Title: Diet-induced plasticity of life-history traits and gene expression in outbred *Drosophila melanogaster* population

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Abstract

Food is fundamental for the survival of organisms, governing growth, maintenance, and reproduction through the provision of essential macronutrients. However, access to food with optimum macronutrient composition, which will maximize the evolutionary fitness of an organism, is not always guaranteed. This leads to dietary mismatches with potential impacts on organismal performance. To understand the consequences of such dietary mismatches, we examined the effects of isocaloric diets varying in macronutrient composition on eight key organismal traits spanning across the lifespan of a large outbred *Drosophila melanogaster* population (n ~ 2100). Our findings reveal that carbohydrate-reduced isocaloric diets correlates to accelerated pre-adult development and boosts reproductive output without impacting pre-adult viability and body size. Conversely, an elevated dietary carbohydrate content correlated to reduced lifespan in flies, evidenced by accelerated functional senescence including compromised locomotor activity and deteriorating gut integrity. Furthermore, transcriptomic analysis indicated a substantial difference in gene regulatory landscapes between flies subject to high carbohydrate vs high protein diet, with elevated protein levels indicating transcriptomes primed for reduced synthesis of fatty acids. Taken together, our study helps advance our understanding of the effect of macronutrient composition on life history traits and their interrelations, offering critical insights into potential adaptive strategies that organisms might adopt against the continual dietary imbalances prevalent in the rapidly evolving environment.

Keywords

Developmental time, Reproductive output, Lifespan, Aging, Gut integrity, Locomotor activity, Isocaloric diet

1. Introduction

The fundamental necessity of an organism is food. It provides all the essential resources and nutrients essential to the survival, growth, maintenance and reproduction. Central to these resources are macronutrients, utilized in distinct yet interconnected functions in an organism's physiology. For instance, proteins primarily serve as building blocks for growth and repair, while carbohydrates and lipids are generally utilized to fuel the energy requirements for cellular and organismal functions (Jequier, 1994; Wu et al., 2014). Therefore, a balanced diet comprising of optimal macronutrient composition is key to maximize the evolutionary fitness of an organism (Altmann, 1991; Brunner et al., 2001; Hawley et al., 2016).

However, the availability of such an optimal diet is not always guaranteed in natural settings, leading potentially to dietary mismatches (Gurven & Lieberman, 2020; Logan & Jacka, 2014; Wiggins & Wilder, 2018). The consequences of this dietary shifts on an organism’s performance and life history traits are difficult to predict based on our current knowledge. Furthermore, a comprehensive study of diet's impact on an organism's performance is difficult, given that it requires exact manipulation of food components and an understanding of their short-term as well as long-term effects on performance. Therefore, conducting such studies in natural settings and using organisms with longer lifespans can be challenging. Additionally, considering that diet may simultaneously influence multiple organismal traits, an effective study requires a system where these traits are well-characterized. It is also crucial to understand how these nutrients are being metabolised, which lead to the changes in the observed phenotypes. In this context, *Drosophila melanogaster* serves as a suitable model system for investigating diet's influence on organismal traits spanning across its life stages. Given its well-understood laboratory ecology, well-characterized traits, and a comparatively short lifespan, *Drosophila* allows for precise dietary component manipulations and longitudinal performance tracking in controlled laboratory settings (Carvalho et al., 2012; Catterson et al., 2010; Jang & Lee, 2018; Piper et al., 2005; Skorupa et al., 2008).

Historically, in studies correlating diet’s influence to organismal performance, the calorific value of diet has gathered considerable attention given the impact of dietary restriction on lifespan across various taxa, including invertebrates (Houthoofd et al., 2005; Jiang et al., 2000; Partridge et al., 2005), rodents (McCay et al., 1935), and humans (Holloszy & Fontana, 2007). However, recent findings highlight that diet's impact is more nuanced, with isocaloric diets varying in macronutrient

composition significantly affecting organismal life history including lifespan (Mair et al., 2005; Shi et al., 2021). While all macronutrients are essential, empirical evidence highlights the critical importance of dietary composition, often depicted as the ratios of macronutrients, for organismal growth, reproduction, and survival (Raubenheimer & Simpson, 1997; Simpson & Raubenheimer, 2009).

However, in-spite of several efforts, knowledge about the precise role of macronutrient composition in diets in influencing organismal performance is still discrepant (Bruce et al., 2013; Lee, 2015; Lee et al., 2008; Lushchak et al., 2012, 2014). These discrepancies across the existing published reports can be attributed to several interconnected reasons. First, a standardized method of varying nutrient composition across reports does not exist, with studies being varied in the choice of ingredients and thereby often introducing confounding effects due to changes in caloric content. Second, many studies examine the impact of diet on a specific trait or a limited set of traits, potentially leading to inconsistencies in the interpretation of trait correlations. Third, the reports often vary in terms of the timing of dietary change implementation relative to the organism's life stage. And finally, strong interaction of diet with genetics (G×E interaction) (Heianza & Qi, 2017; Roche et al., 2005; Ussar et al., 2015) complicates the synthesis of results from different experiments, particularly when most existing studies use inbred *Drosophila* lines with diverse genetic backgrounds. Taken together, these shortcomings highlight the need for a consistent experimental framework to comprehend the effects of diet on traits across various life stages.

To circumvent such challenges, in this study we examined the effect of diet-composition-induced plasticity across eight key life history traits spanning various life stages, thereby aiming to obtain a holistic understanding of the impact of diet on organismal fitness. Using a well-characterized outbred population of *D. melanogaster* (Sarangi et al., 2016) and sampling a large number of individuals during assays, we ensured that our results are free from biases of a specific genotype or a particular inbreeding trajectory. We have utilized isocaloric diet regimes with five different protein-to-carbohydrate ratios to control the confounding effect of diet's calorific value. Our findings suggest a positive correlation between increased carbohydrate content in diet to delayed pre-adult development, compromised reproductive output, and a shortened lifespan. In addition, using transcriptomic studies, we observe significant differences between gene expression profiles between flies fed on different dietary conditions, with aberrant transcriptional upregulation of genes involved in muscle development, being observed in flies fed on diet with higher carbohydrate content. Our results provide crucial insights into how macronutrient composition shapes various life

history traits and their correlations and help advance our understanding of how organisms might adapt to dietary fluctuations in today's rapidly changing world.

2. Materials and Methods

2.1 Maintenance regime of fly populations

The flies used in this experiment were derived from a large (n = ~2100), outbred Baseline Population (BP). This baseline population was derived by equally mixing MB_{1-4} (Melanogaster Baseline) populations, whose detailed ancestry can be found in a previous article (Sarangi et al., 2016; Shenoi et al., 2016). The BP population was maintained at 25°C in constant light, following a 14-day generation cycle for over 30 generations before the current experiments. Throughout this period, the flies were given a baseline diet composed of corn, sugar, and yeast (recipe provided in Table S1). This diet had a calorific value of 714kcal/L and a protein-to-carbohydrate ratio (henceforth, P:C) of 0.4. For routine population maintenance, we collected ~300 eggs in each of seven clear Laxbro© FLBT-20 plastic bottles, each containing ~50ml of food. On the 11th day post egg-collection, the adults were transferred to a large plexi-glass cage (25 cm × 20 cm × 15 cm). After two days, a fresh petri-plate containing baseline media was provided for about 14-hour egg-laying period. On the 14th day post egg-collection, these eggs were then transferred into seven bottles following the above method to initiate the next generation. The adult flies remained in the cage as a backup until egg collection for the subsequent generation.

2.2 Diets

To evaluate organismal performance across different macronutrient compositions, we formulated five isocaloric diets (matching the calorific value of the baseline diet) with distinct P:C ratios: 0.2, 0.25, 0.4, 0.55, and 0.7. These ratios were adjusted by modifying the amounts of sugar and yeast in the food, while maintaining consistent caloric values (see Table S1 for exact recipes). The P:C 0.4 diet matches the baseline, P:C 0.2 represents the maximum carbohydrate and minimum protein, and P:C 0.7 is the converse.

