MSC THESIS

Effect and interaction of sugar and diverse environments on the lifespan of Drosophila melanogaster

GEN84036

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Abstract

Excessive sugar intake is known to be an important risk factor for chronic diseases such as type 2 diabetes and cardiovascular disease. However, the effects of sugar consumption on lifespan are not the same for everyone and are also influenced by genetic, environmental, and physiological factors. In this study, *Drosophila melanogaster* was used as a model organism to investigate how dietary sugar levels, larval density, genetic background, and sex interact to affect lifespan.

Experiments were conducted on three genetically distinct lines (CE2, CE3, CL3) exposed to five sugar levels (12.5g, 25g, 50g, 100g, 200g) under high and low developmental density conditions. Lifespan data for 4,814 flies were analyzed, considering factors like genotype (*Eip75B* and *Liprin-gamma* loci), sex, and environmental stress. Results showed that there were significant differences by line or sex and that all these conditions not only had independent effects but also had a correlational effect on lifespan. In addition, the analysis of two genotypes that appeared to affect lifespan highlighted the independent influence of *Liprin-gamma* on lifespan, while *Eip75B* exhibited significant sugar-dependent interaction. Unexpectedly, flies fed with the highest sugar levels (200g) were found to have the most extended lifespans across all conditions, which may be related to the hyperactivity mitigating metabolic effects of sugar.

This study found that *D. melanogaster* exhibited various optimal sugar conditions for lifespan under different conditions. These findings suggest that generalized dietary guidelines have limitations in applying the same sugar amount to all populations and that personalized nutritional strategies that consider genetic and environmental contexts are needed.

Introduction

Excessive sugar intake is widely recognized as a significant risk factor for various chronic diseases, such as type 2 diabetes and cardiovascular diseases (Rippe & Angelopoulos, 2016). To reduce this risk, the American Heart Association advises limiting daily sugar intake to less than 30 grams to reduce these risks (*How Much Sugar Is Too Much? | American Heart Association*, n.d.).

Despite this advice, sugar consumption in many populations far exceeds the recommended amount. For instance, average daily sugar intake among U.S. adults ranges from 40 grams among Asian Americans to 76 grams among African Americans, showing a widespread pattern of overconsumption across ethnic groups (Figure 1) ((Get the Facts: Added Sugars | Nutrition | CDC). These levels of excessive sugar intake suggest a universal predisposition across all ethnic groups to metabolic disorders.

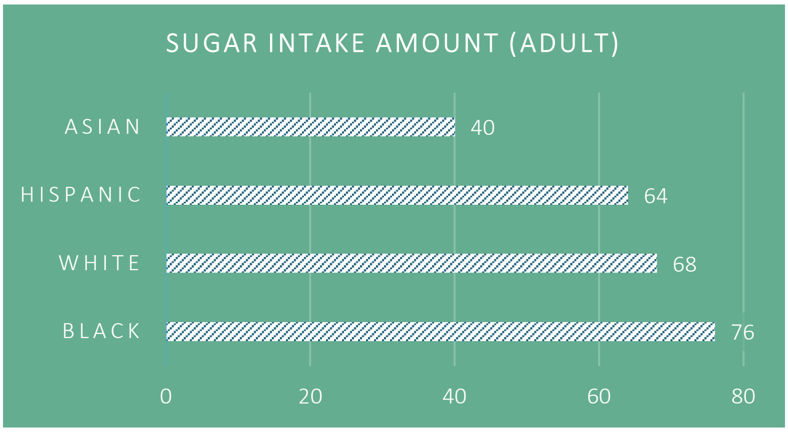

Figure 1 Daily added-sugar intake among U.S. adults from 2017 to 2018, categorized into four ethnic groups. All groups exceeded the recommended daily intake, with one group consuming more than the recommendation and three groups exceeding it by over double (*Get the Facts: Added Sugars | Nutrition | CDC*, n.d.).

Such dietary patterns contribute to the growing prevalence of metabolic syndrome (MetS), a combination of metabolic abnormalities characterized by abdominal obesity, hypertriglyceridemia, low HDL cholesterol, hypertension, and hyperglycemia (*Metabolic Syndrome | Johns Hopkins Medicine*, n.d.; Moore et al., 2019). MetS significantly elevates the risk of developing type 2 diabetes and cardiovascular disease (Mohamed et al., 2023), with individuals experiencing up to a five-fold and a two-fold increase, respectively, within 5-10 years (Jha et al., 2023).

Inequalities and Background of Metabolic Syndrome

Studies on the prevalence of metabolic syndrome show significant differences by ethnicity and sex. For example, Asian women show remarkably higher rates of metabolic syndrome compared to the male population within the same ethnic group. In contrast, African American men report the lowest incidence of metabolic syndrome among all analyzed groups despite their relatively high dietary sugar intake (Adjei et al., 2024).

Sugar consumption alone cannot explain why metabolic syndrome has different incidence rates across ethnicities or sexes. Recent studies suggest that metabolic syndrome is likely influenced by complex interactions such as genetic variations, socioeconomic factors, cultural backgrounds, and access to healthcare (Adjei et al., 2024). Therefore, understanding how these factors interact is important for reducing the incidence of metabolic syndrome in people living in diverse backgrounds.

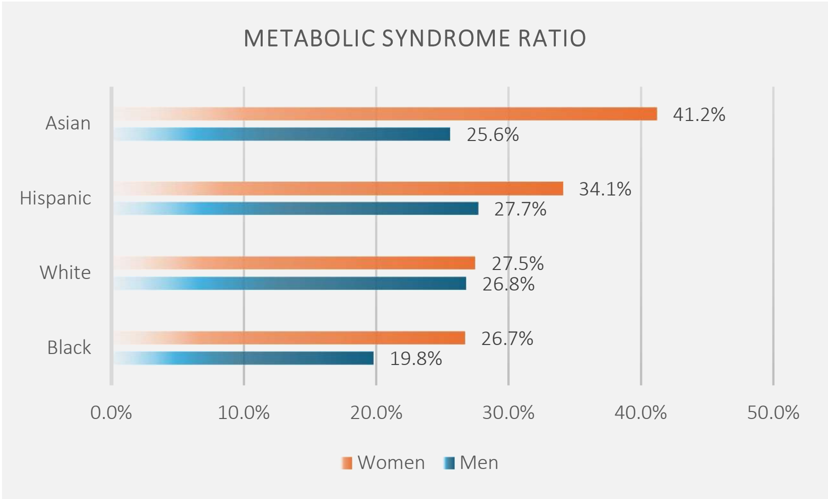

Figure 2 Incidence of metabolic syndrome by ethnicity and sex. Asians (15.7%), Hispanics (6.4%), and blacks (6.9%), there is a significant gap in incidence. However, there is only a slight gap of 0.7% for whites. For men, there is a small difference of about 1% in the incidence rate between all ethnicities except African American males, which shows a 5% lower rate. For women, the incidence is around 15% higher in Asians and 7% higher in Hispanics than black and white females.

Rationale and Objectives of the Study

Although there have been several studies on each factor, there has been no proper study on overall interactions. To confirm whether these interactions exist, an approach that can obtain all information on genetic, dietary, sex, and environmental influences is needed. However, human studies are often restricted by challenges such as population diversity, long-term compliance with dietary interventions, and ethical considerations (Weaver & Miller, 2017). Therefore, model organisms like *Drosophila melanogaster* offer a favorable alternative. With its short lifespan, well-mapped genome, and similar metabolic pathways to humans, *D. melanogaster* is an ideal model for studying these relationships (Ogienko et al., 2022).

Previous research using the *D. melanogaster* has provided critical insights into how genetic and environmental factors influence lifespan. For instance, studies on the chico[1] mutant have shown that mutated phenotypes can extend lifespan in females under normal nutrition conditions by affecting the insulin/insulin-like growth factor (IGF) signaling (IIS) pathway. This research indicated the direct role of genetic differences in lifespan regulation and the importance of genetic variation in understanding longevity mechanisms (Clancy et al., 2001). Furthermore, subsequent studies have shown sex differences in the response to dietary restriction, suggesting that males and females have different nutrient requirements and energy allocation strategies, and differences in the IIS pathway (Magwere et al., 2004).

Subsequent studies have extended these insights by exploring natural genetic diversity. Populations of *D. melanogaster* generated by combining six different strains from multiple geographic origins have been used to investigate how natural genetic variation mediates responses to dietary stressors (Hoedjes et al., 2019). More recently, large-scale genotyping with 3.211 individuals identified six key loci, *hppy*, *Eip75B, mRps22, capu, Liprin-gamma*, and *CG4554*, which play significant roles in lifespan regulation (Joost van den Heuvel (in prep)).

On the other hand, studies have emphasized the critical role of dietary composition in lifespan determination. An experiment tested 25 combinations of sugar and protein levels, including sugar and yeast concentrations ranging from 25 g/L to 400 g/L. Their results demonstrated that high-sugar diets or high-yeast diets consistently reduced lifespan, while optimal lifespan was observed when both nutrient levels maintained intermediate levels (Skorupa et al., 2008). These findings have important implications for modern dietary trends, such as high-protein or ketogenic diets, which often advocate extreme nutrient restrictions.

