International Journal of Entomology Research
www.entomologyjournals.com
ISSN: 2455-4758
Received: 01-08-2022, Accepted: 17-08-2022, Published: 02-09-2022
Volume 7, Issue 9, 2022, Page No. 19-25

The effect of non-nutritive sweeteners on oxidative stress, reproductive fitness and their interrelationship in *Drosophila melanogaster*

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Abstract
Non-Nutritive Sweeteners (NNS) are low-calorie synthetic sweeteners that have negative effects on metabolic, physiological, behavior and reproduction. A present study has been undertaken to understand the effects of NNS on accessory gland traits and reproductive fitness in *Drosophila melanogaster (D. melanogaster)*. Dietary restrictions may lead to an imbalance in the production of Reactive Oxygen Species (ROS) and detoxification, which contribute to nutritional stress. A high level of oxidative stress affects the Accessory gland proteins (Acps) of *D. melanogaster* during reproduction. *D. melanogaster* has a vital role in nutrition research where diet-induced stress responses and reproductive and life-history traits can be examined under defined experimental procedures. In this work, aspartame and sucralose are used as NNS and nutritive sweetener sucrose to examine the effects of oxidative stress (ROS, SOD and CAT) and reproductive fitness of *D. melanogaster*. The results reveal that NNS has a positive correlation between oxidative stress and the reproduction of flies. Aspartame-treated flies showed higher ROS levels and lower SOD and CAT antioxidant enzyme activity. ACPS and reproduction traits were reduced in NNS-treated flies, suggesting that oxidative stress may be susceptible to the cost of reproduction.

Keywords: *Drosophila melanogaster*, oxidative stress, accessory gland proteins (ACPS), reproductive fitness

Introduction

Non-Nutritive Sweeteners (NNS), also called artificial sweeteners are gaining popularity as nutritional assessment tools to aid in the fight against diseases by offering a sweet flavor without the added calories. NNS are commonly used by people in an attempt to reduce their overall daily caloric consumption, lose weight, and maintain a balanced diet [1]. Sweeteners are either nutritive or non-nutritive, and both improve the flavor and texture of food. Carbohydrates and calories are present in nutritive sweeteners (energy). NNS are low or no-calorie alternatives that contain very little or no carbohydrates or energy [2]. To date, with sucrose being the international standard for sweetness, several NNS has been approved by the US Food and Drug Administration (FDA), but aspartame and sucralose are used widely in food and dietary products. Depending on their respective properties, different NNS are absorbed differently and their metabolic fate may elucidate conflicts about their effect on oxidative stress and reproductive fitness in model organisms [3].

Aspartame is a low-calorie synthetic sweetener frequently used in foods, medications and beverages, notably carbonated and powdered soft drinks. When aspartame is taken orally, it is hydrolyzed into methanol which cannot be metabolized in the enterocytes, and then oxidized in the liver into formaldehyde [4]. The metabolic process of methanol to formaldehyde and formic acid is linked to the generation of superoxide anion and hydrogen peroxide, which causes oxidative stress [5]. Many studies have shown that aspartame has negative effects on metabolic, physiological behavior and reproduction of various model organisms [6]. Anbara *et al*., 2020 [7] revealed that long-term exposure to aspartame led to reproductive toxicity and this development causes structural and functional impairments of proteins that regulate and maintain the lower temperature of the testes. Further, the results suggest that aspartame in high doses for a prolonged period reduced sperm parameters and total antioxidant capacity, antioxidant enzyme activities (superoxide dismutase, catalase, and glutathione peroxidase), and pituitary-testis axis hormones cause apoptosis in mature male mice's testis. According to Ashok and Sheeladevi (2014) [8], Wistar strain male albino rats had a significant increase in enzyme activity and a decrease in glutathione reductase and protein thiol, indicating the generation of free radicals. This suggests that aspartame is toxic at the cellular level and that long-term exposure to aspartame can alter the brain's antioxidant status and induce apoptosis. Further, in *Drosophila melanogaster* (*D. melanogaster*) sperm competition is influenced by accessory gland proteins and the emergence of free radicals and variation in reproductive fitness in *Drosophila* is likely to represent a variation in accessory gland proteins. However, this study mainly focuses to examine the impact of NNS on oxidative stress and reproductive fitness in *D. melanogaster*. Sucralose is a sucrose-derived disaccharide with a sweet flavor that is very similar to sucrose, which represents its widespread use in foods and beverages. However, sucralose did not support any early negative effects on metabolic studies when examined in animal studies, but there are controversies about the genotoxic potential and effects of sucralose in animal toxicology [9].

