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A Combined Diet Of High Fat, Sugar, And Salt Affects Development And Aging In Drosophila Melanogaster

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Abstract

Background:

The increasing consumption of high-fat, high-sugar, and high-salt (HFSS) diets, particularly among children, is a growing global health concern due to its association with metabolic disorders, neurodegeneration, and accelerated aging. ***Aim:*** *To investigate the impact of a combined HFSS diet on development and aging using Drosophila melanogaster as a model organism.* ***Methods:*** *Drosophila larvae were exposed to an HFSS diet from the developmental stage to adulthood. Neurobehavioral assays, biochemical analyses (protein, triglycerides, glucose), oxidative stress markers, lifespan evaluation, and DCFDA gut staining were performed. Raman spectroscopy and FTIR were used to confirm the presence of fat, sugar, and salt components. Statistical significance was considered at* $p < 0.05$. ***Results:*** *Larvae fed the HFSS diet showed a significant increase in body weight, triglycerides, glucose, and oxidative stress, with a decrease in protein levels and lifespan. Neurobehavioral impairments were observed, and DCFDA staining confirmed elevated ROS levels in gut tissues. Raman and FTIR analyses validated the presence of HFSS components in the diet.* ***Conclusions:*** *Exposure to a combined HFSS diet during early development negatively impacts metabolism, neurobehavior, and lifespan, suggesting that such diets may contribute to early-onset aging and chronic health conditions. Early dietary interventions are essential for long-term health outcomes.*

Keywords *Drosophila melanogaster, Processed Food Ingredients, Development, Neurobehavioral Impairments, Aging, oxidative stress response*

Graphical Abstract

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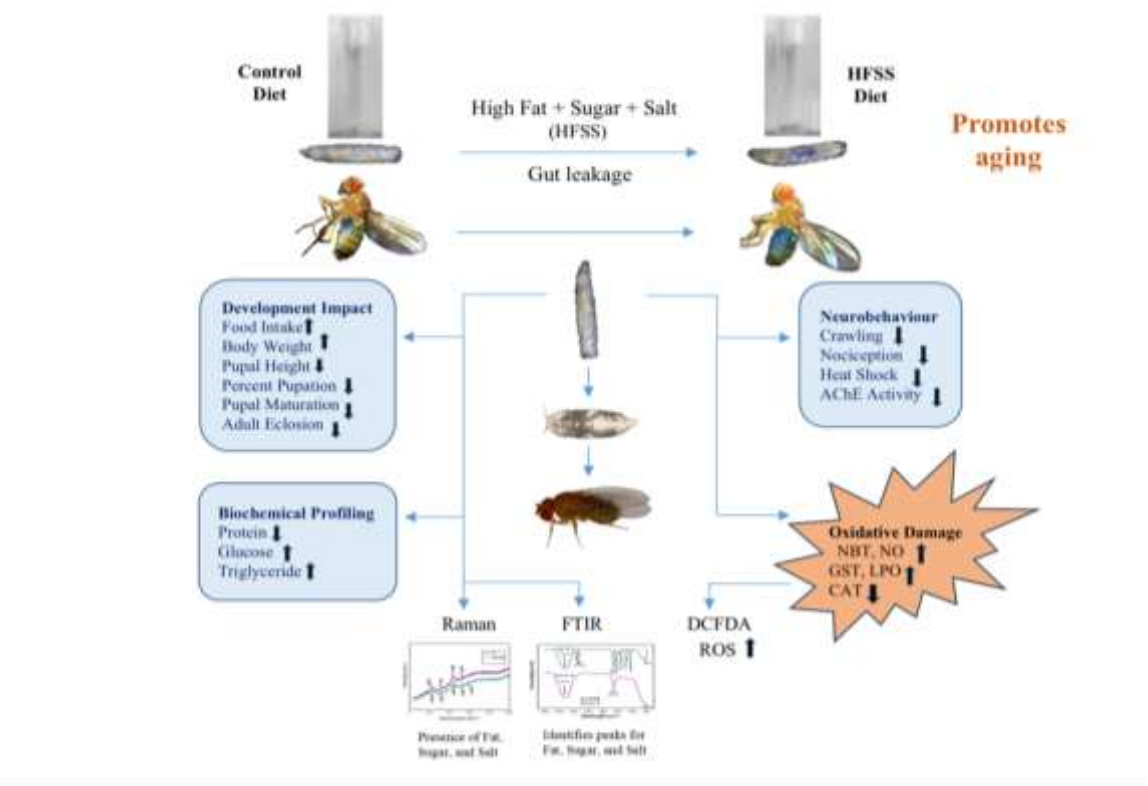

Consumption of Processed Food ingredients, high-fat, high-sugar, and high-salt in D. melanogaster larvae resulted in Developmental, neurobehavioural, gut leakage, and Biochemical alterations. Additionally, exposure to this diet induces oxidative stress and accelerates aging processes, ultimately reducing lifespan.

INTRODUCTION

In previous generations, diets were predominantly nutrient-dense, consisting of fruits, vegetables, whole grains, and lean meats. This pattern of eating contributed to lower rates of lifestyle-related diseases and better overall health [1]. The emergence of convenience meals, which are heavy in calories but poor in vital nutrients, has caused an enormous change in modern diets [2]. This shift, especially during early developmental stages, has alarming consequences, such as the increased prevalence of childhood obesity and other metabolic disorders like diabetes and hypertension in children [3,4].

According to the World Health Organization (WHO), in 2020, over 340 million teenagers between the ages of five and nineteen were categorized as overweight, and over 39 million youngsters under the age of five as well [5]. Research from numerous longitudinal and cross-sectional studies indicates that consuming higher quantities of processed foods containing high fat, sugar, and salt (hereafter referred as HFSS) is associated with a decrease in the overall quality of nutrients and an increased risk of adverse health consequences [6–8].

Nutritional input during the developmental stage is important for physiological trajectories with lifelong consequences. The connective link between HFSS diet exposure during early development and its long-term effects across development to aging remains unexplored. Most research focuses on early metabolic syndromes or aging parameters without examining how early developmental exposure affects age-related outcomes.

In the current study, we aimed to fill this gap by using Drosophila melanogaster as a model organism to investigate the continuous exposure of HFSS diet from early development to aging. Drosophila melanogaster is a good model for understanding the impact of HFSS on health, mainly because of its

genetic similarity to humans, especially in the areas of metabolism, oxidative stress, and neurodegenerative pathways [9–16]. Its rapid life cycle, genetic tractability, and low maintenance make it ideal for studying development and lifespan.

We assessed the effect of an HFSS diet by evaluating neurobehavioral, biochemical, gut damage, and oxidative stress parameters from the larval stage through adult aging. This included changes in fat storage, oxidative stress levels, aging, gut health, and the resulting behavioral changes, such as impaired locomotion and memory deficits. In addition to conventional physiological and behavioral assays, we employed Fourier-transform infrared (FTIR) and Raman spectroscopy to examine the molecular and structural changes in control and HFSS-fed D. melanogaster. Raman spectroscopy, with its ability to provide detailed vibrational information about molecular bonds, further allowed us to detect subtle shifts in metabolic signatures and oxidative stress markers in the tissues of D. melanogaster exposed to the HFSS and Changes in macromolecular compositions, such as lipids, proteins, carbohydrates, are revealed through FTIR spectroscopy, thus allowing for an understanding of how manipulation of the HFSS impacts the biochemical landscape on a molecular level. Additionally, we perform DCFDA staining of the gut in D. melanogaster to evaluate oxidative stress, further complementing our findings on the effects of the diet on oxidative damage. These spectroscopic techniques complement our biochemical and physiological findings, providing a comprehensive picture of the diet's impact on cellular and molecular pathways.

Understanding the role of diet in development is essential for understanding how dietary patterns set up early in life may predispose individuals to adverse health outcomes, including metabolic disorders and chronic diseases later in life. This research presents a comprehensive developmental analysis of aging due to HFSS exposure in a genetically tractable model. Our findings demonstrate that poor nutrition intake during early development not only affects growth but also contributes to long-term effects that reflect aging. These findings highlight the importance of implementing early-life dietary interventions to prevent the rising incidence of lifestyle-related diseases.

