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Influence of non-nutritive sweeteners on stress resistance in *Drosophila melanogaster*

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Abstract
Environmental changes can decrease fitness which is generally called stress. Nutritional variation also causes stress and influences the fitness, survival and evolutionary approach of organisms. In the present study, flies of *Drosophila melanogaster* obtained from Non-Nutritive sweeteners aspartame and sucralose, Nutritive sweetener sucrose and control media have been subjected for physiological stresses such as desiccation resistance, chill-coma recovery, heat-shock resistance and starvation resistance. Results reveal that Non-Nutritive Sweeteners have the least resistance to stress when compared to nutritive sweetener sucrose and control flies. Aspartame treated flies had the least resistance to desiccation and recovery from cold treatment and had the highest mortality rate in starvation resistance when compared with Sucralose, nutritive sweetener sucrose and control. However, Aspartame treated flies recovered soon when subjected to heat-shock resistance. Thus, our findings add to the evidence that adult flies treated with non-nutritive sweeteners have a significant impact on the ability to resist stress, and that the type of stress affects the altered nutritional composition in flies.

Keywords: *Drosophila melanogaster* (*D. melanogaster*), non-nutritive sweeteners (NNS), stress resistance

Introduction
Many species survival, growth, and reproduction are affected by the lack of nutrient availability. Most animals are exposed to times of acute food scarcity, and as a result, stress-enhancing adaptations are expected to evolve over the period. The interaction between nutrition and environment is predominant for stress and behavioral changes of an organism. Stress is referred to as any environmental condition that triggers the fitness of the organisms (Rion and Kawecki, 2007) [35]. Starvation from food deprivation and malnourishment from nutrient imbalance or depletion are examples of nutritional stress. Fluctuations in food availability or composition, oxygen levels and temperature activate chemical processes that allow an organism to exist, adapt and reproduce. Non-Nutritive Sweeteners (NNS) are getting prominence as dietary assessment which helps in fighting against obesity and diabetes mellitus by providing a sweet flavor without the additional calories (Siervo *et al.*, 2014) [40].
Dietary restriction or mild starvation can improve longevity and stress tolerance, revealing the complexity of organismal nutrition intake and utilization (Kapahi *et al.*, 2017; Sisodia and Singh, 2012) [24, 43]. Ectotherm's physiology is highly influenced by their surrounding's temperature. Extreme temperatures can be lethal, but even moderate temperatures have an impact on performance and, ultimately, Darwinian fitness (Huey and Berrigan, 2001; Martin and Huey, 2008; Savage *et al.*, 2004) [20, 30, 38]. Research has revealed that reptiles (lizards) thermoregulatory behavior influences habitat choice, behavioral activity patterns, and physiological performance and survival in the field (Heath, 1965; Porter *et al.*, 1973; Huey, 1991; Bartholomew, 1964; Huey and Stevenson, 1979; Christian and Tracy, 1981) [16, 34, 5, 21, 9].
Recent research has revealed that various species respond to stress in different ways (Yaribeygi *et al.*, 2017) [53]. In general, starvation resistance is associated with a long lifespan, slow development, low egg production, and big body size. Insight into the mechanism of evolution in the laboratory (Rion and Kawecki, 2007) [35]. and natural populations can be achieved by studying the evolution of starvation resistance in the lab. A study found that treating *D.melanogaster* a sucrose-rich diet increased stress and starvation resistance, indicating that food composition may influence stress responses (Lüersen *et al.*, 2019) [28]. Physiological and behavioral responses have assisted model organisms in adapting to and surviving under an expansive range of humidity (Matzkin *et al.*, 2007; Sejian *et al.*, 2018) [31, 39]. oxygen concentration (Romero *et al.*, 2007) [36], temperature (Bochdanovits and de Jong, 2003; Trotta *et al.*, 2006) [6, 51] and food availability (Kolss *et al.*, 2008) [26].
Desiccation resistance is the ability to withstand water loss in dry or drought conditions. Laboratory selection studies for desiccation show that rates of water loss in *D. melanogaster* are strongly related to resistance (Gibbs and Markow, 2001) [12]. Desiccation and starvation resistance in *Drosophila* are two stress-resistant components for which there is significant evidence of genetic variation between wild populations (Hoffmann and Harshman, 1999) [17]. When compared to tropical populations, temperate populations have a higher level of resistance and desiccation but in the case of starvation resistance, the pattern is reversed.
Ectothermic insects give a better understanding of several factors of thermal biology. for example, Heat and cold tolerance have been extensively studied in *Drosophila melanogaster*. *Drosophila* larvae are sensitive in detecting high temperatures (≥39 °C) in both the central nervous system and heavily arborized peripheral neurons beneath the epidermis, according to anatomical observations. (Tracey *et al.*, 2003) [49]. *Drosophila* adults, like larvae, have distinct sensors for discretely cold and warm temperatures. The basis of adult cold sensing is undefined at the molecular level. (Barbagallo and Garrity, 2015) [4].
Chill-coma is like a narcosis condition caused by cold temperatures in several animals. According to Gibert *et al.*,