Due to the nutritional cost of reproduction, diet and mating preferences frequently go hand in hand. Oxidative stress is also produced during mating, courtship, and copulation [10,11]. Consequently, oxidative stress brought on by dietary components like carbohydrate and protein intake along with oxidative stress may change metabolic phenotypes and affect organism survival. In male *Drosophila* flies, the accessory gland of the reproductive system develops from a distinct collection of cells in the genital imaginal disc [12].

This gland's secretions, along with sperms, are transferred to the female during copulation [13]. Secretion has been found to cause physiological changes in the mated female accessory gland, including stimulation of oviposition, egg-laying, reduction in female receptivity to courtship, facilitation of sperm storage, and maintenance of sperm in the mated female [14-16].

The age of the organism is thought to be a significant factor that determines metabolic processes and fitness factors [17,18]. In *D. melanogaster* flies, age-dependent oxidative stress and its effect on reproductive fitness and metabolic processes support the theory that oxidative stress levels increased with age, resulting in a lack of male reproductive fitness [19]. According to a recent study, nutrition has a beneficial influence on the accessory gland and male reproductive success in *D. melanogaster* when provided with a protein and carbohydrate-rich diet. However, the effect of NNS on oxidative stress and accessory gland traits has yet to be studied.

Drosophila studies concern the impact of diet on various outcome measurements including behavior, life span, locomotor activity and fertility. It also has offered an excellent system for modeling several aspects of dietary intervention studies which have many similarities with mammalian species [20]. Hence, the current research has been undertaken in *D. melanogaster* to examine the effect of NNS aspartame and sucralose on oxidative stress and reproductive traits.

Material and Methods

Drosophila melanogaster was used in the present experiment. The experimental flies were provided from the *Drosophila* Stock Centre at Manasagangotri, Mysuru. These flies were maintained on wheat cream agar medium (Control media) and kept in a 12-hour dark, 12-hour light cycle. 10 males and 10 females were placed into culture bottles kept at 22±1ºC with a relative humidity of 70%. This method was replicated 3 times to acclimatize flies to the laboratory environment. Following Delcour's procedure [21], eggs were collected from these flies in the fourth generation. According to Amrutha and Krishna [22], the NNS concentrations for the study were set. 100 eggs were seeded in each culture bottle containing NNS (Aspartame and sucralose treated media), nutritive sweetener sucrose, and control media. Further experiments were carried out with the flies obtained from these media.

Effect of Non-Nutritive Sweeteners on Biochemical assays

Control and treated flies obtained as described above were used in the biochemical assays. Ten flies per vial-cold anesthesia treated were homogenized in 1ml of respective assay buffers and centrifuged at 2500 g for 10 mins at 4ºC. The supernatant obtained was used to measure ROS and antioxidant enzymes (SOD and CAT). A total of three trials were conducted separately for ROS, SOD and CAT for control and treated flies.

Reactive Oxygen Species (ROS)

ROS was measured using 2′7′-Dichlorofluorescein diacetate (DCFH-DA) (Sigma Chemical, St. Louis, MO, USA). The presence of ROS was determined using a spectrofluorometer with excitation at 488 nm and emission at 525 nm. ROS was quantified using a DCF standard curve and expressed as µmoles of DCF formed/min/mg protein [23].