MATERIAL AND METHODS

D. melanogaster rearing

D. melanogaster (ORK strain) was originally obtained from the D. melanogaster Stock Center and kept in a controlled laboratory environment with a 12 hr light/dark cycle at 22 ± 1°C. Adults were fed on standard food. After oviposition, the first instar larvae were collected for the experimental procedures.

Diets of D. melanogaster

The parent flies were reared on a standard diet consisting of cornmeal, sucrose, yeast, and agar-agar. Propionic and methyl paraben were incorporated into food to prevent fungal contamination. Parent flies were allowed to oviposit on the standard diet, and first instar larvae were collected and transferred to the HFSS diet, which consisted of 15% palm oil, 15% sugar, and 0.1 M NaCl [17]. A control set was established simultaneously. All the experiments were conducted on third instar and newly eclosed flies from the control and HFSS groups. All assays, except for the longevity assay, were conducted on third-instar larvae. Every experimental method was carried out in triplicate.

Effect on Larval and Pupal Development

Measurement of food intake

Third-instar larvae from each experimental diet were first rinsed with PBS (n=40). They were then transferred to food containing agar, yeast, and 0.2% (w/v) sulforhodamine B. After 30 minutes of incubation, the larvae were washed with PBS to remove excess stains from their bodies and homogenized in PBS. The amount of food consumed was quantified by measuring the absorbance of the homogenate at 550 nm using a spectrophotometer. The absorbance values corresponded to the total

quantity of food ingested by the larvae [18]. This feeding assay was independently conducted five times to ensure the reproducibility of the results.

Measurement of body weight

To assess changes in body weight, larval weight was measured. Third-instar larvae were collected from control and HFSS conditions and then washed with PBS to remove residual food particles. Subsequently, ten larvae were weighed using a weighing balance, with the weight of the empty Eppendorf tube recorded beforehand to ensure accuracy. This procedure was repeated ten times to ensure reproducibility.

Pupal height

The position of pupation, specifically pupal height, can reflect environmental and physiological factors influencing larval development. In this study, first instar larvae were transferred to both the control diet and HFSS conditions to assess pupation height. The pupal height was measured from the surface of the food to the midpoint of the pupa [19]. This assay was independently repeated ten times per group (n = 200 pupae per condition) to ensure statistical robustness. This measurement provides insight into the effects of diet on larval behavior and developmental outcomes.

Percent pupation

First instar larvae were transferred to the control and HFSS and allowed to feed on their particular diets throughout the larval stage. The larvae proceed to the pupal stage and crawl on the walls of the vial. The number of pupae that developed in each group at this point was counted to evaluate how food affected the efficiency of pupation and larval development. This assay was independently repeated ten times per group (n = 200 pupae per condition) to ensure statistical robustness.

Adult eclosion

The number of adult flies that successfully emerged from pupae was counted to assess the impact of diet on eclosion rates and compared with the control group. Following pupation, the number of eclosed adults in each group was counted, and the vials were checked every day for adult emergence [20].

Effect on Gut Integrity

Trypan blue staining

Trypan blue staining was employed to differentiate between live and dead cells. Dead cells are stained by Trypan blue due to the lack of intact cell membranes, which allows the dye to penetrate, whereas live cells remain unstained. Briefly, third-instar larvae from both control and HFSS groups were collected and rinsed with PBS and subsequently incubated in 0.2% Trypan blue solution for 30 min. Following this incubation, the larvae were thoroughly washed in PBS three times for 10 min each to remove excess dye. Images of the larvae were then captured using a stereomicroscope [21].

Smurf assay

The gut permeability of D. melanogaster was evaluated using the Smurf assay, which uses a blue dye to detect any gut leakage. Two to three-day-old adult flies (n=40) from the control and HFSS diet groups were placed in a medium containing 0.5% (w/v) blue food dye for 2 h [22]. This allows the dye to pass through any compromised areas of the gut. The flies were examined under a stereo microscope.

Neurobehavioral assays

Crawling Assay

Larval crawling is a highly coordinated process driven by interactions between neurons, muscles, and neuromuscular junctions, providing a useful tool for assessing neuronal function. This rhythmic

behavior includes movements like linear crawling, turns, and twists, which reflect underlying motor circuitry and cellular processes. Studies on D. melanogaster larvae show that motor neuron activity and neuromuscular coordination are crucial in generating the precise movements required for crawling, providing insights into nervous system function and developmental biology. In this assay, third-instar larvae (n=20) were placed on a 1% agar-coated petri dish to assess their locomotor activity. After one min of free movement, their crawling path was marked on graph paper, and the speed was measured in millimeters per second (mm/s) to evaluate their motor performance [23].

Pain sensitivity assay

To assess thepain sensitivity response of HFSS groups, third instar larvae were taken and compared with that of control, in which each larva was placed in a petri dish (n=60), and 1.5 µL of 9% hydrochloric acid (HCl) was applied to trigger a nociceptive response. The larvae's behavior, specifically the characteristic "crossed-screw" or rolling movement in response to the noxious stimulus, was observed [24]. The latency between the HCl application and the initiation of this rolling movement was recorded via video capture, allowing for a precise analysis of the nociceptive response across the two dietary groups. This method enabled the comparison of pain sensitivity under different dietary conditions.

Heat shock recovery assay

The heat shock assay in D. melanogaster larvae is employed to assess cellular stress responses, particularly the induction of heat shock proteins critical for cellular protection against thermal stress [25]. Briefly, 20 third-instar larvae were placed on a 1% agar plate in three replicates, which were then immersed in a water bath maintained at 35°C. After a 5-min exposure to this elevated temperature, the plates were removed, and the number of larvae found adhering to the lid of the Petri dish was recorded. This procedure facilitates the examination of stress tolerance mechanisms and the role of genetic pathways in heat resistance.

Floating Assay

To evaluate triglyceride levels in larvae, a buoyancy-based floating assay was performed. Forty-third instar larvae from both the control diet and HFSS groups were collected and washed with PBS. The larvae were then transferred into test tubes containing a 10% sucrose solution. After allowing time for the larvae to settle, the tubes were gently agitated to ensure proper distribution. The number of larvae that floated and those that remained settled were recorded, with floating larvae indicating higher triglyceride levels, demonstrating changes in buoyancy due to increased fat content [26].

Biochemical Estimation

Homogenate preparation

To perform the biochemical assays, homogenates were prepared from third-instar larvae. Briefly, third instar larvae were collected from each experimental food vial (n=100) and thoroughly washed with PBS. The larvae were then transferred into Eppendorf tubes containing PBS, and homogenization was carried out using a motor-driven microcentrifuge homogenizer (Gebetrix TG20) on ice. Following homogenization, the samples were centrifuged at 10,000 rpm for 10 min at 4°C to prevent protein denaturation. The supernatant was carefully collected for subsequent biochemical analyses.

Measurement of body protein level

Protein concentration in the homogenate samples was determined using the Bradford assay, a colorimetric method based on the binding of Coomassie Brilliant Blue dye to proteins, with absorbance measured at 595 nm using a microplate reader.

Measurement of triglyceride and glucose levels

Triglyceride and glucose levels were quantified using commercial assay standard kits (Tulip Diagnostics, India) specific for each metabolite, and the absorbance was read at appropriate wavelengths using a microtiter plate reader according to the manufacturer's instructions. These absorbance values were used to calculate the concentrations of triglycerides and glucose in the samples, based on standard curves generated during the assay.

AChE activity assay

AChE activity was assessed spectrophotometrically, following Ellman's method [27]. The enzyme acetylcholinesterase terminates cholinergic neurotransmission by breaking down acetylcholine into choline and acetic acid. The assay mixture consisted of 100 mM PBS (pH 7.4), 10 mM 5,5'-dithiol-bis-2-nitrobenzoic acid (DTNB), 75 mM acetylthiocholine iodide, and supernatant. After 10 min of incubation at 37°C, absorbance changes were measured at 412 nm within 1 min.

Oxidative stress markers

LPO levels

LPO triggered by free radicals damages polyunsaturated lipids and disrupts membrane function. This process is extensively studied in disease contexts and antioxidant modulation. Various by-products are formed, commonly measured by the thiobarbituric acid reactive substances (TBARS) assay, which detects aldehydic products spectrophotometrically. To determine the Lipid peroxidation level, the method described by [28] was utilized with minor modifications. Briefly, 10mM butyl-hydroxytoluene, 1% O-phosphoric acid, and 0.67% thiobarbituric acid were added to the sample, which was then incubated at 90°C for 45 min. Then its absorbance was measured at 535 nm.