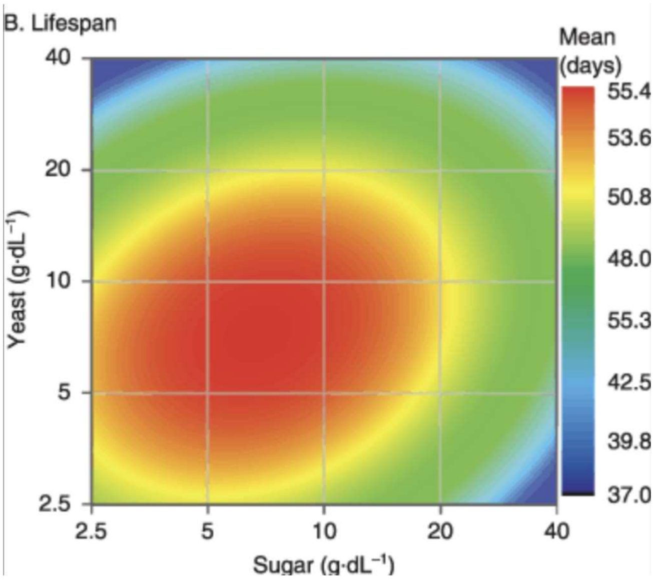

Figure 3 The lifespan of female flies treated with 25 diets after reproduction age. Their lifespan was maximized at around 100/100 g/L yeast/sucrose. They showed a wide range of lifespans under various nutritional combination conditions, and especially when the amount of sugar and yeast was extremely high, or one of them was too low/high, their lifespan was reduced to 66% of the maximum. When yeast concentration is 70g/L, it shows the broadest range of sugar concentrations living longer than 50 days (Skorupa et al., 2008).

In addition, the stress-induced release of heat shock proteins due to the larval density condition may influence the lifespan in adulthood of *D. melanogaster* (Linford et al., 2013).

These studies, one focusing on genetic variation and the other on environmental factors, emphasize the interaction of diet and genetics in determining lifespan. Based on these findings, this study aims to investigate these factors and their combined effects on lifespan.

Experimental design

Based on these foundational studies and reasons, this experiment is designed to evaluate the effects of environmental conditions, sugar intake, and genetic variations on the lifespan of *D. melanogaster*. The experimental factors are chosen to reproducibly reflect the significant influences such as socioeconomic status, cultural background, and genetic variation in each sample group.

Sugar levels (cultural background): 12,5g, 25g, 50g, 100g, and 200g

Five sugar concentrations were selected to create a range of dietary conditions, from suboptimal to excessive intake. This design was influenced by prior studies demonstrating that sugar levels significantly impact lifespan.

Larval Density (Socioeconomic status): High and low developmental density

Two developmental larval population densities were used to evaluate the role of stress from density. Developmental stress is likely associated with changes in metabolic output and lifespan. To account for these conditions, every population line of *D. melanogaster* was reared under high- and low-density conditions during its developmental stage.

Sex (Genetic variation): Female and male

Both male and female flies were included to examine sex-specific responses, given evidence of differing metabolic syndrome ratios in the human study and physiological demands between the sexes in the *D. melanogaster* study.

population lines (Genetic variation): CE2, CE3, CL3

Three genetically distinct population lines were selected to investigate genetic variation among different lines. These lines, developed through long-term EE (evolutionary evolved) experiments, represent diverse geographic origins and genetic backgrounds.

Genetic loci (Genetic variation): *Eip75B* and *Liprin gamma*

Two loci were selected from the six loci identified in large-scale genotyping studies involving CE3 female populations, where they showed independent significance in lifespan regulation and an interaction effect on lifespan (Joost van den Heuvel (in prep)). Genotyped data can measure inter-individual variation, providing powerful tools for elucidating genetic differences between lines and their interactions.

This proposed study design will certainly determine whether genetic locus variation among individuals within the same sugar intake group significantly affects lifespan. It will also elucidate the optimal sugar intake for each *D. melanogaster* population under specific environmental conditions and sexes that maximize lifespan. Furthermore, if multiple loci are successfully analyzed, this study will allow for the identification of locus-to-locus interactions that contribute to lifespan variation across populations.

For these questions, it was hypothesized that when *D. melanogaster* individuals are fed a different-sugar diet from adulthood compared to a standard control diet (70g of yeast, 100g of sugar, 20g of agar, 10 mL of nipagine (antifungal agent), and 3 mL of propionic acid (antibacterial agent) per liter of water), the effects on lifespan will vary depending on the larval conditions they are exposed to and that the optimal sugar level required for longevity in *D. melanogaster* will vary depending on genetic differences, and sex.

This study aims to appropriately apply genetic and environmental factors affecting the lifespan of the D. melanogaster level and to confirm their effects and interactions through phenotyping and genotyping, thereby challenging general dietary guidelines such as '30g daily sugar intake per day' and emphasizing the need for personalized sugar intake strategies.

Method

Fly maintenance and Dietary Condition

The preparation of the experiment began with flies maintained under two density conditions during the development period. In the high-density condition, approximately 1,000 to 1,500 flies were reared in 500 mL bottles (Large Bottle, LB), while in the low-density condition, 200 to 250 flies were reared in 250 mL bottles (Small Bottle, SB). During this period, all flies were fed with a standard control diet consisting of 100g of sugar, 70g of yeast, 20g of agar, 10 mL of Nipagine, and 3 mL of propionic acid per 1L of water. The maternal effects were minimized by standardizing flies during two generations under controlled laboratory conditions (25°C, 50% humidity, and a 12-hour light-dark cycle).

The experiment began when flies from three *D. melanogaster* population lines reached their reproductive age. On the same day, all flies were sex-separated under CO2 anesthesia and randomly assigned to bottles containing one of five sugar diets. Each bottle was prepared with 50 mL of food, containing each sugar amount, 70g of yeast, 20g of agar, 10 mL of Nipagine, and 3 mL of propionic acid per 1L of water. The food was stored at 4°C for up to 4 weeks and prepared in the laboratory for 2-3 hours before using it to prevent temperature-related effects.

After short anesthesia, 100 flies were transferred into each 250 mL bottle, with each sugar condition represented across three lines, two sexes, and two density conditions, resulting in 60 unique combinations. A total of 6,000 flies were used in the experiment, with each condition initially containing 100 flies.

Phenotyping

Throughout the experiment, flies were maintained under controlled laboratory conditions. Food was replaced every 2-3 days to maintain optimal living conditions. Dead flies were collected during each transfer, and their lifespan, sugar concentration, sex, line, and density condition were recorded. Bodies were preserved in deep well plates at -18°C for subsequent genotypic analysis. By the end of the experiment, lifespan data were collected for 4,814 flies, accounting for a 20% loss due to transfer errors and early deaths not by natural cause.

Genotyping

DNA was extracted from the preserved flies using a Dirty & Crude DNA extraction protocol (protocol in Appendix). The Kompetitive Allele Specific PCR (KASP) genotyping method (Protocol in Appendix) was employed to determine the genetic variants of *Eip75B* and *Liprin gamma* in each sample using primers (table 1).

Table 1 Primer sequences of *Eip75B* and *Liprin gamma* used for KASP genotyping

	FAM	HEX	Common
Eip75B	GAAGGTGACCAAGTTCATGCTGGCGCCAAAGCTTTCC (G)	GAAGGTCGGAGTCAACGGATTAGGCGCCAAAGCTTTC (T)	TACAGTTTGTCGCACTGCTTGCATG
Liprin gamma	GAAGGTGACCAAGTTCATGCTGGCCAGGACCTCGACT	GAAGGTCGGAGTCAACGGATTGGCCAGGACCTCGACT	ATTAAATTCGTSCCTGTCTGCCTGG

Data analysis

Data from lifespan (Phenotypic data) and genotyping data were analyzed using R software. One-way ANOVA and Two-way ANOVA were used to test for checking significant differences among experimental conditions. A survival curve was drawn using lifespan data with the Kaplan-Meier method, and other graphical visualizations were drawn to present key findings, showing the relationships between sugar intake, genetic variation, and lifespan.

Result

After all the individuals that died naturally, how sugar intake, environmental factors, and sex interact to influence lifespan in *D. melanogaster* could be confirmed. Then, by examining lifespan as phenotypic data and genotypic data from extracted DNA, the independent and combined effects were analyzed thoroughly.

The initial step of this experiment involved examining the lifespan data of *D. melanogaster*. The survival curve below was constructed using lifespan data collected from three distinct lines of *D. melanogaster* (CE2, CE3, CL3). The survival analysis revealed that the late reproduction line, CL3, exhibited the longest mean lifespan, followed by CE2 and CE3. While CE2 and CE3 initially showed similar survival rates, the CE3 line exhibited a more rapid decline after 75% survival (Figure 4). These findings suggest inherent genetic and physiological differences between the lines, which could influence their responses to dietary and environmental conditions.

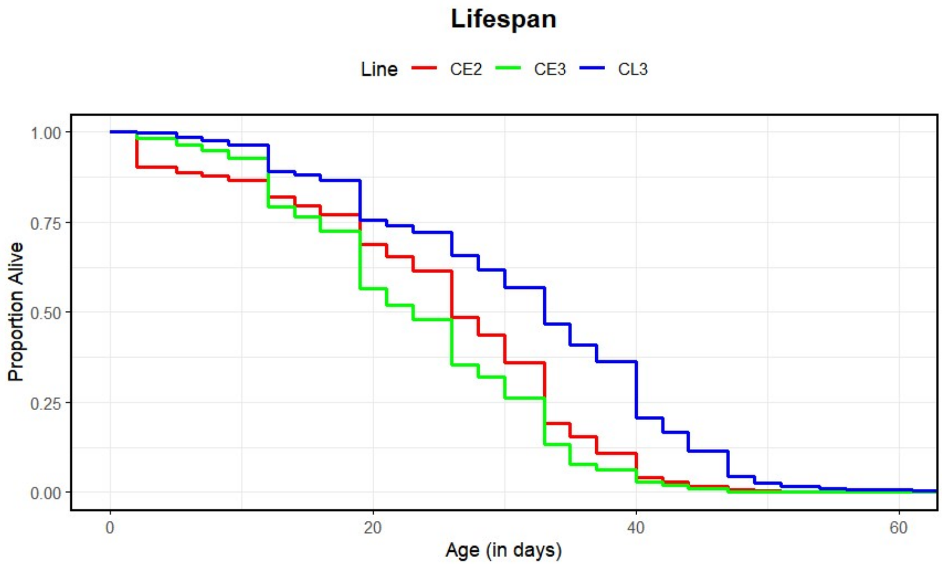

Figure 4 Survival curves of *D. melanogaster* lines. The lifespan differences among the lines were statistically significant (p-value < 0.0001). The CL3 line demonstrated the longest overall survival, while CE3 and CE2 showed similar patterns up to the 75% survival point. Beyond this point, the CE3 line showed a more rapid decrease. By approximately day 40, most individuals in CE2 and CE3 had died, and in CL3, around day 50.