Antioxidant enzymes (SOD and CAT)

SOD activity was measured using pyrogallol (2 mM) (Sigma Chemical, St. Louis, MO, USA), with autoxidation of pyrogallol in 0.1 M Tris buffer (pH 8.2) monitored at 420 nm for 3 minutes and expressed as units of enzyme required to prevent 50% pyrogallol autooxidation [24]. The activity of (H2O2) catalase was assessed by monitoring the change in absorbance at 240 nm for 3 minutes and expressed as millimoles of H2O2 decomposed/min/mg protein using 1 % hydrogen peroxide in 0.05 M phosphate buffer (pH 7) as a substrate [25].

Protein estimation

Protein content in the homogenate was estimated by Lowry's method using BSA (Sigma Chemical, St. Louis, MO, USA) as the standard [26].

Effect of non-nutritive sweeteners on accessory gland size, the numeral of cells (per lobe), and cell size in accessory gland

Accessory glands of control and treated flies (Sucrose, Aspartame and Sucralose) were dissected separately using Medium A [27] and were fixed in 1N HCl for 5 min. The shape of the accessory gland in *D. melanogaster* was 's', 'c' or 'j' formed. Following Ravi ram and Ramesh [28], the area of each gland was estimated by dividing it into smaller regions made up of triangles, trapeziums, and rectangles. Later capturing the photos of the dimension of accessory gland size, immediately these glands were stained using Lacto aceto-orcein 2% and stained for up to 20 minutes and squashed between a glass slide and a coverslip using 45% acetic acid. The total number of main cells in each accessory gland was counted and main cell sizes were measured. A total of fifty replicates from each concentration of dietary medium were used for the experiments.

Relationship between quantity of total protein in unmated males and male diet Sample preparation of unmated males

Accessory glands of unmated control and treated flies (etherized) were dissected separately using insect saline and entomological needles. These glands were maintained in 95% ethanol. The outer membrane of ethanol-fixed glands was removed on a clean micro slide, and secretions of the accessory gland were washed in a 1:1 combination of methanol and chloroform and dried at 37° C in an incubator for 15 minutes. To dissolve the glands and secretions, each sample was added a 100 µL of sample buffer (0.625 M tris-HCL pH 6.8, 1% SDS, 1% b-mercaptoethanol, and 10% glycerol). Using Lowry's method [26], the accessory gland protein was estimated quantitatively collecting ten pairs of accessory glands from each concentration separately.

Quantitative estimation of protein using Lowry's method

Approximately 50 µL of ACPS were obtained from the unmated males (10 days old) as mentioned above and mixed with 5 ml Bradford reagent, which was prepared by mixing 100 mg Coomassie Brilliant Blue G-250 (in 50 ml 95 % ethanol) to 100 ml of 85 % phosphoric acid and then diluting the mixture with 1L distilled water. The solution was allowed to settle for 5 minutes to develop color. The amount of proteins in each sample was determined using a spectrophotometer measuring optical density at 595 nm. As

a standard, bovine serum albumin was used. Fifty trials were carried out for each NNS aspartame and sucralose, nutritive sweetener sucrose and the control and analyzed separately.

Effect of Non-Nutritive Sweeteners on copulation duration, the quantity of protein transferred, fecundity, and fertility

