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PETUNIDIN-3-GLUCOSIDE SUPPLEMENTATION CAUSES SEX-SPECIFIC EFFECTS ON THE LIFESPAN AND MOTOR FUNCTION IN *Drosophila melanogaster*

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ABSTRACT

Petunidin-3-glucoside (P3G), an anthocyanin prevalent in vegetables, fruits, and wine, possesses noteworthy antioxidant properties attributed to its capacity to scavenge free radicals. This property has sparked interest in the potential for anthocyanins to confer benefits on longevity and age-related motor dysfunctions. In this study, the impact of petunidin-3-glucoside (P3G) on the lifespan and motor function in *Drosophila melanogaster* was examined, both in the presence and absence of neurotoxic substances, such as glyphosate or ethanol. Varying concentrations of P3G were incorporated into the flies' diet immediately after hatching. Subsequently, distinct sets of flies were subjected to diverse conditions, including a stress-free environment, ethanol exposure, and glyphosate exposure. The survival rate of the flies was monitored daily, and their motor function was assessed using a negative geotaxis assay on days 1, 5, 10, and 15 post-eclosion. Only male flies exhibited an extended lifespan under normal and ethanol-exposed conditions. While P3G did not induce significant alterations in overall motor function for male and female flies across various conditions, intriguing variations were observed at different time points. In female flies, low concentrations of P3G seemed to exacerbate motor function decline during mid to late adulthood, particularly in the presence of glyphosate. Conversely, middle P3G concentration improved motor function in male flies during early to mid-adulthood, a trend consistent regardless of neurotoxic exposures. Taken together, the findings underscore the potential sex-specific effects of P3G on both lifespan and motor function, with observed benefits predominantly applicable to male flies. Nonetheless, while suggestive, a more in-depth investigation is imperative to comprehensively determine whether the advantageous impact of P3G on motor function is exclusive to the early to mid-adult stage. As such, this study presents a foundation that warrants further investigation.

Keywords: Anthocyanin, longevity, locomotion, glyphosate, ethanol, neurotoxicity.

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INTRODUCTION

Voluntary motor function is crucial to mobile organisms' survival and interaction capabilities (Manjila & Hassan, 2018). However, stressors and neurological disorders can compromise this function, leading to neurodegeneration. The resulting decline in motor function often manifests as muscle weakness, fatigue, compromised balance and coordination, and even muscle spasticity (Nas et al., 2022).

Different stressors, which trigger oxidative stress, lead to varying effects on motor function. Alcohol-induced neurotoxicity results in the inhibition of N-methyl-D-aspartate (NMDA) receptors and stimulation of gamma-aminobutyric acid (GABA) receptors (Guese et al., 2023). This effect reduces synaptic plasticity, contributing to the impairment of motor function (Nas et al., 2022). The impact of alcohol-induced neurotoxicity on motor function varies with age. Early alcohol exposure in young individuals can lead to less sensitivity to alcohol, resulting in various adverse effects (Fernandez et al., 2007).

Another neurotoxic substance that causes motor impairment is glyphosate, a prevalent chemical in herbicides and pesticides, which makes farmers and consumers vulnerable to chronic exposures. Glyphosate induces autophagic cell death and apoptosis in neuronal differentiated PC12 cells (Cattani et al., 2014). Neurotoxic effects stemming from glyphosate exposure manifest in motor function impairments, such as exposure to herbicides and pesticides containing glyphosate elevates glutamate levels in the synaptic cleft, leading to altered glutathione (GSH) levels and increased lipoperoxidation, excitotoxicity, and oxidative stress, which results in sluggishness, resting tremors, and limb rigidity (Guese et al., 2023).

Petunidin-3-glucoside (P3G), an anthocyanin found in various dietary sources, including vegetables, fruits, and wine, exhibits a notable free-radical scavenging capacity attributed to its distinctive ring orientation, which mitigates oxidative stress (Lacorte et al., 2021). While previous research speculates that P3G has the potential to preserve, enhance, and restore motor function by promoting axonal regeneration, there is still no study conducted to prove this (Nas et al., 2023). Nevertheless, within this framework, P3G, one of the prevalent anthocyanins in food, remains relatively unexplored. To date, a conspicuous gap exists in the literature regarding the isolated effects of P3G on motor function.

Drosophila melanogaster commonly known as the fruit fly, was employed as the model organism in this study to demonstrate the effects of P3G on lifespan and motor function.

MATERIALS AND METHODS

Preparation of Petunidin-3-glucoside

Petunidin-3-glucoside (P3G) was obtained from AS Polyphenols (Norway). The powdered anthocyanin was dissolved in distilled water to prepare a 1.0 mg/mL stock solution concentration. Since light affects the stability and degradation of anthocyanins, the prepared stocks of P3G were covered by foil to maintain their concentrations. The P3G solutions were then stored at 4 ^{o}C until further use.

