 2A; Table S8). In contrast, males showed more modest effects. Multivariate analyses indicated a significant mitonuclear interaction (Pillai's trace = 0.038, $F_{2,157}$ = 3.14, p = 0.046), but neither mitochondrial nor nuclear genotype had strong main effects. Univariate analyses revealed that protein intake was affected by the mito × nuc interaction ($F_{1,158}$ = 5.60, p = 0.019), while carbohydrate intake was significantly affected by developmental diet ($F_{2,158}$ = 3.61, p = 0.029) (Figure 2B; Table S8). There were no significant three-way interactions in males. The same results obtained via MANOVA were also confirmed with linear mixed models, with the exception of the diet effect in male carbohydrate-intake (Tables S9-S10).

When examining for coevolution effects (Table S11), we find significant effects in overall protein responses ($F_{2,380}$ = 4.039, p = 0.045). Interestingly, we find in females that mitonuclear mismatch affects carbohydrate consumption in a diet-dependent manner (coev × diet: $F_{2,215}$ = 5.032, p = 0.01), whereas for males it affects protein consumption no matter the diet (coev: $F_{1,164}$ = 5.054, p = 0.026, Table S12). The same results were also confirmed in the linear mixed model analysis (Tables S13)

We also examined adult dry weight as a proxy for body size (Figure 2C,D; Table S14, supplementary code sections 4.3 – 4.5). As expected, females were significantly heavier than males ($F_{1,368.47}$ = 1294.30, p < 0.001). Nuclear genotype ($F_{1,368.28}$ = 12.13, p < 0.001) and developmental diet ($F_{2,368.24}$ = 20.48, p < 0.001) both significantly influenced weight, with flies reared on high-protein diets being heavier than those on standard or high-carbohydrate diets. A significant mitonuclear interaction was detected ($F_{1,368.09}$ = 8.71, p = 0.003), and effects also varied by sex: the mito × sex ($F_{1,368}$ = 6.19, p = 0.013) and mitonuclear × sex interactions ($F_{1,368.47}$ = 6.70, p = 0.010) were both significant. Furthermore, there was a significant three-way interaction between mitonuclear genotype and diet ($F_{2,368.06}$ = 4.99, p = 0.007), and a four-way interaction involving mitonuclear genotype, diet, and sex ($F_{2,368.09}$ = 3.49, p = 0.032), suggesting that the effects of early nutrition on body size depend on a combination of genetic background and sex.

Sex-specific models revealed that females were particularly sensitive to mitonuclear interactions. While developmental diet had a strong main effect on weight ($F_{2,209.01}$ = 10.14, p < 0.001), the mitonuclear interaction ($F_{1,209.01}$ = 20.08, p < 0.001) and mitonuclear × diet interaction ($F_{2,209.02}$ = 9.04, p < 0.001) were also highly significant. In males, body weight was significantly influenced by nuclear genotype ($F_{1,159}$ = 8.64, p = 0.004) and developmental diet ($F_{2,159}$ = 12.51, p < 0.001), but no significant interactions were detected (Table S14).

When examining for an effect of intergenomic matching on body mass (Table S15, supplementary code section 4.6), we find a significant three-way interaction between genomic coevolution, dietary regime and sex ($F_{2,379}$ = 3.43, p =0.033). This suggests a significant impact of mitonuclear coevolution on female weight, dependent on the developmental diet (coev × diet: $F_{2,214}$ = 8.84, p < 0.001), whereas for males only an effect of development diet was detected (diet: $F_{2,164}$ = 11.98, p < 0.001).