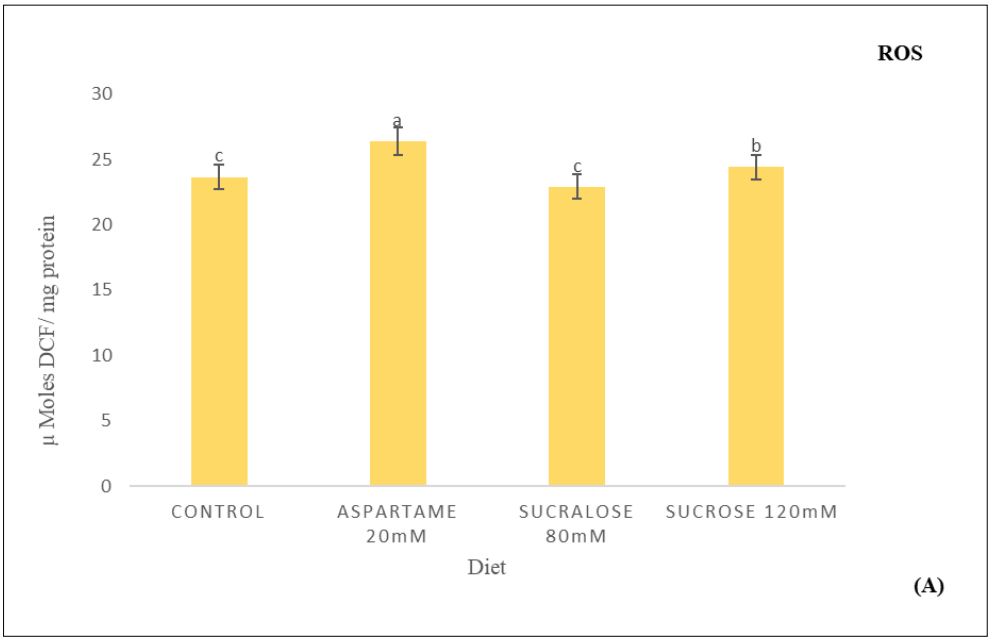
To study the relationship between NNS accessory gland proteins, copulation length, fecundity, and fertility, A 5-6-day-old virgin female and an unmated male from each medium were placed in an Elens Wattiaux mating chamber for 1 hour. Within an hour, unpaired pairs were discarded. If mating occurred, the duration of copulation was recorded (time between initiation of copulation to termination of copulation of each pair). Mated females were placed in a new vial containing wheat cream agar medium after copulation, and vials were changed every 24 hours until they died. The number of eggs laid and the number of progenies produced was also recorded. To quantify protein, the mated males were dissected. Fifty trials were carried out for each of the control and treated flies and analyzed separately.