Drosophila culture and husbandry

The parental generation of Oregon-R strain *D. melanogaster* was obtained from the Bloomington Stock Center (USA). The fruit flies were stored in 25 mm × 95 mm culture vials containing 5 mL of culture media at 25 ^{o}C. The culture media was made with 1,000 mL distilled water, 15.4 g agar powder, 500 g sweet potato, 10 g yeast, and 400 µL propionic acid. The sweet potato was boiled and mashed using a masher, then added to the boiling distilled water. The yeast and agar were then added to the boiling mixture. When a change in the consistency of the mixture was observed, the mixture was allowed to cool to around 60 ^{o}C. Eight drops of propionic acid were then added to the cooled-down mixture to prevent the growth of unwanted microorganisms. After, 5 mL of this mixture was added to each vial and allowed to solidify.

Sublethal assay

The P3G concentrations were 200 µg/mL, 100 µg/mL, and 50 µg/mL. The different concentrations of P3G stock solution were administered to twenty newly-eclosed male and twenty newly-eclosed female *D. melanogaster* through supplementation in yeast paste. The yeast paste was made with 0.2 g of Baker's yeast and 0.25 mL of the P3G solution. The number of live fruit flies was recorded every 24 hours for five days of supplementation. The sublethal concentration was determined at which ≥ 90% of the fruit flies were alive after three days (Nas et al., 2021). With this, 50 µg/mL was selected as the high dosage concentration of P3G for the experiment. This solution was diluted to 2-fold and 4-fold concentrations, and all three concentrations were administered in the experimental treatments through supplementation in food media. Each assay included three trials consisting of 20 biological replicates each.

Lifespan assay

Different concentrations (0, 12.5, 25, and 50 µg/mL) of P3G solution were added to the yeast paste and placed on top of the food media in each vial. Each vial with various treatments of P3G contains 20, which were maintained at 25 ^{o}C. The survival percentage was computed as the fraction of alive fruit flies over the total number of flies, which was recorded daily until all the fruit flies died.

Negative geotaxis assay

D. melanogaster was subjected to the negative geotaxis assay every five days. Each group of *D. melanogaster* was transferred to an empty vial with a marking at 8 cm above the bottom surface. Each vial was tapped on a table to let the flies fall to the bottom, and the number of flies able to climb above the 8-cm mark was noted after 10 seconds (Ali et al., 2011). This assay was repeated 10 times at 1-minute intervals. The number of flies that passed the 8-cm mark was recorded as the percentage of total flies without motor deficits.

Glyphosate stress

Glyphosate, Roundup® (Monsanto, Philippines), was diluted to a concentration of 0.0002 µg/mL as described in a previous study (Ambramson and Joykutty, 2020). The experimental methods for introducing glyphosate stress to *D. melanogaster* were adapted from previous studies (Muller et al., 2021). This solution was incorporated into the fly's food by mixing 23.96 µL of the diluted glyphosate into the yeast paste. Freshly prepared glyphosate-containing yeast paste was placed in the *Drosophila* vial every three days before transferring *D. melanogaster* to fresh culture media. Exposure to glyphosate was done until all flies died. Chronic glyphosate exposure was induced in *D. melanogaster* daily post-eclosion following the previously mentioned protocol for the negative geotaxis and lifespan assays.

Alcohol stress

Chronic alcohol exposure was induced in *D. melanogaster daily* post-eclosion following the previously mentioned protocol for the negative geotaxis and lifespan assays. For each setup, 0.5 mL of 95% ethanol was sprayed on the tissue cover of the vial facing the *D. melanogaster*. After 10 minutes of exposure, the tissue covers were changed. This procedure was done every 24 hours until all *D. melanogaster* in the vials died.

Statistical analyses

Data were presented as mean ± SD. Lifespan data were analyzed using a log-rank test and Bonferroni for post-*hoc* multiple comparisons test using Online Analysis for Survival Application 2 (OASIS 2). For the negative geotaxis assay, the Kruskal-Wallis Test was conducted with the Mann-Whitney U test for the post-*hoc*. Differences were considered statistically significant when $p < 0.05$. Statistical analyses were conducted using SPSS version 26.0 (IBM Corp., Armonk, NY).

RESULTS

Sublethal assay

Shortly after eclosion, mature adult flies were placed on a diet comprising sweet potato

culture medium and yeast paste mixed with varying concentrations (0, 50, 100, or 200 µg/mL) of P3G. The survival rates were meticulously determined after five days: 100% for the control group, 90% for the 50 µg/mL concentration, and 85% for the 100 µg/mL and 200 µg/mL concentrations, as shown in Figure 1.

