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Effects of Caffeine on Heat Resistance of Unmated and Mated Males and Females in *Drosophila melanogaster*

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Abstract

The amount and quality of nutrients consumed by organisms have a strong impact on stress resistance, life history traits and reproduction. The balance between energy acquisition and expenditure is crucial to the survival and reproductive success of animals. The ability of organisms to adjust their development, physiology or behaviour in response to environmental conditions. The availability of the food and nutrition is important factor for the flies or organism which copes with the various types of environmental stress. In the present study the flies of *Drosophila melanogaster* flies are cultured in the wheat cream agar media and caffeine media to understand the effect of caffeine on the Heat resistance. The results reveals that the caffeine fed flies had the greater resistance to the heat than the Wheat cream agar media which had the second least resistance to heat as well as the diet having highest concentration of caffeine had less resistance of heat than any other. Further in the present study, females are more sensitive to the heat resistance than those of the males which show greater tolerance to the heat, in all the three diets. Further among mated and virgin flies, virgin males and females were more resistant to the heat than those of mated males and females in all the three diets. Hence these studies suggests that caffeine slightly enhances the heat resistance results in *D. melanogaster*.

Keywords: Nutrition, *Drosophila melanogaster*, heat resistance, mated, virgins.

Introduction

Life-history features like illness susceptibility, fertility, reproduction, lifespan, and stress resistance are significantly influenced by the quantity and quality of nutrients consumed by organisms. Studies that examine the effects of diet frequently evaluate how people react physiologically and morphologically when exposed to various nutrient quality and quantity. In order to adapt to environmental changes, organisms have evolved a variety of techniques, such as phenotypic plasticity and adaptability (Lynch and Gabriel, 1987; Stearns, 1989; Meyers and Bull, 2002) [16, 27, 17] Extreme environmental factors and stress may have a negative impact on an organism's physiology and life-history features.

The genetic variations in stress tolerance leads to adaptive change relies on the frequency of environmental challenges faced by the organism and the corresponding physiological costs (Hoffmann *et al*., 1991) [12] Environmental stress is defined as the lack of normal nutrients caused by inadequate or unsuitable food resources, and it has been proposed that stress related to few resources has an effect on populations of most species (White, 1993) [29]. Selection most likely influences stress resistance features either directly or indirectly because *Drosophila* stress resistance traits frequently differ across latitudinal clines (Sisodia and Singh, 2010) [23]. Numerous elements influence an organism's ability to withstand stress. A response to climatic fluctuations may involve physiological stiffening, coma, or the creation of chemicals that enable an organism to withstand temperature extremes. (Sørensen *et al*., 2003, 2005; Lalouette, 2007) [25, 26, 14] Also, an organism may compensate for nutritional stress and reduced body size by extending its growth period or altering its energy allocation to growth, hence postponing the reproductive period (Reichling, 2000; Lobe, 2006) [21, 15].

Because the climate changes significantly with geographic factors, geographic gradients are of particular relevance in the study of climatic adaptation. Although a number of environmental factors may have an effect on an individual's physiology, temperature is believed to be one of the greatest and is therefore of significant selection significance (Clarke, 2003; Hoffmann *et al*., 2003a) [10, 13] It is possible that temperature has a significant influence on the distribution and development of species, but it is less certain which aspects of the thermal environment operate as the primary drivers of thermal selection. Although the exact method of adaptation is unknown, it is commonly acknowledged that temperature selection is extremely important and that *D.melanogaster* is likely to be subjected to stressful situations. Caffeine has been shown to induce the heat shock response (HSR), a protective mechanism that helps cells cope with stress, including elevated temperatures. This induction is mediated by the heat shock transcription factor HSF-1 and leads to the upregulation of heat shock proteins (HSPs) such as HSP-4, HSP-6, and

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HSP-16. These proteins function as molecular chaperones, assisting in protein folding and preventing aggregation, thereby promoting proteostasis Fredholm *et al*. (1999)

Furthermore, caffeine treatment in *C. elegans* has been associated with enhanced survival in models of neurodegenerative diseases, suggesting that caffeine-induced HSR activation may confer protective effects against protein misfolding and aggregation.Caffeine is indeed considered a drug—specifically, a central nervous system stimulant. It's the most widely consumed psychoactive substance in the world, found naturally in coffee, tea, and cacao plants, and added to many sodas, energy drinks, and medications. Wu *et al*. (2017). To studies the various effects of caffeine on different physical and physiological prospects of the model organisms body. A caffeine supplement was used with appropriate amount and concentration (250mg) of drug and was provided for the drosophila species. Various parameters were observed and calculated accordingly

Materials and Methodologies

"The caffeine powder" a pre-workout supplement (orange flavour) was purchased from www.amazon.com, and used as a dietary component in the experimental setup.