As suggested above, the differences in survival rates across lines clearly illustrate the inherent variations among the lines. At the same time, it also shows a substantial lifespan gap among individuals within the same line. Despite belonging to the same line, significant inter-individual differences exist.

Independent effect of factors on lifespan

Sugar level – lifespan

Lifespan was significantly affected by sugar intake. Increasing sugar levels were associated with longer mean lifespans ($p < 0.0001$), although the relationship was non-linear. The lowest mean lifespan was observed at 12.5 g/L (21.8 days), while the highest was at 200 g/L (30.5 days). Differences between intermediate sugar levels (25g/L to 100g/L) were minor compared to extreme sugar conditions (Figure 5).

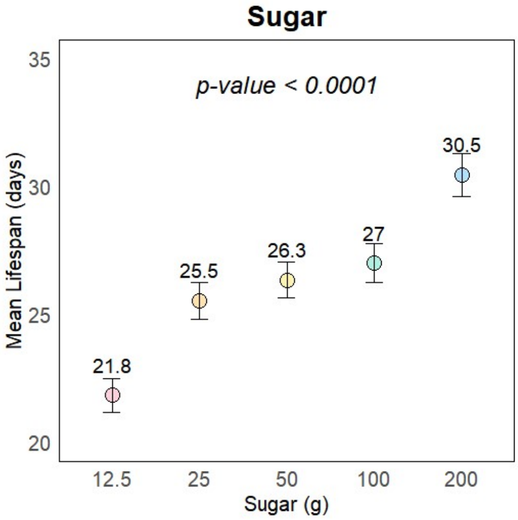

Figure 5 Interaction between sugar and mean lifespan. Mean lifespan increases with sugar concentration, showing the lowest value at 12.5g (21.8 days) and the highest at 200g (30.5 days). The progression is non-linear, with more considerable differences observed between extreme concentrations than intermediate sugar levels.

Line – lifespan

As we already seen in the first result section, significant differences are observed in the mean lifespan of each line (p-value < 0.0001), with the CL3 clearly showing a longer lifespan compared to the other two lines, a 6.4 day longer lifespan relative to the CE2 line and an 8.2 day to CE3 line (figure 6).

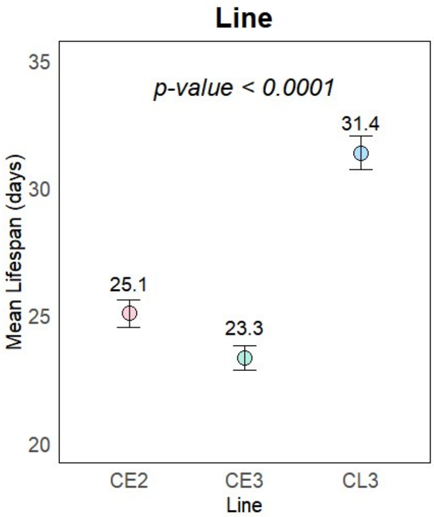

Figure 6 Mean lifespan differences across three lines showed significant (p-value < 0.0001), with CL3 exhibiting the longest mean lifespan (31.4 days) and CE3 the shortest (23.3 days).

Sex – lifespan

Sex also shows a significant role in lifespan by the difference observed between females and males (p-value < 0.0001). Females lived significantly longer than males, with mean lifespans of 28.7 and 23.7 days, respectively (figure 7).

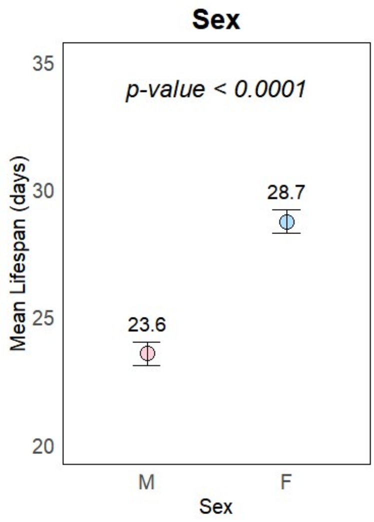

Figure 7 Mean lifespan comparison between sexes shows remarkable difference, with females living longer than males. This gap indicates the influence of sex-specific physiological or genetic factors on lifespan.

<u>Density – lifespan</u>

Developmental density significantly influenced lifespan (p-value < 0.0001). Flies reared in less crowded conditions (low density, SB) had a mean lifespan of 28.8 days, which was 5.4 days longer than those reared in more crowded conditions (high density, LB) (figure 8).

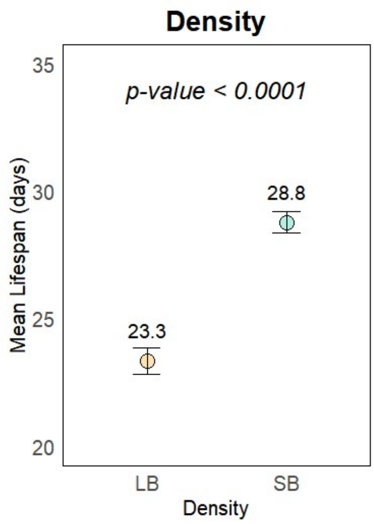

Figure 8 Developmental density conditions impact lifespan, with flies raised in low-density conditions (SB) living longer than those in high-density conditions (LB). At the level of developmental density used in this study, either a high-density condition acts as a stressor that harms lifespan, or low-density has a positive effect on lifespan.

<u>Eip75B – lifespan</u>

Eip75B, previously studied for its potential involvement in lifespan regulation mainly in female *D. melanogaster*, was analyzed in this study using genotyping. However, unlike other factors mentioned earlier, the *Eip75B* locus was found to have no statistically significant effect on lifespan. This locus includes three genotypes: GG, GT and TT. While there were Although there were observable differences in mean lifespan between genotypes, the p-value was 0.0973, failing to meet the threshold of statistical significance ($p < 0.05$).

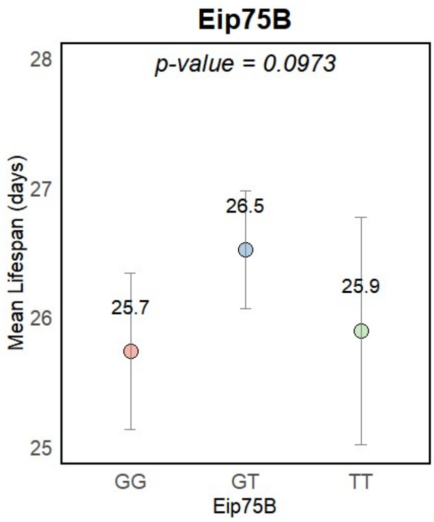

Figure 9 Genotypes of the *Eip75B* locus (GG, GT, TT) showed no statistically significant difference in lifespan (p = 0.0973). The GT genotype exhibited the longest mean lifespan (26.5 days), followed by TT (25.9 days) and GG (25.7 days). The gap between the longest-living heterozygote genotype and shortest-living homozygote GG genotype was only 0.8 days.

Liprin-gamma – lifespan

Liprin-gamma, another locus previously studied and analyzed in this study, exhibited a significant association with lifespan. Statistical analysis confirmed that individuals with the CC and CT genotypes lived significantly longer than those with the TT genotype. CC and CT genotypes lived an average of 26.5 and 26.7 days, respectively, while those with the TT genotypes lived 25.7 days. The lifespan difference between the longest-living CT genotype and the shortest-living TT genotype was 1.6 days.

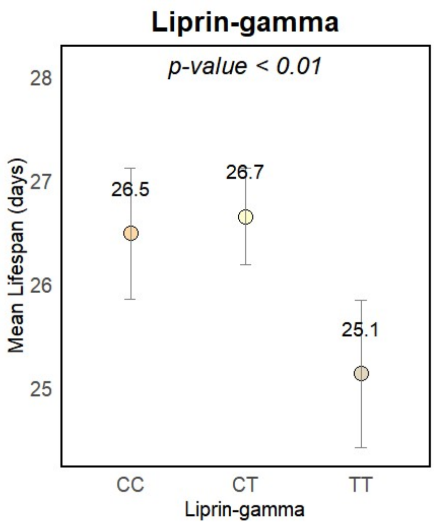

Figure 10 *Liprin-gamma* locus was confirmed to have the genotypes of CC, CT, and TT and demonstrated statistically significant differences in lifespan. The lifespan difference between the longest-living CT genotype and the shortest-living TT genotype was 1.6 days. However, the observed effect size was relatively smaller compared to other independent factors such as sugar levels, sex, density, and line.

In this sub-result, the above results re-confirm what previous studies found and open the possibility that all these factors can significantly affect lifespan in the subgroups and interact with each other.

Sugar condition dependent effects on lifespan

To study the dependent effects of various factors on lifespan, a two-way ANOVA was conducted for each factor. This analysis mainly focused on the interactions between sugar intake amount as a controllable factors and other factors. The analysis assessed whether each factor influenced lifespan, whether interactions between factors existed, and the significance of these interactions.