NO levels

For the NO assay, nitrite, a stable end product of nitric oxide metabolism, was measured in the supernatant as an indicator of Nitric oxide production. This was done using the Griess reagent, which reacts with nitrite to form a purple azo dye. The samples were first incubated with Griess reagent, and the absorbance was measured at 540 nm using a microtiter plate reader. The concentration of NO was determined by comparing the absorbance values to a standard curve generated from known concentrations of sodium nitrite. This assay provides insight into the inflammatory response and oxidative stress levels in the larvae [29].

CAT levels

Catalase activity was assessed using the method described by Sinha. In this method, the sample was allowed to react with dichromate-acetic acid, followed by the addition of hydrogen peroxide (H_2O_2). The amount of chromic acid formed is directly proportional to the H_2O_2 concentration, which was measured at 570 nm [30].

GST activity

GST activity was assessed using 1-chloro-2, 4-dinitrobenzene (CDNB) as a substrate, which was combined with reduced glutathione and PBS to make a premix to which 10 µl of the sample was added, and its absorbance was measured at 340 nm [31].

NBT levels

For the NBT assay, a 0.2% Nitroblue tetrazolium solution was used to detect the formation of reactive oxygen species in the supernatant of homogenized larvae from both the control and HFSS groups. After adding the NBT reagent to the sample, the mixture was incubated for 1.5 h, followed by the addition of dimethyl sulfoxide (DMSO) to solubilize the formazan produced by the reduction of NBT. The optical density was measured after a total incubation period of 2 h at a wavelength of 560 nm using a microplate reader to quantify ROS levels [32].

DCFDA staining

The production of reactive oxygen species in the gut of third-instar D. melanogaster larvae wasmeasured using DCFDA (2', 7'-dichlorodihydrofluorescein diacetate) staining. To maintain tissue integrity and morphology, the larval guts were dissected under a stereomicroscope and placed in 4% paraformaldehyde for 24 h of fixation at 4°C [33]. To permeabilize the membranes and allow dye entrance, the tissues were washed three times using PBST and PBS following the fixing period. After that, the guts were incubated in the dark for 30 min. at room temperature with DCFDA to prevent photobleaching. The levels of ROS in the guts of larvae fed an HFSS and those fed a control were observed under fluorescence microscopy using ZEISS Celldiscoverer.

RAMAN Spectroscopy

The hemolymph from third instar larvae (n=10) was carefully collected and subsequently spread onto a glass slide. After allowing the hemolymph to air dry, Raman spectroscopy analysis was conducted on a Renishaw inVia spectrometer (wavelength 532 nm) to examine the molecular composition and identify any diet-induced structural changes in the sample.

FTIR Analysis

Haemolymph from third-instar larvae exposed to both diets was subjected to FTIR analysis to see the changes in functional groups. Larvae (n=were first washed with PBS to remove any residual food particles and transferred to a small centrifuge tube. All procedures were carried out on ice, with a small incision made at the tube's base, and the larval cuticle ruptured using a microneedle. After removing the cap from the smaller tube, the 1.5 mL centrifuge tube was filled with the 0.5 mL tube containing the dissected larvae. The mixture was centrifuged for 10 min at 4°C at 5000 rpm, and the supernatant was collected for FTIR analysis (Bruker Tensor-3, software Opus) [34].

Lifespan

Flies that emerged from control and HFSS diets were separated by sex into males and females. Each group consisted of 200 flies, distributed across 10 vials (20 flies per vial) to maintain consistency. Flies were monitored every three days, transferred to fresh vials, and mortality was recorded until all had died. Survival data were used to calculate mean, median, and maximum lifespan, with maximum lifespan defined as the average lifespan of the final ten percent of deaths.

Statistical Analysis

All experiments were performed in triplicate to ensure the reliability and reproducibility of the results. The data are presented as mean values with their corresponding standard deviations. For statistical analysis, GraphPad Prism 5 Software was used. The statistical comparisons were made using an unpaired t-test to assess the significance of the differences between experimental groups.

RESULTS

Effect on Larval and Pupal Development

Effect of diet on body weight

The HFSS larvae exhibit a significant increase ($p=0.0006$) in body weight and show a 24±0.1632% variation compared to larvae maintained on the control diet (Fig. 1A). These observations suggest that an HFSS induces an obesogenic effect in larvae. Body weight is a crucial parameter for assessing overall health, growth, and development and indicates nutritional status. The increase in body weight likely results from metabolic dysregulation caused by the HFSS, mimicking dietary conditions associated with obesity and related metabolic disorders.

Measurement of food intake

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The food intake assay is used to quantify the amount of food consumed by larvae, providing insights into feeding behavior and dietary preferences. In our study, larvae reared on the HFSS diet demonstrated a significant 36±0.02% increase (p= 0.3392) in food intake compared to the larvae fed on the control diet (Fig. 1B). This suggests that the larvae preferred the HFSS, likely due to its palatability. The HFSS may enhance its sensory appeal, leading to overconsumption. While these ingredients contribute to the diet's tastiness, they are associated with negative health outcomes, such as obesity and metabolic disorders.

Effect on Pupal Height

Larvae raised on HFSS exhibited a significant reduction in pupal height compared to those in the control group. The distance from the food surface to the pupa HFSS-fed pupae was notably shorter, indicating impaired pupal development, with a significant decrease ($p < 0.001$) of 49.30±0.35% observed between the HFSS and control diet groups (Fig. 1C). This difference suggests that the HFSS negatively impacts pupal growth, due to the altered nutrient composition, which could affect developmental processes. Additionally, Pupae developed from larvae of HFSS exhibited significant deformities compared to the control group. The control group pupae appeared normal and healthy, whereas the HFSS pupae displayed irregular morphological features and a high incidence of developmental irregularities to eclode properly (Fig. 1F,G). The rates of pupation and eclosion were markedly lower in the HFSS group, with pupation rates reduced by 23.45±3.54%, ($p < 0.001$) and eclosion rates decreased by 37.37±3.99% ($p=0.0002$) compared to controls (Fig. 1D,E). These abnormalities suggest that the nutritional composition of the HFSS characterized by elevated levels of fat, sugar, and salt adversely affects the developmental processes critical for proper pupation and eclosion. The impaired eclosion and deformation observed in HFSS pupae may be indicative of metabolic disturbances caused by the unbalanced diet, emphasizing the detrimental effects of a high-fat, high-sugar dietary regimen on normal developmental outcomes.

