Title: Nutritional stress adaptation shapes host microbiome stability and life history dynamics

Running title: Adaptive microbiota dynamics to dietary stress

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Author contributions: PKW performed 16S sequencing and analysis and wrote the manuscript. GM performed all fly experiments. GSK helped with data analysis and performed critical revision of the manuscript. EN conceived the study, designed experiments, analyzed data, and wrote the manuscript.

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***Conflict of interest*:** The authors declare no conflicts of interest.

Data Accessibility Statement

All 16S Illumina raw reeds are available in the NCBI Sequence Reads Archive (SRA) BioProjectID PRJNA1233815. Computer code to reproduce all analyses is available as a GitHub repository at https://github.com/nochet/selPhenotyping/ and analysis steps laid out in a ProjSteps.md file.

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Abstract

Dietary environments can influence host-microbe evolution by shaping adaptive fitness responses. However, host-microbe dynamics on evolutionary time scales remain under-characterized. We studied *Drosophila melanogaster* experimentally evolved under three nutrient regimes: constant high (CH), deteriorating (DA), and fluctuating (FA) availability, alongside unselected controls (C). DA and FA increased longevity relative to CH, with DA enhancing late-life survival, though all selected groups showed higher mortality than C, suggesting fitness costs of dietary adaptation. Sex-specific effects emerged, with FA favoring early-life female survival and DA supporting late-life female longevity. Microbial richness and composition shifted with selection and time. While Firmicutes and Proteobacteria dominated generally, selection regimes showed higher Firmicute and lower Proteobacteria abundances compared to C, correlating with dysbiotic microbiomes starting early in adulthood. DA flies maintained a relatively stable microbial diversity over time, potentially supporting longevity. In contrast, CH and FA both exhibited low microbial diversity that destabilized with age. Indicator species analysis revealed treatment- and age-specific genera, with CH and FA showing greater dysbiosis, marked by potentially pathogenic taxa such as *Leptotrichia* and *Mobiluncus*. Our findings highlight the importance of early life stages in maintaining microbiome stability and host fitness, suggesting dietary stress drives distinct microbiota patterns and survival strategies.

Keywords: experimental evolution, nutritional environment, longevity, microbiota dynamics, adaptive response

Introduction

Microorganisms are deeply integrated into host biology across taxonomic and functional scales, playing critical roles in organismal adaptation and fitness (Gould et al. 2018). Rapidly changing environmental conditions increase the pressure for hosts to adapt to new conditions faster than their genomes can evolve. Microbial genomes, which evolve more rapidly than their hosts', represent a potential adaptive variation, enabling hosts to respond more quickly to environmental changes. For example, microbiotas can contribute functional hologenomes capable of modulating host genomic responses to environmental change (Petersen et al., 2023). Indeed, variation in microbiome composition has been linked to allele frequency shifts in genes associated with local adaptation in *Drosophila* (Rudman et al. 2019). More broadly, microbiotas can influence host life-history traits by mediating development (de Weerth 2017), reproduction (Rowe et al. 2020; Suyama et al. 2023), survival (Aleman and Valenzano 2019; Christensen et al. 2024), and defense against pathogens (Spragge et al. 2023; Christensen et al. 2024).

Microbiotas facilitate various vital functions for their hosts, including nutritional intake (Leitão-Gonçalves et al. 2017), metabolic regulation (Wong et al. 2014), and maintaining fertility under poor diet (Gnainsky et al. 2021). For instance, in *Drosophila melanogaster*, environmental microbes enhance oogenesis by promoting germline stem cell proliferation and egg maturation through increased ovarian cell division and repression of apoptosis (Suyama et al. 2023). During diapause in a ground-nesting solitary bee species, immature stages maintain a core microbiome of Actinobacteria (*Streptomyces* and *Nocardiodes*) that inhibit pathogenic fungal growth while supporting fungal groups that increase cold tolerance and overwintering survival (Christensen et al. 2024). In some amphibians, environmental and host-derived microbiomes shape defense against pathogens, such as the skin surface bacterium *Janthinobacterium lividum*, which protects the frog *Rana muscosa* from a lethal skin fungus (Harris et al., 2009). These examples illustrate the adaptive roles of beneficial microbe-host interactions in diverse environmental contexts.

Nutritional environments play a dominant role in shaping the composition, richness, and abundance of beneficial microbiota, ultimately determining host fitness and life-history outcomes. For example, *Drosophila* harbor microbial communities shaped by diet rather than host genotype, as seen in the facultative symbiosis between the host and *Lactobacillus plantarum* in which microbial production of N-acetyl-amino acids from diet enhance host growth

(Martino et al. 2018). Similarly, in honeybees, gut microbiotas biosynthesize essential amino acids critical for host nutrition (Parish et al. 2022). Environmental microbiomes can also shape host immunity, as shown in amphibians, where climatic variation impacts microbial species richness and functional traits (Kueneman et al. 2019). Thus, by directly modulating microbial composition and function, environmental conditions can have profound indirect effects on host adaptability and resilience to stressors.

Intrinsic host factors, including host age, physiology, and life-history strategies, also shape microbiome composition and function. In humans, age-related changes in microbiota parallel physiological shifts; younger individuals harbor microbiomes enriched with Bacteroidetes, while older adults exhibit increased prevalence of *Streptococcus* (Zhang et al. 2021). Similarly, age-related shifts in *D. melanogaster* behavior, locomotion, and cognition coincide with shifts in microbiome dominance (Chiang et al. 2022), pointing to a potentially interactive role of host and microbiota effects on key organismal traits. Microbiotas can also mediate life-history trade-offs. For example, in seasonal killifish, gut microbiomes regulate longevity and aging rate, reflecting trade-offs shaped by environmental conditions and microbial dynamics (Aleman and Valenzano 2019). These examples highlight the intricate interplay between microbiota and host biology, with long-term implications for adaptation and fitness.