<u>Sugar - line interaction for lifespan</u>

Table 2 Two-way ANOVA result of Sugar - Line

	Df	Sum Sq	Mean Sq	F-value	P-value
Sugar	4	35780	8945	79.92	< 2e-16***
Line	2	51472	25736	229.96	< 2e-16***
Sugar : Line	8	19818	2477	22.74	< 2e-16***
Residuals	4518	505640	112		

The two-way ANOVA results (Table 2) indicate that both sugar intake and population strain independently and significantly influence lifespan ($p < 0.0001$). Furthermore, their interaction also showed a significant effect on lifespan ($p < 0.0001$). Among these factors, line had the greatest impact, reflecting substantial lifespan differences among the three strains tested in this study.

The CE2 line does not show a significant effect of sugar intake on lifespan (p-value = 0.233). The outstanding difference observed in the CE2 line is the highest mean lifespan for this group, in 25 g/L sugar, making a difference compared with the proportional trends observed in the other lines (figure 9).

All individuals were raised on a 100g sugar condition during their developmental period, and the CE2 line showed a greater lifespan advantage when their sugar intake was reduced to 25 g/L (26.2 days) than when they remained constant (24.3 days). This lifespan gap suggests that sugar intake change stress did not significantly affect the lifespan of the CE2 group, indicating that stresses of sugar intake have a minimal effect on lifespan in the CE2 group. It also suggests that the increase in mean lifespan at 25 g/L (50 g/L) sugar and 200 g/L sugar was based on different mechanisms.

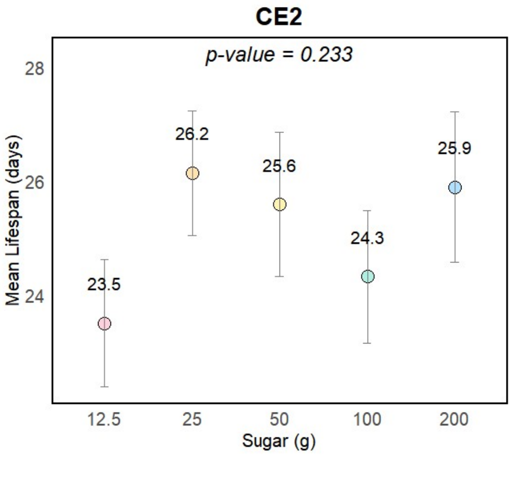

Figure 11 The CE2 line shows no significant relationship between sugar intake and lifespan, with the highest mean lifespan observed at 25g of sugar.

The CE3 and CL3 lines exhibited a significant correlation between sugar intake and lifespan (p-value < 0.0001). Despite differences in the mean lifespan observed at the highest and lowest sugar levels between the two lines, a shared trend emerges; both lines show the longest lifespan at the highest sugar level (200g) and the shortest lifespan at the lowest sugar level (12.5g) (figure 10).

For the CE3 line, the mean lifespan ranges from 17.3 days (12.5g/L sugar) to 28.9 days (200g/L sugar), giving an 11.6-day difference. Meanwhile, the CL3 line exhibited a broader gap, with a lifespan increasing from 24.1 days (12.5g/L sugar) to 39.4 days (200g/L sugar), representing a 15.3-day difference. These trends show that both the CE3 and CL3 lines have high sugar requirements and have not yet developed life-shortening diseases such as metabolic syndrome at the 200 g/L sugar level.

Therefore, the significant differences in lifespan patterns among the lines can be attributed to the distinct response of the CE2 line to sugar intake compared to the CE3 and CL3 lines.

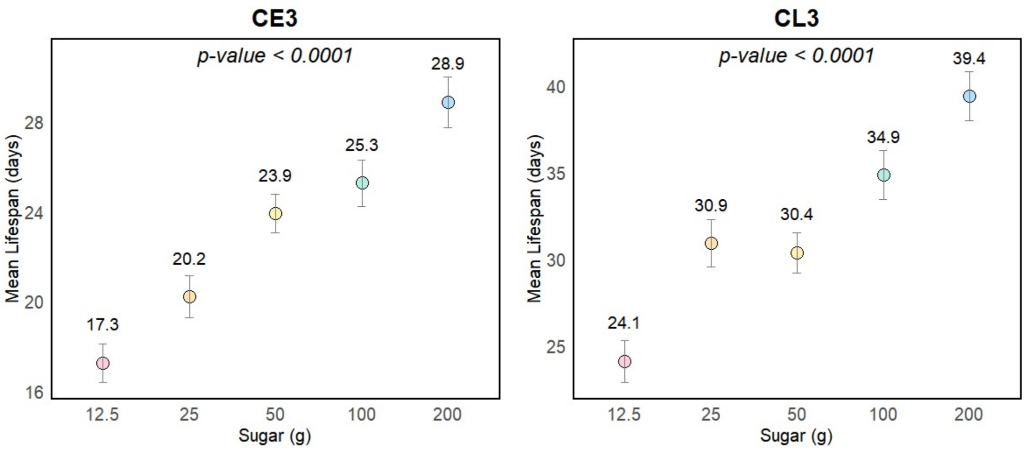

Figure 12 The CE3 and CL3 lines are significant (p-value < 0.0001) and demonstrate a strong positive correlation between sugar intake and lifespan. For both lines, the shortest lifespan is observed at the lowest sugar level (12.5g), while the longest occurs at the highest (200g).

<u>Sugar – sex interaction for lifespan</u>

Table 3 Two-way ANOVA result of sugar - sex

	Df	Sum Sq	Mean Sq	F-value	P-value
Sugar	4	35780	8945	77.28	< 2e-16***
Sex	1	29733	29733	256.87	< 2e-16***
Sugar : Sex	4	23648	5912	51.07	< 2e-16***
Residuals	4523	523549	116		

The two-way ANOVA results (Table 3) for sugar and sex indicate that both factors independently and significantly influence lifespan (p < 0.0001), like the effects observed with sugar and line. However, a key difference was in their interaction, which had more than double the significant effect on lifespan compared to sugar-line interaction. This suggests the potential involvement of sex-specific metabolic or physiological mechanisms under varying sugar conditions.

Both sexes show a significant relationship between sugar intake and lifespan (p-value < 0.0001). The gap between the maximum lifespan of both sexes is very minor, females (30.7 days) and males (30.5 days) in 200g of sugar. However, the difference between sexes was more pronounced at lower sugar levels, with women having almost twice as long a lifespan as men at 12.5 g/L sugar intake (figure 11). This could mean that females have a mechanism that allows them to survive longer in low-sugar conditions. This mechanism(s) seems to be more active in females or almost inactive in males drastically affecting their lifespan, especially in extremely low sugar conditions.

Additionally, males are more negatively affected by low amounts of sugar intake than females, showing a sharper reduction in lifespan. In contrast, females exhibited relatively consistent lifespans across the three lower sugar levels (12.5g, 25g, and 50g), indicating tolerance to low-sugar environments. This sex-specific variation in mean lifespan clearly shows the same sugar stress can be managed differently in males and females.

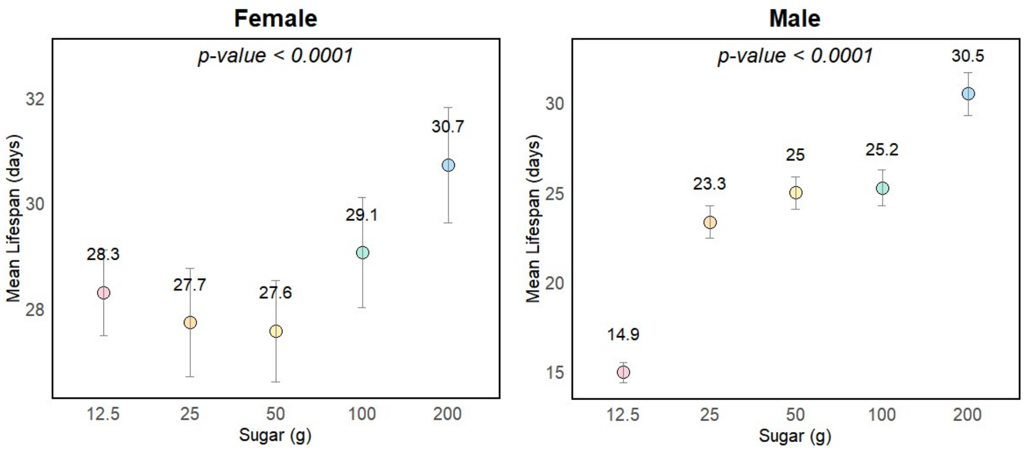

Figure 13 Males and females exhibit distinct responses to sugar intake, with significant differences in lifespan observed across levels but as different patterns. Both sexes show comparable lifespans at 200 g/L sugar (~30 days). However, females consistently outlive males at lower sugar levels, particularly at 12.5 g/L, where the gap reaches around 14 days. This highlights a substantial adverse effect of low sugar intake on males and females.

Sugar – density interaction for lifespan

Table 4 Two-way ANOVA result of sugar - density

	Df	Sum Sq	Mean Sq	F-value	P-value
Sugar	4	35780	8945	74.370	< 2e-16***
Density	1	31640	31640	263.060	< 2e-16***
Sugar : Density	4	1279	320	2.658	0.0311*
Residuals	4523	544011	120		

The ANOVA results for sugar and density (Table 4) show that both factors independently have strong and significant effects on lifespan. ($p < 0.0001$). While their interaction was statistically significant ($p = 0.0311$), the effect size was apparently smaller compared to earlier interactions in this study (sugar-line/sugar-sex). This indicates that the combined influence of sugar and density on lifespan is relatively minor.

In all sugar conditions, the SB group exhibits longer lifespans than the LB group, expressing the significant impact of developmental density stress on lifespan (p-value < 0.0001). The SB condition displayed relatively consistent lifespans across middle sugar levels (25g, 50g, and 100g). In contrast, the LB group exhibits a proportional relationship between sugar intake and lifespan. Across all sugar levels, SB flies lived longer by approximately 5 to 8 days compared to LB flies, with the gap remaining substantial and relatively consistent even at the highest/lowest sugar levels (figure 12).