Establishment of Stock: The *Drosophila melanogaster* flies used in this study were sourced from the *Drosophila* stock centre, Department of Studies in Zoology, University of Mysore, Manasagangothri, and Mysore. The collected flies were cultured in glass containers containing wheat cream agar media composed of 100g jaggery, 100g rava powder, and 10g agar dissolved in 1000ml boiling distilled water, with 7.5ml propionic acid added to inhibit fungal growth. The flies were maintained under laboratory conditions at a temperature of 22 ± 1°C, and a 12:12 hour light-dark cycle and humidity level of approximately 70% RH.

Establishment of Experimental Stock

Wheat Cream Agar Media (Control Media): This was made using 100g of jaggery, 100g of rava powder, and 10g of agar in 1000ml of boiling distilled water, along with 7.5ml of propionic acid.

2.5g of caffeine media was prepared using 100g of jiggery, 90g of rava powder, 2.5g of caffeine powder, 10g of agar powder in 1000ml of boiling distilled water, along with 7.5ml of propionic acid.

5g of caffeine media was prepared using 100g of jiggery, 80g of rava powder, 5g caffeine powder, 10g of agar powder in 1000ml of boiling distilled water, along with 7.5ml of propionic acid.

10g of caffeine media was prepared using 100g of jiggery, 60g of rava powder, 10g of caffeine powder, 10g of agar powder in 1000ml of boiling distilled water, along with 7.5ml of propionic acid. These flies were maintained under the laboratory condition mentioned above and used to study starvation resistance in *D. melanogaster*.

Experimental Procedure

Experimental Procedure Heat Resistance: To study the heat resistance, five days old virgin's flies (male and female) and the mated flies (male and female) were obtained from the wheat cream agar, 2.5g, 5g and 10g of caffeine media were used. Ten flies (unmated male/unmated female, mated male/mated female) were observed by transferring them to empty vials each vial containing five flies. These vials were kept at 37^0C (sub lethal temperature) in water bath and resistance to heat of each fly was observed in 10 minutes interval until its death by taking out the vials each time from the water bath. A total of 10 flies were observed for each of the wheat cream agar, 2.5g, 5g and 10g of caffeine media. Separate experiment was carried out for mated and unmated flies.

Result

Figure 1: Indicates the effect of caffeine on heat resistance, it showed the mean and standard error value of heat resistance in unmated female and unmated male flies cultured in Wheat cream agar media and 2.5g, 5g, and 10g caffeine media. According to this data, cold resistance was very less in 10g caffeine media, more or less similar in wheat cream agar media and 2.5g and 5g caffeine media. Further, this data also showed that unmated males had more heat resistance than unmated female flies.

The above heat resistance data subjected to two-way ANOVA followed by Tuckey's Post hoc test showed significant variation in time taken by unmated female and male flies cultured wheat cream agar media, 2.5g, 5g, and 10g caffeine media in to survive in high temperatures. However, insignificant variation in cold resistance was noticed in interaction between treatment and sex. Tukey's post hoc test showed significant variation in heat resistance in unmated female flies of 5g caffeine media and unmated male flies of wheat cream agar 2.5g and 10g caffeine media.

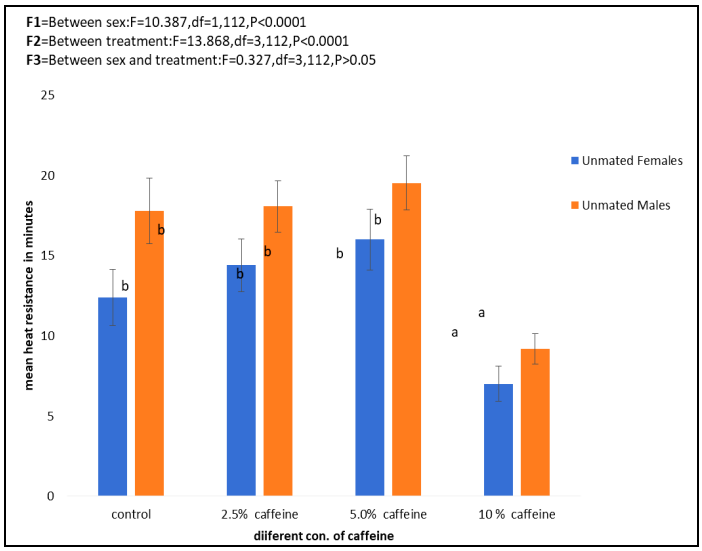

Fig l: Effect of caffeine on heat resistance in unmated female and unmated male of *Drosophila melanogaster.*

The different letters on the bar graph indicates the significant variation at 0.05 levels by Tukey's Post Hoc test.