2001, *Drosophila* species living in more tropical climates are less resistant to cold temperatures than those living in temperate climates. Hoffmann *et al.*, 2002 [19] have reported increased resistance to cold with high-latitude populations of *Drosophila melanogaster* on the eastern coast of Australia. This geographical clinal variation gives indirect evidence of affirmation for thermal adaptation within a species (Sisodia and Singh, 2010) [44].

On this subject, *Drosophila melanogaster* is a good modal organism since it can be raised in the lab, have short generation periods, and are genetically and developmentally well characterized. *Drosophila's* global spread is based on its ability to adapt to a variety of environmental circumstances (Stephan and Li, 2007) [46]. The physiological ecology and variation in stress resistance assess the physiological adjustments and changes in energy allocation when given different diet regimes. Nutrition has been a significant focus in research of environmental variables that impact stress-induced acquired and heritable characteristics due to the ease with the diet of the fly which can be altered in the laboratory (Rion and Kawecki, 2007) [35].

Despite the increasing usage of NNS, metabolic diseases like obesity and type 2 diabetes continue to be at a high rate over the past decades (Hales *et al.*, 2017) [14]. In rats, sucrose-nutritive and NNS consumption has been related to a down regulation of corticotropin-releasing factor (CRF) expression in the brain which leads to metabolic effects of Nutritive sweeteners (Ulrich-Lai *et al.*, 2007) [50]. A study recently reported that young mice who habitually consumed oral sucralose directly after weaning had a heightened preference for sweetened water and increased weight gain at approximately 15 weeks of life (Rosales-Gomez *et al.*, 2018) [37]. The effects of nutritive and non-nutritive sweeteners in *D. melanogaster* on environmental stress responses remain unknown.

Materials and Methods

Experimental stock

The experimental collection of *D. melanogaster* was obtained from the Drosophila Stock Centre in Manasa gangotri, Mysuru. The flies were developed in different cultured bottles using wheat cream agar medium (100 g of jaggery, 100 g of wheat powder, 8 g of Agar- Agar was boiled in 1000 ml of double-distilled water and 7.5 ml of propionic acid was added at last). In a 12-hour dark, 12-hour light cycle, twenty flies (10 males and 10 females) were introduced into culture bottles at a temperature of 22±1ºC with a relative humidity of 70%. This procedure was carried out for three generations to acclimatize flies to lab conditions. In the fourth generation, eggs were collected from these flies using Delcour's procedure (1969). 100 eggs were seeded to each culture bottle containing control, nutritive sweetener sucrose and NNS aspartame and sucralose media (Treated media). The NNS doses for aspartame and sucralose and nutritive sweetener Sucrose were set at 20mM, 80mM, and 120mM, respectively, for the study (Krishna *et al.*, 2021) [1]. The flies obtained from these control and treated media were used for the present experiments.

Desiccation resistance

Desiccation resistance was studied on 4-5 days old virgin flies, and up to 10 flies of each treated media were evaluated. To measure resistance, flies from each vial were transferred to a new vial containing a disc of dry filter paper and covered with muslin gauze cloth secured with an elastic band. Desiccation vials were kept at 25ºC under constant light and were observed for the number of dead flies seven hours after the flies were originally transferred and then at half-hourly intervals until all the flies had died. Five replicates were carried out. The method of Kennington *et al*, (2001) [25] was used to measure desiccation resistance.

Starvation resistance

Starvation resistance was evaluated on 4-5 days old virgin flies, and up to 10 flies were measured of each treatment medium, similar to desiccation resistance. To measure starvation resistance, flies from each vial were transferred to a new vial containing 7 ml of 1% agar and plugged with cotton to prevent desiccation. The number of dead flies in starvation vials was recorded 40 hours after the flies were transferred, and subsequently at 6-hourly intervals until all of the flies had deceased. A total of five replicates were conducted for the experiment.