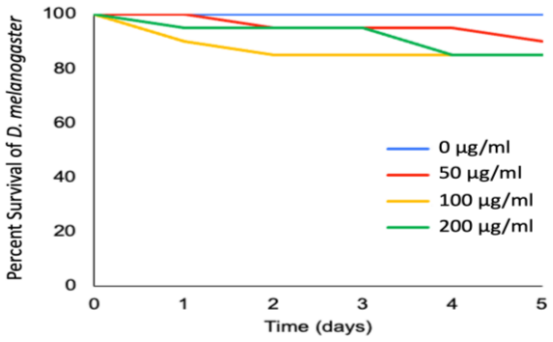

Figure 1. Sublethal concentrations of petunidin-3-glucoside after 5 days

Given the predefined criterion of maintaining a survival rate exceeding 90%, the sublethal threshold of P3G was deduced to be 50 µg/mL specifically for *D. melanogaster*. Consequently, this concentration of 50 µg/mL was adopted as the high dose, whereas concentrations of 25 µg/mL and 12.5 µg/mL were designated as the medium and low doses, respectively.

Lifespan without stress

The investigations revealed that P3G did not result in a lifespan extension for female flies ($p > 0.05$), as illustrated in Figures 2A & 2B. In contrast, in the case of male flies, exposure to a P3G concentration of 12.5 µg/mL led to a remarkable lifespan increase of 1.67 days, equivalent to an 8.5% extension ($p = 0.0010$), as depicted in Figure 2C & 2D. Intriguingly, higher concentrations of P3G; 25, 50 µg/mL, did not yield a statistically significant impact on the lifespan of male flies ($p > 0.05$).

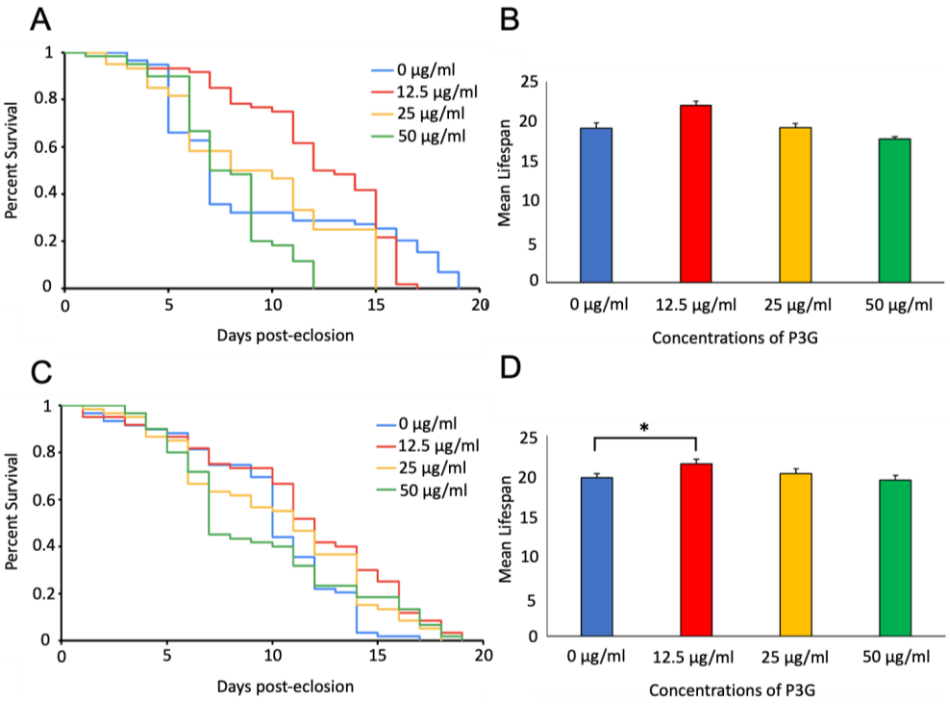

Figure 2. Effects of P3G on lifespan in *Drosophila* in the absence of stressor. Twenty male and twenty female flies treated with varying concentrations of P3G (0, 12.5, 25, and 50 µg/mL) were examined for **A**- daily survival of female flies post eclosion, **B**- mean lifespan of female flies, **C**- daily survival of male flies post eclosion and **D**- mean lifespan of male *Drosophila melanogaster* under normal conditions. Asterisks (*) denote significance at $p < 0.05$

Lifespan with glyphosate exposure

Under chronic glyphosate exposure, the lifespan of adult male and female *D. melanogaster* was not affected by P3G supplementation, as shown in Figure 3. The mean lifespan of the female flies fed with P3G was comparable with that of the untreated flies exposed to glyphosate ($p > 0.05$). This consistent pattern was similarly observed in male flies, regardless of the diverse concentrations of P3G administered ($p > 0.05$).