Here, we exposed a population derived from the *Drosophila Synthetic Population Resource* (DSPR) to 65 generations of dietary selection followed by 38 generations of inbreeding in common garden to investigate the effects of long-term dietary selection on microbiota richness and host longevity under stressful nutritional conditions. The dietary regimes included deteriorating nutrient availability (DA), fluctuating nutrient availability (FA), and constant high nutrient availability (CH) (see Methods). We hypothesized that adaptation to these dietary environments would result in distinct microbial communities and survival patterns shaped by the interplay of diet, microbiota, and host physiology. Specifically, we predicted that: 1) the DA regime would enrich for microbial taxa promoting somatic maintenance and longevity, supported by lower pro-inflammatory dynamics; 2) the FA treatment, characterized by cyclical resource availability, would prioritize reproduction over longevity, leading to divergent microbial compositions and increased pro-inflammatory taxa compared to DA; and 3) the CH treatment would exhibit stable microbiota compositions with reduced microbial diversity, reflecting selection against traits favoring resource storage or stress resilience. Additionally, we anticipated that age-related shifts in microbiota would interact with these dietary regimes,

123 revealing unique dynamics in survival across treatments. This experiment provides insight into
124 how environmental pressures, mediated by diet, shape the evolution of host-microbiota
125 interactions and their consequences for fitness under stress.
126
127 **Materials and Methods**
128 **Study population**
129 The study population was derived from a long-term, experimentally evolved population of
130 *Drosophila melanogaster* (Ng'oma et al. 2019). The base population was established by mass-
131 intercrossing 835 recombinant inbred lines from the B population (pB) of the Drosophila
132 Synthetic Population Resource (DSPR) for five generations in common garden (Figure 1A).
133 From this genetically diverse base, 36 populations were established and subjected to one of
134 three dietary selection regimes (described below) for 65 generations (Figure 1B). Following
135 selection, populations were inbred for 38 generations (~19 months) under common garden
136 conditions to generate Selection Inbred Lines (SILs).
137

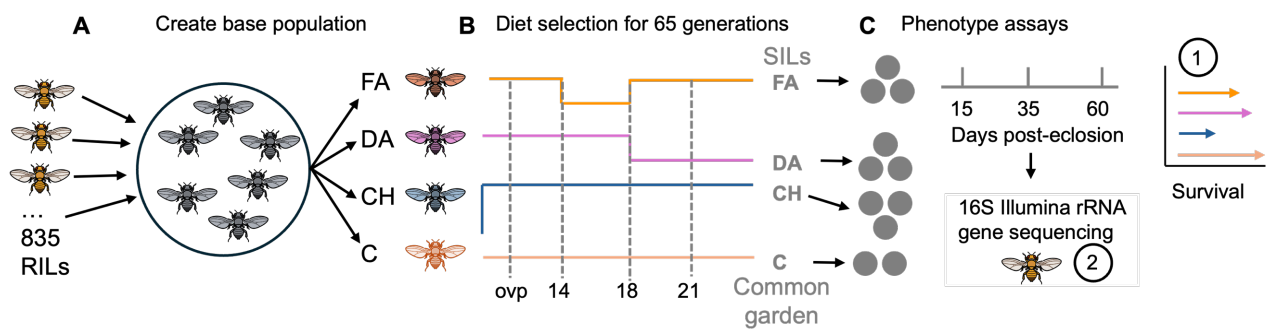

Figure 1: Study design. **A.** A total of 835 recombinant inbred lines of the DSPR were interbred into a genetically heterogenous population which was subjected to experimental evolution for 65 generations. **B.** Three selection treatments involved diet alternations over a 21-day cycle per generation involving fluctuating (FA), deteriorating (DA) and constant high (CH) nutrient availability. An unselected group resulting from the base population was used as a control group (C). **C.** Multiple replicate selection lines maintained in common garden were assayed at three time points for survival and microbiome analysis at 15, 35, and 60 days post-eclosion.

138 For this study, six randomly selected SILs from each selection regime FA, DA, and CH (i.e.,
139 fluctuating, deteriorating, and constant high availability, respectively) were used alongside a
140 non-selected control (C) population (maintained at N > 4,000 flies). Each SIL was split into six
141 replicate vials (24 females and 6 males per vial): three vials were assigned to microbiome
142 assays, and three to lifespan estimation (Figure 1C). Microbiota sampling was performed on

143 females only to capture microbiomes of naturally mating individuals while avoiding effects of
144 male mating pressure. Whole flies were collected at three time points post-eclosion (15, 35, and
145 60 days) based on prior studies indicating key variability in lifespan and fecundity responses to
146 diet. At each time point, four females were flash-frozen and stored at -80°C for analysis. Surface
147 sterilization was omitted as environmental microbiomes in vials closely resemble gut
148 microbiomes in *Drosophila* (Douglas 2018).
149
150 **Selection treatment regimes**
151 Selection effects were analyzed using three diet regimes adapted from Bass et al. (2007) and
152 Skorupa et al. (2008), and previously used in our studies (Stanley et al. 2017; Ng'oma et al.
153 2019, 2020). Relative to the control diet (C, 200 g/L yeast, 50 g/L sucrose), the restricted (DR)
154 diet contained 0.5x less yeast and 1x sucrose. The high sugar (HS) diet included 1x yeast, but
155 7.2x sugar compared to the C diet. Thus, DR differed from C only in protein content, while HS
156 differed in sugar content. The C diet was considered optimal for growth and reproduction and
157 provided a reference for nutritionally stressful diets DR and HS due to restriction or oversupply
158 of key nutrients (previously characterized in Ng'oma et al. (2019). The FA treatment alternated
159 nutrient availability as follows: flies received C until day 14 post-oviposition (po), DR from days
160 14–18 po, and C again until day 21 po when eggs were collected for the next generation. The
161 DA treatment provided C until day 18 po, followed by DR until day 21 po. The CH treatment
162 provided HS until day 21 po. Diets were prepared with inactivated SAFPro Relax + YF 73050
163 yeast (Lesaffre Yeast Corp., Milwaukee, USA), stored at 4°C, and used within 14 days. Evolved
164 lines were held on a common Nutri-fly molasses formulation (Genesee Scientific) based on a
165 Bloomington Drosophila Stock Center Nutri-fly molasses protocol (12.4 g/L yeast, 61.3 g/L
166 yellow cornmeal, and 75.2 ml/L molasses). Across all assays (experimental evolution, survival
167 and microbiome assays), flies were maintained in a temperature-controlled chamber at 23°C
168 under continuous light. These conditions mirrored those under which the DSPR was originally
169 generated and maintained (King et al. 2012). Flies were supplied with fresh food three times per
170 week (Monday, Wednesday, Friday).
171
172 **Phenotype measurement and 16S sequencing**
173 We measured lifespan and microbiota composition (species diversity and richness) in flies
174 subjected to 65 generations of dietary selection followed by 38 generations of inbreeding in
175 common garden. For lifespan, three replicate vials per SIL, each with 24 females and 6 males,
176 were monitored thrice weekly (Monday, Wednesday, Friday) until all flies died. For microbiota

analysis, vials with the same initial composition were used for periodic female collections. DNA was extracted from pools of 2–4 whole flies using the Qiagen DNeasy Blood & Tissue Kit (Cat. No 69504) following the Quick-Start Protocol. Samples were manually homogenized with a glass pestle in ATL buffer, incubated for 4 hours, and processed per protocol. DNA yield and quality were assessed using a NanoDrop Spectrophotometer (ND-1000) and Qubit 4 Fluorometer with the Qubit 1X dsDNA HS Assay Kit (Cat. No Q33231). Samples were normalized as needed with elution buffer and sequenced on a MiSeq instrument (PE250-v2 Nano flow cell, 25 cycles) targeting the V4 region of the bacterial 16S rRNA gene with primers 515F and 807R. Sequencing was performed at the University of Missouri Genomics Technology Core.