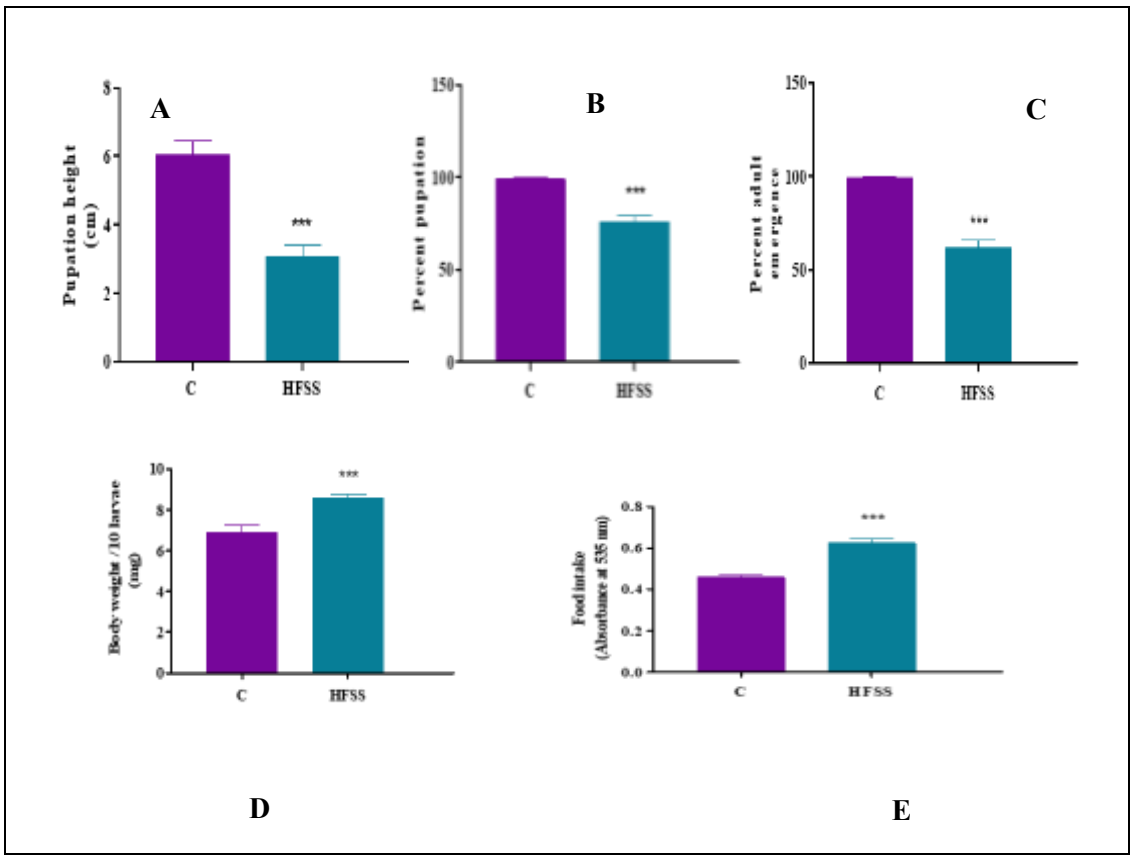

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Fig. 1 Effect of HFSS on larval and pupal development: (A) The Body weight of HFSS larvae significantly increases. Data represented as the mean body weight of 10 larvae per replicate for each treatment group. **(B)** Food intake is significantly increased in HFSS larvae, indicating that the dietary composition may lead to higher consumption levels compared to control larvae. Statistical analysis was performed using an unpaired t-test (*** indicates a highly significant difference with $p < 0.001$)

Effect on Gut

Trypan blue staining

Trypan blue staining is commonly used to assess cell viability by detecting non-viable cells with compromised membrane integrity, as the dye selectively permeates and stains dead or damaged cells. In our study, we observed that larvae fed an HFSS exhibited significant blue staining in the gut tissue, indicating cellular damage and compromised gut integrity. In contrast, larvae fed a control diet showed no signs of staining, suggesting the absence of gut damage compared to controls (Fig. 2A). These findings strongly suggest that the HFSS induces gut damage in D. melanogaster larvae, likely due to its high fat, sugar, and salt content, which can lead to increased cellular stress and loss of membrane integrity in the gut epithelium.

Smurf Assay

In the Smurf assay, we observed gut integrity in both male and female D. melanogaster subjected to control and HFSS regimens. Notably, both male and female adults fed an HFSS diet exhibited signs of compromised gut barrier function, characterized by gut leakage, when compared to their control diet compared to controls (Fig. 2B,C). This gut leakage is indicative of a disrupted intestinal epithelial barrier, which could be associated with increased intestinal permeability.

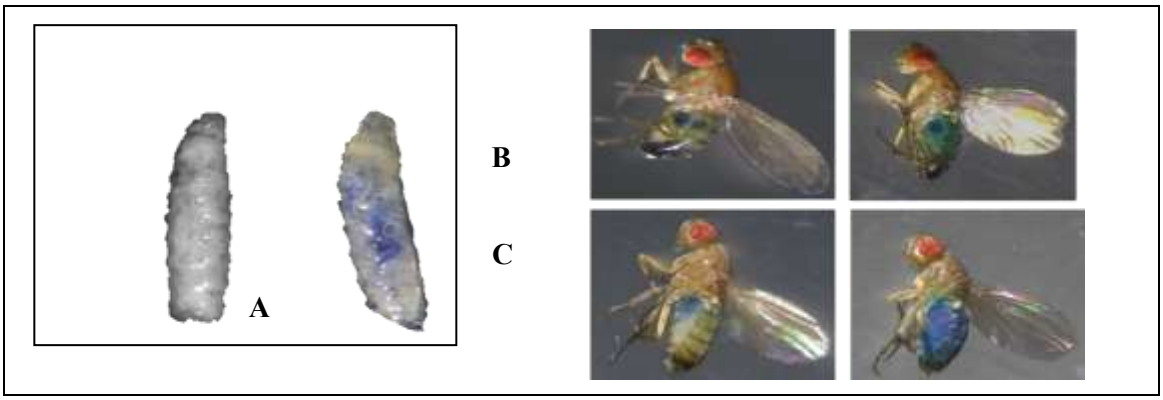

Fig. 2 Effect on gut: (A) Increased trypan blue staining, reflecting gut integrity, shows visible gut damage in HFSS larvae. **(B)** In adult male flies exposed to the HFSS, gut leakage is observed using the Smurf assay. **(C)** HFSS-fed female flies also exhibit gut leakage in the Smurf assay, indicating a comparable disruption in gut barrier integrity, where flies with compromised gut barrier function show the spread of a blue dye from the gut into the body, signifying a loss of intestinal permeability. These findings suggest that the HFSS leads to a breakdown of gut barrier function in both male and female flies, resulting in increased gut permeability.

Neurobehavioral assays

Crawling Assay

A crawling assay further assessed neuronal function and motor activity in D. melanogaster larvae. We observed a marked difference in the crawling speed and movement patterns between larvae fed an HFSS and those on a control diet. Larvae reared on the HFSS diet exhibited significantly reduced ($p < 0.001$) crawling speeds, with a 51.45±0.09% decrease compared to the control diet group (Fig. 3A,B). This impaired locomotion can likely be attributed to two key factors: first, a potential neuronal dysfunction caused by the HFSS, which may disrupt proper motor signal transmission; and second, the increased body weight observed in HFSS larvae as a result of the high-fat, sugar, and salt composition of the diet. The excessive weight gain, indicative of diet-induced obesity, further hampers their mobility. These findings suggest that the HFSS negatively affects both neuronal health and physical function, leading to reduced motor activity in D. melanogaster larvae.

Pain sensitivity assay

A nociception assay was conducted to assess potential neuronal damage in larvae by measuring their response to painful stimuli. We found that larvae fed on HFSS exhibited a significant increase ($p<0.001$) of 281±1.529% in nociceptive responses estimating latency time compared to those fed a control diet (Fig. 3C). This considerable increase in nociceptive response in HFSS-fed larvae suggests an impairment in neuronal function, possibly indicating delayed or altered transmission of neuronal signals. The increased nociception observed in HFSS larvae may be indicative of neuronal damage or dysfunction, potentially caused due to the composition of the HFSS. This diet-induced alteration in neuronal signaling could impair the larvae's ability to process pain stimuli efficiently, leading to slower and exaggerated nociceptive responses. These findings suggest that prolonged exposure to an HFSS may negatively impact neuronal health and function in D. melanogaster larvae.

Heat shock assay

To evaluate the stress response, we performed a heat shock assay and observed a significant decrease ($p<0.001$) of 68.85± 0.521% in the stress response in larvae fed an HFSS compared to those on a control diet (Fig. 3D). This reduction suggests that the HFSS larvae exhibit diminished activation of heat shock proteins (HSPs) and other stress-related pathways in response to heat stress. The impaired stress response indicates that the HFSS's high-fat, sugar, and salt composition may compromise the larvae's ability to effectively manage cellular damage and protein misfolding caused by elevated temperatures. Consequently, this diet-induced reduction in stress response could lead to increased susceptibility to heat-induced damage and decreased overall stress resilience in HFSS-fed larvae.

AChE activity

A significant downregulation of AChE activity was noted when compared to control suggesting a potential mechanism of action involving enzyme inhibition. Insufficient AChE activity results in acetylcholine buildup, disrupting normal neurotransmission and potentially harming neuronal damage. AChE activity was reduced by 37.43±0.020% as compared to the control ($p=0.0023$) (Fig. 3E).

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Floating Assay

A floating assay assessed triglyceride levels in HFSS larvae. The number of larvae floating on a 10% sucrose solution showed a significant difference of 200±0.583% from the control group ($p < 0.001$), which indicates higher lipid levels in the HFSS group (Fig. 3F, G). This flotation behavior suggests that HFSS larvae have increased lipid content. To further confirm these findings, biochemical assays for protein, triglyceride, and glucose levels were performed.