2.3 Assays

For all assays, 100-150 eggs from the baseline population were collected in plastic bottles containing 50 ml of the respective food types to generate the experimental flies. Throughout the study, we ensured consistent environmental conditions, maintaining constant light, a temperature of 25°C, 80–90% humidity, abundant nutrition, and an uncrowded rearing environment. Chronological age of the flies was also uniformly controlled across assays. Our investigation focused on measuring four sets of important life-history traits related to the flies' preadult development, reproductive output, lifespan, and functional senescence across the aforementioned five dietary regimes.

2.3.1 Preadult development and survivorship assay:

For this assay, exactly 100 stage-synchronized (Prasad et al., 2000) eggs were collected from the baseline flies in each of five replicate bottles containing 50 ml food of a specific experimental regime. The same is repeated for all the five experimental food regimes. The bottles were maintained under the environmental conditions, which were identical to the baseline population. After the pupae had darkened, the bottles were regularly checked at 2-h intervals and the number of eclosing males and females was recorded against the corresponding eclosion time. We continued these observations until no flies eclosed for over 48h from a bottle. From this data, we computed mean time to eclosion, preadult survivorship and sex ratio of the emerged adults.

2.3.2 Fertility assay:

To understand the effect of diet on reproductive output, we examined the number of viable offspring produced by the females reared on different diet regimes. On the 12th day from egg-collection, 2 pairs of experimental male and female flies were introduced into a vial with 6ml of food. They were allowed to lay eggs for 24 hours and then the adults were discarded. 30 such vial setups served as replicates for each food regime. The number of adults that eclosed from each vial was used to compute per capita female fertility. To determine whether variations in fertility data correspond to differences in body size across the dietary regimes, we used wing length of adult flies as a proxy for body size (see Supplementary Text S1 for details).

2.3.3 Lifespan and functional senescence assays:

Subsequently, we investigated the effect of nutrition on the fly physiology as the fly ages. To check this, we measured lifespan, and quantified male activity and female gut health at old age to test the state of these functional traits in flies reared under different diet regimes. For measuring lifespan, on the 12th day from the day of egg collection, we separated the experimental flies by sex under light CO_2 anesthesia and placed them in same-sex groups of 8 individuals into glass vials containing 6mL food of respective diet regime. We conducted 10 such replicates per sex for each of the five diet regimes. We monitored the vials daily at a particular time (arbitrarily set at 1200 noon) and they were transferred into fresh food vials every alternate day, till the last individual died. The number of days one fly lived from the time of the assay setup is considered its lifespan.

To quantify the senescence status of activity, we monitored locomotor activity of 34-day old male flies using Drosophila Activity Monitoring (DAM) Systems. We recorded their activity for 1 hour 10 minutes. For analysis, the first 10 minutes of data was excluded and the next 1 hour of data was used to calculate fly activity. Activity of 32 male flies were measured per diet regime.

To check the gut health, 34-day old female flies were put in a cohort of 10 individuals into glass vials with approximately 2 ml of fly food colored with 0.07 ml of brilliant blue dye (Arun Colour Chem Pvt. Ltd.) at a concentration of 2.5% (wt/vol). 10 such replicate vials were made per diet regime. The flies were allowed to stay in the vial for 1 day and then they were checked under the microscope for a Smurf phenotype (Martins *et al.* 2018). The number of flies which turned Smurf was recorded to compute the proportion of Non-Smurf flies for each vial. We computed arcsine of the square root of the proportions (following Zar, 1999) and subjected to statistical analysis.

2.3.4 RNA extraction and Library Preparation

On the 13th day post egg collection, 12 female flies were collected for a particular biological replicate and snap frozen in Trizol solution. Three such replicates were made for each of the diet regimes P:C 0.25, 0.4, 0.55 and 0.7. RNA isolation was performed using standard Phenol Chloroform extraction methods and RNA quality determined on Agilent Bioanalyzer System 4000. RNA libraries were performed post polyA enrichment using the NEBNext Ultra II RNA Library Prep Kit for Illumina, using standard protocol. Libraries were sequenced using a 150BP paired end chemistry on Novaseq6000.

2.4 Statistical and bioinformatic analysis

2.4.1 Analysis of life-history traits

The data from the developmental time and lifespan assays were subjected to a two-way fixed factor ANOVA with diet (P:C=0.2,0.25,0.4,0.55,0.7) and sex (=M, F) as fixed factors. For the rest of the assays *i.e.*, preadult survivorship, sex ratio, fertility per female, male activity and Smurf assay, data was subjected to a one-way fixed factor ANOVA with diet (P:C=0.2,0.25,0.4,0.55,0.7) as the fixed factor. If the main effect was significant, then a post-hoc analysis was done using Tukey's HSD test. All the above statistical analysis was done using STATISTICA® v5 (StatSoft Inc., Tulsa, Oklahoma). The graphs were made using RStudio.

2.4.2 RNA-seq analysis

Raw FASTQ files were subject to initial quality control analyses using the FASTQC software (https://www.bioinformatics.babraham.ac.uk/projects/fastqc/). FASTQ files were then trimmed using Trim-galore (https://www.bioinformatics.babraham.ac.uk/projects/trim_galore/) and aligned to the *Drosophila* genome (dm6) using hisat2 (Kim et al., 2019). The Seqmonk program (https://www.bioinformatics.babraham.ac.uk/projects/seqmonk/) was used to generate raw counts and differential gene expression performed using DESEq2 (Love et al., 2014). PCA and volcano

plots were generated in R using ggplot2. Genes were first filtered on padj<0.05 and Fold-change (FC)>2 and sorted based on FC. The top 40 differentially expressed (DE) genes between P:C 0.4 and P:C 0.25, P:C 0.4 and P:C 0.7 and the top 5 DE genes between P:C 0.4 and P:C 0.55 were selected, and unique genes amongst the list of 85 genes (final total of 77 genes) were used for heatmap analyses which were clustered using the Complete agglomeration method. The gProfiler software (Raudvere et al., 2019) was used for Gene Ontology analysis using a custom background (all expressed genes) and Bonferroni $p_{adj}<0.05$ was used as the cutoff for significant GO-terms.

3. Results

Reduced carbohydrate proportion in isocaloric diet accelerates developmental speed without affecting pre-adult survivorship or sex ratio

To understand how dietary composition affect organismal performance, we first looked into developmental timing, *i.e.* the time taken for an egg to develop into an adult *Drosophila*, when subject to different diets. We report a significant effect of macronutrient composition of diet on the developmental time in flies (Figure 1, $F_{4,38} = 79.67$, $p < 0.0001$). Our results indicate that flies reared on an isocaloric diet with a P:C ratio of 0.7 were found to develop faster compared to flies reared on a diet with a P:C ratio of 0.2 (Tukey's $p = 0.0001$), 0.25 (Tukey's $p = 0.0001$), and marginally faster than the flies reared on P:C ratio of 0.4 (Tukey's $p = 0.057$). Remarkably, despite a minor decrease in dietary carbohydrate content, flies reared on a diet with a P:C ratio of 0.4 exhibited significantly accelerated development compared to those on a P:C ratio of 0.25 (Tukey's $p = 0.0001$). Similar to previous reports, we too observe a shorter eclosion time for female flies in comparison to males ($F_{1,38} = 7.79$, $p = 0.008$), however, the food x sex interaction was not significant ($F_{4,38} = 0.57$, $p = 0.687$). Overall, our findings suggest that, despite equal calorific values, the relative proportions of dietary carbohydrates and proteins play a critical role in the egg to adult developmental time of *Drosophila melanogaster*. Specifically, we report that reducing carbohydrate content and increasing protein content in the diet is positively correlated to pre-adult developmental rate. We further found that pre-adult survivorship (Figure S1, $F_{4,19} = 0.528$, $p = 0.716$) and the sex ratio of the eclosing adults (Figure S2, $F_{4,19} = 0.958$, $p = 0.452$) were comparable across the experimental diets.