This ultimately means that when *D. melanogaster* spends its developmental period at a relatively high density, it receives more stress at almost the same level across all sugar levels compared to low density. This uniform lifespan difference could imply that the mechanisms by which they respond to stress for density exist independently of the mechanisms that operate under sugar stress.

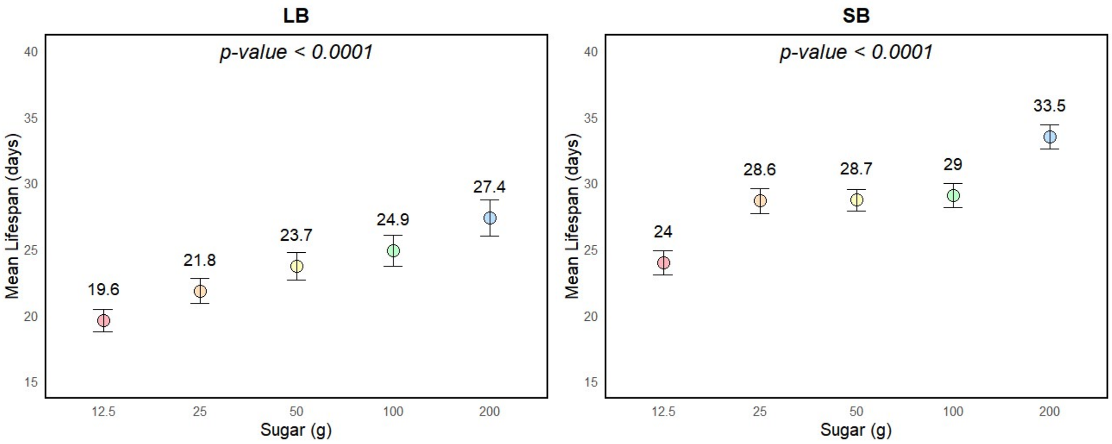

Figure 14 In both LB and SB groups, lifespan significantly correlated with sugar intake (p-value < 0.0001). SB flies consistently lived longer than LB flies across all sugar levels, with a gap of 5-8 days. While SB flies showed stable lifespans at mid-range sugar concentrations (25g, 50g, 100g), LB flies exhibited a proportional increase in lifespan.

Sugar – lifespan with Eip75 genotype

Table 5 Two-way ANOVA result of sugar – *Eip75B*

	Df	Sum Sq	Mean Sq	F-value	P-value
Sugar	4	35780	8945	70.922	< 2e-16***
Eip75B	2	1617	809	6.411	0.00166**
Sugar : *Eip75B*	8	5482	685	5.434	7.75e-07***
Residuals	4518	569830	126		

Result of the two-way ANOVA for sugar and *Eip75B* (Table 5) confirm that these factors interact significantly to regulate lifespan. While *Eip75B* was not significant as an independent factor in the one-way ANOVA, the two-way ANOVA result indicated that *Eip75B* has a significant and independent effect on lifespan ($p < 0.01$). In addition, the interaction between sugar and *Eip75B* locus is found showing significance ($p < 0.0001$), suggesting that the role of *Eip75B* in lifespan regulation related to sugar intake. This finding emphasizes the strong interaction between these two factors.

The plots for each genotype of *Eip75B* show a significant relationship between sugar intake and lifespan (p-value < 0.0001). GG and GT genotypes are almost the same as the entire sugar-lifespan trend, which suggests that GG and GT flies are consistently similarly influenced by sugar concentration in the population. However, the TT genotype presents a unique pattern. At 25g/L sugar, TT flies achieve 29.3 days lifespan, which is significantly higher than GG and GT genotypes in the same condition. However, at 100g, the TT genotype gets a noticeable drop in lifespan compared to the other genotypes (figure 13).

When each 100g/L sugar condition is considered a control for each genotype, it can be assumed that the specific advantage of the TT genotype is expressed at the 25-50 g/L sugar level. This advantage of the TT genotype is no longer shown at 12.5 g/L sugar, which may be because this sugar amount is insufficient to sustain this mechanism. In addition, the lifespan of all genotypes increases at 200 g/L sugar, which may mean this sugar amount corresponds to the point where the effect is much stronger than the advantage or disadvantage caused by having a specific genotype.

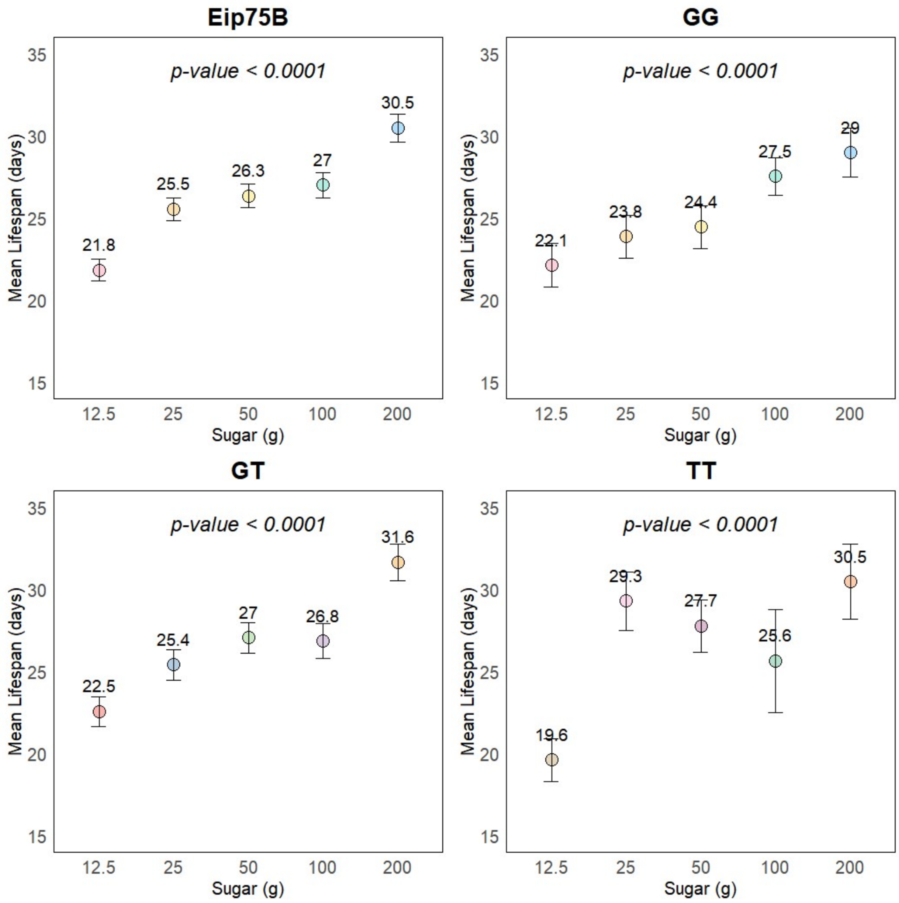

Figure 15 The *Eip75B* genotypes (GG, GT, TT) exhibit a significant correlation between sugar intake and lifespan (p-value < 0.0001). GG and GT genotypes follow similar trends, while the TT genotype shows unique patterns. At 25g, it achieves a lifespan comparable to 200g, however, at 100g, it has a huge drop before recovering at 200g. At 12.5g, all genotypes display reduced lifespans, with TT being the shortest.

sugar-lifespan with Liprin-gamma genotype

Table 6 Two-way ANOVA result of sugar – *Liprin-gamma*

	Df	Sum Sq	Mean Sq	F-value	P-value
Sugar	4	38425	9606	75.507	< 2e-16***
Liprin-gamma	2	1609	805	6.325	0.00181**
Sugar : *Liprin-gamma*	8	1847	231	1.815	0.06953
Residuals	4722	600743	127		

The ANOVA result for sugar and *Liprin-gamma* indicate that both sugar ($p < 0.0001$) and *Liprin-gamma* ($p < 0.01$) have significant and independent effects on lifespan (Table 6). However, in contrast to the *Eip75B* locus, the interaction between sugar and *Liprin-gamma* is not significant. This suggests that *Liprin-gamma* locus and sugar intake do not interact and that it acts in different way from *Eip75B*.

The lifespan trends associated with *Liprin-gamma* genotypes show less variation compared to *Eip75B*. Although a drop in lifespan is observed in the TT genotype at 100g sugar compared to the CC or CT genotypes, no distinctive lifespan difference is observed at the other sugar levels. Therefore, the effect of *Liprin-gamma* on lifespan is relatively minor.