Recovery time (RT) from cold treatment

Flies obtained as above were aged for 5 days before being transferred into empty glass vials and maintained at 5° C. For this experiment, 50 flies were transferred separately into each control and treated media. The duration of cold treatment was 16 h. Adults were placed in a Petri dish at room temperature to measure recovery time. Initially, the flies were in a chilled coma and unable to move. Transferring to room temperature allows for a gradual recovery, beginning with the ability to move the tarsi, then the legs, and finally to stand up. We recognized a fly recovered from a chill coma when it could stand on its legs, the fly was then collected from the Petri dish, and the time changed from the initial noted as an estimate of Recovery Time (RT). The mean RT for each group was measured and used as a basic observation. Ayrinhac *et al*, (2004) [2] procedure was followed for this experiment.

Heat shock survival

In an empty food vial, flies obtained as above were heat-shocked. The stoppers were moistened with tap water to avoid desiccation. In incubators, the vials were evenly distributed in racks. One group was heated for 1 hour at 37°C, followed by 1 hour at 25°C to allow the flies to recover before being heat-shocked for 1 hour at 40°C. The other group was exposed directly to a temperature of 40°C for 1 hour. Five vials containing ten flies of each treated media were used. After the heat shock, the flies were transferred to fresh food vials and allowed to recover for 24 hours at a temperature of 25 ° C before being observed for survival (ability to walk).

Results and Discussion

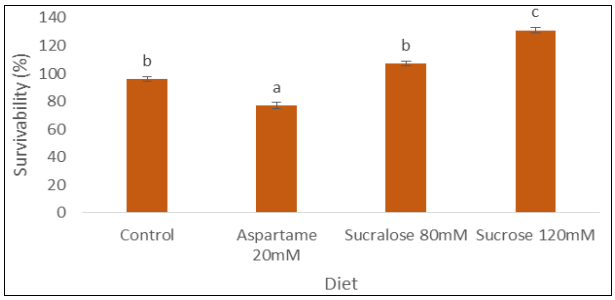

Fig 1: Effect of Nutritive and Non-Nutritive Sweeteners on cold resistance in adult flies of *D. melanogaster*. Different letters on the bar graph indicate significance at 0.05 level by Tukey's Post Hoc test.

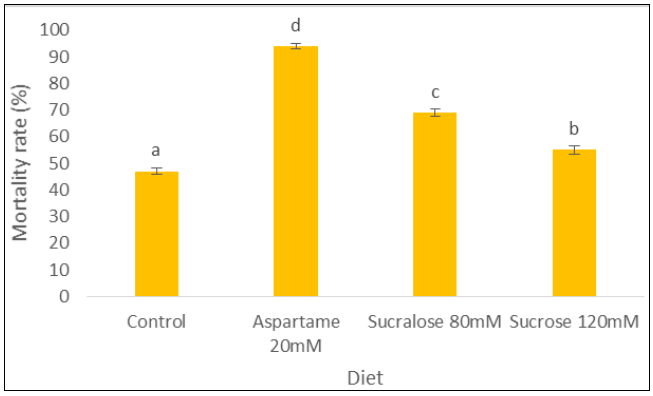

Fig 2: Effect of Nutritive and Non-Nutritive Sweeteners on desiccation resistance in adult flies of *D. melanogaster*. Different letters on the bar graph indicate significance at 0.05 level by Tukey's Post Hoc test.

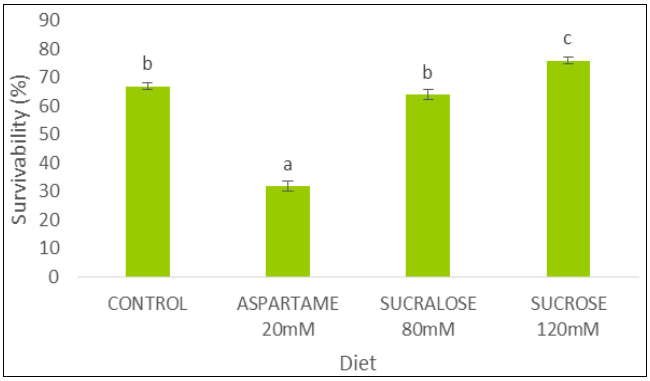

Fig 3: Effect of Nutritive and Non-Nutritive Sweeteners on heat-shock resistance in adult flies of *D. melanogaster*. Different letters on the bar graph indicate significance at 0.05 level by Tukey's Post Hoc test.