Data Analysis

Lifespan

We analyzed lifespan data (N = 2,427 individuals; 2,242 deaths, 185 censored) using survival models. Preliminary Kaplan-Meier analysis visualized global survival patterns and sex-filtered survival at key time points (15, 35, 60 days). To assess evolved differences in mortality risk after 65 generations of selection, we fitted a Cox proportional hazards model: `~ regime + strata(sex)` and `~ regime*sex`. Stratification allowed sex-specific baseline hazards while maintaining proportionality (Mills 2010; Gordon 2021). To evaluate shifts in baseline hazards due to selection, we tested eight parametric survival models (e.g., Gompertz, generalized gamma) using the **flexsurv** and **muhaz** R libraries. The generalized gamma and Gompertz models provided the best fit (Appendix Figure 1a). Our recent study testing effects of larval nutrition on adult phenotypes in this population (Jones et al. 2024) showed that patterns of survival can vary significantly across shorter periods of time or adult stages. To understand evolved patterns of survival in each selection treatment, we examined percentile survival differences in ten-day intervals in which each of our sampling time points formed the median: 10-20, 30-40, and 55-65 days, post eclosion. We tested for significant differences between treatments in each time interval I1, I2 and I3 using either analysis of variance (Anova) or Kruskall-Wallis tests followed by an appropriate post-hoc test (Turkey's HSD or Dunn's Test, respectively)

Microbiome analysis

We processed Illumina reads using the DADA2 pipeline (Callahan et al. 2016), leveraging its amplicon sequence variant (ASV) approach to achieve higher resolution and reproducibility in microbial community profiling. ASVs provide exact biological sequences, avoiding the limitations of traditional OTU clustering. Reads were quality-filtered and trimmed at positions 225 (forward) and 200 (reverse), and chimeras were removed. Taxonomy was assigned using the Silva database (v138.1). Samples with fewer than 3,000 reads were excluded. To ensure a more accurate representation of microbial communities, taxa identified as Wolbachia were removed (Simhadri et al. 2017). Sequences were imported into **phyloseq** (McMurdie and Holmes 2013) for analysis and visualization. Read counts were normalized using a log-transformation: `log(1 + x)/sum(log(1 + x))`, to obtain relative abundances (i.e., the proportion of specific microbial groups within samples). This approach combines two commonly used normalization equations, log(1 + x) and (x)/sum(x), into one step and effectively scaling data to relative abundances while revealing subtle differences (McKnight et al. 2019).

Indicator species analysis (ISA) was performed using **indicspecies** (Cáceres and Legendre 2009) to identify taxa enriched in specific treatments or time points. Alpha diversity (Shannon and observed richness) was visualized with **phyloseq**, capturing richness and evenness. Beta diversity was assessed using principal coordinate analysis (PCoA) based on UniFrac distances obtained from phylogenetic trees generated by **ape** (Paradis et al. 2004). **vegan** (Oksanen et al. 2015) was used for PERMANOVA to test group differences in centroids and dispersion, and PERMDISP to evaluate variability in dispersion. Visual inspection aided interpretation of whether differences reflected means, dispersion, or both.

Results

Evolution of longevity following diet selection

Lifespan performance in the non-selected base population (C) served as a benchmark for evaluating selection treatments (Figure 1). Summary survival patterns are presented in Supplementary Table S1. Overall, DA- and FA-selected populations lived significantly longer than CH-selected flies (Log-Rank tests: CH vs DA, $p = 7.6e-45$; CH vs FA, $p = 3.3e-27$; CH vs C, $p = 4.5e-35$). DA flies also outlived FA flies ($p = 4.1e-5$), but the control group exhibited the longest lifespan, significantly exceeding both DA ($p = 0.025$) and FA ($p = 2.5e-07$) regimes (Figure 2a). Early adulthood (~38 days post-eclosion) showed higher male survival in controls

(Figure 2c&e), whereas greater female mortality under CH conditions likely reflected metabolic stress from the high-sugar diet.

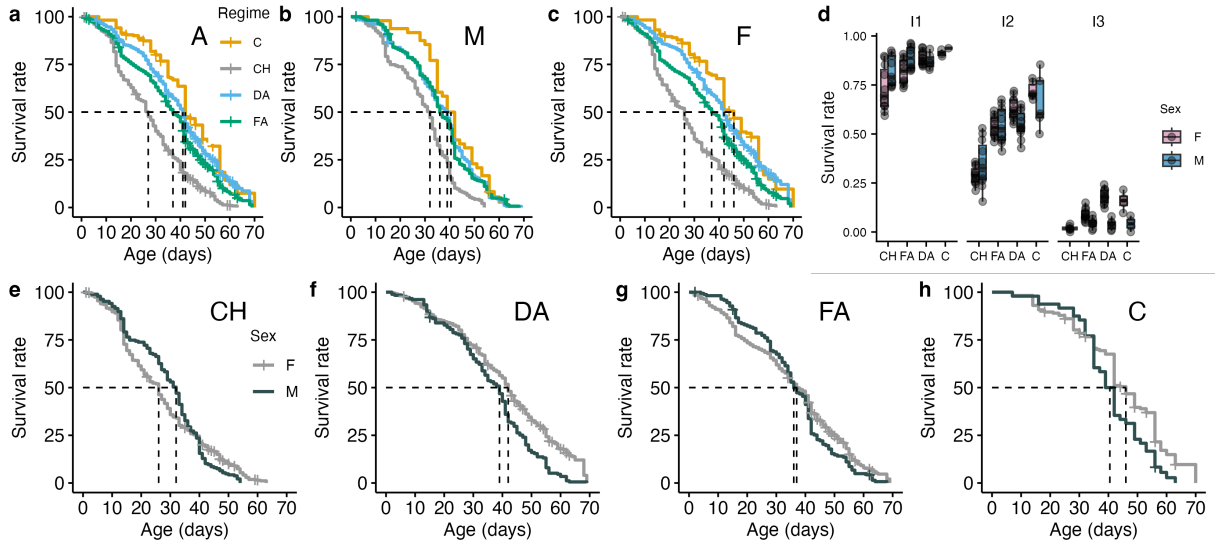

Figure 2: Survival performance in each treatment: **a** sexes together, **b** males, **c** females**,** **d** in bins of 10 days in which the sampling time point is the median (only bins containing assayed time points of 15, 35 and 60 days are shown (I1=10-20 days, I2=30-40 days,

To understand evolved patterns of survival in each selection treatment, we examined percentile differences in survival in ten-day intervals in which each of our sampling time points formed the median (I1: 10–20 days, I2: 30–40 days, I3: 55–65 days, Figure 2g). Two key patterns emerged:

1. *Selection regime differences*: DA and FA regimes showed reduced early mortality in I1 and I2, favoring longevity, but diverged in I3, with FA experiencing higher late mortality while DA maintained slower rates (Supplementary Table S2). Control flies exhibited the lowest mortality across I1 and I2. In contrast, CH flies suffered heavy mortality across all intervals.
2. *Sex-based variation*: CH and FA treatments favored male survival in I1 and I2, reversing in I3 with higher female survival. Conversely, DA and control regimes showed greater female survival later in life (I2, I3; Figure 2e&g, Supplementary Figure S1), suggesting adaptation to dietary stress through reduced early mortality (DA, FA) or combined early and late-life mortality reduction (DA, control).