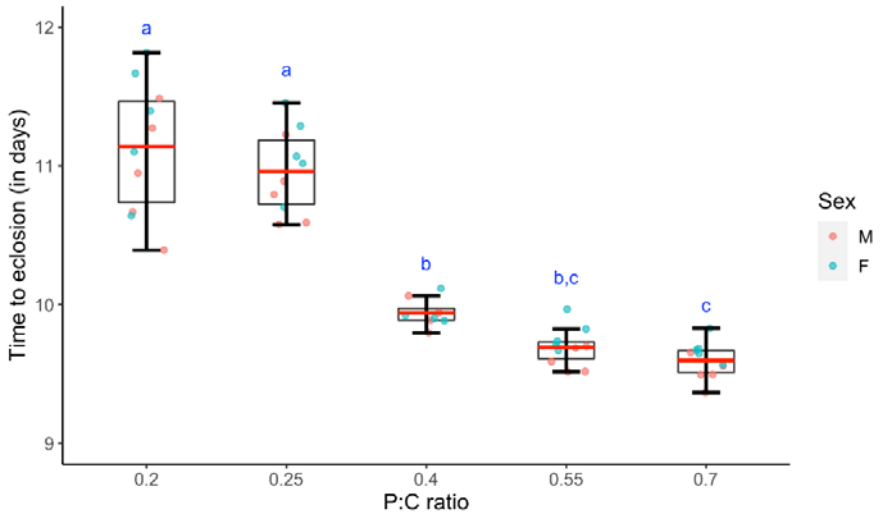

Figure 1. Effect of diet on development. Mean (±SEM) development time of individuals reared on

different diets is plotted against the diet which they were reared on. The box plot represents the time taken to eclosion. The red line, lower and upper edge of the box, lower and upper end of the whiskers represent the mean, 25th, 75th, 5th, 95th percentile of the development time data. The scatter points denote the mean of the developmental time of the individuals in each bottle. Different lower-case alphabets denote statistically significant differences (p<0.05).

Higher dietary carbohydrate proportions reduce reproductive output without affecting body size

We report significant differences in viable offspring produced per adult pair, composed of one male and one female, in relation to dietary proportion of protein and carbohydrate (Figure 2, $F_{4,145}$ = 86.530, p < 0.0001). Interestingly, even slight increase in carbohydrate content (and thereby, decrease in protein content) was negatively correlated to the reproductive output, with flies reared on an isocaloric diet with a P:C ratio of 0.2 producing fewer number of viable offspring than flies reared on a diet with a P:C ratio of 0.25 (Tukey's p = 0.00001). In contrary, flies reared on increased protein concentrations display higher fertility, with a significant increase seen between diets with protein to carbohydrate ratio 0.55 and 0.4 (Tukey's p=0.00004), and an even greater change between P:C 0.70 and P:C 0.55 (Tukey's p= 0.00001). In terms of wing length as a measure of body size, flies were comparable across experimental diets (Figure S3), except for those on the lowest protein-to-carbohydrate ratio, which were smaller. Taken together, our results reveal a remarkable reproductive plasticity exhibited by *Drosophila* across varying P:C ratios in isocaloric diets, independent of body size.

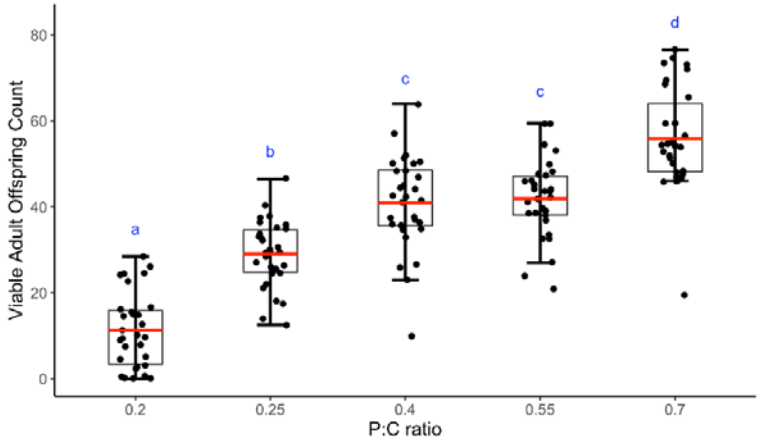

Figure 2. Effect of diet on fecundity. Mean (±SEM) number of viable adult offspring sired by a male-female pair reared on different diets is plotted against the diet which they were reared on. The box plot represents the fecundity of the females reared on diets with different P:C ratios. The red line, lower and upper edge of the box, lower and upper end of the whiskers represent the mean, 25th, 75th, 5th, 95th percentile of the fecundity data. The scatter points denote the number of eggs laid by each female. Different lower-case alphabets denote statistically significant differences (p<0.05).

High carbohydrate content in diet shortens fly lifespan

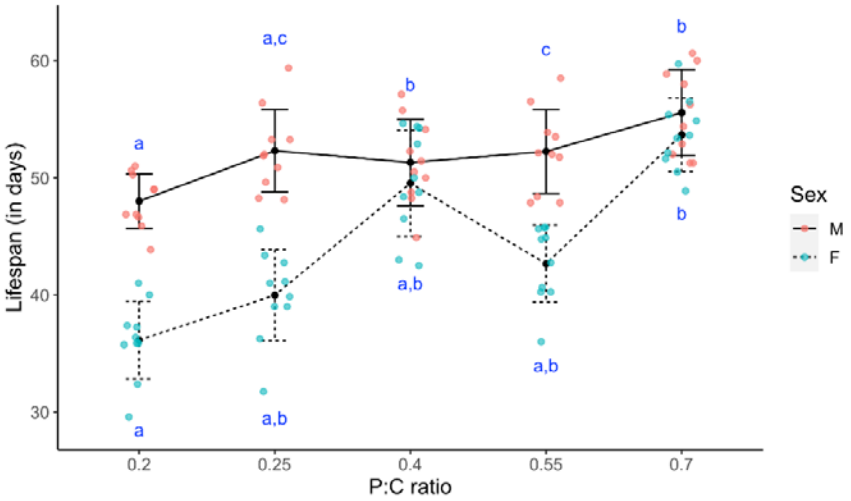

Figure 3. Effect of diet on lifespan: Mean (±SEM) lifespan of the flies reared on different diets. The box plot represents the lifespan of flies reared on diets with different P:C ratios. The red line, lower and upper edge of the box, lower and upper end of the whiskers represent the mean, 25th, 75th, 5th, 95th percentile of the lifespan data. The scatter points denote the mean of the lifespan of 10 flies of each sex housed together. Different lower-case alphabets denote statistically significant differences ($p<0.05$).

We next evaluated the effect of varying macronutrients on the lifespan of *Drosophila* and observe a significant effect of varying carbohydrate to protein ratio on longevity of flies(Figure 3, $F_{4,90}$ = 35.24, $p < 0.0001$), with males living significantly longer than females ($F_{1,90}$ = 111.86, $p < 0.0001$). We observe that a diet with a high P:C ratio of 0.7 prolonged the lifespan of flies significantly when compared to those reared on a diet with a low P:C ratio of 0.2 in both males (Tukey's p = 0.0001) and females (Tukey's p = 0.0001). It is also notable that lifespan of male and female flies was comparable when flies were reared at high P:C ratio (= 0.7), whereas with diets having high carbohydrate proportion (P:C = 0.2 and 0.25), female lifespan was substantially lower than that of males (Figure 3). This led to a significant food × sex interaction ($F_{4,90}$ = 11.05, $p < 0.0001$). Overall, our results indicate that given comparable calorific value diets, on one hand we observe a significant and negative correlation of lifespan to the amount of carbohydrate in diet, and on the other a positive correlation with the amount of protein.