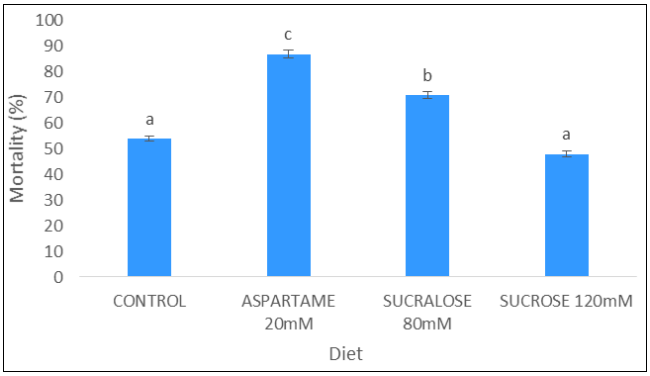

Fig 4: Effect of Nutritive and Non-Nutritive Sweeteners on starvation resistance in adult flies of *D. melanogaster*. Different letters on the bar graph indicate significance at 0.05 level by Tukey's Post Hoc test.

Results and Discussion

Recent developments in environmental stress and fitness show remarkable progress have been achieved in evolutionary biology as well as advances in molecular and physiological mechanisms. To induce plastic adaptive responses, animals, including *Drosophila*, can recognize and utilize their nutritional situation (Tatar *et al.*, 2003; Partridge *et al.*, 2005) [48, 33]. The carbohydrate-protein ratio may be altered by changing the components of the fly diet (Bruce *et al.*, 2013) [7]. Changing the nutrients in flies can cause a variety of reactions, making it difficult to identify particular nutritional changes linked to adaptive responses (Lee *et al.*, 2007) [27]. In an ecological, evolutionary, and physiological context. In the present study, at varying concentrations in different types of NNS media, the results provide a different perspective on NNS.

Desiccation resistance

Flies are more susceptible to dehydration than humans (desiccation). In this study, adult flies have been used for desiccation resistance in laboratory-reared populations. Here, desiccation resistance is quantified as the time until death under the condition. In the present study, Desiccation resistance affected flies significantly by different nutritional diets. Flies treated with sucrose have higher desiccation resistance than flies treated with aspartame and sucralose. Mortality differed significantly among NNS and Nutritive media treated flies. Aspartame treated flies showed the least desiccation resistance when compared to sucralose, sucrose and control. The desiccation resistance data subjected to One-way ANOVA followed by Tukey's post-hoc test confirmed that desiccation resistance varied significantly between control and treated media. Tukey's post hoc test revealed that desiccation resistance had a significant difference between treated media. The tolerance to desiccation was highest in sucrose than in NNS. The fat body of the *Drosophila* acts as both the adipose tissue and the liver of the fly. When the fly is treated with sucrose-based media, this tissue specializes in fat storage and metabolism, and its lipid content increases. Carbohydrates also serve as the primary source of energy in adult flies during desiccation stress (Marron *et al.*, 2003) [29]. Increased accumulation of carbohydrates during larval feeding could result in enhanced desiccation resistance of the newly eclosed fly. Hence, our results agree with other research done by Parkash *et al.*, (2008) [34] showing a positive correlation between lipid accumulation and desiccation resistance. A research study by Hoffman and Harshman, 1999 [17], has reported the strong connection between desiccation and starvation resistance considering the positive effect of increased carbohydrate storage. Among NNS, aspartame has least resistance to desiccation. This is because aspartame's components (phenylalanine and aspartic acid) have important functions in neurotransmitter modulation and excitatory neurotransmitter production in the central nervous system. Methanol, the other component of aspartame, and its metabolites produce a variety of toxic derivatives (Fahira reshman *et al.*, 2015) [11]. Desiccation induced gene expression alterations have been demonstrated in various studies, however, the mechanisms behind these changes remain unknown.

Starvation resistance

Selection experiments are a good approach to learn more about physiological traits like starvation resistance. A study reviewed that starvation is correlated with an increase in larval development time, higher adult lipid content and larger adult body size in *D. melanogaster* (Harshmann and Harshman, 1999) [17]. The Starvation resistance was observed high in flies treated with nutritive sweetener (sucrose) than flies treated with NNS treated media. Mortality varied substantially among NNS. Flies treated with aspartame showed high mortality during starvation resistance when compared to sucralose, sucrose and control. The starvation resistance data subjected to One-way ANOVA followed by Tukey's post-hoc test confirmed that

starvation resistance varied significantly between control and treated media. Tukey's post hoc test revealed that starvation resistance had a significant difference between treated media.