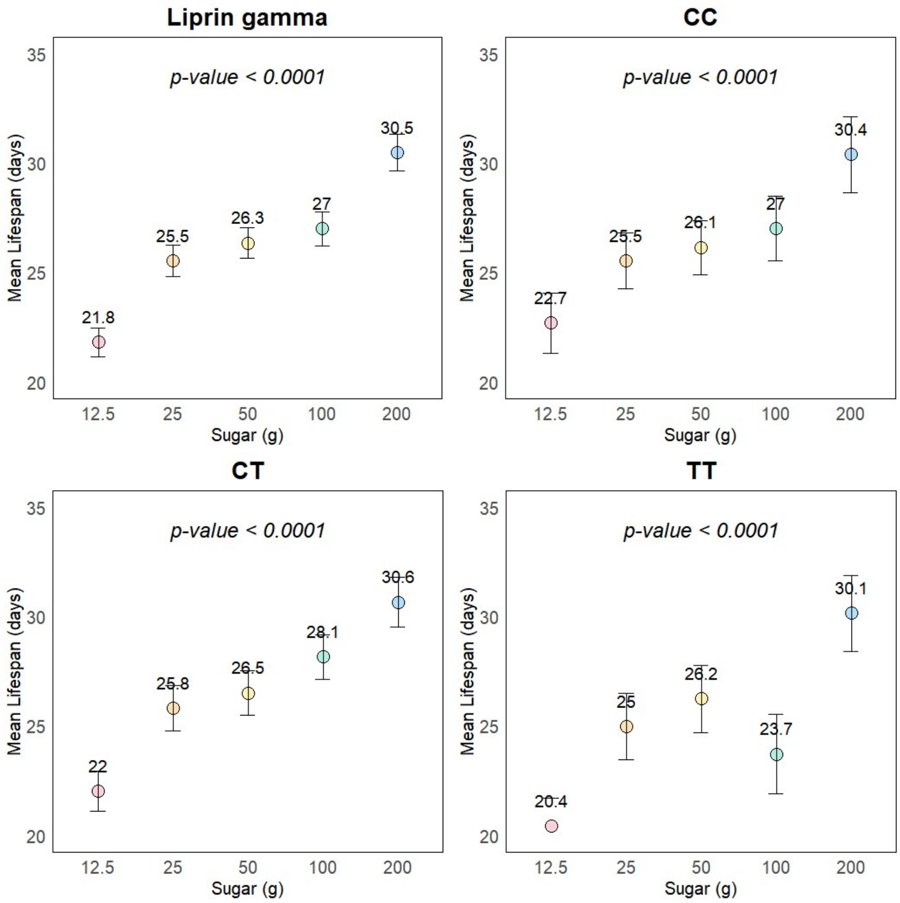

Figure 16 Lifespan trends for *Liprin-gamma* show a consistent increase in sugar levels for almost all except one notable drop at 100 g sugar in the TT genotype.

This section mainly examined how each independent factor interacts with five sugar conditions to influence lifespan. While the previous section centered on the singular effects of each factor, the focus here was on their interactions with sugar. The two-way ANOVA results revealed that line and sex exhibited stronger interactions with sugar than density, suggesting a greater sensitivity to sugar variations. Notably, *Eip75B*, which was insignificant in the one-way ANOVA, became significant when interacting with sugar, whereas *Liprin-gamma*, though significant independently, showed no significance in interaction with sugar.

Incorporated effect and calculation for optimal sugar amount

In the earlier section, the effects of each factor on lifespan were analyzed separately, and there were significant differences in lifespan depending on which subgroup they belonged to. Every individual used in the experiment had multiple of these factors simultaneously. These factors may operate through independent mechanisms or interact with each other.

This study hypothesized that the optimal sugar condition would vary under various factors of the individual, and to confirm this, the longest-living sugar amount was calculated based on the average lifespan of individuals in each condition and visualized in a heatmap (Figure 15). The heatmap clearly shows the differences in optimal sugar amounts, often driven by sex and environmental density. In some cases, individuals of the same population line exhibited markedly different optimal sugar levels under varying stress conditions. Conversely, there were cases where individuals of the same line showed the same optimal sugar concentration despite differences in the other factors. These patterns suggest that certain conditions, such as genetic background or population line, may have a stronger effect on lifespan than others. For example, in the CL3 line, 200 g/L sugar was identified as an optimal sugar level that maximized lifespan, mainly in males, suggesting that they may have a genetic background optimized for higher sugar concentrations. In contrast, individuals of the CE2 line consistently showed optimal lifespans at relatively lower sugar concentrations (12.5 – 50 g/L) in most cases.

Moreover, the results suggest that genotypic differences, particularly in *Eip75B* and *Liprin-gamma*, exerted stronger influences on females than on males.

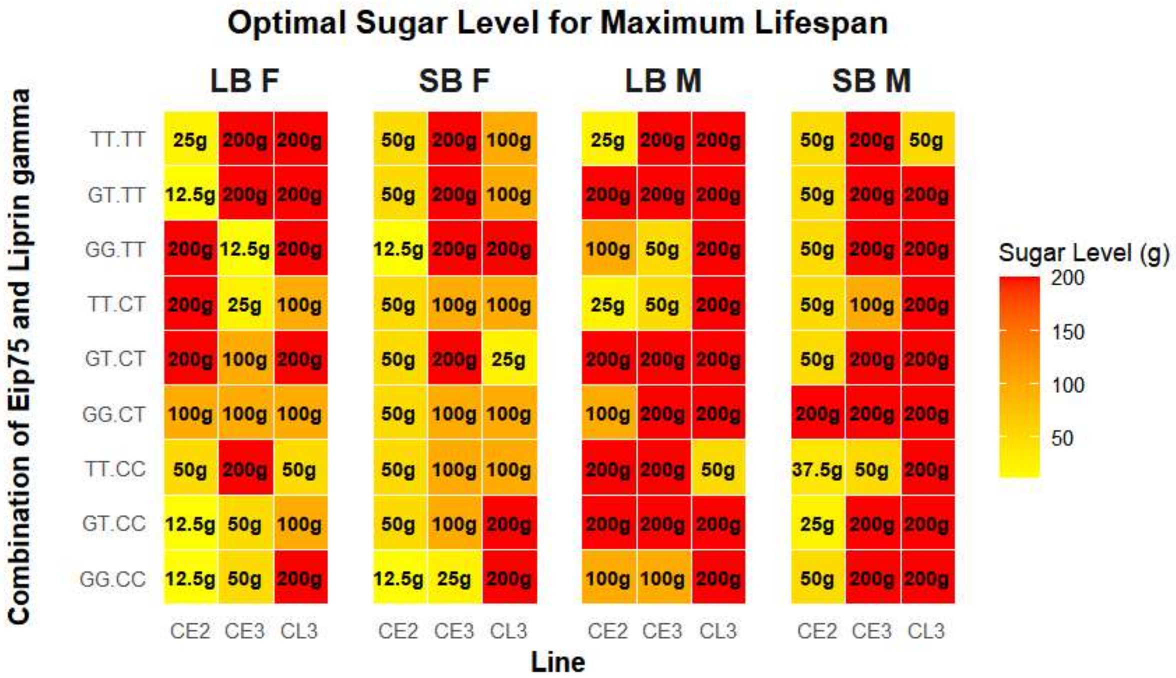

Figure 17 The heatmap shows optimal sugar levels for an average lifespan with different genotypes, sexes, densities, and line combinations. Most conditions favor 200g sugar, particularly in CL3. Lower levels like 12.5g and 25g are optimal in some CE2 and CE3 cases. The SB M panel includes a unique 38.5 midpoint, reflecting dual-optimal conditions at 25g and 50g. This result emphasizes how genotype and environment influence sugar-lifespan relationships.

Interaction between Genotype and Lifespan across different sugar levels

<u>Eip75B</u>

Only the 100g sugar group did not show significant differences among genotypes among the five sugar conditions. In all other sugar groups, statistically significant differences were observed. The reason for the lack of lifespan variation at 100g sugar has yet to be studied, but it may be due to long-term intergenerational maintenance at this sugar concentration. This stabilization might have minimized genotype-specific variability in lifespan. Alternatively, 100g of sugar could represent a threshold where *Eip75B*'s role in the IIS shifts, alleviating its influence on lifespan differentiation.

Under the 12.5g sugar condition, the GT genotype showed the most extended mean lifespan, but the gaps between genotypes were relatively small. At 25g and 50g sugar levels, the TT genotype exhibited the most extended mean lifespan, showing a 5-day and 3-day longer life, respectively, compared to the GG genotype, which had the shortest mean lifespan. However, at 100g sugar level, as already mentioned, the mean lifespans of all genotypes were gathered, with no significant differences observed. At 200g sugar, all genotypes experienced an increase in lifespan to over 30 days, with no significant differences between them.

A detailed analysis reveals distinct genotype-specific trends. The GT genotype showed consistent mean lifespan values across the 25-100 g/L sugar range, indicating a certain level of stability regardless of sugar levels. The results of the GG genotype gave an increase in mean lifespan as sugar intake increased, suggesting a preference for higher sugar levels. Conversely, the TT genotype slightly declined in mean lifespan as sugar levels increased from 25g to 100g. Interestingly, at 200g sugar, the TT genotype demonstrated a marked recovery in their lifespan. This substantial increase may reflect a metabolic shift or activation of compensatory mechanisms at higher sugar levels, possibly linked to *Eip75B*'s regulatory role in insulin signaling pathways (Herboso et al., 2015).