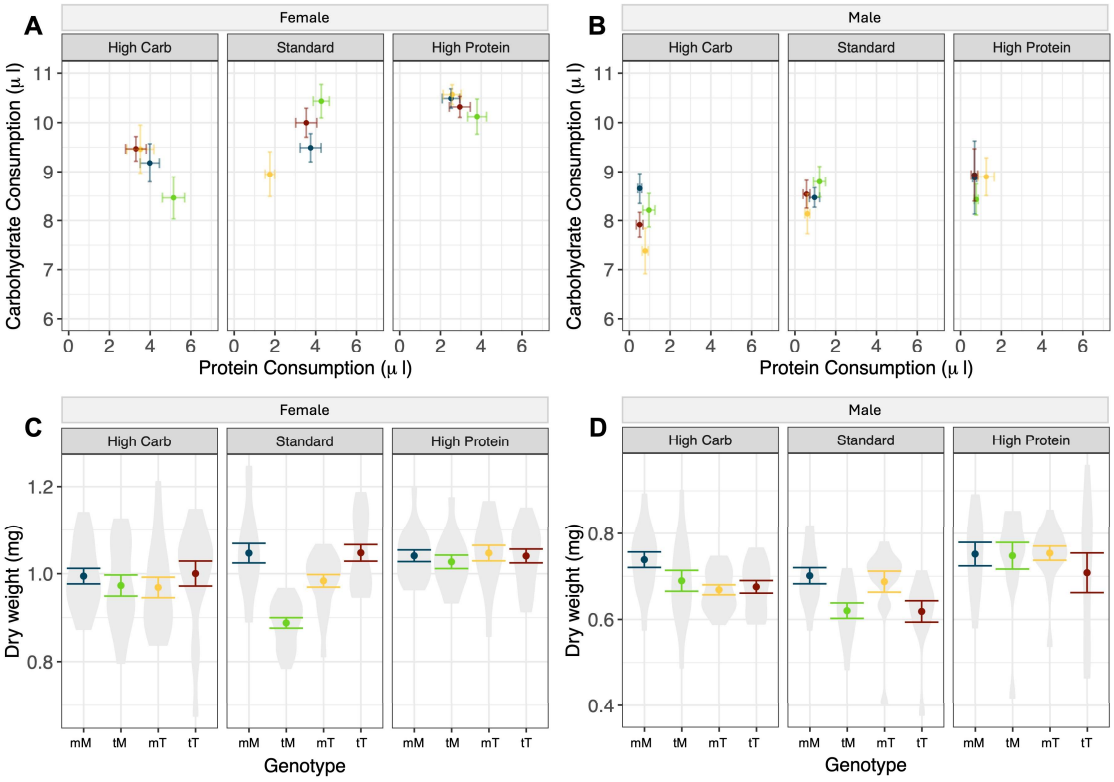

Figure 2: Effects of developmental diet and mitonuclear genotype on adult body size and nutrient consumption. (A–B) Macronutrient consumption profiles of adult females (A) and males (B) reared on the same diets. Flies were offered a binary choice between protein and carbohydrate solutions in a capillary feeding assay, and consumption (µl) was recorded over three days. Each point represents the mean intake ± SEM of protein and carbohydrate across replicates. **(C–D)** Dry body weight (mg) of triplet adult females (C) and males (D) reared on one of three developmental diets differing in yeast:sugar ratios. Points show mean dry weight ± SEM for each mitonuclear genotype (mM, tM, mT, tT), with overlaid violin plots indicating the distribution of individual measurements. Dry weights were obtained after nutritional preference assay.

Discussion

Mitonuclear incompatibility delays development, especially under dietary stress

Our data reveal that mitonuclear mismatch significantly delays development in *Drosophila melanogaster*, and this effect is exacerbated under protein- and carbohydrate-biased diets (Figure 1A; Tables 1, S2; Supplementary code sections 2.1-2.2). These findings are consistent with previous studies showing that disrupted mitonuclear coordination impairs OXPHOS performance, affecting energetically demanding stages like larval growth (Mossman et al., 2016, Rank et al., 2020, Meiklejohn et al., 2013, Hoekstra et al., 2018). Although investigating the mechanistic basis of these GxGxE effects was beyond the scope of this study, the developmental outcomes observed likely arise from a combined influence on mitochondrial function (where mitonuclear epistasis modulates OXPHOS performance) and on nutrient-sensing pathways (where early-life nutritional cues shape metabolic signalling and developmental strategy). Recent analyses of these fly populations raised on a standard diet revealed that adult cybrids exhibit altered OXPHOS activity compared to their coevolved counterparts. This was characterised by higher O_2 consumption rates in cybrids, likely reflecting respiratory compensation to restore ATP levels under mitonuclear inefficiency (Bettinazzi et al., 2024).

Mitochondria are crucial metabolic hubs, connecting catabolic and anabolic pathways. Any mitochondrial defect can therefore have a detrimental effect not only on energy supply, but also in the provision of the building blocks necessary for growth. If low-nutrient conditions (or metabolic dysfunction due to mitonuclear epistasis) cause a greater share of TCA substrates to be allocated to OXPHOS rather than biosynthesis, development may be delayed (Jacobs et al., 2020). As such, the mitonuclear-diet-mediated impact on developmental rate described in this study might reflect a metabolic rewiring which enhances catabolic processes (to compensate for mitonuclear inefficiency) at the expense of anabolic processes, resulting in the delayed larval development observed in cybrid lines.