Carbohydrate accelerates aging and impairs late-life physiology and behavior

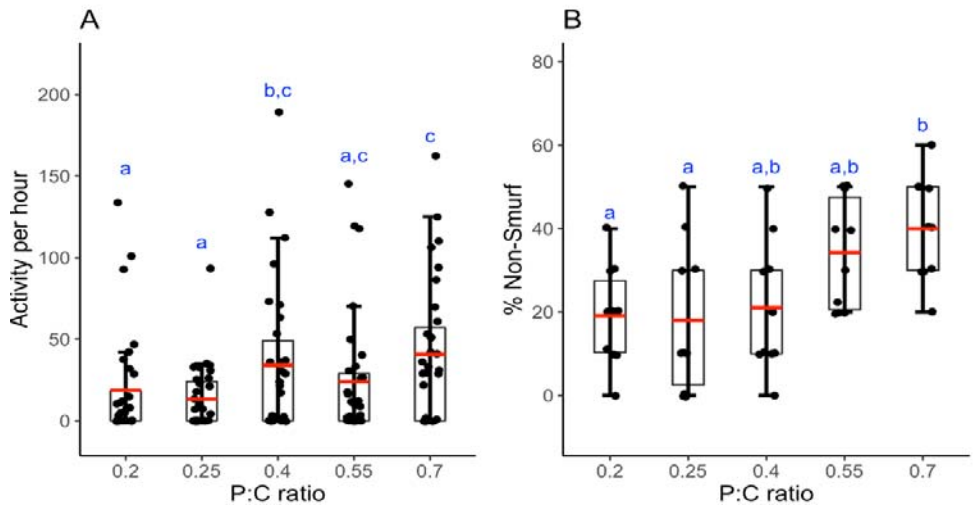

Figure 4. Effect of diet on late-life fitness. (A) Mean (±SEM) locomotor activity of male flies reared on different diets. (B) Mean (±SD) percentage of female flies with intact gut lining. The red line, lower and upper edge of the box, lower and upper end of the whiskers represent the mean, 25th, 75th, 5th, 95th percentile of the data. The scatter points denote the (A) late-life locomotor activity of a male and (B) percentage of females which remained non-smurf (i.e. healthy) in each vial. Different lower-case alphabets denote statistically significant differences (p<0.05).

Consistent with the effect of macronutrient composition on lifespan, our experiments also reveal the role of varying protein-to-carbohydrate ratios on late-life locomotor activity of males (Figure 4A, $F_{4,150} = 2.779$, $p = 0.028$) and gut physiology of female flies (Figure 4B, $F_{4,45} = 4.518$, $p = 0.0037$). We report that the functional status of both these traits was better in flies that consuming food with higher P:C ratio. Male flies reared on a high protein diet with a P:C ratio of 0.7 were more active than those reared on a high carbohydrate diet with a P:C ratio of 0.25 (Tukey's $p = 0.0314$). Females reared on a low carbohydrate diet with a P:C ratio of 0.7 showed increased gut integrity when compared to females reared on a high carbohydrate diet with (P:C 0.25) (Tukey's $p = 0.0091$). Our studies, thereby, suggests a correlation between increased dietary carbohydrate to reduced late-life activity in male flies and compromised gut health in female flies.

Impact of protein-to-carbohydrate (P:C) ratios on gene expression levels in *Drosophila melanogaster*

We further investigated the effect of varied protein-to-carbohydrate ratio isocaloric diets on gene expression profiles, we performed transcriptomic analysis of adult female flies. Principal

component analysis (PCA) indicates a distinct separation on PC1, between flies fed on diets having a P:C ratio of 0.25 and P:C ratio of 0.7, suggesting highly dissimilar transcriptional profiles (Figure 5A) across the expressed genes (Table S2). Meanwhile, expression profiles of flies fed on diets with P:C ratio of 0.4 and 0.55, while equidistant from the other two conditions (P:C 0.25 & P:C 0.7) on the PCA plot, tend to cluster together, thereby indicating that transcriptional profiles of the intermediate diet (P:C 0.55) mirrors that of the control (P:C 0.4) (Figure 5A). This is confirmed by the sparse number of differentially expressed (DE) genes (25) between the control (P:C 0.4) vs P:C 0.55 conditions (Table S4, Figure 5D). On the other hand, we report a total of 160 DE genes in P:C 0.25 and 287 DE genes in P:C 0.7, when compared to the control diet (Figure 5C, Table S3; Figure 5E, Table S5). Interestingly, we find a shift in the regulation profiles between the P:C 0.25 and P:C 0.7 when compared to the control, with 95% of the genes being upregulated in the former as compared to 11.8% in the latter (88.2% downregulated) (Figure 5C, 5E). Heatmap analysis of the top 77 DE genes (detailed methodology for choosing the genes provided in the methods section) suggest distinct differences in the gene expression profile across the test groups (Figure 5B). This indicates a rewiring of the gene regulation machinery in response to change in protein-to-carbohydrate ratio of the diet, while also alluding to a dosage-dependent effect for the cause of the phenomenon. To investigate further into the category of genes being differentially expressed in different test groups, we performed Gene Ontology analysis (Table S6, S7). We find that for the P:C 0.25 condition (high carbohydrate, low protein diet), genes which are involved in muscle development are upregulated. Whereas, for the P:C 0.75 condition, we find that genes involved in fatty acid synthesis process (including long-chain fatty acyl CoA synthetases), were significantly downregulated, indicating a transcriptome poised for reduced expression of fatty acids. Taken together, our study here uncovers a distinct diet-dependent modulation of gene expression profiles in adult female flies.

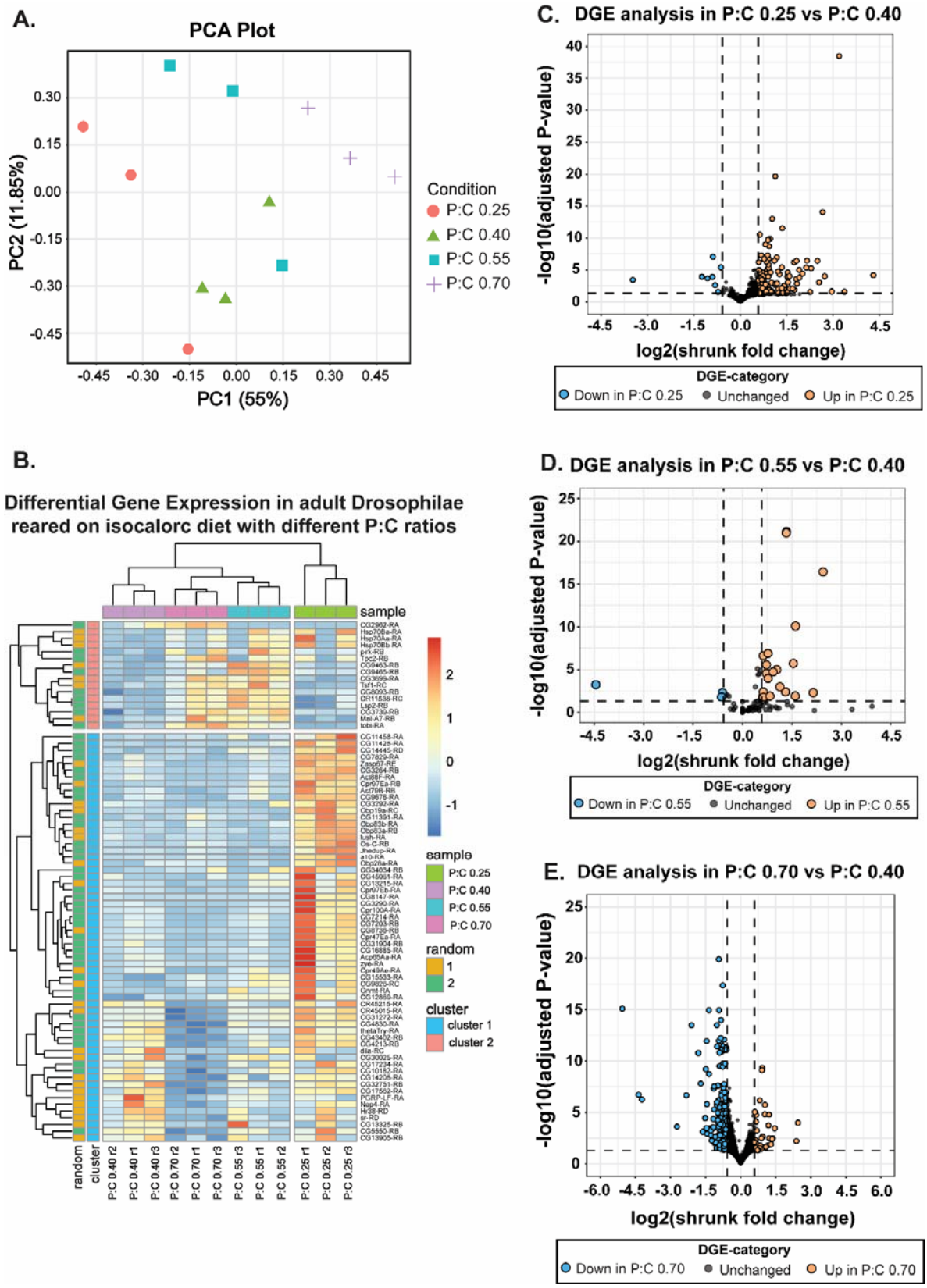

Figure 5. Impact of diet on gene expression profile. (A) Principal Component Analysis (PCA) plot showing the gene expression data of flies maintained on four distinct diet regimes: P:C ratios of 0.25, 0.4, 0.55, and 0.7. (B) Heatmap showing the expression patterns and clustering of the 77

differentially expressed genes from each category. (40 genes between P:C 0.4 and P:C 0.25, 40 genes between P:C 0.4 and P:C 0.7, and 5 genes between P:C 0.4 and P:C 0.55, duplicates excluded) (C, D, E) Volcano plot comparing gene expression levels between flies reared on the baseline diet (P:C = 0.4) and the carbohydrate-rich diet (P:C = 0.25) (C), the intermediate diet with a P:C ratio of 0.55 (D) and the protein-rich diet with a P:C of 0.7 (E).