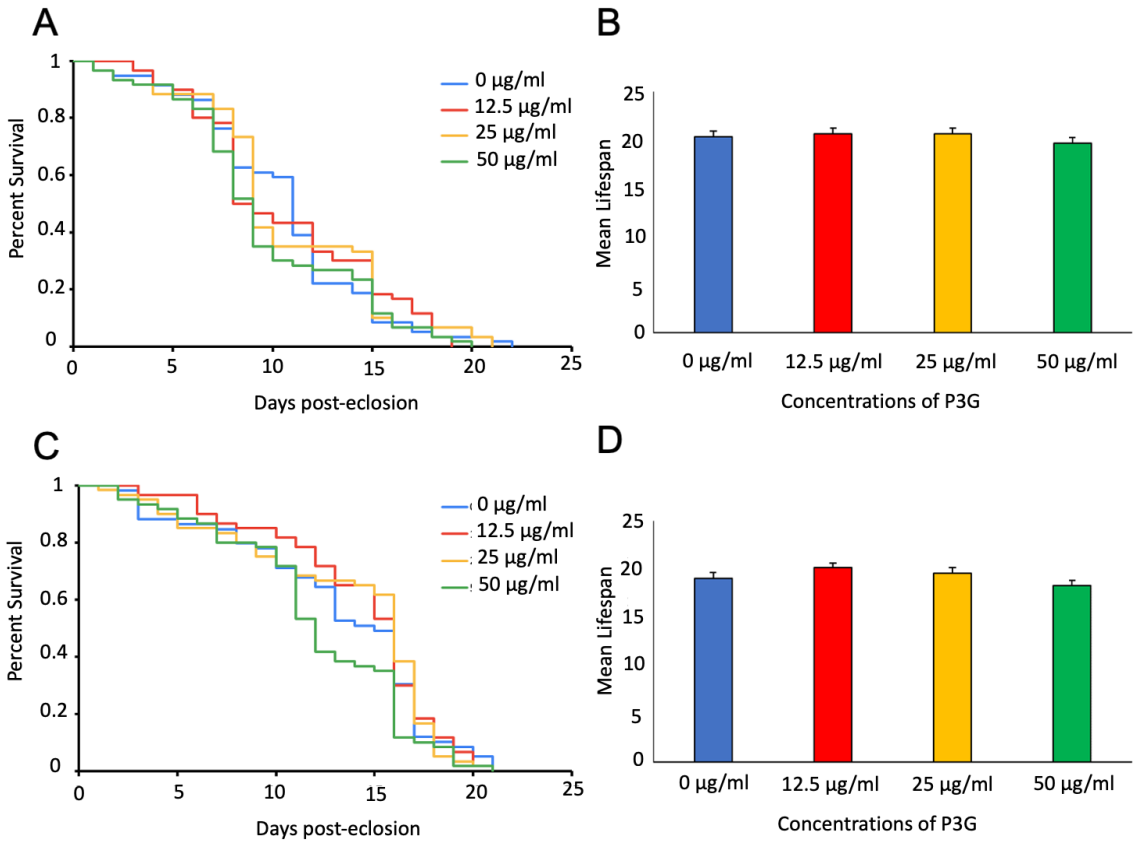

Figure 3. Effects of P3G on lifespan in *Drosophila* in the presence of glyphosate. Twenty male and twenty female flies treated with varying concentrations of P3G (0, 12.5, 25, and 50 µg/mL) were examined for **A-** daily survival of female flies post eclosion, **B-** mean lifespan of female flies, **C-** daily survival of male flies post eclosion and **D-** mean lifespan of male *Drosophila melanogaster* under glyphosate exposure. Asterisks (*) denote significance at $p < 0.05$

Lifespan with ethanol exposure

In prolonged alcohol exposure, an intriguing divergence became evident: male *D. melanogaster*, but not their female counterparts, showcased a noteworthy extension in lifespan upon consumption of elevated doses of P3G (25 and 50 µg/mL), as illustrated in Figure 4. Regardless of the varying P3G amounts administered, the average lifespans of female flies remained uniformly akin to the untreated group, as shown in Figures 4A & 4B. In contrast, among male flies exposed to alcohol and provided with 25 and 50 µg/ml of P3G, a statistically significant extension in lifespan of 1.57 days (equivalent to 8.7%) and 2.22 days (equivalent to 12.2%), respectively, was distinctly observed ($p < 0.05$), as shown in Figure 4C & 4D.