Figure 2: The data showed the mean and standard error value of heat resistance in mated female and mated male flies cultured in Wheat cream agar media and 2.5g, 5g, and 10g caffeine media. According to this data, cold resistance was very less2.5g, in 5g and 10g caffeine media, more in control than in caffeine media. Further, this data also showed that mated males had more heat resistance than mated female flies. The above heat resistance data subjected to two-way ANOVA followed by Tuckey's Post hoc test showed significant variation in time taken by unmated female and male flies cultured wheat cream agar media, 2.5g, 5g, and 10g caffeine media in to survive in high temperatures. However, insignificant variation in cold resistance was noticed in interaction between treatment and sex. Tukey's post hoc test showed significant variation in heat resistance in unmated female flies of 5g caffeine media and unmated male flies of wheat cream agar 2.5g and 10g caffeine media.

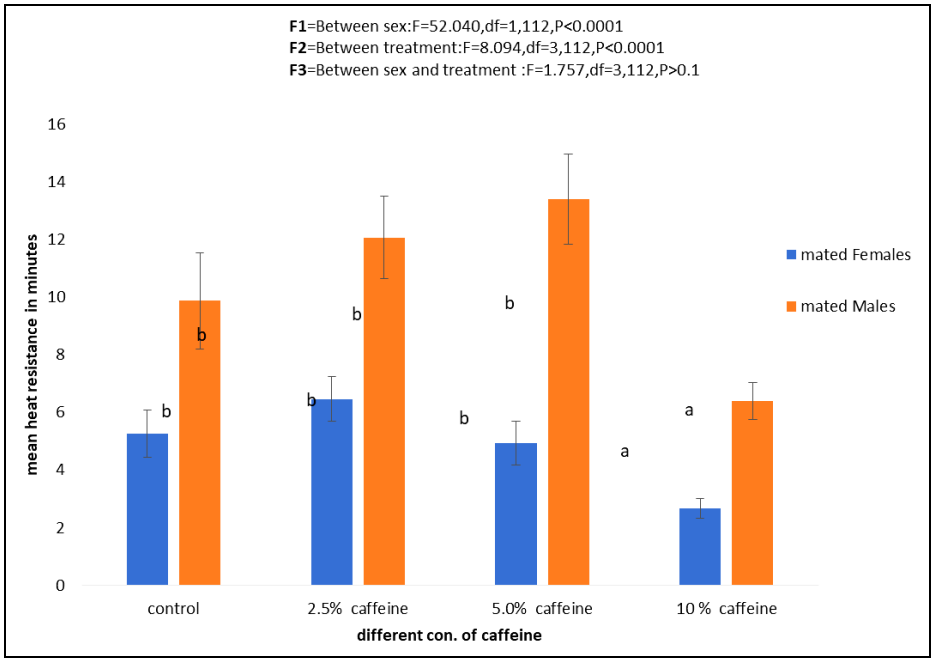

Fig 2: Effect of caffeine on heat resistance in mated female and mated male of *Drosophila melanogaster*

The different letters on the bar graph indicates the significant variation at 0.05 levels by Tukey's Post Hoc test.

Figure 3: This graph showed the mean and standard error value of heat resistance in mated female and mated male flies cultured in Wheat cream agar media and 2.5g, 5g, and 10g caffeine media. According to this data, heat resistance was very less in 10g caffeine media when compared to control media, more or less similar in wheat cream agar media and 2.5g and 5g caffeine media. Further, this data also showed that unmated females had more heat resistance than mated female flies.

The above heat resistance data subjected to two-way ANOVA followed by Tuckey's Post hoc test showed significant variation in time taken by unmated female and male flies cultured wheat cream agar media, 2.5g, 5g, and 10g caffeine media in to survive in without food. However, insignificant variation in cold resistance was noticed in interaction between treatment and sex. Tukey's post hoc test showed significant variation in cold resistance in unmated female flies of 5g caffeine media and mated female flies of wheat cream agar 2.5g and 10g caffeine media.