4. Discussion

Food provides essential calories for vital activities in an organism, but its significance extends beyond its caloric content (Simpson & Raubenheimer, 2012). Macronutrients obtained from food play a crucial role in growth, development, and maintenance of bodily functions (Raubenheimer & Simpson, 1997; Simpson & Raubenheimer, 2009). However, excessive or inadequate intake of macronutrients can negatively impact organismal health and performance (Lee, 2015; Lee et al., 2008). Our study systematically investigates how altering the relative proportion of carbohydrate and protein in isocaloric diets affects various life-history traits and gene expression profile in *Drosophila melanogaster*. Using a large outbred population (n ~2100), we explore the complex relationship between diet and organismal performance across multiple phenotypes pertaining to development, reproduction and survival. Previous research primarily used isogenic lines, making it unclear if those results extend to populations with standing genetic variations in natural settings, particularly given substantial genotype × environment interactions observed in other studies (Heianza & Qi, 2017; Roche et al., 2005; Ussar et al., 2015).

In order to examine the influence of diet composition (protein to carbohydrate ratio) on development, we quantified the time taken for flies to develop from embryonic stage to adult eclosion. We observed that an increased proportion of carbohydrates in the diet resulted in a longer developmental period for the flies. This aligns with previous studies that showed a delay in developmental time due to a decrease in protein content (Lee et al., 2008; Matzkin et al., 2011). A possible explanation for this finding can be found in an earlier study by Almeida de Carvalho & Mirth (2017), who reported that larvae regulate their food consumption in order to achieve an intrinsic threshold level of protein intake. As a result, when raised on an isocaloric high-carbohydrate diet with a low P:C ratio, larvae need to feed for a longer duration to obtain sufficient protein intake, while under a low-carbohydrate diet with a high P:C ratio, larvae reach their protein-intake goal more quickly, resulting in a shorter overall pre-adult developmental time. Despite a substantial (~48h) difference in developmental time between flies reared on diets with different compositions, pre-adult viability was not impacted by diet. This suggests that even under the experimental diet with the lowest P:C ratio, flies were able to satisfy their necessary protein requirements for pre-adult growth and development, albeit at a later time point. Additionally, no difference in sex ratio was observed across our experimental diets, indicating that no diet-induced developmental plasticity was present under these dietary regimes, a phenomenon that is often seen in various organisms (reviewed in Rosenfeld & Roberts, 2004).

Pre-adult development time is known to play a crucial role in shaping an organism's body size and morphology, especially in insects (Atkinson & Sibly, 1997; Davidowitz et al., 2004; Nijhout, 2003; Nylin & Gotthard, 1998). Previous studies have indicated that extending the pre-adult period leads to an increase in body size, while shortening it results in a decrease in body size (Davidowitz et al., 2004). However, in our study, we did not observe a substantial difference in body size, neither in males nor females, across the experimental isocaloric food regimes with varying protein to carbohydrate ratios. This suggests that, as previously explained, the flies reared on a low P:C ratio diet required a significantly longer time to attain their protein requirement. However, in the process, the excess carbohydrate that they consumed did not lead to additional growth. This illustrates that the correlation between pre-adult development time and body size can be context-specific and dependent on various factors. This raised the question of whether the surplus carbohydrate intake is prioritized for reproductive purposes instead of growth on high-carbohydrate diets (Lee et al., 2008). To test this hypothesis, we investigated the reaction norm of reproductive output across the experimental diet regimes.

Interestingly, even though flies reared on different experimental diets had similar body sizes, we observed a significant effect of diet on the number of viable offspring produced by adult *Drosophila* pairs (henceforth termed as reproductive output; Figure 3). An increase in the relative proportion of carbohydrates in the diet resulted in a significant decrease in reproductive output. The observed discrepancy of reduced reproductive output on a carbohydrate-rich diet, despite comparable body sizes, aligns with findings from a prior study. This research indicated that elevated sugar intake leads to cholesterol accumulation, subsequently reducing fertility (Brookheart et al., 2017). In addition to the detrimental effect of excessive carbohydrate consumption on reproduction, dietary protein is known to stimulate ovarian stem cell proliferation and oogenesis (Drummond-Barbosa & Spradling, 2001), resulting in greater reproductive output. These findings are supported by several studies that have reported a reduction in female fecundity when dietary protein content is decreased or sugar intake is increased (Lee, 2015; Lushchak et al., 2014; Savola et al., 2021). They also underscore the observed difference in fertility of flies reared on diets with different P:C ratios in the current study, with a staggering more than four-fold increase in the number of offspring observed on the isocaloric experimental diet with the lowest carbohydrate content compared to that with the highest carbohydrate content.

A negative correlation between reproductive output and lifespan is a widely recognized phenomenon across species, including *Drosophila* (Flatt & Kawecki, 2007; Hunt et al., 2004; Rose, 1984). This trade-off is thought to originate owing to resources directed towards reproduction, reducing available resources for somatic maintenance and repair, leading to accelerated senescence

and reduced lifespan (Kirkwood & Rose, 1991). However, in a study on *Drosophila*, ablating the germ line had no effect on longevity (Barnes et al., 2006), suggesting that the association between these two traits can be uncoupled. Interestingly, the association between reproduction and lifespan can even be positively correlated if any factor impacts both the traits in the same manner independently. Carbohydrate-rich diets have been found to reduce lifespan, as well as lower egg-laying capability in fruit flies with low protein diets (Lushchak et al., 2014). Our current study on *Drosophila* also showed that with decreasing P:C ratio, lifespan decreases. Further, it is interesting to note that the signature is more pronounced in females than males, consistent with earlier findings that females are more vulnerable to the adverse effects of high-sugar diets in fruit flies (Chandegra et al., 2017). Supporting the notion of the findings of our study, several studies have demonstrated that a high-sugar diet can lead to cardiomyopathy, elevated oxidative stress, and diabetes-like phenotypes, thereby compromising the lifespan of adult *Drosophila* (Ecker et al., 2017; Musselman et al., 2019; Na et al., 2013; Palanker Musselman et al., 2011), whereas the lifespan of flies increased with increasing protein consumption (Lee, 2015; Savola et al., 2021). However, our results are in contrast to other studies that found no difference in lifespan between flies reared on high protein and high carbohydrate diets (Watada et al., 2020) or where they found that consuming high carbohydrate and low protein maximizes lifespan (Bruce et al., 2013). Given these conflicting findings, a more targeted investigation is needed to understand the mechanistic underpinnings of the impact of macronutrients on lifespan, which is beyond the scope of the current study. Instead, we examined the effects of macronutrient composition on health span by studying the functional senescence of adult locomotor activity and gut physiology.

Lifespan serves as a crucial measure of organismal evolutionary fitness. However, it provides an incomplete binary view of organismal performance. A more informative approach is to examine the gradual decline in functional status with age, or functional senescence, of an organism's various traits. This perspective is mirrored in human gerontology research, which has recently shifted its focus from extending lifespan to extending the period of healthy, active, and disability-free living, referred to as health span (Partridge, 2016). In our study, we found that male flies reared on a low carbohydrate, high protein diet exhibited greater activity levels at a later age. Similarly in females, with increasing P:C ratio, we observed a delayed decline in gut lining integrity, as indicated by the higher proportion of flies exhibiting the non-Smurf phenotype (Figure 4B). These findings are consistent with prior research, which found that increasing sugar content in the diet increased the proportion of flies displaying a leaky gut phenotype (Pereira et al., 2018, however see Galenza & Foley, 2021). Taken together, our findings suggest that increasing the relative proportion of carbohydrate and decreasing the proportion of protein in the isocaloric diet accelerates functional senescence in flies, leading to a shorter lifespan.