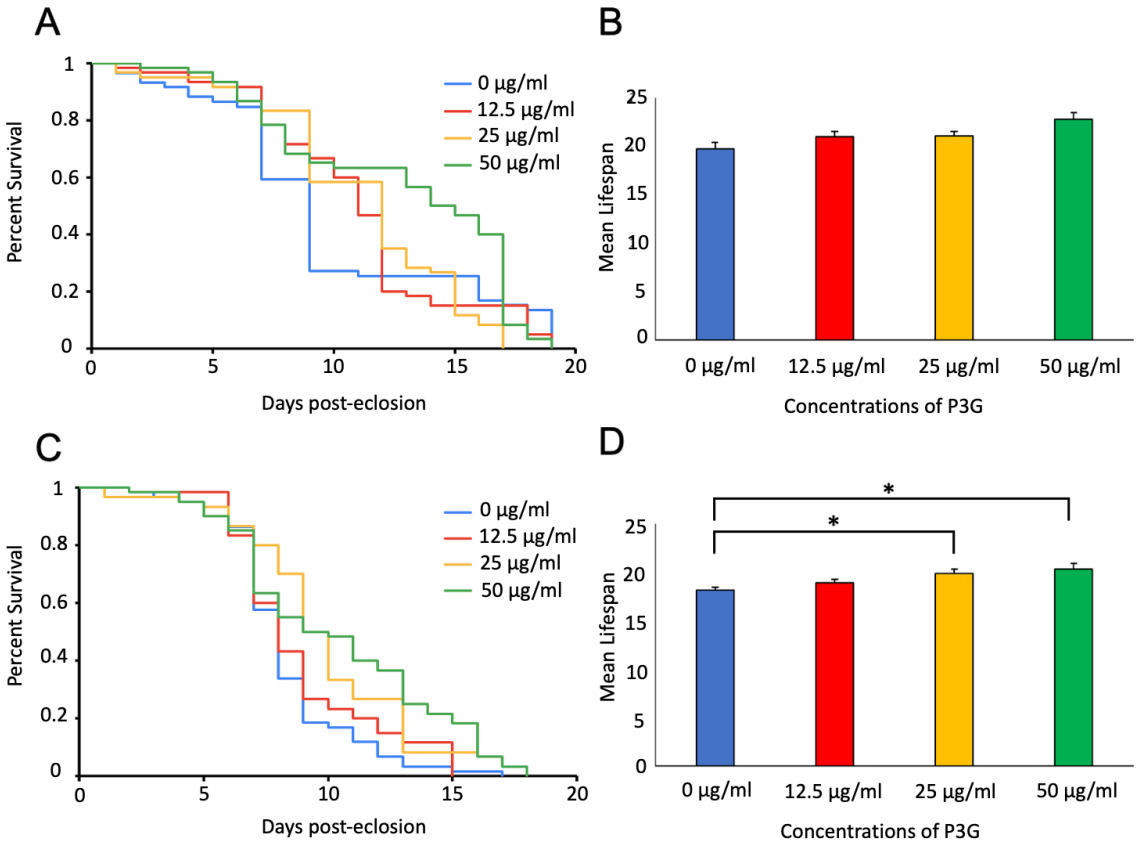

Figure 4. Effects of P3G on lifespan in *Drosophila* in the presence of ethanol. Twenty male and twenty female flies treated with varying concentrations of P3G (0, 12.5, 25, and 50 µg/mL) were examined for **A**- daily survival of female flies post eclosion, **B**- mean lifespan of female flies, **C**- daily survival of male flies post eclosion and **D**- mean lifespan of male *Drosophila melanogaster* under ethanol exposure. Asterisks (*) denote significance at $p < 0.05$

Motor function under normal conditions

Motor function assessment in *Drosophila* was carried out using a negative geotaxis assay, where an individual fly's ability to climb a vial was gauged. Under non-stressful conditions, male and female flies treated with P3G displayed climb pass rates similar to those untreated.

Upon examination, the climb pass rates of female flies exposed to different P3G concentrations exhibited no significant changes on day 1 and day 5 post-eclosion. Nevertheless, for those treated with 12.5 µg/mL P3G, a notable decline of 37% and 40.3% was witnessed on days 10 and 15, respectively ($p < 0.05$), as depicted in Fig. 5A. Notably, the overall climb pass rates throughout the lifespan of female flies were comparable between the P3G-treated and untreated groups, as illustrated in Figure 5B.

In the case of male flies, those administered with 25 µg/mL P3G experienced a remarkable climb pass rate increase of 26.2% and 22.9% on days 5 and 10, respectively ($p < 0.05$), as shown in Figure 5C. Conversely, males given 12.5 µg/mL P3G displayed a significant 81.9% reduction in climb pass rate on day 15. However, it is noteworthy that the average climb pass rates across the entire lifespan of male flies, whether given P3G or not, remained comparable, as shown in Figure 5D.