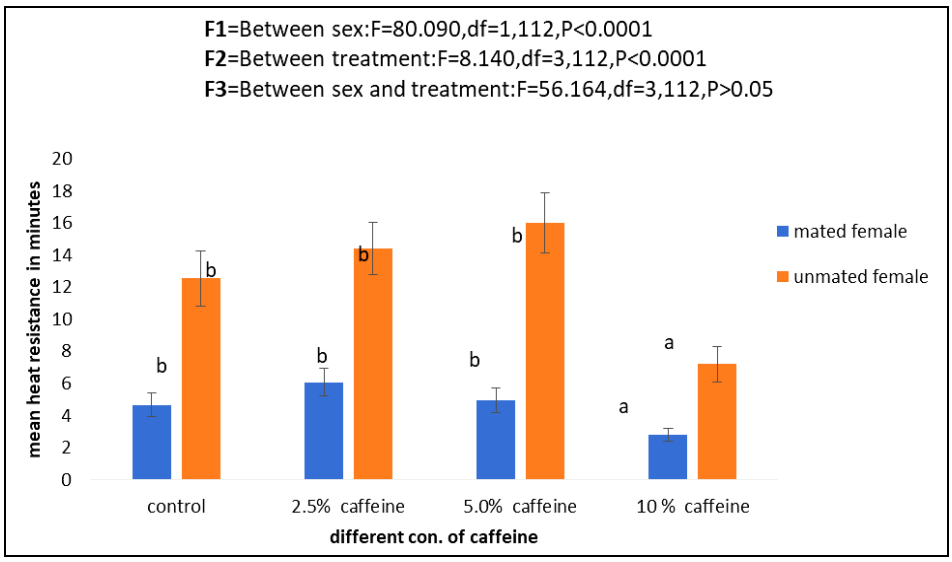

Fig 3: Effect of caffeine on cold resistance in mated female and unmated female of *Drosophila melanogaster*

The different letters on the bar graph indicates the significant variation at 0.05 levels by Tukey's Post Hoc test.

Figure 4: showed the mean and standard error value of heat resistance in mated female and mated male flies cultured in Wheat cream agar media and 2.5g, 5g, and 10g caffeine media. According to this data, heat resistance was greater in 5g and 10g caffeine media, more or less similar in wheat cream agar media and 2.5g caffeine media. Further, this data also showed that unmated males had more heat resistance than mated male flies.

The above heat resistance data subjected to two-way ANOVA followed by Tuckey's Post hoc test showed significant variation in time taken by unmated female and male flies cultured wheat cream agar media, 2.5g, 5g, and 10g caffeine media in to survive in high temperature. However, insignificant variation in cold resistance was noticed in interaction between treatment and sex. Tukey's post hoc test showed significant variation in heat resistance in unmated male flies of 5g caffeine media and mated male flies of wheat cream agar 2.5g and 10g caffeine media.

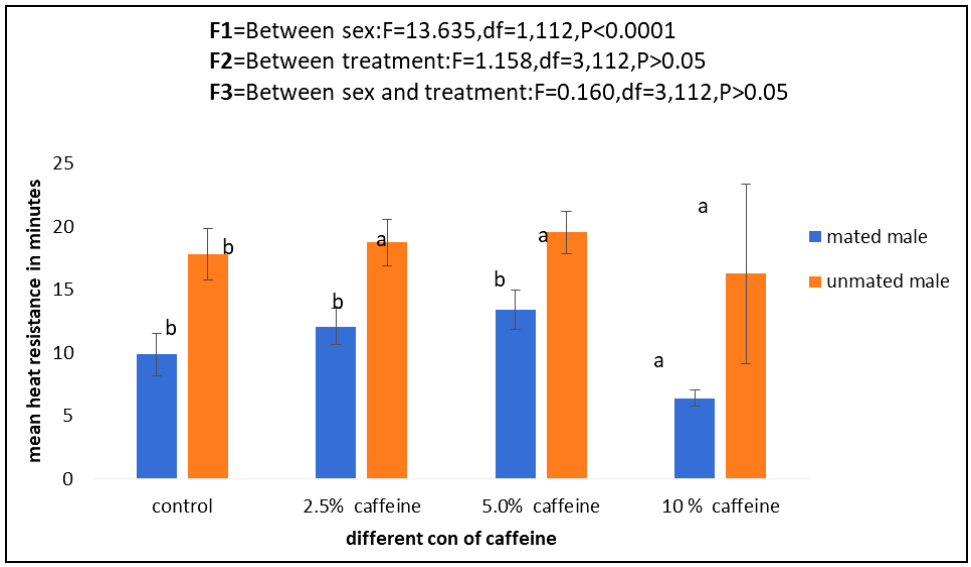

Fig 4: Effect of caffeine on heat resistance in mated male and unmated male of *Drosophila melanogaster*

The different letters on the bar graph indicates the significant variation at 0.05 levels by Tukey's Post Hoc test.