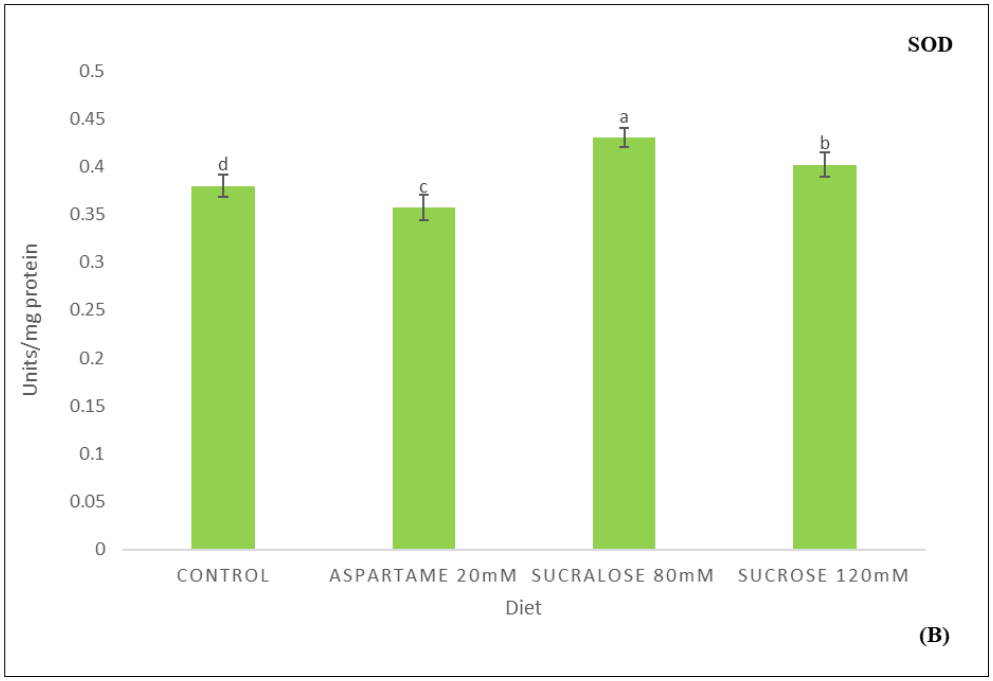
Statistical analysis

One-way Analysis of Variance (ANOVA) followed by Tukey's Post-hoc test was done for a span of copulation, Accessory gland protein (number and size of the main cell, size of accessory gland, Amount of Accessory gland protein, transferred amount of ACPS), sperm traits (spermathecae, seminal receptacle, total sperm transferred) and oxidative stress enzymes. All the above information was done by using Statistical Product and Service Solutions-20 program.

Results

Oxidative stress





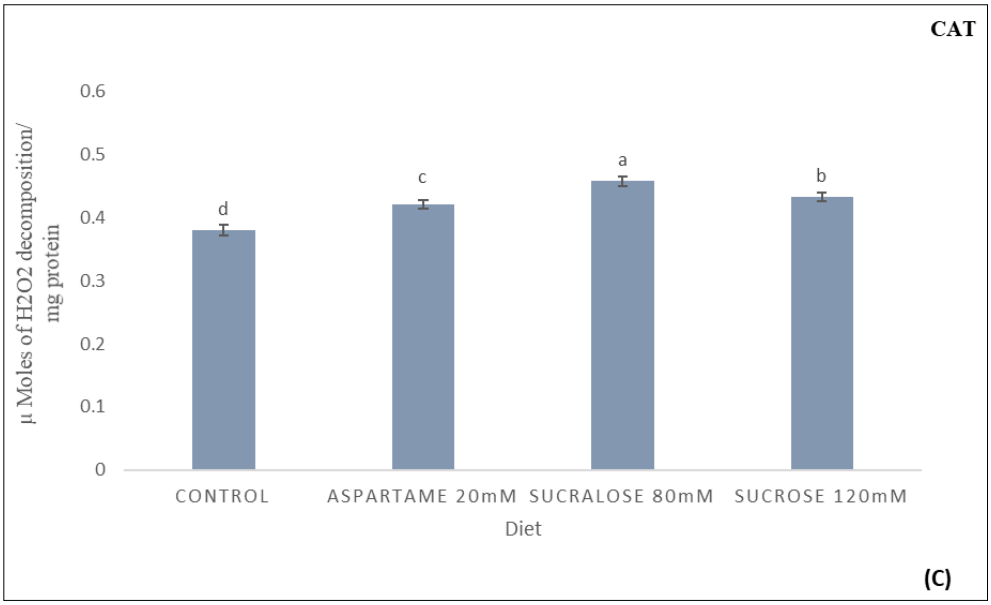


Fig 1: (A, B, C) Effect of NNS on ROS (A), SOD (B) and CAT (C) activities in *D. melanogaster*. (ROS, *df=3,196 f=9353.80:* SOD, *df=3,196 f=7530.12:* CAT, *df=3,196 f=4344.85; p<0.001)*. Different letters in the figure indicate significance at 0.05 levels by Tukey's Post-hoc Test.`

Table 1: Effect of Non-Nutritive Sweeteners diet on accessory gland traits in *Drosophila melanogaster*

Parameters	Df	Control	Aspartame treated media	Sucralose treated media	Sucrose treated media	F-value
Number of Accessory Gland cells (Unmated)	3,196	1481±1.085^{b}	1104.57±1.25^{d}	1193.14±1.053^{c}	1380.32±1.67^{a}	4746.036**
Accessory Gland cell size (mm)	3,196	0.066±0.000098^{d}	.000066±.0000011^{a}	0.000702±.0000094^{b}	0.0066±.000074^{c}	2697.689**
Accessory Gland size (cm2)	3,196	0.334±0.00016^{a}	0.324±0.00033^{d}	0.338±0.00010^{c}	0.335±0.00011^{b}	443.606**
Quantity of Protein in Unmated males (mg/male)	3,196	15.80±0.0154^{d}	14.29±0.033^{a}	14.64±0.011^{b}	15.58±0.021^{c}	1105.848**