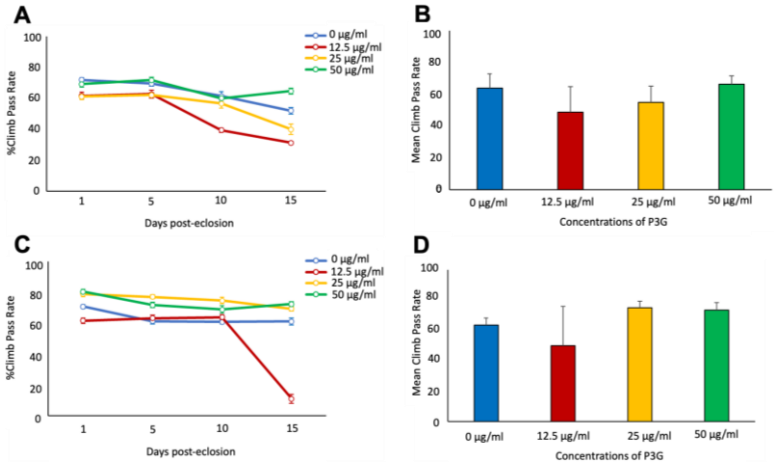

Figure 5. Effects of P3G on motor function in *Drosophila* in the absence of stress. Twenty male and twenty female flies treated with varying concentrations of P3G (0, 12.5, 25, and 50 µg/mL) were examined for **A**- daily climb pass rate of females, **B**- mean climb pass rate of females, **C**- daily climb pass rate of male, and **D**- mean climb pass rate of male *Drosophila melanogaster* under normal condition. Asterisks (*) denote significance at $p < 0.05$

Motor function under glyphosate stress

The observations revealed intriguing outcomes among female flies exposed to 12.5 µg/mL P3G, showing a significant 21.2% and 53.8% reduction in climb pass rate on days 10 and 15 of glyphosate exposure ($p < 0.05$), as depicted in Figure 6A. Notably, 25 µg/mL of P3G yielded a distinctive pattern, enhancing the climb pass rate by 27.8% on day 5 but subsequently leading to a substantial 90.3% reduction on day 15 ($p < 0.05$). Despite these fluctuations, the cumulative climb pass rates across the entire lifespan of female flies remained comparable even with P3G supplementation ($p > 0.05$), as depicted in Figure 6B.

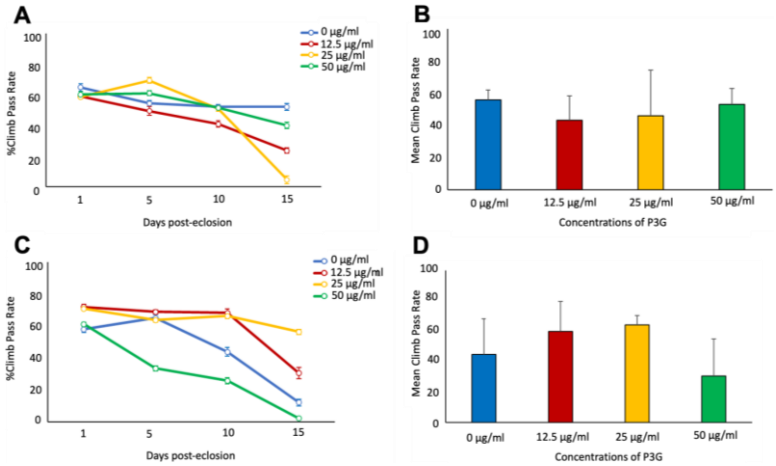

Figure 6. Effects of P3G on motor function in *Drosophila* in the presence of glyphosate. Twenty male and twenty female flies treated with varying concentrations of P3G (0, 12.5, 25, and 50 µg/mL) were examined for **A**- daily climb pass rate of females, **B**- mean climb pass rate of females, **C**- daily climb pass rate of male, and **D**- mean climb pass rate of male *Drosophila melanogaster* after glyphosate exposure. Asterisks (*) denote significance at $p < 0.05$

A noteworthy trend emerged among male flies: those provided with 12.5 and 25 µg/mL P3G exhibited significant improvements in climb pass rate on days 10 and 15 of glyphosate exposure ($p < 0.05$), as illustrated in Figure 6C. Specifically, the 12.5 µg/mL group displayed a remarkable 54.5% and 158.3% increase in climb pass rate on days 10 and 15, respectively. Similarly, the 25 µg/mL group demonstrated an impressive 50% and 366.7% climb pass rate increase on days 10 and 15, respectively ($p < 0.05$). Intriguingly, male flies treated with 50 µg/mL P3G experienced a 47.7% reduction on day 5% and 40.9% on day 10 ($p < 0.05$). Notwithstanding these variations, the overall average climb pass rates of male flies exposed to varying P3G concentrations closely mirrored those not supplemented with P3G ($p > 0.05$), as shown in Figure 6D.

Motor function under alcohol stress

The observations unveiled an intriguing pattern among female flies exposed to varying P3G concentrations during 15 days of ethanol exposure: their climb pass rates remained similar to those untreated ($p > 0.05$), as illustrated in Figure 7A. Likewise, the average climb pass rates spanning the observation period showed comparability ($p > 0.05$), as depicted in Figure 7B.