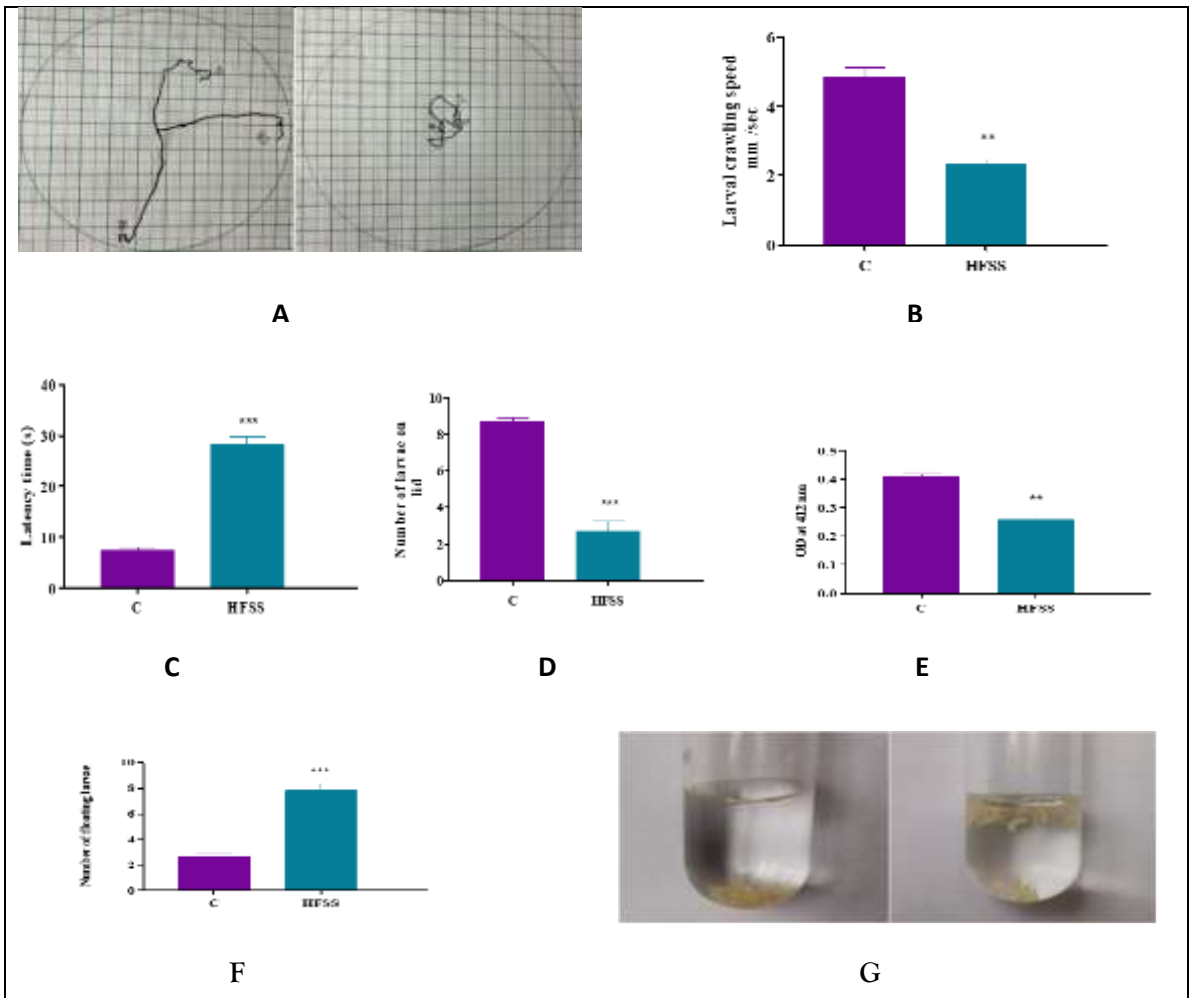


Fig. 3 Effect on neurobehavior: **(A)** Images of control larvae (left side) and HFSS larvae (right side) during crawling behavior illustrate clear differences in locomotor patterns. **(B)** The analysis revealed a significant reduction in the crawling speed of HFSS. The slower crawling speed reflects impaired neurobehavior. **(C)** The latency time of HFSS larvae is significantly increased in HFSS. **(D)** HFSS larvae showed a significant reduction in their tolerance to thermal stress in the heat shock assay. **(E)** AChE level significantly decreases in HFSS. Statistical analysis was performed using an unpaired t-test, with ** and *** indicating significant differences at $p < 0.01$ and $p < 0.001$, respectively, compared to the control group. **(F)** Picture of the floating assay, control (left), and HFSS (right) larvae. **(G)** Larvae fed an HFSS diet exhibit significantly enhanced floating behavior.

Biochemical and Oxidative stress Parameters

Assays for protein, triglyceride, and glucose levels were performed to investigate the biochemical alterations in larvae. The results demonstrated significant differences between the HFSS-fed larvae and those fed on the Control. Protein levels were notably reduced in the HFSS group compared to the control group. The HFSS diet resulted in a 43.21±0.023% reduction in protein content ($p=0.0003$). This reduction in protein levels may be attributed to the nutritional imbalance in the HFSS, which is characterized by high fat, sugar, and salt content and may lead to altered protein metabolism or degradation pathways in developing larvae (Fig. 4A). Triglyceride levels were initially assessed using a flotation assay, which suggested an increase in lipid accumulation in HFSS-fed larvae. To confirm these findings, a quantitative triglyceride assay was conducted, revealing a significant elevation of 222±5.64% in triglyceride levels in the HFSS group compared to the control ($p < 0.001$). This increase in triglycerides is consistent with the high-fat content of the HFSS, which promotes lipid storage in tissues (Fig. 4B). Glucose levels were similarly elevated in the HFSS-fed larvae, as determined by glucose assay. The HFSS's high fat and sugar content likely contributed to dysregulated glucose metabolism, resulting in a significant increase in 60.3±5.638% glucose levels compared to the control group ($p=0.0003$). This hyperglycemia state could further exacerbate metabolic stress, contributing to the observed biochemical changes in the larvae (Fig. 4C). These findings suggest that HFSS induces significant metabolic disruptions in protein, lipid, and carbohydrate metabolism, highlighting the potential long-term effects of dietary imbalances on organismal development.

LPO levels

LPO activity, a marker for oxidative stress, showed a significant increase in HFSS, indicating potential cellular and tissue damage. The LPO activity was elevated by 25±1.04% ($p=0.0065$), suggesting a notable rise in oxidative stress (Fig. 4D).

NO levels

In D. melanogaster larvae, oxidative stress can lead to an increase in nitric oxide production in the gut as part of the cellular response to maintain homeostasis and combat oxidative damage. ROS stress in D. melanogaster can induce NO production in the gut. This is part of a complex immunological communication system that involves the gut, fat body, and hemocytes, increasing the NO by 11.69±0.009% ($p=0.0353$). A decrease in NO was observed (Fig. 4E). The decrease in NO activity in HFSS-fed flies suggests that the high-fat, sugar, and salt diet may be impairing nitric oxide production, potentially leading to negative effects on neurological function and overall well-being.

CAT levels

A significant decrease of 20.97±1.66%was observed in catalase activity, indicating that the enzyme is utilized to break down hydrogen peroxide. Less production of catalase may be due to the accumulation of ROS, leading to suppression of the cells' ability to handle oxidative stress, which may lead to negative effects on overall health. ($p=0.0026$) (Fig. 4F).