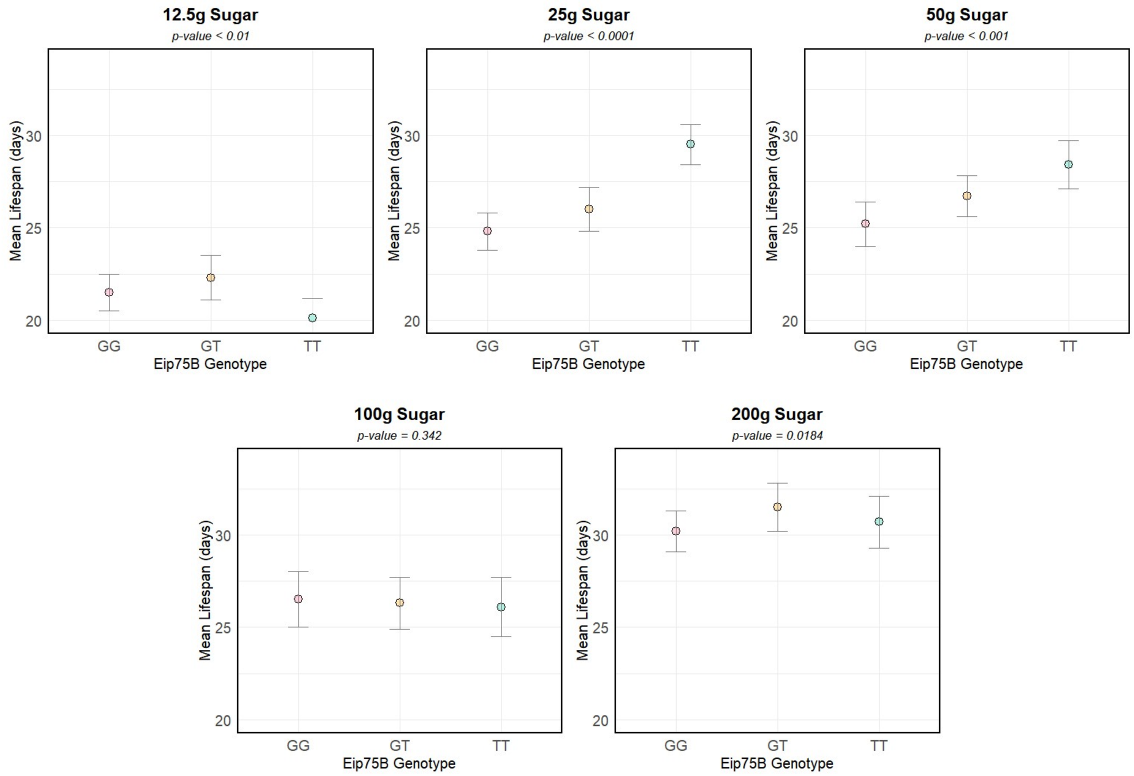

Figure 18 The figures depict the relationship between *Eip75B* genotypes and mean lifespan across five sugar levels. Significant differences between genotypes were observed in four panels (p-value < 0.05) except 100g sugar (p-value = 0.342).

<u>Liprin gamma</u>

Liprin-gamma showed statistical significance only at the 12.5g and 100g sugar conditions, with the 100g sugar level being significant (p-value < 0.0001). No significant differences were observed at the other sugar levels, especially 25g, 50g, and 200g, and lifespan variations across genotypes were minimal.

At 12.5g sugar, the TT genotype exhibited the lowest mean lifespan, while the CC and CT genotypes showed similar survival durations. The mean lifespan of CC and CT genotypes was approximately 2 days longer than that of TT genotypes. For the 100g sugar condition, the distinction between genotypes became more pronounced than the other sugar groups. The TT genotype again showed the shortest mean lifespan, while CC and CT genotypes displayed approximately four days longer lifespans.

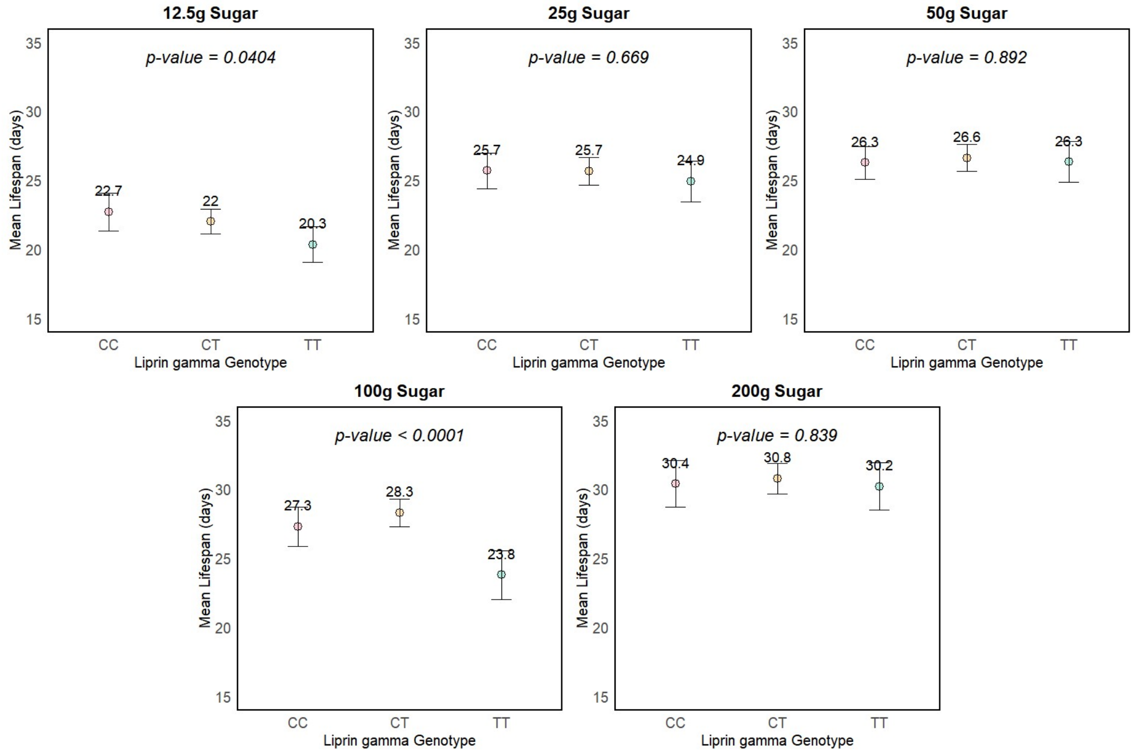

Figure 19 *Liprin-gamma* shows significant differences at 12.5g and 100g sugar. TT genotypes consistently have the shortest lifespan across all sugar amounts, with CC and CT genotypes living 2-4 days longer. No notable differences are observed at 25g, 50g, or 200g sugar levels.

Loci-loci interaction on lifespan

Figure 18 (left) plot is from Joost van den Heuvel's preliminary work, and it showed a correlation between *Liprin-gamma* and Eip75B with significance (p-value < 0.0001). When *Eip75B* had a TT genotype, and *Liprin-gamma* had CT or TT genotypes, lifespan differences of more than seven days were observed.

Based on all samples, the results confirmed the significance between the genotypes of *Liprin-gamma* and *Eip75B*. However, it was less significant than previous work, and the lifespan gap between each genotype-genotype combination did not stand out (Figure 18 (middle)). Additional analysis was conducted with the same population line with the same conditions (100g sugar, female) as Joost's previous work. While significance was not achieved, the p-value was near 0.05, and similar patterns were observed in the *Eip75B* TT genotype. A significant difference was identified when *Eip75B* had the GT genotype, showing a drastic lifespan reduction of approximately eight days in the *Liprin-gamma* TT genotype (Figure 18 (right)).

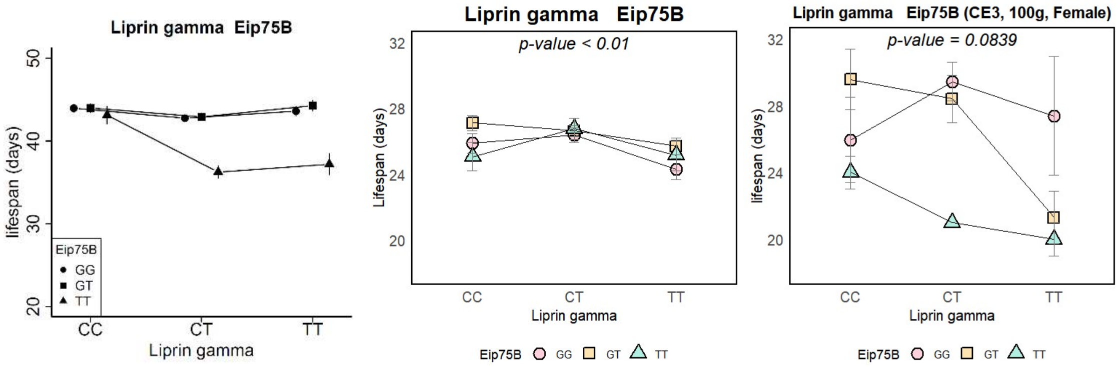

Figure 20 The three plots examine the interaction between *Liprin-gamma* and *Eip75B* genotypes. The left interaction is significant (p-value < 0.0001), especially showing lifespan gaps between the other combinations and *Eip75B* TT and *Liprin-gamma* CT/TT genotypes. The broader dataset (middle) confirmed the significance but with less distinction. Under the same condition as the left panel's condition, a similar pattern in the *Eip75B* TT genotype and different patterns on other genotypes were seen in the right panel.

Discussion

Non-proportional lifespan responses in CE2 line

Unlike CE3 and CL3 lines, which displayed proportional changes in lifespan with increasing sugar levels (Figure 12), the CE2 line exhibited a distinct non-proportional response (Figure 11). When CE2 line separated by sex, a clear sexual dimorphism was shown with females and males responding differently to sugar concentration (Figure 21, left). This finding is consistent with previous studies on sex-based lifespan differences in dietary sugar responses, suggesting underlying genetic mechanisms (Chandegra et al., 2017). In this previous study of sexual dimorphism effect on lifespan, they used the fruit flies from Dahomey stock, suggesting that the mechanism by which sugar influences lifespan may operate in a similar way in CE2 and Dahomey stocks.