Prompted by the significant influence of diet composition on key life-history traits, including the preadult developmental period, reproductive output, aging, and lifespan, we expanded our exploration to effects at a sub-organismal level. With the understanding that the traits mentioned above are polygenic, we conducted an untargeted transcriptomic analysis of whole-fly samples. The findings reveal a robust diet-induced plasticity in gene expression (Figure 5), corroborating with earlier studies (Haro et al., 2019; Whitaker et al., 2014). Notably, our investigations involved a large, outbred *D. melanogaster* population with substantial standing genetic variation. The consistency across biological replicates attests to the robustness of our data and signal clarity, bolstering the reliability of our results and confidence in the observed gene expression patterns. Gene ontology analysis of differentially expressed genes indicates overexpression of several genes under a high-carbohydrate diet (P:C 0.25). This overexpression might be attributed to the upregulation of the target of rapamycin (TOR) pathway, a key regulator of multiple transcription factors. TOR pathway is conserved across the species, including *Drosophila* (Tatebe & Shiozaki, 2017), and it is a known downstream target of insulin and insulin-like peptide signaling (IIS) pathway, which is upregulated under high-sugar diet (Teleman, 2009). An upregulation of genes related to muscle development was also detected. Consistent with this, past experiments have demonstrated that the TOR signaling pathway can enhance muscle-specific transcription factors in mammalian systems (Risson et al., 2009) and the Insulin receptor/Foxo signaling plays a crucial role in activating muscle protein in *Drosophila* (Demontis & Perrimon, 2009). Conversely, the gene expression profile of the low-carbohydrate, high-protein diet (P:C 0.7) reveals a general transcriptome downregulation (Figure 5B, 5C, 5E). Notably, genes implicated in fatty acid synthesis, including long-chain fatty acyl CoA synthetases, which have been linked with obesity and associated metabolic disorders under high fat diet (Bjermo & Risérus, 2010; Sampath & Ntambi, 2011), were also downregulated under high-protein diet. Although proposed here, a systematic investigation of the precise molecular mechanisms behind these diet-induced transcriptional changes is needed, which is outside the scope of the current study. We recognize that diet impacts may vary across tissues (Ellis et al., 2015), suggesting that future research could focus on a tissue-specific analysis of our findings. Further exploration of the molecular mechanisms triggering these gene expression changes could reveal new pathways and molecular entities involved in dietary adaptations. Ultimately, these findings provide promising avenues for elucidating the molecular basis of dietary adaptations and unraveling the complexities of gene regulation in response to diverse nutrient compositions.

5. Conclusion

Our study highlights the significant effects of macronutrient composition on various life history traits - development, reproduction, and survival of *Drosophila*. Specifically, our results indicate that a high proportion of carbohydrates in the diet negatively impacts development, reproduction, and lifespan. Moreover, our findings demonstrate that carbohydrate accelerates aging and impairs late-life physiology and behaviour. These results have implications for understanding the role of diet in shaping the life history traits of organisms. Notably, we detected distinct diet-induced variations in gene expression. Further in-depth investigations of the molecular pathways, such as Insulin signaling (IIS), target of rapamycin (TOR) signaling, and related pathways involved in energy homeostasis will provide us the key to understand underlying mechanisms behind the observed diet-induced plasticity in life-history, physiology, and behaviour. Such understanding holds the potential for devising therapeutic strategies targeting age-related diseases.

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Conflict of Interest statement

The authors declare no conflict of interest.

Author contributions: AM, CM and ST conceived the ideas and designed methodology of the life-history assays in this study; AM, CM, CS, NK, ST collected the data; AM analysed the data. SK and ST conceived the ideas and designed methodology of the transcriptomics part in this study; SK collected the data; SK analysed the data. AM, SK and ST led the writing of the manuscript. All authors contributed critically to the drafts and gave final approval for publication.

Data availability statement

Life-history data will be available on Dryad Digital Repository upon acceptance of the manuscript. The raw data for the RNAseq experiment is submitted in GEO repository, and can be accessed using the Accession number GSE241397.