Values are Mean ± SE. Different letters in table suggests Significant at 0.05 level (P < 0.05) by Tukey's post hoc test; **Significant at 0.001 level (P < 0.001).

Table 2: Relationship between different parameters with males treated with different NNS in *Drosophila melanogaster*

Parameters	Df	Control	Aspartame treated media	Sucralose treated media	Sucrose treated media	F-value
Copulation duration (min)	3,196	17.84±0.116^{c}	14.58±0.207^{a}	16.11±0.175^{b}	16.61±0.135^{b}	68.994**
Amount of Protein in mated males (µg/male)	3,196	11.90±0.055^{a}	12.38±0.058^{c}	12.15±0.042^{b}	11.77±.053^{a}	26.033**
The amount of protein transferred to the mated female	3,196	3.90±0.056^{c}	1.85±0.062^{a}	2.49±0.047^{b}	3.81±0.062^{c}	307.469**
Spermthecae	3,196	34.03±0.108^{d}	15.24±0.082^{a}	16.36±0.086^{b}	24.45±0.215^{c}	4180.661**
Seminal receptacle	3,196	178.94±0.794^{d}	83.48±0.554^{a}	90.18±0.801^{b}	111.96±0.641^{c}	3814.438**
Total sperm transferred	3,196	6474.50±4.92^{d}	3130.44±9.61^{a}	3299.05±7.77^{b}	4716.25±11.32^{c}	31558.011**

The copulation period was measured in minutes; the number of sperm are counted and the amount of ACPS in µg/male or µg/ pair of glands. Different letters in table suggests Significant at 0.05 level (P < 0.05) by Tukey's post hoc test; **Significant at 0.001 level (P < 0.001). The quantity of protein transferred was calculated by subtracting the protein of mated males from unmated males.

Results

Oxidative Stress

Figure 1 illustrates the mean value of ROS, SOD, and CAT. The data was analyzed on flies treated with different NNS and nutritive diets. It was observed that aspartame-treated flies and sucrose-treated flies had increased ROS levels when compared with sucralose and control (1A). Furthermore, when comparing treated flies and control, aspartame-treated flies showed a significant decrease in antioxidant enzymes SOD and CAT. One-way ANOVA followed by Tukey's Post-hoc test indicated significant differences in ROS levels between diets in *D. melanogaster*.

Antioxidant parameters

Figures 1B and C show superoxide dismutase (SOD) and catalase (CAT) activities in control and treated flies. The activity of antioxidant enzymes SOD and CAT increased in flies treated with sucralose. Aspartame-treated flies showed decreased antioxidant enzyme activities when compared to sucralose and control flies. One-way ANOVA followed by Tukey's post hoc test indicated significant differences in SOD, and CAT levels between diets in *D. melanogaster*.

Reproductive fitness

Table 1 shows the mean values for accessory gland traits in unmated males. The number of main cells increased in aspartame and sucralose-treated males when compared with sucrose and control-treated males. One-way ANOVA followed by Tukey's post hoc test revealed significant differences between the sweeteners. while the size of the main cells decreased in sucralose and aspartame treated male flies and increased in sucrose treated and control male flies comparatively. Tukey's post hoc test showed significant variation between the sweeteners. The size of the accessory gland was larger in sucralose, sucrose, control and smaller in aspartame-treated flies. Tukey's post hoc test revealed significant variation between the sweeteners. whereas the quantity of Acps in unmated males was least in aspartame and sucralose when compared with sucrose and control unmated males. One-way ANOVA followed by a Tukey's post hoc test was done on the above data and showed significant variation in the mean number of main cells in the accessory gland was found to be significantly greater in aspartame and sucralose when compared to the sucrose and control.