Metabolic rewiring in response to energy shortage (or mitochondrial stress) is orchestrated, among others, by the energy-sensors AMP-activated protein kinase (AMPK). Under condition of low intracellular ATP, AMPK promotes catabolism and mitochondrial biogenesis, while suppressing anabolic processes to restore energy balance. This is partly achieved through inhibition of mammalian target of rapamycin (mTOR) activity, which decreases protein synthesis and cell growth (Herzig & Shaw, 2018). As shown in recent studies, AMPK activation (and its mediated inhibition of IIS) can restrict food consumption, larval growth, and moulting in various insects, including *Drosophila*, *Bombix and Hyphantria* species (Yuan et al., 2020,

Zou et al., 2022). It is therefore plausible that mitonuclear-dictated early-life metabolic adjustments of this kind may underpin both the elevated mitochondrial respiration (i.e. AMPK-mediated mitochondrial biogenesis) found in cybrids (Bettinazzi et al., 2024) , as well as their slower development rate (i.e. AMPK-mediated shift towards catabolism) we report here, particularly evident under early-life dietary stress. Our future work will aim to test this hypothesis by measuring the expression specific genes associated with mitochondrial biogenesis, mitochondrial stress, and nutrient-sensing pathways in our lines (see (Hunter-Manseau et al., 2024)). Interestingly, despite these developmental delays associated with mitonuclear mismatch, egg-to-adult viability remained relatively stable across genotypes (Figure 1B; Table S6; Supplementary code section 3.1), suggesting that mismatched combinations prolong development to preserve survival rather than resulting in outright developmental failure (Chippindale et al., 1994, Prasad et al., 2000). These findings suggest that mitonuclear compatibility does not merely affect absolute performance but alters developmental strategy in response to environmental stressors.

Life-history trade-offs between early-life fitness and lifespan have long been recognized. In addition to the commonly observed fertility cost of a long life (Zwaan et al., 1995), traits such as developmental time and growth rate can also be constrained. Development time is a trait under strong selection in nature, as quick development can provide advantages, such as reducing exposure to predation, increased larval competitive ability, and earlier access to ephemeral resources (Prasad & Joshi, 2003). However, as exemplified in fly populations under various selection regimes (selected for either development time or age-of-reproduction), there might be a cost of rapid development. Although the nature of trade-offs can vary considerably across studies (Prasad & Joshi, 2003) they could involve multiple components of fitness, including larval survival, body size, fecundity, stress resistance, and lifespan (Chippindale et al., Partridge & Barton, 1993, Yadav & Sharma, 2014),

Complementary work on the same lines indicated that, under normal dietary conditions, fertility is also compromised in cybrid lines (Bettinazzi et al., 2024). However, nothing is known about the effect of mitonuclear mismatch on lifespan. As selection for slower development has been associated with beneficial effects on lifespan (Lind et al., 2017) it is therefore possible that the altered developmental strategies herein described for cybrids might have far-reaching effects later in life. Further studies will be needed to uncover any potential mitonuclear-driven trade-offs in life-history traits in these lines.

Early diet and genotype shape adult feeding behaviour

Our results show that both developmental diet and mitonuclear genotype influence adult macronutrient preference in *Drosophila melanogaster*, with flies exhibiting some compensatory feeding: individuals reared on high-protein diets consumed more carbohydrate as adults and vice versa (Supplementary code section 4.7). This behaviour is consistent with known nutrient-specific appetites and homeostatic regulation (Corrales-Carvajal et al., 2016, Rodrigues et al., 2015, Dobson et al., 2023). However, the strength and direction of these responses varied with genotype and sex (Figure 2A,B, Tables S4-S10). These findings diverge from those of Davies et al. (2018), who reported that adult females selected similar protein-to-carbohydrate intake ratios regardless of larval diet (Davies et al., 2018), whereas males adjusted intake in response to developmental protein restriction. In contrast, we found that female flies altered their adult feeding behaviour in a developmental diet-dependent manner, but only in certain genotypes. This discrepancy may reflect genetic background differences or the effects of mitonuclear mismatch, which may constrain optimal dietary choice decisions. Our results also extend these findings by showing that developmental effects are not universally "cancelled out" in adulthood—as suggested by Davies and colleagues—but instead can persist, particularly when metabolic coordination is compromised.