GST activity

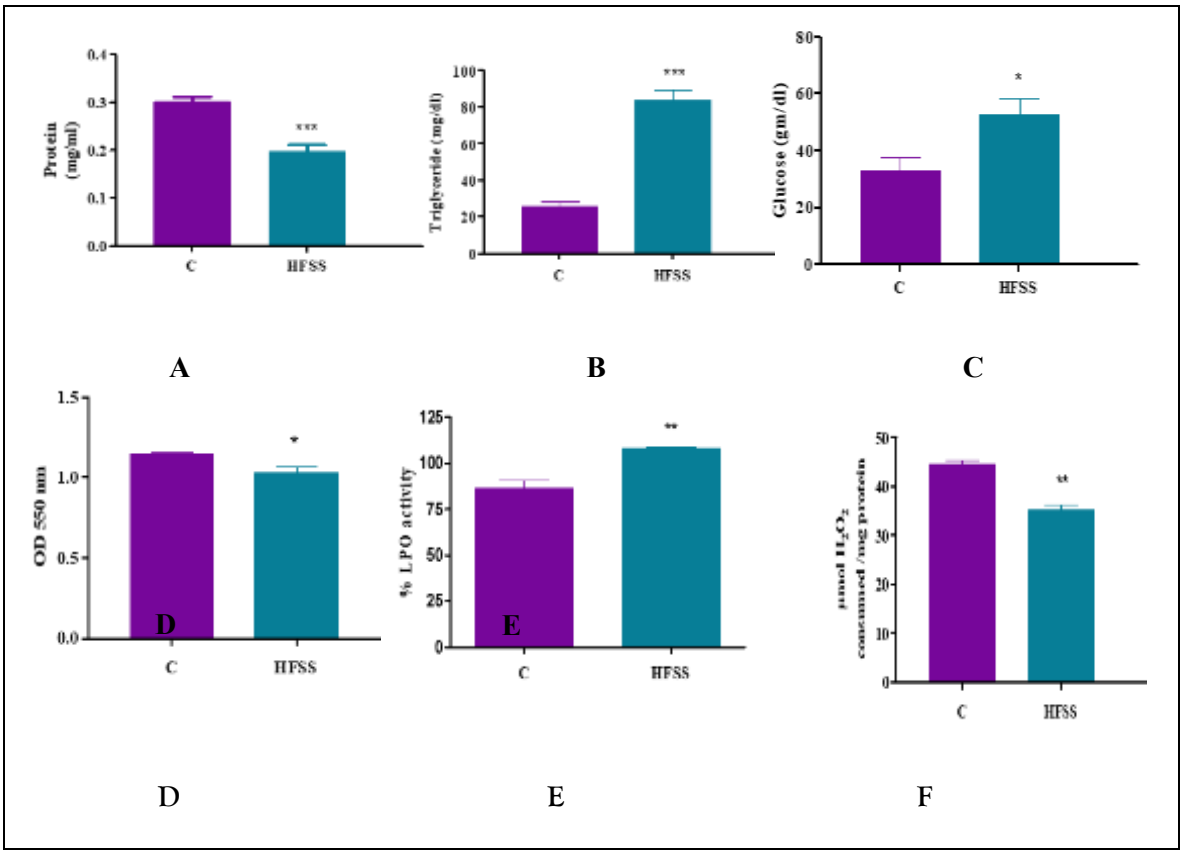
GST activity is reduced in the HFSS diet, indicating a compromised antioxidant defense system due to diet-induced oxidative stress. The HFSS diet increases reactive oxygen species, leading to oxidative inactivation of the Glutathione-S-transferase enzyme and a decrease in glutathione by 81.25±1.20% ($p=0.01413$) (Fig. 4G). Elevated sugar and salt levels exacerbate metabolic stress, inducing inflammation and potentially suppressing GST synthesis, collectively impairing the ability to counteract oxidative damage.

NBT levels

A significant increase in NBT activity indicated the amount of superoxide radicals. The absorbance values in this assay reflect the concentration of the formazan product, which in turn correlates with the extent of oxidative stress and the generation of ROS. A higher absorbance value indicates greater ROS production, signifying more oxidative stress in larvae. ROS are generated when the natural antioxidant defenses of the body are overwhelmed by potentially destructive substances. Therefore, the positive correlation between ROS production and absorbance values is a key feature of this study. NBT assay shows a significant increase in absorbance due to oxidative stress in D. melanogaster larvae, with an increase of 60±0.0019% and a very strong statistical significance ($p < 0.001$). This result suggests that oxidative stress leads to a marked increase in the production of reactive oxygen species (ROS) in the larvae (Fig. 4H).

DCFDA staining

DCFDA, or 2,7'-dichlorofluorescein diacetate, is a cell-permeable fluorogenic dye used to quantify intracellular levels of ROS production. ROS oxidizes the non-fluorescent DCFDA into the highly fluorescent compound 2',7'-dichlorofluorescein (DCF), a quantifiable index of the magnitude of oxidative stress within cells. It was shown that D. melanogaster larvae fed an HFSS exhibit elevated levels of DCF fluorescence compared to the control, suggesting increased levels of ROS (Fig.4 I). The increase in fluorescence indicates larvae fed an HFSS are experiencing increased oxidative stress, likely as a consequence of the metabolic shift caused by the high-fat and high-sugar content of such diets. The increased ROS generation may reflect mitochondrial dysfunction, lipid peroxidation, or other oxidative damage pathways activated in response to dietary excess. Thus, these findings suggest that HFSS triggers a considerable oxidative burden in D. melanogaster larvae, which may subsequently affect cellular homeostasis and overall metabolic health.



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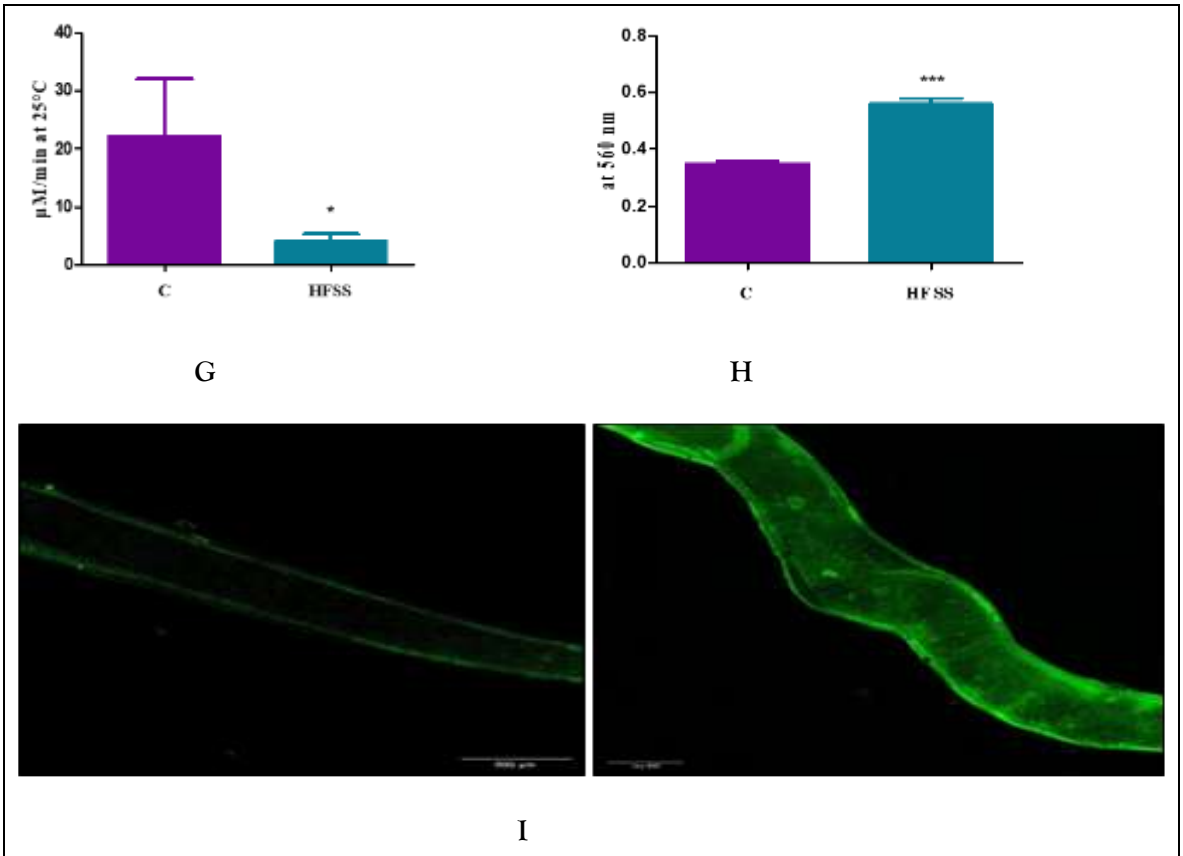

Fig. 4 Effect on Biochemical changes: **(A)** Biochemical analysis reveals a significant decrease in protein levels in HFSS-fed larvae compared to controls. **(B)** Triglyceride assays show a marked elevation in fat storage in HFSS-fed larvae. **(C)** Glucose assays demonstrate significantly higher glucose levels in larvae fed an HFSS diet. **(D) Effect of Oxidative stress:** Lipid peroxidation activity revealed a significant increase in lipid peroxidation in the HFSS diet when compared to the control. **(E)** Nitric Oxide was assessed, which revealed a decrease in NO activity in HFSS-fed flies compared to control **(F)** Catalase analysis revealed a decrease in catalase activity in HFSS larvae. **(G)** A significant decrease in GST level was observed **(H)** A significant increase in NBT was observed. **(I)** DCFDA staining shows increased ROS production in the gut of larvae exposed to an HFSS (right side), indicated by intense green fluorescence. This visual evidence emphasizes oxidative stress and its role in gut damage under dietary stress conditions.