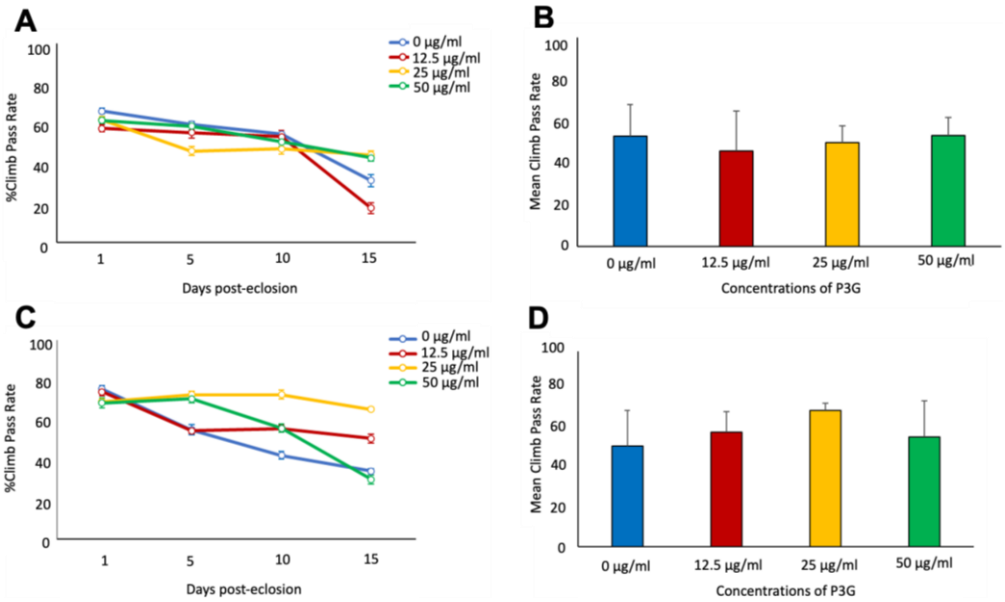

Figure 7. Effects of P3G on motor function in *Drosophila* in the presence of ethanol. Twenty male and twenty female flies treated with varying concentrations of P3G (0, 12.5, 25, and 50 µg/mL) were examined for **A**- daily climb pass rate of females, **B**- mean climb pass rate of females, **C**- daily climb pass rate of male, and **D**- mean climb pass rate of male *Drosophila melanogaster* after ethanol exposure. Asterisks (*) denote significance at $p < 0.05$

Meanwhile, male flies subjected to alcohol exposure exhibited a notable enhancement in their climb pass rates when treated with 25 µg/mL P3G. This group displayed significant increases of 32.7%, 73.8%, and 91.2% on days 5, 10, and 15, respectively ($p < 0.05$), as depicted in Figure 7C. Similarly, male flies administered with 12.5 µg/mL P3G showcased improvements of 33.3% and 50% in climb pass rate after days 10 and 15 ($p < 0.05$). Likewise, those given 50 µg/mL P3G experienced climb pass rate increases of 29.1% and 33.3% on days 5 and 10, respectively. Despite these varying enhancements observed on different days post-P3G treatment, the average climb pass rates of male flies exposed to ethanol, whether supplemented with P3G or not, were comparable, as shown in Figure 7D.

DISCUSSION

Sex-specific effects of P3G on lifespan

The outcomes bring intriguing insights into the nuanced effects of P3G on the lifespan of *D. melanogaster*. Notably, the gender-specific response observed underscores the complex interplay between P3G and physiological factors influencing longevity.

In the female cohort, the lack of a significant extension in lifespan upon P3G supplementation is a noteworthy finding. Despite the antioxidant properties attributed to anthocyanins, which could potentially confer longevity benefits, the results did not reveal such an effect in female flies (Nas et al., 2021; Nas & Medina, 2023; Nas, 2023). This emphasizes the intricate interactions between bioactive compounds and gender-specific biological systems. Factors such as hormonal dynamics or genetic variations may contribute to the lack of response observed in females.

Conversely, the effect of P3G on male flies presents a contrasting narrative. The notable extension in lifespan upon exposure to a specific concentration of P3G (12.5 μg/mL) suggests a potential avenue for enhancing male longevity. This observation follows previously reported effects of anthocyanins on male *Drosophila*, which points to a distinct susceptibility of male flies to the beneficial effects of P3G on lifespan (Zhang & Dai, 2022; Han et al., 2021). The absence of significant effects at higher concentrations may hint at saturation effects or potential antagonistic interactions, warranting further exploration.

In scenarios of chronic glyphosate exposure, the consistent lack of impact on lifespan across both male and female flies upon P3G supplementation suggests the resilience of these organisms to the tested concentrations. This resilience could be attributed to the intricate interplay between glyphosate toxicity and P3G's antioxidative potential.

The divergence in response under chronic alcohol exposure adds another layer of complexity. The extension in the male lifespan upon P3G supplementation contrasts with the lack of significant impact observed in females. This intriguing gender-specific response necessitates probing into underlying mechanisms that could contribute to the observed differences. Hormonal factors, metabolic variations, or even differences in detoxification pathways may play pivotal roles.