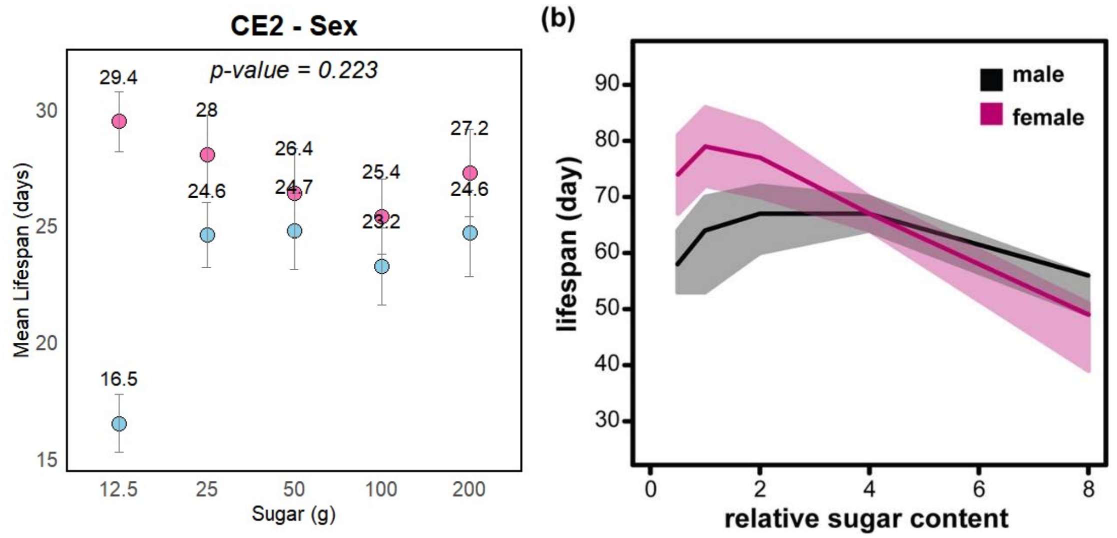

Figure 21 (Left) Average lifespan graph of CE2 line individuals sorted by sex. Females show the longest lifespan (29.4 days) at the lowest sugar level (12.5 g), while males show the shortest lifespan (16.5 days). Females show a decreasing pattern of lifespan up to 100g of sugar and then slightly increasing in 200g of sugar, while males maintain an equivalent lifespan at the remaining levels. (Right) Graph showing sexual dimorphism in lifespan according to sugar level in *D. melanogaster*. The section corresponding to 0.5x – 4x sugar (25g – 200g of sugar) of this graph shows a pattern similar to that shown in the experimental results of CE2 line. (1x = 50g of sugar / 1 L of water, the graph used sugar concentration of 0.5x - 8x) (Chandegra et al., 2017)

Lifespan Increase at the highest sugar level

In this experiment, lifespan showed a consistent increasing trend with sugar concentration, giving the highest lifespan averages at 200g sugar. This was not simply observed in one specific condition or group, but when all fruit flies were divided into each detailed standard and applied, it was confirmed that they had the longest average lifespan very uniformly. This result contrasts with earlier studies cited in the introduction, which indicated lifespan declines at sugar concentration exceeding 100g.

During the experiment, the flies in the 200g sugar group were found to fly very quickly and showed hyperactivity in moving inside the bottle. They showed much more movement (flying, crawling) than the other sugar groups, which can consume calories from sugar. In fact, a study in *D. melanogaster* using a Western-style diet high in saturated fat, sugar and salt showed that regular flight training significantly counteracted the adverse effects of the diet such as triglyceride increasing and mitochondrial dysfunction (Murashov et al., 2020). Therefore, it is possible that the excess calories from sugar in the 200g group were commonly counteracted by the hyperactivity acting as a therapeutic intervention.

Limitation of experiment

Sample loss and experimental setup

This experiment started with 6,000 flies, randomly assigning 100 *D. melanogaster* individuals to each of the 60 conditions. However, only 4,814 flies were identified during the collecting steps for phenotypic analysis, indicating around 20% of the sample was lost during the experiment due to accidental loss in handling or early death by the experimenter. As a result, genotyping and phenotyping were not performed on these missing individuals, inevitably impacting the statistical analysis and conclusions. Future studies should consider increasing the initial sample size to accommodate potential loss to address these issues. To achieve a reliable number of 100 individuals per condition after losses (Kennedy, 2021), the starting sample size should be approximately 125 flies per condition.

Lack of replicates and statistical reliability

The absence of replicate bottles limited reliability. Replicate bottles were not included because there were practical limitations in allowing one experimenter to handle 60 different conditions. Including replicate bottles would allow for comparisons between bottles and obtaining averages between them, which would reduce variability and increase the reliability of the data.

Unregulated Dietary intake

Food consumption by individual flies was not measured, introducing potential variability in dietary intake across groups. If an effective method to control the amount of food provided to each fly, or a reliable way to track their food intake were implemented, accurate and consistent results would be made.

Conclusion

The experiment tested two hypotheses: When *D. melanogaster* individuals are fed a sugar diet different from the standard control diet after their reproductive age, the effects on lifespan will vary depending on the conditions they are exposed to. Furthermore, the optimal sugar level required for longevity in *D. melanogaster* will differ depending on genetic differences, sex, and developmental density conditions.

The results demonstrated that despite being raised on the same sugar level during the development period, variations in the adult diet can lead to significant differences in lifespan. The optimal sugar conditions for a longer lifespan differed according to internal (genotypic/sex) and external (sugar/density) factors.

In this experiment, reducing sugar intake showed consistent effects under certain conditions, but it does not always work in the same way. Because there is considerable genetic variation even within the same species, and various environmental or sex-specific factors can cause noticeable differences between population lines. These findings suggest the limitations of applying uniform dietary guidelines to all individuals. Instead, more effective dietary recommendations may need to consider an individual's genetic background and environmental condition. While this experiment did not investigate all previously studied candidate loci related to lifespan, further research is required to validate these associations and assess the feasibility of this approach in human models.

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Appendix

Appendix 1

1. Physical and crude DNA extraction

Solutions:

Squishing buffer 1L contains

- 100mL of 1M Tris HCL pH 8,0
- 20mL of 0.5M EDTA
- 50mL of 5M NaCl
- 830mL of MilliQ

For long-term use, autoclave

Protocol:

1. Fill two deep 96-well plates (with fly sample) with 3-5 beads per well.
2. Put a plastic lid on it and close it down with a hammer in one direction. Use duct tape to secure the lid to the deep well plate so it does not fall off.
3. Use aluminum plate holders for the shaking machine, turn the screw clockwise to secure it firmly, and fix it.
4. Make sure that they are tightly stuck in the machine parts. Set shaking to 30x per second for one minute. Turn plates and repeat.
5. Centrifuge 2 plates 1 min at ±3000 rpm, and check whether any fly leftover is stuck to the lid. If there is leftover, repeat centrifugation.
6. Add 200 µL of squishing buffer to each well and close the plate with the lid again using a hammer and duct tape.
7. Put plates in a water bath at 56°C degrees for half an hour, then spin shortly at 3000 rpm, 1 min maximum for the debris on the lid, and incubate for another 30 minutes in the water bath.
8. Spin shortly again.
9. Open the lid, pull 100 µL up, and add that to the half-skirted plate (DNA stock).
10. Dilute 10x by adding 10 µL of DNA stock to 90 µL of distilled water (MilliQ) in the new half-skirted plate: 10x diluted DNA.
11. Dilute 100x by adding 10 µL of 10x diluted DNA to 90 µL of distilled water (MilliQ) in the new half-skirted plate: 100x diluted DNA.

Appendix 2

A test sample should be prepared before conducting KASP. For test samples, the required materials and reagents are as follows.

1. Materials
   A. PCR plate 96well (½ skirt) (transparent, 10x), Cat No. 652290, Greiner Bio-One
   B. 96×0.1 ml plate, 96 well plate (0.1 mL) (white, box of 25), Cat No. B17489, Bioplastics
   C. Microseal® 'B' seals, Cat No. MSB1001, BIO-RAD
   D. PCR plate ALU seal, Cat No. 676090, Greiner Bio-One
   E. PCR 8-strip tubes (0.2 ml) WITH caps, Cat No. 608271, Greiner Bio-One
   F. 1000 ㎕ Graduated Tip, Cat No. S1111-6701, Starlab
   G. 200 ㎕ Graduated Tip, Cat No. S1111-1706, Starlab
   H. 10 ㎕ Graduated Tip, Cat No. S1111-3700, Starlab
   I. 0.5 mL tube (Autoclaved)
   J. CFX96™ Real-Time System, BIO-RAD
   K. Centrifuge
   L. Use the same or equivalent material as the above item.
2. Reagents
   A. KASP mix (Mastermix)
   B. Primers (COM/FAM/HEX), Biolegio
   C. Distilled Water
   D. Use the same or equivalent reagent as the above item.
3. Test samples
   A. Take out all the ingredients required for the experiment in advance and thaw them at room temperature.
   B. If the DNA sample is stock or 10x diluted DNA, dilute it to 100x.
      i. Use a multi-channel pipette, and add 90 ㎕ DW to all 96 wells (½ skirt plate).
      ii. Use a multi-pipette to dilute 10 ㎕ DNA into each well by 1/10.
      iii. Repeat the above process if the DNA sample needs to be diluted again.
   C. Mix primer and KASP mix (master mix).
      i. Each primer is used after dilution to 10x in DW. Dilute all kinds of primers to 10x.
      ii. Mix KASP mix(master mix) and 10x diluted primers firmly in a 0.5 ml tube according to the number of plates.

	One plate (㎕)	Two plates (㎕)
FAM	8,8	4,4
HEX	8,8	4,4
COM	17,6	8,8
KASP MIX (master mix)	1100	550

   D. Dispense 5 ㎕ of the mixture of primers and the KASP mix(master mix) to 96 well plate(white).
   E. Dispense 5 ㎕ of 100x diluted DNA into 96-well plates while changing the pipette tips.
   F. When each 100x diluted DNA sample and primer mixture are loaded to 10 ㎕ per well, attach the micro seal 'B' seal well to the plate and proceed with the centrifuge shortly.

Conducting Test

1. Turn on the CFX96™ Real-Time System machine and adequately position the plate for analysis.
2. Check if the status of the test protocol to be performed and the sample to be tested is set to unknown.
3. Close the lid of the machine with the 'lid close' button and click when the start button is activated.
4. Set the name in the folder for saving the result and save it to start the analysis.

Tips

* After checking the signal by HEX and FAM and if the signal is not strong enough to give a clear result, readjust their amount later when preparing the sample again.

** If the result is unclear (not dispersed enough), increase cycle numbers to analyze it.