These findings suggest that dietary stress adaptation involves distinct survival strategies, with DA and FA regimes prioritizing early-life resilience and DA further extending late-life survival.

Sex significantly influenced mortality rates, with rank-order effects varying by selection regime (Figure 2e-h, Supplementary Figure S2). To evaluate the impact of dietary selection on mortality relative to the control (C) group, we computed hazard ratios using Cox proportional hazards models accounting for sex. All selection regimes (CH, FA, DA) showed higher mortality risk compared to controls (Table 1, Appendix Figure 1b, Supplementary Table S3), as indicated by positive coefficients. The DA coefficient was marginally significant ($p = 0.052$), suggesting that while DA approached control-like outcomes, it achieved them via potentially distinct adaptive strategies. Notably, males in the DA and FA regimes exhibited nearly identical survival trajectories (Appendix Figure 2c), suggesting that despite differing selection pressures, they underwent similar adaptive responses to diet stress, resulting in comparable survival outcomes.

Sex-specific differences within regimes were evident and suggested interactions between sex and selection treatment (Figure 2, Appendix Figure 2). Modeling survival as a function of regime-by-sex interaction revealed significant effects in most categories, except for C males and DA males (Table 1, Supplementary Table S3). CH flies displayed significantly high mortality risk for both sexes ($p < 0.0001$). Overall, these results highlight significant adaptation in DA and FA regimes but not in CH, with sex playing a critical role in shaping survival responses.

Table 1: Patterns of mortality risk in lifespan adaptation to diet selection.

Variable	**Coeff.**	**HR**[1]	**95% CI**[1]	**p-value**
Regime + strata(sex)				
CH	0.99	2.69	2.29, 3.17	<2e-16
DA	0.16	1.18	1.00, 1.39	0.0515
FA	0.40	1.48	1.26, 1.74	<9.16e-07
Sex				
M	0.23	1.26	1.15, 1.39	<1.68e-06
Regime * sex interaction				
C * F	-0.64	0.53	0.42, 0.66	3.33e-08
CH * F	0.44	1.56	1.31, 1.86	6.52e-07
DA * F	-0.50	0.61	0.51, 0.73	9.51e-08
FA * F	-0.18	0.84	0.70, 1.00	0.0457
C * M	-0.22	0.81	0.58, 1.12	0.1941
CH * M	0.49	1.64	1.33, 2.02	4.67e-06
DA * M	-0.02	0.98	0.79, 1.21	0.8501
FA * M	—	—	—	—

[1]HR = Hazard Ratio, CI = Confidence Interval

Microbial composition across selection regimes and time points

To assess microbial composition, we analyzed the average relative abundance of phyla across time points and selection regimes (Figure 3). Proteobacteria and Firmicutes strongly dominated microbial composition in all samples (Figs. 3, 4). Proteobacteria and Firmicutes were the most abundant across all regimes but exhibited distinct temporal trends. In the control group (C), Firmicutes were initially low, increasing at the second time point before slightly decreasing at the final time point (Figure 4a). In contrast, all selection treatments began with higher Firmicutes levels and declined similar declines with age (Figure 4a). These results are also consistent with the relative abundance profiles before being averaged (Supplementary Figure S3).

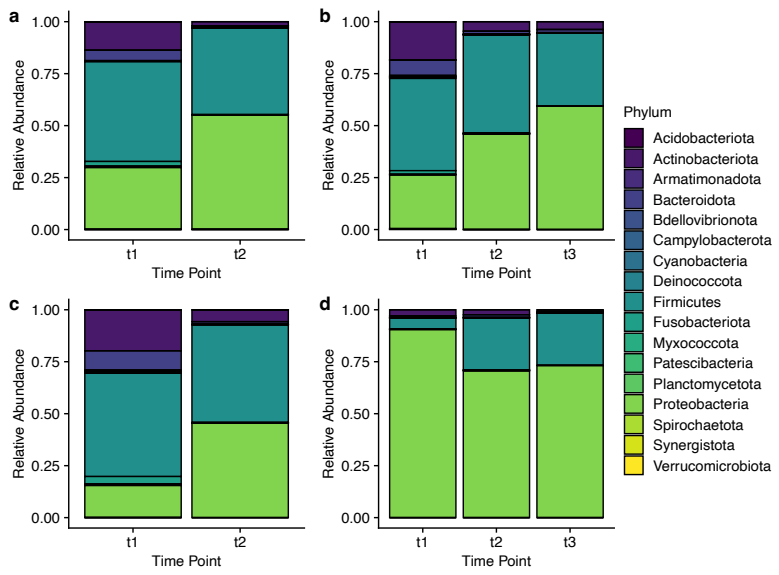

Figure 3: Phylum-level microbial relative abundance over time (t1-t3) for each of the four dietary regimes, including **a.** constant high sugar, **b.** decreasing nutrient availability, **c.** fluctuating nutrient availability, and **d.** control non-selected group.

Proteobacteria was most abundant in the control group, showing a decline at the second time point followed by a partial recovery at the third (Figure 4b). In contrast, the FA regime had the lowest initial Proteobacteria levels but exhibited a marked increase over time (Figure 4b). The DA and CH regimes showed steady increases in Proteobacteria abundance as they aged (Figure 4a-b). Minor phyla, including Actinobacteria, Bacteroidota, and Fusobacteriota, were more abundant in the selection treatments than in the control group but generally declined with age (Figure 4c-d). These results highlight divergent microbial dynamics between control and selection regimes over time.