These findings underline the multifaceted nature of P3G's effects on lifespan, influenced by intricate interactions with gender-specific physiological processes and stressors. The gender dimorphism observed underscores the importance of considering sex-specific responses when assessing the potential health benefits of bioactive compounds. The study not only advances the understanding of the influence of P3G on lifespan but also highlights the need for further exploration into the underlying mechanisms governing these responses. Such investigations could pave the way for more targeted interventions and contribute to the broader understanding of longevity modulation.

Sex-specific and time-dependent effects of P3G on motor function

The comprehensive analysis of motor function in *D. melanogaster* reveals sex-specific and time-dependent effects of P3G supplementation. The negative geotaxis assay, employed to evaluate climbing ability, explains how P3G influences fly behavior under various conditions.

Notably, under non-stressful conditions, P3G-treated male and female flies exhibited comparable climb pass rates to their untreated counterparts. This suggests that P3G does not inherently impact motor function without external stressors, indicating a lack of acute effects on climbing ability.

However, distinct patterns emerged when investigating the effects of P3G on motor function under different stressors. Among female flies, exposure to varying P3G concentrations did not significantly change climb pass rates in early adulthood. Yet, intriguingly, supplementation with 12.5 μg/mL P3G resulted in a noticeable decline in climb pass rates in mid and late adulthood. This reduction in climbing ability raises questions

about the potential interactions between P3G and stressors, such as glyphosate exposure. Despite these fluctuations, the cumulative climb pass rates over the entire lifespan remained similar between P3G-treated and untreated female flies.

Conversely, male flies displayed responses that differed based on stressors and P3G concentrations. Noteworthy improvements in climb pass rates were observed in male flies treated with 25 µg/mL P3G during early adulthood and mid-adulthood, regardless of the stressor. The findings follow similar results observed in crude anthocyanin extracts on the locomotor activity of *Drosophila* (Filaferro et al., 2022; Long et al., 2009; Mahesh et al., 2022). However, in the context of chronic ethanol exposure, male flies demonstrated remarkable enhancements in climbing ability upon treatment with 25 µg/mL P3G. This suggests a potential interaction between P3G and alcohol-induced stressors that enhances motor function. These findings underscore the intricate interplay between P3G, gender, length of exposure, and stress conditions.

Furthermore, the effects of P3G under glyphosate exposure demonstrated gender-specific responses. Female flies exhibited reduced climb pass rates during mid-adulthood and late adulthood, suggesting an interaction between P3G and glyphosate that negatively impacts motor function. Conversely, male flies responded positively to varying P3G concentrations under glyphosate exposure, showcasing significant improvements in climbing ability. A study previously reported that mulberry extract, a plant rich in anthocyanin, protected neurons against glyphosate-induced toxicity, which may explain the improvement in the motor behavior of male flies at specific time points (El-Baz, 2018). However, these contrasting responses highlight the potential sexual dimorphism in the interaction between P3G and glyphosate-induced stress.

In the context of chronic ethanol exposure, male flies experienced enhanced climb pass rates upon P3G supplementation. This pattern of improvement aligns with the results observed under other stressors, indicating a consistent positive effect of P3G on male motor function. One study suggests that anthocyanin-rich *Basella alba* extract protected the neurons of another invertebrate model from ethanol-induced excitotoxicity for a short period, which may explain the observations (Nas et al., 2020). In other studies, anthocyanin-rich extracts prevented motor impairments in female animals (de Silva et al., 2023). However, female flies exposed to ethanol and treated with P3G exhibited climb pass rates comparable to untreated flies, indicating a potential sex-specific aspect to the interaction between P3G and ethanol stress.

The findings underscore the complexity of P3G's effects on motor function in *Drosophila*, revealing intricate interactions with stressors and gender-specific responses. These results contribute to the understanding of how P3G may influence behavior under diverse physiological conditions, providing valuable insights for further exploration into the mechanisms underlying these responses.

CONCLUSION

The impact of petunidin-3-glucoside (P3G) on the lifespan of *Drosophila melanogaster* has unveiled intriguing outcomes. Low P3G concentration led to a significant extension of male flies "lifespan in the absence of stress and high P3G concentration when the male flies were exposed to ethanol. In contrast, P3G did not produce a significant extension of female flies" lifespan at any concentration tested under various conditions. The complexity of P3G's effects on motor function were observed, emphasizing the importance of sex and timing of exposure. While P3G did not consistently extend lifespan in both genders, the mid-concentration of P3G exhibited significant improvements in the motor function of the male flies under different environmental conditions. These findings suggest that P3G may confer potential health benefits on lifespan and motor function in the male population. This underscores the

multifaceted nature of P3G's interactions with physiological conditions, warranting further exploration into underlying mechanisms. Ultimately, this research raises questions on how P3G may modulate motor function in a context-dependent manner.

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