Table 2 explains the interaction between copulation duration and quantity of protein and sperms transferred to mated females in *D. melanogaster*. The copulation duration and sperm transferred to mated females were least in aspartame and sucralose treated flies when compared to sucrose and control flies. One-way ANOVA followed by Tukey's post hoc test revealed significant variation between the sweeteners. While the amount of accessory gland protein in mated males increased in aspartame and sucralose treated flies when compared to sucrose and control flies. Tukey's post hoc test showed that the accessory gland protein transferred to mated females varied significantly between sweeteners. Control and sucrose-treated female flies showed insignificant variation in Tukey's post hoc test between diets.

Discussion

NNSs have replaced sugar as a sweetener in today's advanced world and have been approved as safe and acceptable for use in food by the US FDA. NNS has been associated with increased risk factors for physiological, biochemical, clinical, and metabolic variables, all of which have been connected to an increased risk of cardiovascular disease and type 2 diabetes [1]. In the present study, *D. melanogaster* was used in the experiments to analyze the potentially toxic effects of different NNS on oxidative stress and reproductive fitness. Oxidative stress is defined as an imbalance between oxidants and antioxidants [29,30]. In previous studies, parental investment in reproduction was restricted because oxidative damage has been linked to reduced biological function, including reproduction [31, 32]. The objective of this research is to better understand how NNS affects oxidative stress and reproductive fitness. The LD_{50} doses of NNS aspartame and sucralose, the nutritive sweetener sucrose, were determined, and oxidative stress resistance and reproductive fitness assays were performed [22]. The results show that aspartame-treated flies have an increased level of ROS production and a decreased level of antioxidant enzymes (SOD and CAT) (Fig1). The generation of ROS in aspartame could be attributed to the methanol generated during the aspartame metabolism microsomal oxidizing system, which metabolizes methanol and has been identified as a source of free radical generation [33-35]. Aspartame's toxicity resulted in an increase in hydrogen peroxide generation and a decrease in mitochondrial viability. Cells utilize antioxidant defense mechanisms based mostly on enzymes such as superoxide dismutase (SOD) and catalase (CAT) to defend themselves from ROS-induced cellular damage. Variations in the antioxidant enzymes SOD and CAT may be a compensatory response to the increased hydrogen peroxide levels in flies treated with aspartame and sucralose. Our results confirm the findings of Ashok *et al***.** [36], who found that aspartame can contribute to the development of oxidative stress.

In *D. melanogaster*, the effect of NNS aspartame and sucralose on the male reproductive system has been studied. The reproductive system of male *Drosophila* consists of two accessory glands that produce a set of cells in the imaginal disc referred to as the main cells (96%) and secondary cells (4%). Accessory gland proteins are a complex protein combination that is present in seminal fluid. The synthesis of accessory gland proteins occurs in main cells [9]. The amount of ACPS produced by male *D. melanogaster* is known to depend on the number and size of the main cells [37]. During copulation, these ACPS are transferred to the females. ACPS in mated females causes physiological and behavioral changes such as increased egg production, ovulation, and decreased sexual receptivity [38].

Although studies have shown that stress resistance and longevity can be related experimentally, in most cases, higher stress resistance is associated with a shorter lifespan [39]. Two studies in captive zebra finches (*Taeniopygia guttata*) suggest that oxidative stress is a proximate cause of reproduction [40, 41]. Variation in antioxidant enzyme activity can only be interpreted as ROS production [42]. In zebra finches, the increased reproduction resulted in body mass loss in both sexes and the impact of reproduction led to the development of resistance to oxidative stress. A recent study in *D. melanogaster* flies demonstrated the influence of oxidative stress on reproductive fitness and the relationship between the two [43]. A detailed review of Tables 1 and 2 illustrates that males treated with aspartame and sucralose have smaller main cells which produce a lower amount of protein synthesis, while males treated with sucrose control flies have larger main cells and synthesize high protein in the gland. As a result, males with significant amounts of accessory gland proteins will have a large amount of stored secretion, and so gland size and protein are dependent on each other. Hence, a study in *D. melanogaster* suggests that the number and size of the accessory gland's main cell, as well as gland size, play a vital role in ACPS synthesis.