Raman spectroscopy

Raman spectroscopy is a powerful technique used to analyze molecular vibrations, including those in biological samples, and can be particularly useful for studying changes in the molecular structure associated with diets, such as the impact of a high sugar and fat diet. The HFSS induces notable biochemical changes in the hemolymph of D. melanogaster, as reflected in the Raman spectra. The peaks at 567 cm^{-1} and 1099 cm^{-1} (peak is often associated with skeletal vibrations of C–C bonds and C-O-C bonds) likely represent the molecular signatures of lipid and carbohydrate components, which are key to understanding the metabolic impacts of the HFSS diet. The analysis of these peaks can help investigate how the body reacts to such diets, potentially contributing to obesity, insulin resistance, or other related metabolic disorders. The characteristic features of Raman spectra are attributable to the existence of hydrocarbon chains, and they were observed at 1452 cm^{-1} and 1322 cm^{-1}, respectively, which were caused by C–C and C–H stretching modes and the scissoring and twisting vibrations of CH_2 and CH_3 groups. Besides, each group's lipids were found to have specific characteristic bands. The peak at 799 cm^{-1} is more pronounced, indicating a higher presence of tyrosine residues in proteins or

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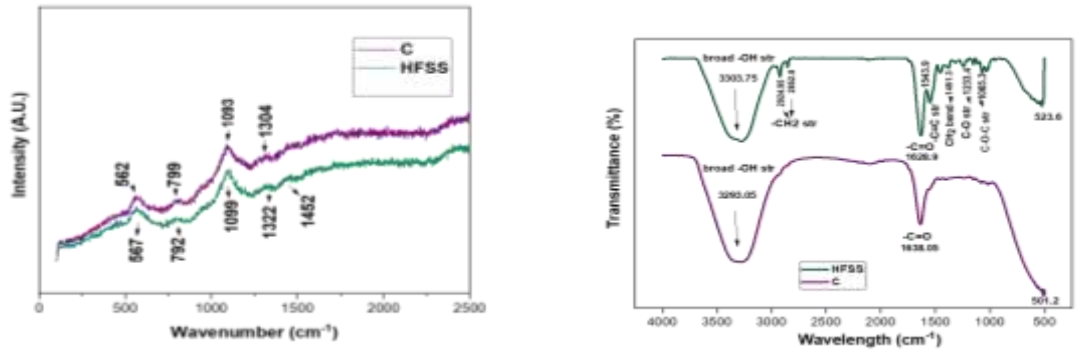
more stable nucleic acid structures. This suggests better protein integrity and nucleic acid stability, which may correlate with healthy metabolism. The peak at 799 cm^{-1} is weakened, indicating potential protein degradation or altered DNA structure due to dietary stress. Lower tyrosine-related signals suggest a reduction in certain protein expressions or metabolic shifts caused by the high-fat/ high-sugar diet. From this analysis, one can only analyze the presence of fats, salt, and sugar, as this is a surface analyzing technique. For detailed analysis use of transmission spectroscopy to qualify and quantify fats, salt, and sugar will be useful (Fig. 5A).

FTIR analysis

The FTIR spectra were recorded in the 500–3500 cm^{-1} range and characterized using a broker analyzer. In the above spectra, there is a comparison between control and HFSS diet groups, a strong peak at approximately 1628 cm^{-1} indicated the presence of esters (C=O stretching), Carbonyl groups are often found in triglycerides and other lipids, further confirming increased fat content. Additional peaks at 3303.75 cm^{-1} correspond to the O-H stretching vibrations due to water. In the HFSS which includes fat, sugar, and salt similar peaks were observed at 1065.17cm^{-1}, 1233.4 cm^{-1}, and 1451.3 cm^{-1} linked to sugar-related functional groups, such as C-O or C-H vibrations from carbohydrates, Indicating higher sugar content in the HFSS diet group. Increased -CH_2 stretching (2924.95 and 2852.8 cm^{-1} in the HFSS sample) indicates a higher presence of lipids, consistent with the high-fat diet. Appearance of C=C stretching (1543.9 cm^{-1} in the HFSS sample) Suggests unsaturated fats or lipid oxidation byproducts, which are common in high-fat diets, indicating higher sugar content in the HFSS diet group. Also, the intensity of –CH_2 bend, C=C stretching, CH_2 bending, C-O, and C-O-C stretching in the HFSS diet is high as compared to the control, suggesting that fat, salt and sugar content in the HFSS diet is high as compared to the control (Fig. 5B)

Lifespan

Aging was significantly affected by dietary conditions in our study, where the HFSS led to a substantial reduction in lifespan compared to the control. Female flies exhibited a 68.03±31.96% reduction in lifespan relative to control females (Fig. 6A,B), while males showed a 60.15±39.84% decrease (Fig. 6C,D). These findings suggest that the HFSS accelerates aging, with a more pronounced effect on



A **B**

Fig. 5 (A) Raman Spectroscopy: Raman spectra of haemolymph from HFSS diet-fed *Drosophila* showed peaks at 567 and 1099 cm^{-1}, indicating elevated lipid and carbohydrate content. Additional peaks at 1322 and 1452 cm^{-1} reflected increased hydrocarbon chains, suggesting diet-induced metabolic changes. **(B) FTIR:** Spectra (500–3500 cm^{-1}) showed increased lipid and sugar content in HFSS diet flies, with prominent peaks for esters (1628 cm^{-1}), O-H (3303.75 cm^{-1}), C-H/C-O (1065–1451 cm^{-1}), and -CH_2/-C=C stretching. These patterns confirm elevated fat and carbohydrate levels in the HFSS group.

females than males. Females have a higher vulnerability, and it might also be attributed to sex-specific differences in hormonal control, metabolic processing, or oxidative stress, suggesting that HFSS diets interact with the aging pathways differently according to sex.