Based on prior studies linking microbial composition to immune function (Shen et al. 2018; Gibbons et al. 2021), we hypothesized that Proteobacteria and Firmicutes could influence hosts positively or negatively depending on their relative proportions. We calculated phyla ratios as age-specific indicators of treatment effects (Figure 4f-g). In the selection regimes, the

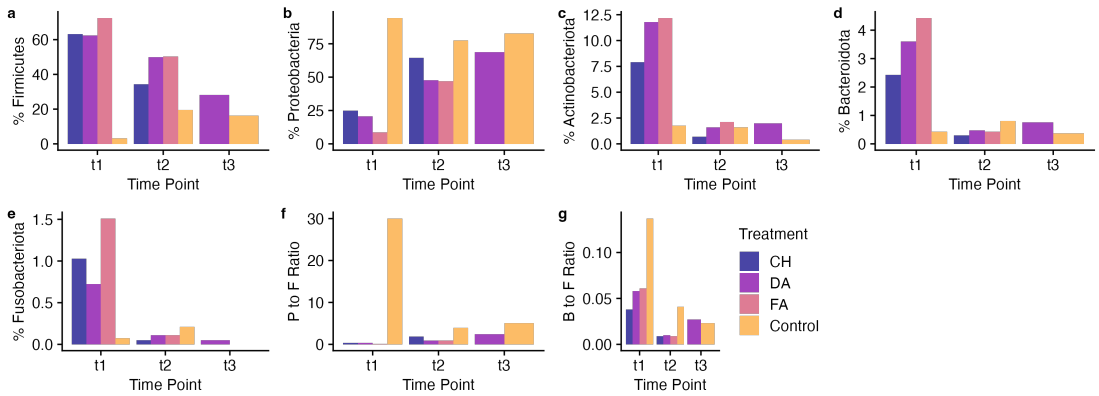

Figure 4: Changes in the percentage of phyla over time (t1-t3) among dietary regimes (a-e): **a.** Firmicutes, **b.** Proteobacteria, **c.** Actinobacteriota, **d.** Bacteroidota, and **e.** Fusobacteriota. Phyla ratios over time between dietary treatments (f-g): **f.** Proteobacteria to Firmicutes, **g.** Bacteroidota to Firmicutes. Time points: t1, 15 days; t2, 35 days; and t3, 60 days.

Proteobacteria-to-Firmicutes ratio generally increased over time (Figure 4f), while the Bacteroidota-to-Firmicutes ratio decreased between T1 and T2, except for DA increasing between T2 to T3 (Figure 4g). The control group began with higher ratios than the treatments and exhibited a general decline, suggesting an age-related increase in Firmicutes relative to Proteobacteria and Bacteroidota. These patterns reveal contrasting dynamics between Proteobacteria and Bacteroidota that are influenced by Firmicutes.

To explore functional significance of observed relative abundance dynamics, we performed an indicator species analysis at the genus level. We identified 40 genera significantly different across time points (Supplementary Table S4a) and 8 across selection regimes (Supplementary Table S4b). Of the 40 time-related genera, 85% (34) were enriched at T1, one at T3, five at both T1 and T3, and none at T2, indicating significant early-life microbial changes that may prime later traits like lifespan. Among the 8 treatment-related genera, we found: *Fructilactobacillus* in CH; *Mobiluncus* in FA; *Leptotrichia* in CH + FA; *Levilactobacillus*, *Acinetobacter*, and *Neisseria* in CH + DA + FA; *Lactobacillus* in CH + FA + C; and *Halomonas* in DA + FA + C. This pattern suggests a mix of treatment-specific and conserved functional effects across regimes.

Taxon diversity across time points and dietary treatments

To assess the effects of selection treatments on microbial diversity over time, we calculated observed alpha diversity (ASV richness) and the Shannon-Wiener Index. ASV richness significantly differed within DA samples across time points (Wilcoxon rank-sum: T1 vs. T2, p =

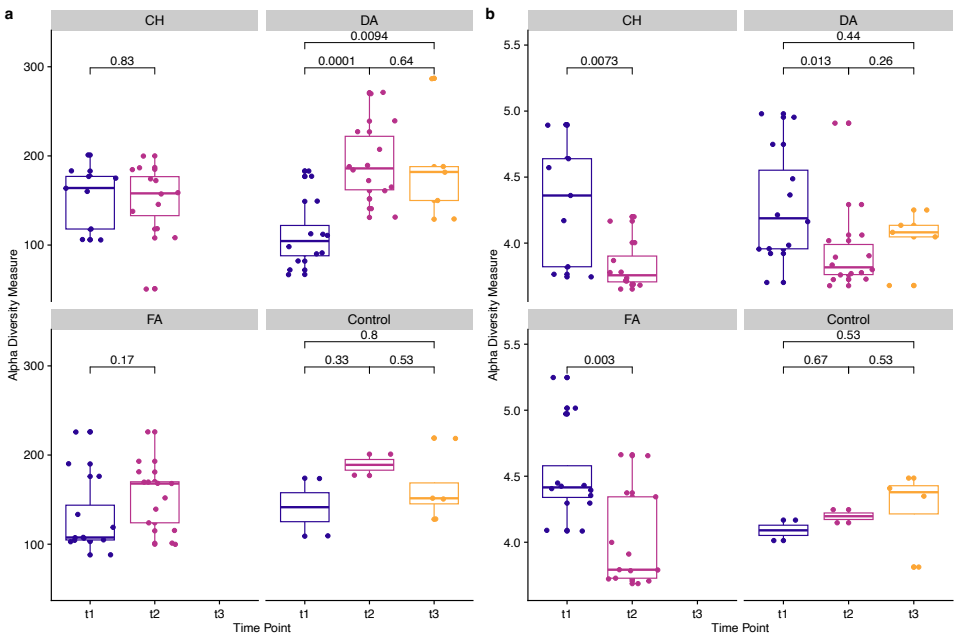

Figure 5: Alpha diversity between time points (t1-t3) across all dietary regimes. **a.** Observed diversity, **b.** Shannon-Weiner diversity. *P*-values between time points denote Wilcoxon rank-sum tests.

0.0001; T1 vs. T3, $p = 0.0094$; Figure 5a). The Shannon Index was significantly different between T1 and T2 in all regimes (CH: $p = 0.0073$; DA: $p = 0.013$; FA: $p = 0.003$; Figure 5b), but not between T2 and T3 or T1 and T3. These findings suggest selection-related diversity changes occur early in life, with CH and FA treatments lacking T3 data due to limited lifespan.