The number of main cells in the accessory gland and the nutrients influenced the amount of ACPS. According to Ravi Ram and Ramesh [28], the secretory activity of the accessory gland's main cell may influence the number of ACPS in *D. nasuta*. the accessory gland of *D. melanogaster* contains the main cells involved in accessory gland formation, it is thought that variations in the amount of ACPS in unmated males treated with different diets could be due to differences in the size of the accessory gland, the number/cell size of the accessory gland's main cell, or the accessory gland's secretory activity with the male diet [37]. In the present study, it was found that aspartame and sucralose decreased the number of main cells in the accessory glands and the size of the main cells, resulting to produce lesser

ACPS than those treated with sucrose and control flies (Table 1). This reveals that NNS had toxic effects on *D. melanogaster* male accessory gland traits. This is the first study of its kind to study the toxic effects of NNS on *D. melanogaster* male reproductive traits.

Copulation in *Drosophila* species varies between species and strains of the same species [44, 45]. Nutrition also has an impact on the copulation duration [46]. A careful analysis of table 2 in the present study reveals an interrelationship between diet, copulation time, and the amount of protein and sperm transferred to mated females. Aspartame-treated males copulated least and transferred a significantly lesser quantity of ACPS and sperms into the females than those treated with sucralose, sucrose and control flies (Table 2). Aspartame-treated males have low caloric values that lead to the inability of flies to digest and store the energy of certain compounds that helps in the attraction of females to copulation. Thus, the inner and outer mitochondrial membranes are disrupted by high amounts of free radical generation, resulting in oxidative stress, which inhibits sperm motility. Factors such as the size and age of the fly also affect the copulation duration. The longer the time of copulation, the more ACPS and sperms are transferred to unmated females [37]. Male flies treated with aspartame in the current study had the shortest copulation time compared to those fed sucrose and a control diet. This implies that nutrition (carbohydrate) in the diet has a significant impact on the reproductive fitness of male *D. melanogaster*. Furthermore, the mated female of aspartame-treated flies had the least amount of accessory gland proteins and sperm transferred (Table 2). It was revealed that the quantity of accessory gland proteins and sperms transferred to aspartame-treated mated females was the least when compared to mated females of sucrose and control diet. Hence, this study confirms that nutrition plays a vital role in copulation duration, fecundity and fertility of *D. melanogaster*. This result supports the work of Amrutha and Krishna [22] working on *D. melanogaster* have found that NNS intake decreased survivability and reproductive traits. This suggests that NNS has a detrimental effect on oxidative stress and reproductive fitness studies in *D. melanogaster*.

Conclusion

The present study highlights the effect of oxidative stress on reproduction and their interrelationship in *D. melanogaster* when treated with NNS aspartame and sucralose. The results of the current study suggest that aspartame can facilitate oxidative stress. Aspartame-treated flies have a negative impact on reproductive success traits. Thus, according to the findings of this study NNS has adverse influences on oxidative stress and reproductive fitness. Further research is needed to determine the impact of parental nutrition on offspring immunity.

Acknowledgments

The authors are thankful to the SC/ST Cell, University of Mysore, Mysore for supporting us financially. We also thank the Chairperson, Department of Zoology, *Drosophila* Stock Center and Stress Biology Lab, Department of Zoology, University of Mysore, Mysuru, Karnataka, for providing facilities to carry out the above work. The authors are grateful to anonymous reviewers and the editor for their comments to improve the quality of the manuscript.

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