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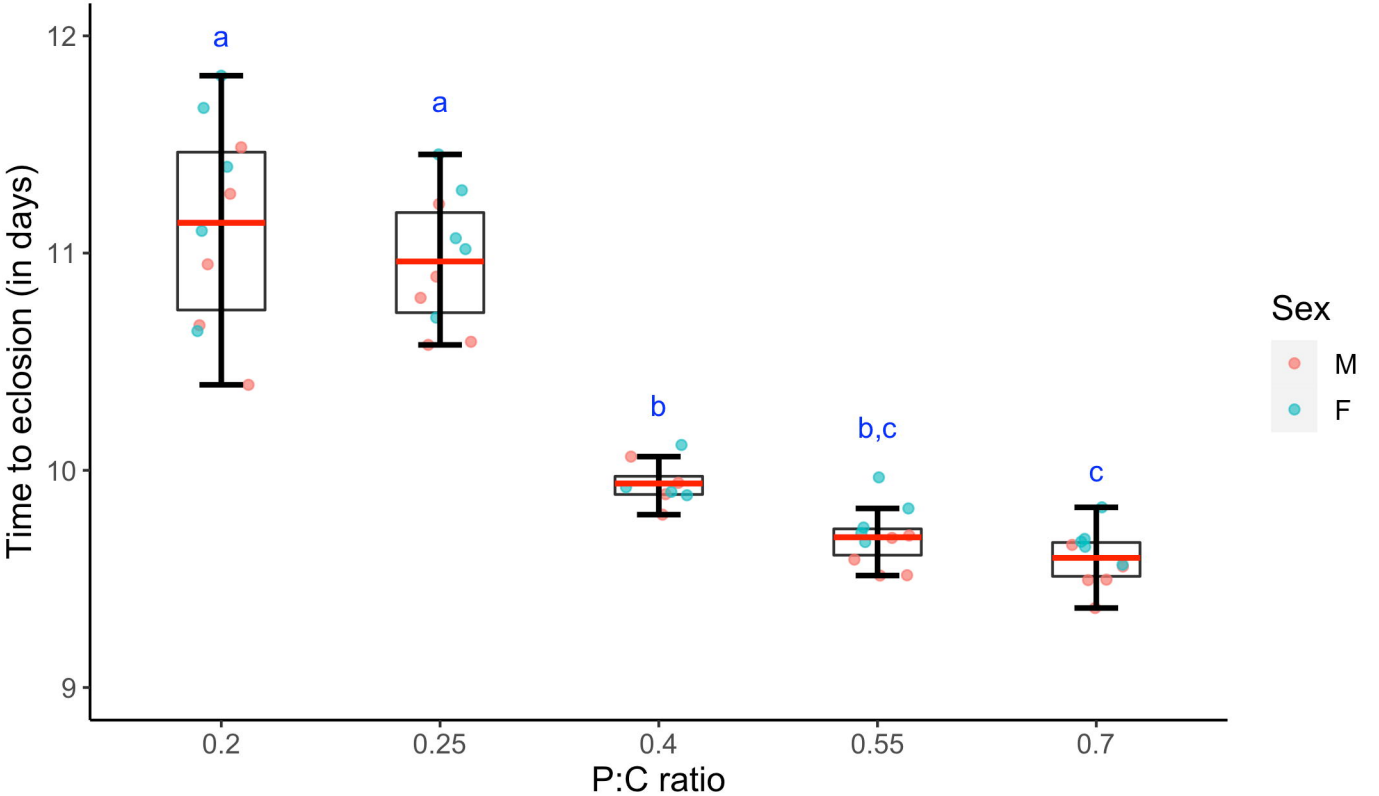
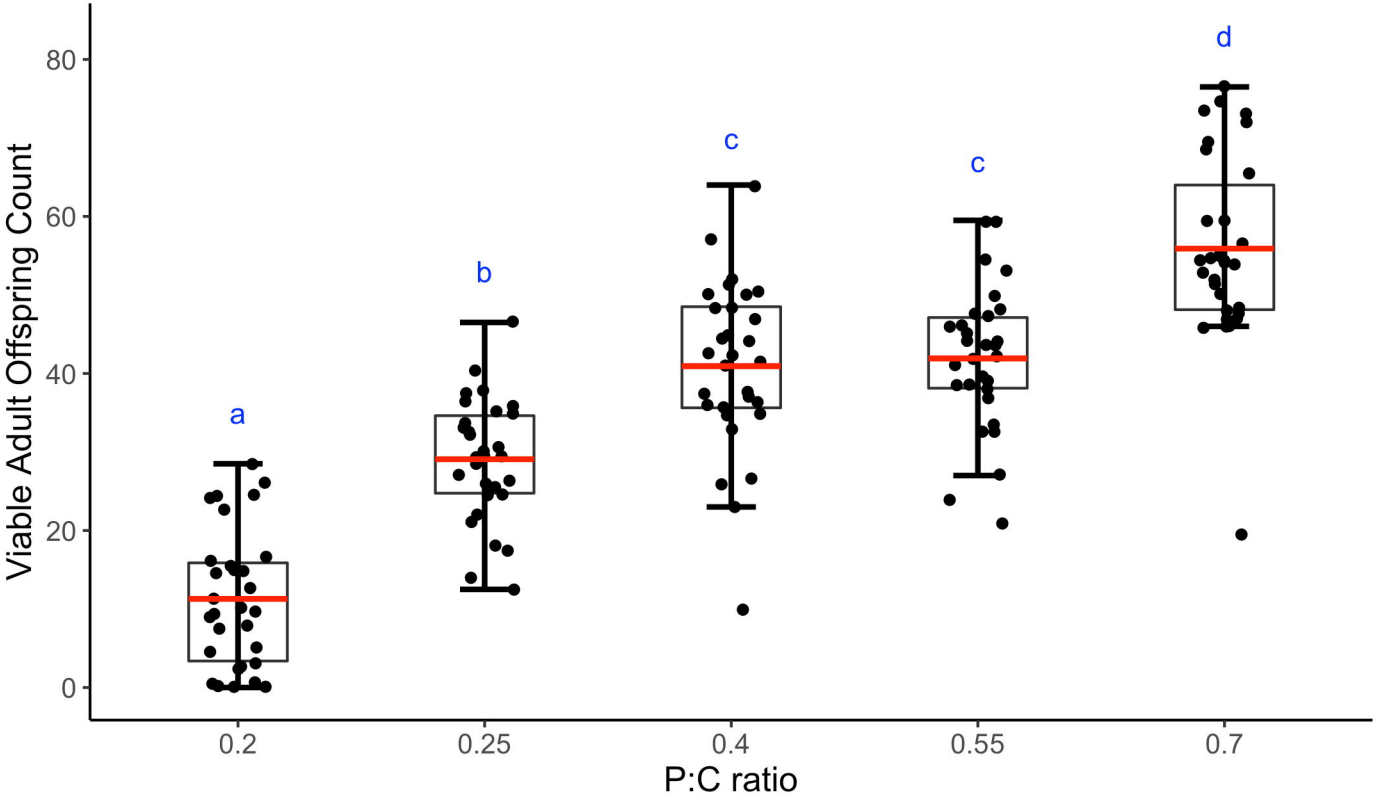
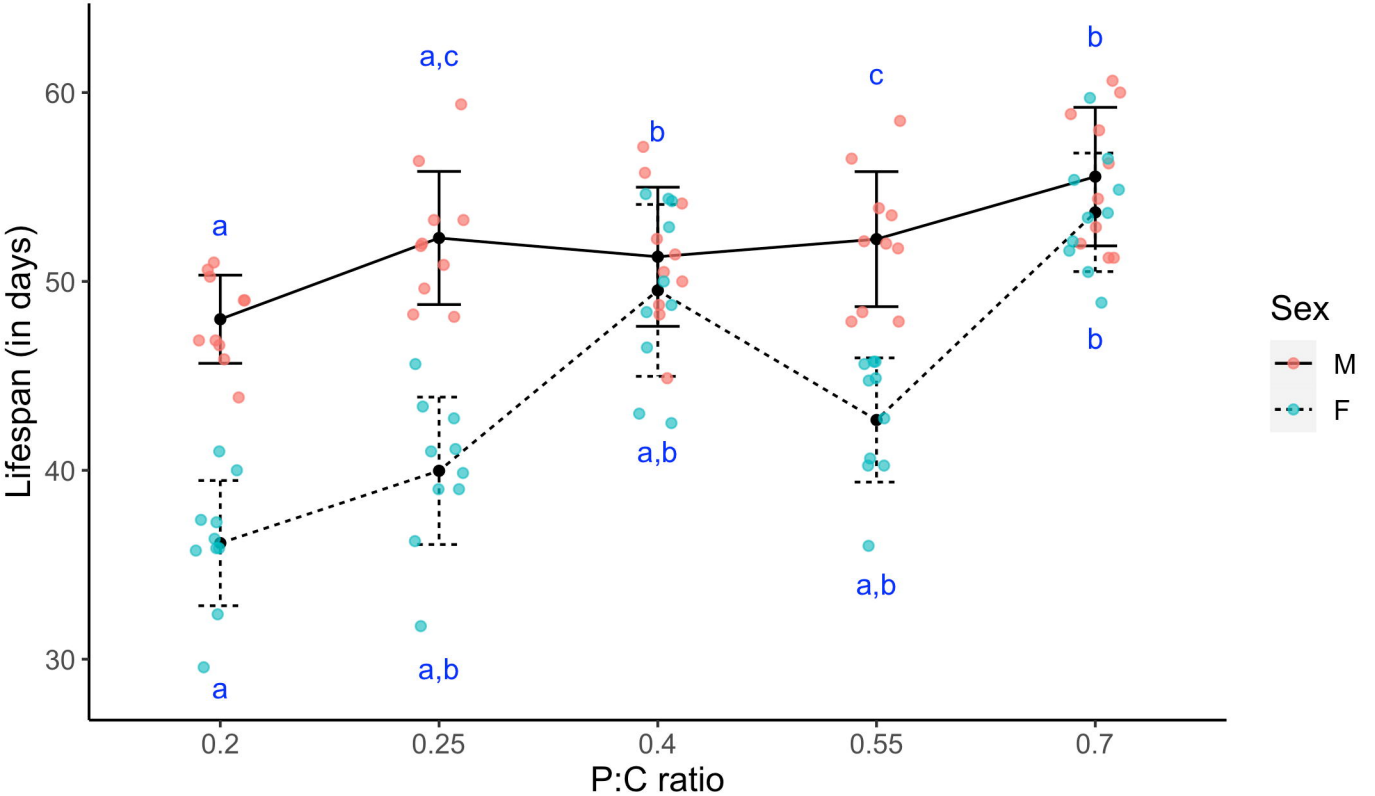
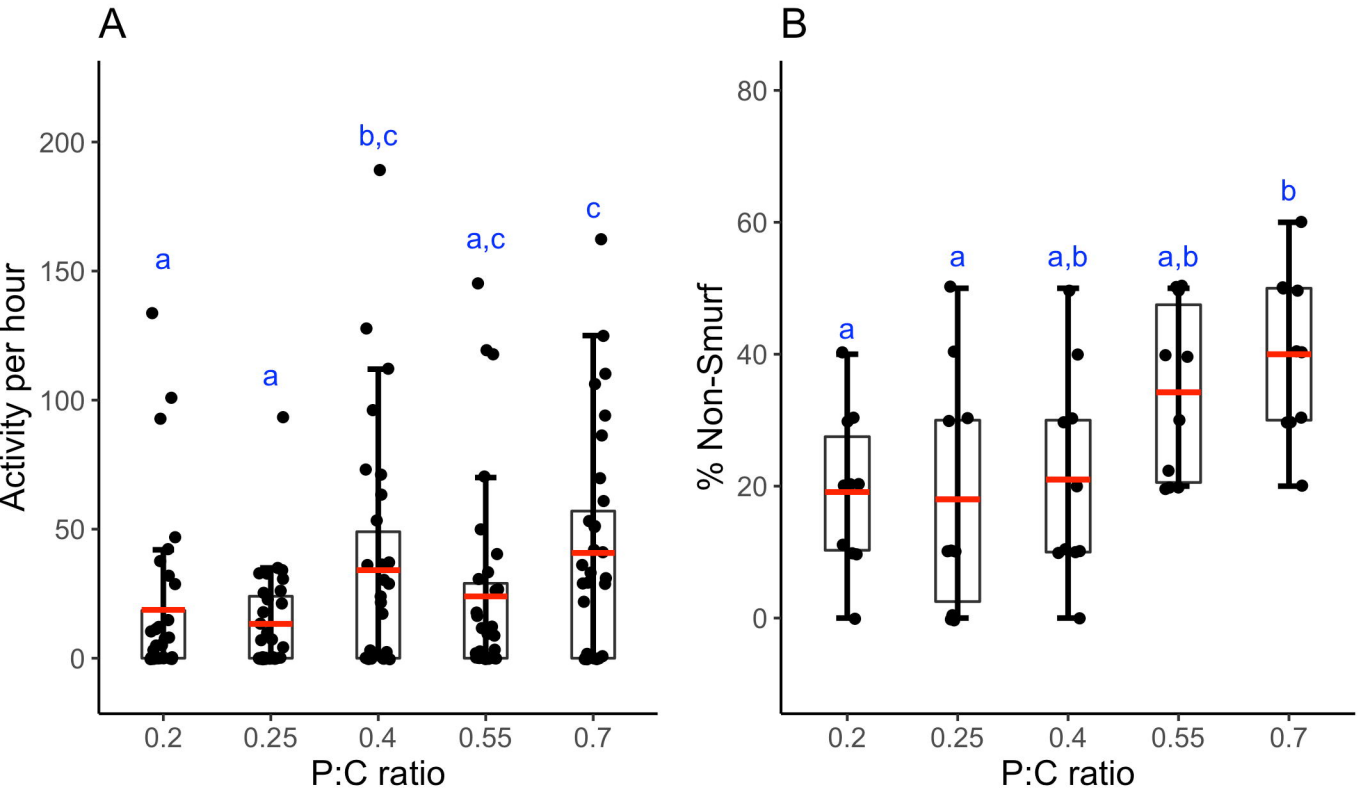
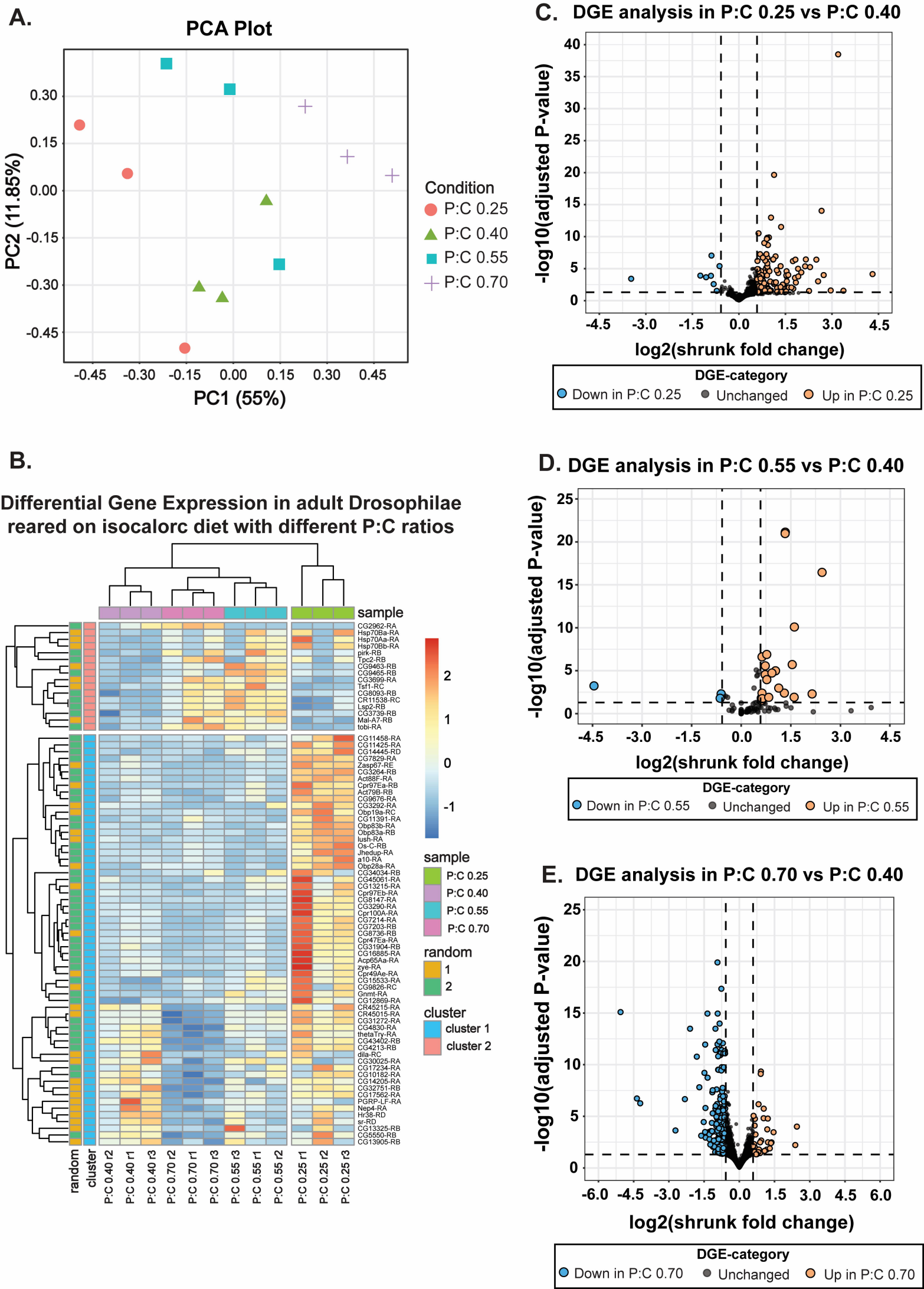