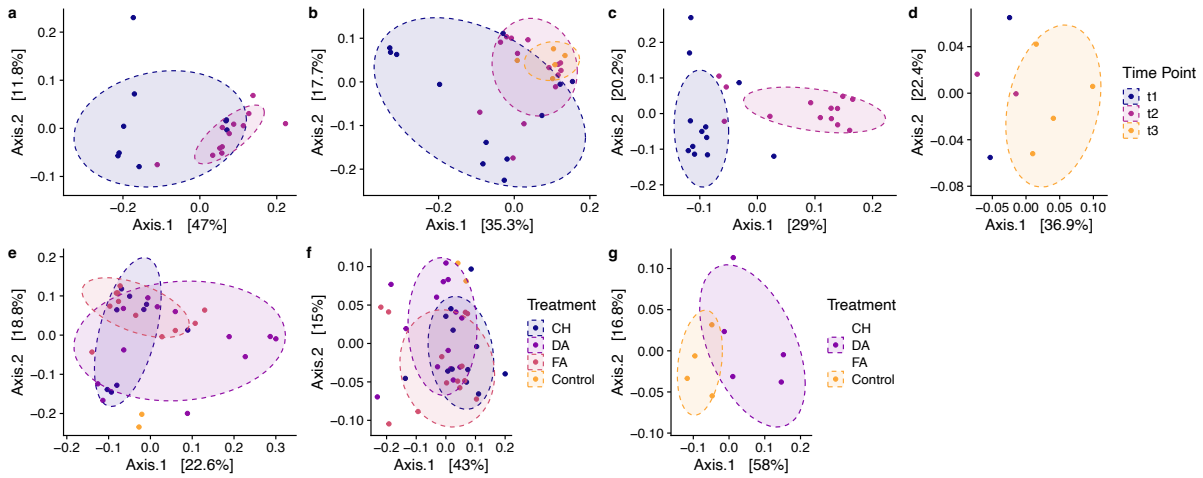

Figure 6: Beta diversity metric showing species diversity between time points (a-d) and across all dietary regimes (e-g). Note: For 6g, CH and FA groups did not reach t3, hence no comparisons.

We next examined between-group beta diversity using PCoA. Shifts in microbial composition were observed from T1 to T2 across treatments (Figure 6). At T3, the DA and C treatments diverged significantly. PERMANOVA confirmed significant differences by selection regime (r^2 =

335 0.12061, p = 0.001) and time point (r^2 = 0.11005, p = 0.001). these differences were due to
336 group means rather than dispersion (PERMDISP; CH vs. C: p = 0.015; DA vs. C: p = 0.003; FA
337 vs. C: p = 0.001). Significant differences were also observed between T1 and later time points
338 (T1 vs. T2: p = 0.001; T1 vs. T3: p = 0.001).
339
340 **Discussion**
341 We hypothesized that adaptation to stressful diet environments selects for distinct strategies in
342 survival, microbial community composition, and richness. To test this, we compared various se-
343 lection treatments against an unselected progenitor population (DSPR) from which the SILs
344 were derived. Although none of the selection treatments surpassed the control in longevity, the
345 DA treatment nearly matched it by generation 65. It is important to note that, after experimental
346 evolution, all selected lines were reared in a common nutritional environment - different from the
347 diets used in the selection regimes - which may influence the patterns we report. Nonetheless,
348 our previous studies have characterized key fitness traits (lifespan and fecundity) in component
349 diets across inbred (Stanley et al. 2017), outbred (Ng'oma et al. 2019, 2020), and evolved
350 (Ng'oma et al., unpublished) DSPR lines, and the patterns we observe here are consistent with
351 those findings. For instance, the DR diet prolonged lifespan by 37.5% in DSPR RILs (Stanley et
352 al. 2017), while the HS diet markedly reduced both lifespan and fecundity (Ng'oma et al. 2019).
353 These outcomes align with our observations: the DA selection treatment, which provided longer
354 exposure to the DR diet each generation, was associated with an extended lifespan, and similar
355 effects were seen in the CH treatment where a high-sugar environment was maintained. To-
356 gether, these findings support the expectation of persistent evolved patterns in response to
357 stressful dietary environments.
358
359 *Effect of diet selection on longevity*
360 Notwithstanding the context outlined above, the CH treatment was particularly detrimental,
361 especially for females, suggesting either incomplete adaptation or weak selection pressure, with
362 the latter likely contributing to slower evolution by depleting beneficial alleles. Where new
363 mutations are expected, stronger selection should lead to faster adaptation as it would favor
364 fitter mutation-genotypes to survive (Hartl and Clark 2007; Stephan 2021). In this study, we
365 expect negligible mutation-driven evolution. Rather, genetic variation should primarily be
366 maintained by recombination. In this case, stronger selection can lead to slower evolution, as
367 intense selection can rapidly deplete beneficial alleles leaving few options for further adaptation
368 (Ueda et al. 2017). This is especially possible in a complex trait like longevity where beneficial

369 combinations may be disrupted by recombination. Thus, reduced survival in the CH regime
370 could indicate greater levels of metabolic stress imposed by the high sugar diet such that
371 reproduction is severely depressed early, around the peak reproductive age (the first 3 weeks
372 post-eclosion) as genetic variation for longevity is removed with those that die early for multiple
373 other reasons.
374
375 The DA and FA treatments closely paralleled the control in longevity, with higher survival around
376 T1 (15 days pe, Figure 2a), reducing early mortality and maintaining lifespan variation. It should
377 be noted that males in the CH regime experienced significantly reduced mortality in the interval
378 around median lifespan (Figure 2b), but this advantage was probably late to significantly
379 improve median survival.
380
381 *Sex-based effects of selection on longevity*
382 Sex-specific diet responses, which are typically expected in *Drosophila*, revealed differing
383 evolved strategies. First, the FA selection shows a pattern of greater male survival earlier and
384 lower survival later which may support sustained reproduction in younger females and incurring
385 lower survival (Figure 2f). This pattern is less pronounced in the C regime (Figure 2h). Second,
386 males of the DA treatment survived poorly earlier in life compared to females which can lead to
387 observed reduced female reproduction, potentially necessitated by cues of a worsening
388 nutritional environment. These patterns suggest that DA and FA selection set different survival
389 patterns in early life. Longevity analysis suggests that adaptation to stressful diet alternations
390 may occur by reducing early mortality (DA, FA) or balancing higher early-life mortality with lower
391 mortality later in life.
392
393 *Effect of diet selection on microbial community composition*
394 Given that flies acquire microbiotas through mother-larvae transmission and from their diet
395 medium (Fischer et al. 2017; Guilhot et al. 2023), we anticipated selection-specific host-
396 microbiota interactions under a common nutritional environment. The DA regime was expected
397 to enrich longevity-promoting, low-inflammatory taxa, while the FA treatment, with its cyclical
398 resource availability, was predicted to shift the microbiota toward reproductive prioritization, with
399 increased pro-inflammatory taxa. In contrast, the CH treatment was projected to maintain a
400 stable but less diverse microbiome, indicating selection against resource storage or stress
401 resilience traits. Additionally, we expected age-related microbial shifts to interact with these
402 regimes, resulting in distinct survival strategies. These expectations were generally conformed

as microbial richness shifted significantly with selection and fly age, although selection regimes differed from the control group.