In the present study, starvation resistance is measured as the time till death under the condition. It is found that starvation resistance was highest in sucrose and sucralose treated flies when compared with aspartame treated flies. Control flies showed equal response to starvation resistance as sucrose treated flies. This shows that NNS showed the least resistance to starvation when compared to nutritive sweetener sucrose. Since nutritive sweeteners (Sucrose) contain some calories and break down into glucose when starved. The sucrose treated flies contain *Drosophila* fat body (FB) which stores excess energy in the form of triglycerides, similar to white adipose tissue in mammals, and it is involved in metabolism, glycogen storage, and nutrient sensing, similar to the mammalian liver (Wigglesworth, 1949) [54]. NNS has zero or low calories, which means fewer triglycerides in the body that results in early starvation and death of the flies. However, Starvation resistance is said to be reduced with prolonged dietary restriction in flies and Chandegra *et al.*, 2017 [8] observed that *Drosophila melanogaster* flies raised on a sucrose-rich diet were more resistant to starvation, confirming our results that diet composition can impact stress response. In NNS, due to calorie restriction, decreased metabolic rate seems to be part of the plastic response to starvation (Partridge *et al.*, 2005) [33]. There is also some evidence suggesting a genetic correlation between starvation resistance and oxidative stress resistance (Harshman *et al.*, 1999) [17]. Understanding the ecological significance of starvation resistance in drosophila has made little progress. The natural selection of starvation resistance remains unclear, and the extent to which population variations in starvation resistance are likely to have contributed to natural starvation resistance selection have to be progressed.

Recovery time (RT) from cold treatment

There are some possible physiological explanations behind cold treatment recovery when flies are treated with nutritive and non-nutritive sweeteners. The general aim behind nutritive and NNS-fed flies is that nutritive sweetener-treated flies consume a carbohydrate-rich diet, and carbohydrate has been shown to increase fat storage in flies (Mayntz *et al.*, 2005) [32]. Literature review on previous studies has recognized a positive connection between cold temperature resistance and lipid content in *Drosophila* flies, starvation resistance and desiccation stress (Hoffman *et al.*, 2001; Ballard *et al.*, 2008; Parkash *et al.*, 2008) [18, 3, 34]. Thus, it is logical to assume that the flies treated with nutritive sweeteners recover faster from cold treatment due to high-calorie deposits. Recovery time of the flies from cold resistance was substantially affected by flies treated with the NNS diet. Flies treated with aspartame recovered more slowly than flies treated with control, sucralose and nutritive sweetener (Sucrose). The cold resistance data subjected to One-way ANOVA followed by Tukey's post-hoc test confirmed that recovery time varied significantly between control and treated media. Tukey's post hoc test revealed that resistance to cold varied significantly between treated media. Our experimental result also strengthens the earlier studies proving that nutritive sweetener (Sucrose) treated flies recovered faster than NNS (Aspartame and Sucralose). Among NNS, aspartame treated flies had the least recovery time than sucralose treated flies. Aspartame is made up of two amino acids, aspartic acid and phenylalanine which might serve as a complicated energy source to metabolize when compared to nutritive sweetener sucrose control flies. This is because metabolizing amino acids need more complex catabolic processes (Singh *et al.*, 2017) [43].

Heat shock survival

NNS has a substantial impact on heat shock resistance. The result showed that Flies treated with aspartame have fast recovery from heat shock than flies treated with sucrose and sucralose. Among NNS, sucralose has the least resistance to heat shock when compared with aspartame and control. The heat shock resistance data subjected to One-way ANOVA followed by Tukey's post-hoc test confirmed that heat shock resistance varied significantly between control, nutritive and NNS media. Tukey's post hoc test revealed that heat shock resistance had a significant difference between treated media. Our findings revealed that flies treated with aspartame was the highest in resistance to heat than flies treated with sucralose, nutritive sweetener sucrose treated flies and control flies. Flies treated with protein media (Aspartame) recover from heat shock faster than those treated with carbohydrate-rich medium (Sucrose). Though the physiological reason for the enhanced resistance to heat shock in aspartame-treated flies is unknown, one hypothesis is that it may be connected to the activity of heat shock proteins, which are known to interact with a variety of stressors (Sorenson *et al.*, 2003; Jones and Candido, 1999; Sinclair *et al.*, 2007; Tammariello *et al.*, 1999) [46, 23, 42, 48]. The earlier study suggests that Hsp70 is upregulated in flies treated with protein-rich media. Further studies are required for proving the hypothesis.

Conclusion

The present study in *D. melanogaster* reveals that NNS diet regimes significantly reduce resistance in desiccation, starvation, cold treatment and heat shock survival stresses suggesting that NNS affects fitness in *D. melanogaster*. In the future, much more research on diet quality and their relation to genetic stress and trans generational effects in *Drosophila* species will be helpful in large interest.

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Conflict of Interest

The authors report no conflict of interests.

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