Time to eclosion (in days)
12
11
10
9
a
a
b
b,c
c
0.2
0.25
0.4
0.55
0.7
P:C ratio
Sex
M
F


Viable Adult Offspring Count
0
20
40
60
80
a
b
c
c
d
0.2
0.25
0.4
0.55
0.7
P:C ratio


Lifespan (in days)
60
50
40
30
a
a,c
b
c
b
a
a,b
a,b
a,b
b
0.2
0.25
0.4
0.55
0.7
P:C ratio
Sex
M
F


A
Activity per hour
200
150
100
50
0
a
a
b,c
a,c
c
0.2
0.25
0.4
0.55
0.7
P:C ratio
B
% Non-Smurf
80
60
40
20
0
a
a
a,b
a,b
b
0.2
0.25
0.4
0.55
0.7
P:C ratio


A.
PCA Plot
PC2 (11.85%)
PC1 (55%)
Condition
P:C 0.25
P:C 0.40
P:C 0.55
P:C 0.70
B.
Differential Gene Expression in adult Drosophilae reared on isocalorc diet with different P:C ratios
sample
random
cluster
P:C 0.40 r2
P:C 0.40 r1
P:C 0.40 r3
P:C 0.70 r2
P:C 0.70 r1
P:C 0.70 r3
P:C 0.55 r3
P:C 0.55 r1
P:C 0.55 r2
P:C 0.25 r1
P:C 0.25 r2
P:C 0.25 r3
cluster 1
cluster 2
C. DGE analysis in P:C 0.25 vs P:C 0.40
-log10(adjusted P-value)
log2(shrunk fold change)
DGE-category
Down in P:C 0.25
Unchanged
Up in P:C 0.25
D. DGE analysis in P:C 0.55 vs P:C 0.40
-log10(adjusted P-value)
log2(shrunk fold change)
DGE-category
Down in P:C 0.55
Unchanged
Up in P:C 0.55
E. DGE analysis in P:C 0.70 vs P:C 0.40
-log10(adjusted P-value)
log2(shrunk fold change)
DGE-category
Down in P:C 0.70
Unchanged
Up in P:C 0.70