The microbiome composition of the selection regimes differed markedly from the control group. Survival patterns across dietary regimes were strongly aligned with distinct microbial richness and diversity. The longest-living group, C, exhibited lower richness in Firmicutes and higher richness in Proteobacteria compared to the selection regimes, particularly early in life post-eclosion. This aligns with prior studies showing that fruit fly microbiomes are primarily dominated by Firmicutes and Proteobacteria (Staubach et al. 2013; Adair et al. 2018; Lee et al. 2019). The dietary selection treatments likely contributed to the increased number of phyla observed in our study.

One explanation for this increase is the colonization of pathogenic groups in the selection regimes, leading to evolutionary stress and dysbiosis. Studies have shown that early increases in pathogenic abundance can inhibit the development of other microbiota, disrupting the protective roles of beneficial microbes (Spragge et al. 2023). For example, host microbiomes in human and mouse guts can compete with pathogens for nutritional resources, with niche exclusion being slower if pathogenic species establish dominance early (Spragge et al. 2023). Additionally, compositional differences in the microbiome may be influenced by lab and strain effects. Inconsistencies in fruit fly microbiomes are often attributed to dietary variation (Arias-Rojas and Iatsenko 2022) and microbial load (Lee et al. 2019), both of which impact lifespan. For instance, Lee et al. (2019) reported increased lifespan in lab strains with higher colonization of commensal bacteria. Keebaugh et al. (2019) found that diet composition, such as the addition of nutritional yeast, altered microbial load and lifespan outcomes by increasing environmental microbe diversity. Conversely, other studies found no impact of microbial load on lifespan (Ren et al. 2007). These findings highlight the complex interplay between diet, microbiome composition, and host state, particularly the role of pathogenic colonization and dietary interventions in shaping microbiota and their effects on lifespan.

Microbial selection effects depend on host stage

Adaptation strategies in response to dietary treatments appear to favor the optimization of microbial community dynamics in younger host stages. For selection regimes that facilitated more flies surviving to old age, such as DA and FA (to a lesser extent), maintaining microbial composition in older flies seemed crucial for survival. In past research (Je et al. 2013; Na et al.

437 2014), the early period is important for establishment and proper succession of microbial
438 richness in adult flies. These patterns observed in microbiome analysis align with the observed
439 patterns in survival. For instance, selection treatments associated with longer lifespan (C, DA,
440 FA) all reduced early mortality compared with the least longevity treatment, CH (discussed
441 earlier). Thus, adaptation strategies to these treatments (especially longevity) likely optimizes
442 microbial community dynamics in younger host stages. For selection regimes that facilitated
443 more flies to survive to old age (i.e. DA, and FA to a lesser extent), it seems that the ability to
444 adapt depended on maintenance of microbial composition at older age.
445
446 *Major microbial groups coordinated by diet selection*
447 The dominant phyla identified in this study were Proteobacteria, Firmicutes, Actinobacteria,
448 Bacteroidota, and Fusobacteriota. Among these, Proteobacteria and Firmicutes were most
449 abundant across all regimes, though they exhibited distinct temporal trends. Previous research
450 has established correlations between certain phyla and immune responses. For instance, Shen
451 et al. (2018) reported a positive association between Bacteroidota (formerly Bacteroidetes) and
452 both immunoglobulin G and cytotoxic T cell production as well as a positive correlation of the
453 ratio of Bacteroidetes to Firmicutes and immunoglobulins G and M. Similarly, Gibbons et al.
454 (2021) observed increased cytokine and immunoglobulin production linked to the ratio of
455 Proteobacteria to Firmicutes.
456
457 In our study, the Bacteroidota-to-Firmicutes ratio generally decreased over consecutive time
458 points, suggesting that as fruit flies age, they may experience an increase in antibody
459 production but a reduction in cytotoxic T cell activity. Additionally, the Proteobacteria-to-
460 Firmicutes ratio declined in non-selected flies but increased in dietary regime flies, pointing to
461 potential differences in immune responses across treatments. However, none of the dietary
462 regimes exhibited levels of these ratios as high as the non-selected group, suggesting that the
463 dietary regimes negatively impacted immune response and survival in adult flies.
464
465 Notably, non-selected flies began with exceptionally high levels of Proteobacteria, a core
466 microbiome member in fruit flies alongside Firmicutes (Staubach et al. 2013; Wong et al. 2013;
467 Adair et al. 2018; Lee et al. 2019; Chiang et al. 2022). The dietary regime flies, on the other
468 hand, appeared to experience dysbiosis, potentially linked to their increased microbial diversity.
469 This imbalance may negatively affect immune function, gut health, and overall host health.
470 Specifically, higher levels of Firmicutes in the selection regime flies could contribute to reduced

immune responses and poorer survival outcomes compared to non-selected flies. These findings highlight the complex interplay between microbiome composition, immune function, and host survival, with Firmicutes emerging as a key player in shaping these dynamics.

Selection and dynamics of Proteobacteria and Bacteriodota

Our results reveal an inverse relationship between Proteobacteria and Bacteroidota in our selection groups, a pattern that likely has important implications for host fitness. Overgrowth of Proteobacteria has been associated with metabolic disturbances and inflammation (Mukhopadhya et al. 2012; Shin et al. 2015), whereas Bacteroidota are generally linked to healthier microbiomes (Zafar and Saier Jr 2021). The lower abundance of Proteobacteria in our selected flies relative to C may indicate antagonistic interactions that contribute to their reduced lifespan under nutritional stress, but not under normal diet. Similar inverse dynamics have been reported in mice, where high-fat diets and inflammatory conditions favor Proteobacteria at the expense of Bacteroidota (Bradley and Pollard 2017; Méndez-Salazar et al. 2018). Moreover, the ability of certain probiotics to modulate this ratio (Gryaznova et al. 2022) supports a mechanistic link between microbial balance, dietary factors, and host survival. Collectively, these observations suggest that shifts in microbiome composition could play a critical role in mediating the effects of our selection regimes.

Indicator species analysis

Our indicator species analysis revealed significant structural changes in the microbiome early in adulthood across all treatments, with 34 of 40 microbial groups undergoing turnover at T1, indicating early succession dynamics during microbiome establishment. This aligns with prior research showing that *Drosophila melanogaster* larvae and pupae harbor rich environmental microbial communities, but newly eclosed adults acquire minimal microbiota, establishing their communities through food sources shortly after emergence (Blum et al. 2013; Broderick et al. 2014). In contrast, only eight microbial groups were enriched in at least one treatment, suggesting relative stability in selection-specific microbiomes. These findings highlight the role of stage-specific microbial shifts and conserved dynamics in long-term dietary adaptation, where early-life succession and later stability influence host physiology and traits such as lifespan.