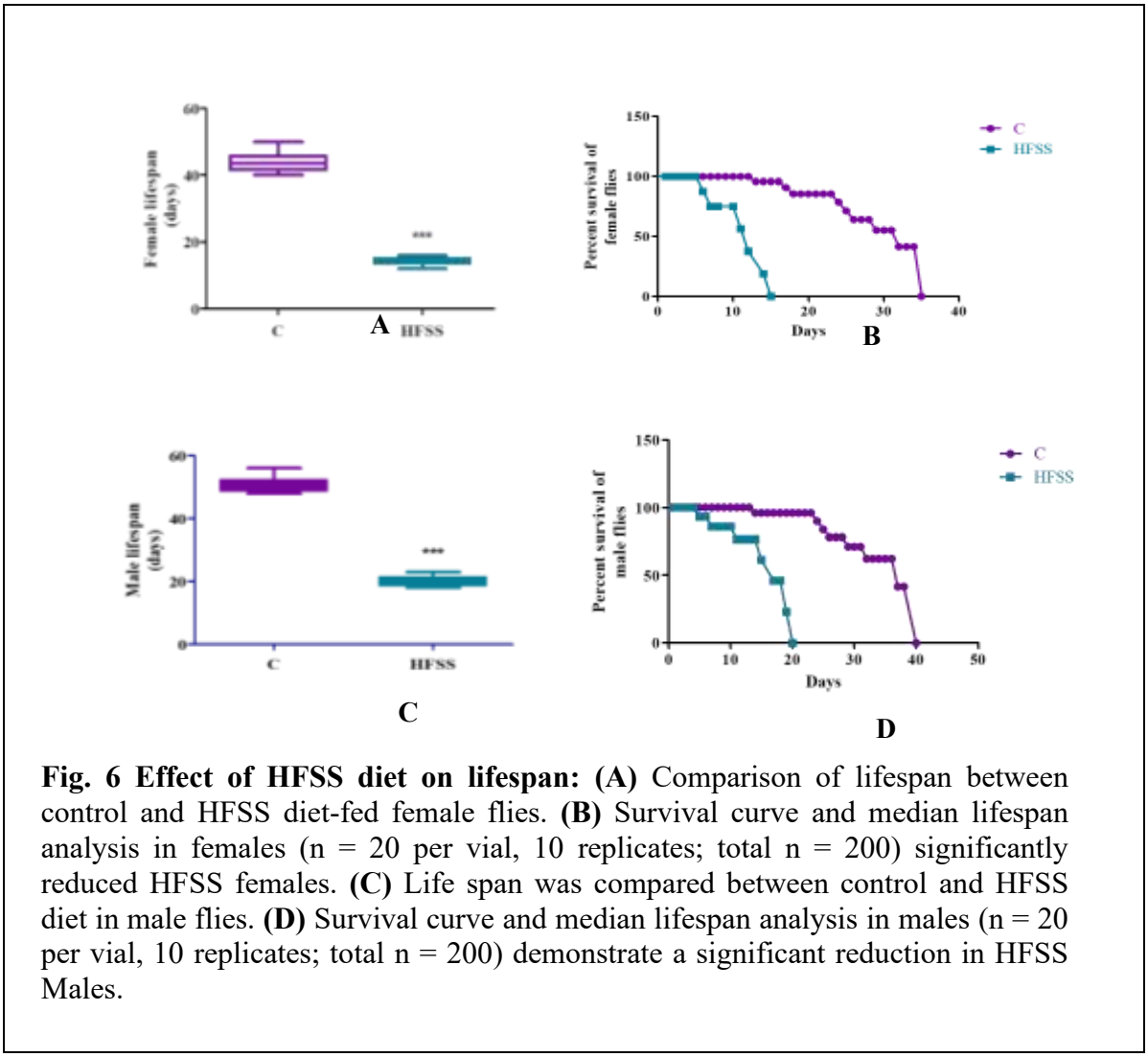


Fig. 6 Effect of HFSS diet on lifespan: (A) Comparison of lifespan between control and HFSS diet-fed female flies. **(B)** Survival curve and median lifespan analysis in females (n = 20 per vial, 10 replicates; total n = 200) significantly reduced HFSS females. **(C)** Life span was compared between control and HFSS diet in male flies. **(D)** Survival curve and median lifespan analysis in males (n = 20 per vial, 10 replicates; total n = 200) demonstrate a significant reduction in HFSS Males.

DISCUSSION

As the popularity of HFSS foods increases, health concerns are also rising. Significant links between HFSS consumption and negative effects on aging, developmental growth, biochemical changes, neurological and behavioral disorders, increased oxidative stress, and intestinal damage [35]. Additionally, delayed developmental progression was identified in D. melanogaster larvae exposed to HFSS, emphasizing the potential disruption of essential growth pathways. Further underscored the broader implications of dietary exposure during critical developmental periods. A delay in pupation and development due to HFSS foods indicates potential disruption of crucial growth pathways and broader implications of dietary exposure during critical developmental periods [17,36]. The observed delay in pupation and development under high-fat, sugar, and salt conditions highlights the potential disruption of essential growth pathways, which could harm immediate health and accelerate the aging process [25].
A study highlighted critical findings suggesting that HFSS foods can lead to metabolic dysregulation, a condition often associated with accelerated aging processes [37]. D. melanogaster larvae that were fed HFSS exhibited significant weight gain and fat accumulation, indicating a pre-obese state, which is a risk factor for chronic diseases such as cardiovascular disorders and diabetes. Additionally, research supports

this conclusion, emphasizing the importance of implementing early dietary interventions to promote a healthier lifestyle [38].

D. melanogaster is a valuable model organism for studying behavioral and neurological changes, allowing for the evaluation of pain perception and responses to harmful stimuli [39]. HFSS impacts the neurobehavioral development of larvae, such as their cross-crew movement patterns, indicating effects on sensory and motor pathways[13,40–42]. The slow crawling and curved paths of HFSS larvae at an early age highlight the effects of diet on neurological development, oxidative stress, neuroinflammation, impaired motor function, and heightened sensitivity to pain. This emphasizes the importance of a healthy diet for optimal aging. These injuries are associated with increased sensitization in these systems, as seen in mammalian models [43–45]. The gastrointestinal tract is essential for nutrient absorption, immune function, and overall organismal health, and its structural and functional integrity is crucial for these roles [46]. The HFSS diet adversely affects gut health, resulting in leaky gut and a compromised gut barrier function in D. melanogaster larvae. Maintaining a balanced diet is essential for preserving gut integrity and preventing inflammation.

The study reveals that exposure to HFSS diets increases reactive oxygen species (ROS) levels in D. melanogaster larvae, which is a significant indicator of oxidative stress. This type of stress plays a crucial role in cellular aging and may worsen cardiovascular issues and mortality rates. Additionally, the research found that oxidative stress induced by HFSS can lead to metabolic imbalances and behavioral changes in the larvae. It also contributes to cellular deterioration, affecting both structure and function [36,47]. The findings suggest that dietary interventions could help mitigate oxidative damage and aging effects in D. melanogaster, similar to mechanisms observed in mammals. Most notably, the study reported a marked increase in ROS fluorescence intensity in the larvae exposed to HFSS, highlighting the pro-oxidant effects of this diet. Overall, these results underscore a potential link between diet-induced oxidative stress and physiological damage, which may contribute to systemic inflammatory responses, mitochondrial dysfunction, and impaired gut barrier function.

Our research demonstrates that increased ROS levels in D. melanogaster larvae indicate oxidative stress, a significant effect of HFSS exposure. The cause of many diseases, including neurodegenerative disorders, has been associated with oxidative stress, which is a crucial component of cellular aging [48]. Hyperlipidemia and hyperglycemia in children are linked to significantly elevated oxidative stress levels, which can exacerbate cardiovascular morbidity and mortality [49]. This oxidative stress has been shown to mediate the development of metabolic syndrome in adults, highlighting its critical role in the pathophysiology of cardiovascular and metabolic consequences of obesity [50]. Oxidative corrosion of lipids, proteins, and DNA can result from increased ROS production, which ultimately affects cell function and raises the chance of death [36,47]. The observed metabolic dysregulation and behavioral alterations in our study seem to be caused by HFSS-induced oxidative stress, which may be connected to cellular deterioration in both structure and function.

This research utilized Raman and FTIR spectroscopy to analyze the biochemical composition of D. melanogaster larvae exposed to HFSS diets, providing a novel approach to assessing molecular changes induced by dietary stress. Raman spectroscopy revealed spectral signatures corresponding to fat, sugar, and salt, offering a non-destructive, label-free method to evaluate biochemical alterations in vivo. These findings suggest that HFSS exposure leads to significant metabolic disruptions, confirming our biochemical assay results. Furthermore, FTIR spectroscopy confirmed these observations by identifying specific peaks associated with structural changes in fat, sugars, and salts, reinforcing evidence of macromolecular modifications due to HFSS consumption. This study represents the first application of Raman spectroscopy in this specific context, offering unique insights into the molecular disruptions induced by HFSS diets. Previous studies have employed Raman spectroscopy on whole anesthetized flies, where individual flies were carefully positioned on a slide and subjected to laser scanning with repeated measurements for accuracy [51]. Our approach differs significantly; instead of whole-body analysis, we utilized Raman and FTIR spectroscopy on the hemolymph of D. melanogaster, providing a more direct and sensitive method to detect diet-induced metabolic changes. This innovative approach allows for a more precise understanding of how HFSS diets influence metabolic health, oxidative stress,

and cellular function at the molecular level. Our findings open avenues for future research utilizing vibrational spectroscopy to assess diet-induced biochemical alterations in metabolic disease models.

CONCLUSION

This study highlights the significant effects of HFSS consumption on the development, health, and aging of D. melanogaster larvae. Our results indicate the detrimental metabolic effects of the HFSS diet by demonstrating that exposure to HFSS resulted in significantly increased body weight, higher triglyceride levels, and decreased lifespan. Crucially, HFSS-induced oxidative stress was linked to indications of accelerated aging in addition to intestinal barrier disruption and locomotor impairments. These markers of aging-such as oxidative damage and protein aggregation, suggest that an HFSS may drive age-related declines earlier in the life cycle. By emphasizing the long-term consequences of HFSS, our study highlights the need for balanced, nutrient-dense diets, particularly during critical developmental periods, to promote healthy aging and reduce the risk of chronic health issues associated with early dietary imbalances.

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