Although we did not assess larval choice directly, our findings align well with studies indicating that early dietary decisions critically influence long-term physiological outcomes (Almeida de Carvalho & Mirth, 2017, Rodrigues et al., 2015). Protein intake during the larval critical weight period is known to strongly influence developmental timing, adult size, and metabolic phenotype (Koyama et al., 2020). Consequently, early-life nutritional exposures may not only shape immediate developmental outcomes but also programme neural and endocrine pathways governing nutrient sensing and behavioural preferences in adulthood. Our results thus highlight how developmental nutritional environment, mediated through mitonuclear interactions, can have lasting effects on behavioural plasticity, underscoring the intricate interplay between early-life nutritional history, genetic background, and adult dietary behaviour.

Evolutionary implications of diet–mitonuclear interactions

Our study underscores the evolutionary significance of mitonuclear interactions as key determinants of phenotypic plasticity in response to nutritional environments. While adaptive flexibility in the face of nutritional stress is crucial for organismal fitness (Cormier et al., 2021, Dobson et al., 2023), we provide evidence that mitonuclear interactions impose significant physiological constraints - potentially requiring mismatched (cybrid) genotypes to undergo

substantial internal physiological changes to achieve their optimal metabolic state. Unlike coevolved genotypes, where we predict that developmental and behavioural phenotypes are underpinned by efficient metabolic coordination, cybrids exhibit altered developmental timing and nutritional choices, indicating distinct metabolic strategies, which have the potential to manifest as maladaptive outcomes. Indeed, since nutritional preferences inherently reflect complex internal metabolic requirements (Corrales-Carvajal et al., 2016), it would be inappropriate to label one dietary choice universally superior to another without direct fitness comparisons. Instead, our findings suggest that cybrid genotypes may adopt alternative physiological trajectories that, while distinct from coevolved genotypes, could still represent effective strategies to mitigate metabolic inefficiencies.

These genotype-dependent physiological adjustments have significant evolutionary implications, particularly in populations experiencing hybridisation, genetic introgression, or rapid environmental shifts (Hill et al., 2019, Hill, 2015). Mitonuclear incompatibilities could drive physiological divergence by prompting mismatched individuals to reconfigure metabolic networks and regulatory pathways, potentially influencing evolutionary trajectories and facilitating novel adaptive outcomes under nutritional stress. Given that females typically have greater metabolic demands due to reproductive investment, the heightened sensitivity we observed in females to mitonuclear mismatch and developmental diets suggests asymmetric selective pressures on mitochondrial function. Such sex-specific metabolic demands could shape evolutionary responses, potentially driving divergent life-history strategies and sex-biased selection pressures in populations encountering frequent genomic mismatches.

Moreover, recent work by Strilbytska et al. (2024) has demonstrated that dietary choice itself can impose physiological costs, affecting lifespan and reproductive success even when nutrient intake seems optimised (Strilbytska et al., 2024). Our results extend this insight by indicating that mitonuclear mismatches might amplify these physiological costs, necessitating greater internal physiological recalibration in mismatched genotypes. Consequently, the evolutionary persistence of mitonuclear incompatibility within populations may favour genotypes that can efficiently recalibrate their metabolic physiology in response to nutritional challenges, rather than those relying heavily on behavioural flexibility alone. Additionally, the developmental delays observed in cybrids, rather than being purely detrimental, may reflect alternative life-history strategies that prioritise metabolic recalibration over rapid developmental progression. Slower developmental rates might provide additional time for these genotypes to adjust internal physiological states, potentially mitigating oxidative damage, enhancing stress tolerance, or even improving longevity. Such outcomes would be consistent with evolutionary scenarios where metabolic inefficiencies trigger compensatory

physiological adaptations rather than outright detrimental effects. Nonetheless, whether such metabolic rewiring is truly adaptive remains uncertain, and the more parsimonious interpretation is that mitonuclear mismatch is primarily deleterious. Future work should address this gap by testing for impacts on other fitness-related traits.

In conclusion, our study highlights the intricate evolutionary interplay between mitonuclear genome compatibility, metabolic plasticity, and nutritional behaviour. If planetary environmental conditions continue to fluctuate and tend towards an increase in mean temperatures over time, the capacity of genotypes to physiologically recalibrate in response to mitonuclear mismatches will likely become an increasingly critical component of adaptive resilience. Future studies explicitly integrating fitness metrics and detailed metabolic profiling will be crucial to clarify how these physiological adjustments shape evolutionary outcomes in rapidly changing nutritional environments.

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