Similarly, prior studies emphasize early adulthood as a critical period for establishing microbial diversity and longevity in flies (Brummel et al. 2004). Consistent with this, our diversity measures showed increased microbiome diversity early in adulthood, particularly in the

selection regime treatments. However, members of some bacterial genera identified in our analysis, such as *Porphyromonas* and *Atopobium*, are associated with inflammation and disease in humans (Caselli et al. 2019; Salliss et al. 2021). An exception is *Lactobacillus*, reported to inhibit these harmful groups (Moses et al. 2015; Mendi et al. 2016).
These results suggest that selection regime flies may have been exposed to harmful bacteria throughout adulthood, contributing to inflammation and reduced survival. While the DA group sustained more survivors, the other two dietary regimes appeared less effective at managing infections. This underscores the complex interplay between microbiome dynamics, immune function, and longevity, shaped by temporal and treatment-specific factors.

Although our design resulted in fewer flies in the CH and FA treatments reaching T3, our findings reveal that adaptation to stressful dietary environments leads to distinct microbial community dynamics, sex-specific survival patterns, and longevity outcomes. Selection regimes such as DA and FA improved early survival and maintained greater lifespan variation compared to the CH regime, which exhibited high early mortality, particularly in females. These patterns underscore the complex interplay between dietary stress, genetic adaptation, and microbial composition. Microbiome analysis highlights significant shifts during early adulthood, consistent with the critical role of this period in microbiome establishment and its influence on longevity. Selection regimes increased microbial diversity but also introduced pathogenic taxa, contributing to dysbiosis and reduced survival compared to the control group. The inverse relationship between Proteobacteria and Bacteroidota, particularly in selection regimes, may reflect antagonistic microbial interactions linked to dietary stress during adaptation. These findings underscore the importance of both stage-specific microbial succession and long-term microbial community stability in shaping host fitness and survival.

Overall, our study provides new insights into the evolutionary and ecological dynamics of diet-microbiome interactions, highlighting how dietary stressors can shape host-microbiome relationships and influence complex traits like longevity. Future research should explore the mechanistic links between microbial composition, immune function, and host physiology to better understand the adaptive responses to dietary perturbations.

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Appendix

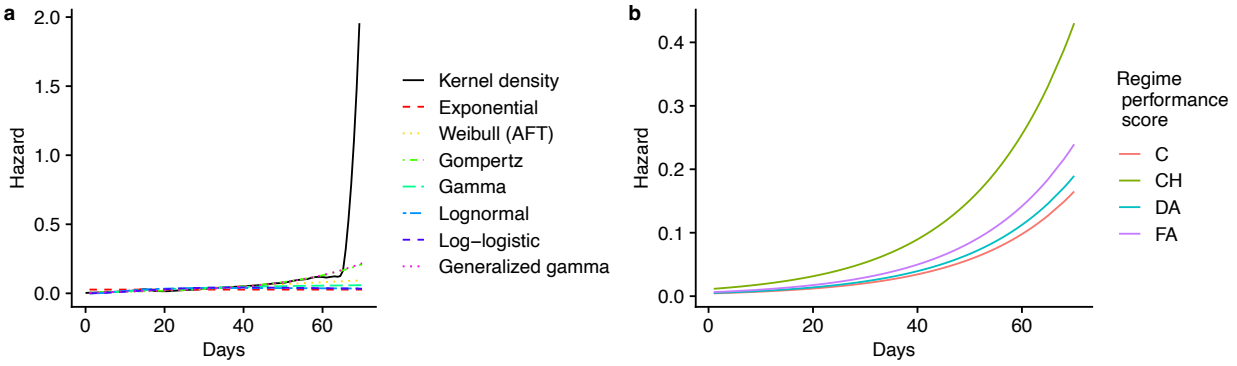

Figure A1: Survival dynamics over time: **a.** Comparison of kernel density and parametric hazard function estimates. The black line represents the non-parametric kernel density estimate of the hazard function, while colored lines correspond to hazard functions estimated from various parametric survival models. The Generalized gamma and Gompertz fit the kernel estimate best suggesting a good fit to the data. **b.** Predicted hazard functions from a Gompertz survival model across different regimes. Each colored line represents the estimated hazard function for a specific regime, showing how the instantaneous risk of mortality changes over time. Diverging hazard trajectories suggest differences in survival patterns between regimes.

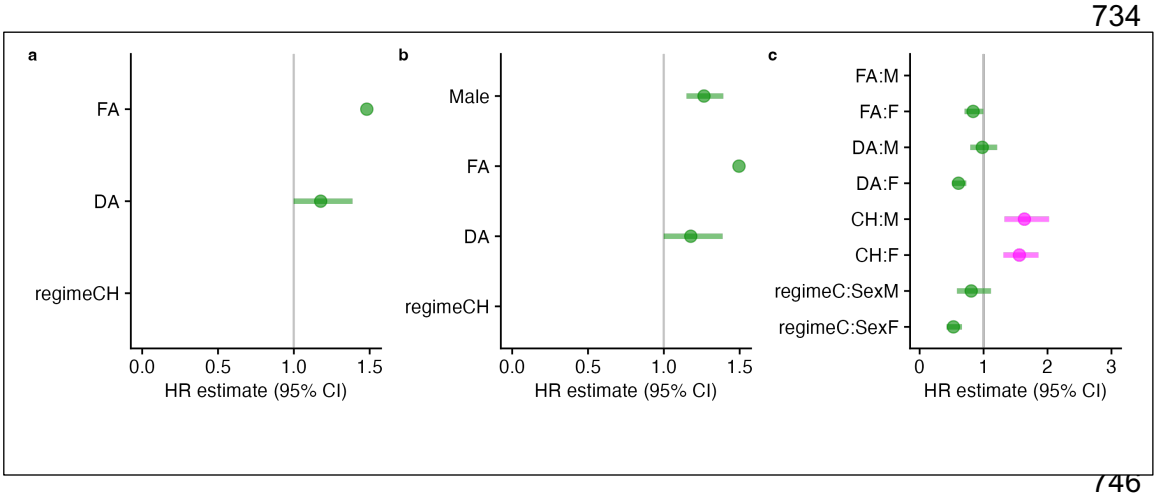

Figure A2: Hazard ratio estimates from Cox proportional hazards models evaluating the effect of selection regime and sex on survival. (a) Model stratified by sex, (b) Model with additive effects of regime and sex, (c) Model with interaction between regime and sex. Points represent hazard ratio (HR) estimates with 95% confidence intervals. HR > 1 indicates increased mortality risk relative to the reference category. Overall, these results suggest that dietary adaptation strongly influences survival, with `CH` and `FA` reducing lifespan relative to `C`, while `DA` showed a weaker effect. Males consistently exhibited higher mortality risk across regimes.