Discussions

Variation in stress related traits in insects in other organisms has been widely studied because it underlies the ability of insects to adopt and counter the effects of changing climatic conditions. Nutrition and sex and genetic variations are also known to influence a species ability to tolerate thermal stress. Temperature rises more gradually in nature than in the majority of lab studies, and extremities often do not vary significantly from day to day. Therefore, the majority of organisms that have previously encountered high temperatures will have triggered the heat shock response and the genetic diversity in resistance to brief exposure to a high temperature that is important for adjusting to a warmer environment. Survival in the presence of high temperatures depends on the capacity to withstand the physiological stress, with selection acting on genetic variation for the amount of a heat shock protein produced, for its amino-acid sequence, or for the thermal stability of structural or enzymatic proteins. (Koushik and Krishna, 2014) [1].

The availability of the food and nutrition is important factor for the flies or organism which copes with the various types of environmental stress. In the present study, the results (Figure 1, Figure 2, Figure 3, and Figure 4) revealed that significant variation in the Thermal stress or heat resistance in the three different diets. It shown that the flies fed with caffeine had slightly greater resistance to heat or thermal stress than those of the flies fed with wheat cream agar media which had the second least resistance to the heat as well as the high concentration of caffeine diet fed flies had less resistance to heat as the wheat cream agar media fed flies. This suggest the nutrient availability, quality in the food was influenced on the variation in the heat resistance of the flies. The nutraj pre-workout powder is rich with caffeine which may provide the energy to withstands the heat resistance in the *D.melanogaster* and as well as caffeine supplemented di*et al*so enhances the heat resistance whereas the wheat cream agar media may still need the extra energized food to cope up with thermal stress. And we also explain the study result that flies developed on caffeine supplemented diet flies cope up with heat shock faster than flies developed on Wheat cream agar diet. The physiological explanation for an increased heat knockdown tolerance among flies developed on protein enriched medium is unknown. Our study also supported by the Sisodia and Singh, (2012) [25], who also found that the flies raised on the protein enriched diet had higher heat resistance than the flies fed with carbohydrates. It is unknown what physiological factors contributed to flies raised on protein-enriched medium having a higher heat knockdown resistance. One option might be associated with the production of heat shock proteins, which are known to be crucial for dealing with various types of stress (Srensen *et al*. 2005; Sinclair *et al*. 2007) [22] Heat shock protein synthesis is also a component of the physiological processes underpinning fast heat hardening. In addition, according to Anderson *et al*. (2010) Hsp 70 is more highly expressed in fly larvae raised on protein-enriched media as opposed to larvae raised on protein-deficient medium. This may be related to our study results because the experimental diet is rich in the protein and carbohydrates content. However we do not quantify the Hsp's proteins in the flies of our experiment.

The pattern of sexual dimorphism in stress resistance has been shown to differ considerably depending on strain, mating status, age and assay condition (Goenaga *et al*., 2012) [4]. The several study, also shown that the normally the females are having the greater stress tolerance than the males. In our study

among the male and females, The Figure1 and Figure 2 revealed that the males were had the greater resistance to heat than those of the female flies in all the three diets. The several studies also shown the females had the greater stress resistance than the males. Our results suggests that, may due to the females are, ingested more food and thus accumulated greater quantity of lipids compared with males as well as the females have the greater fat and the protein content which is efficiently used for the energy metabolism than the males (Carvalho *et al*. 2006; Lee *et al*. 2013) [8] However due to the effect of caffeine in sustainable concentrations, this process had reversed. This may result the males are greater resistant to heat than the females.

And also male and female flies have sex specific difference in how they regulate stress response pathway particularly heat shock proteins, caffeine is a stressor and stimulant in males it may up regulate protective mechanism like HSPs or antioxidant enzymes more effectively compared to females. Males have higher basal levels or a stronger inducible response of certain stress related genes giving them a survival advantage under heat stress.

Male flies generally have a smaller body size which can mean faster metabolism and possibly more efficient heat dissipation. Males have a stronger neuromodulator response to caffeine enhance their behavioural or physiological response to heat. Under caffeine ad heat stress, females may be less able to redirect energy towards survival compared to males. (MN WU 2009)

As well as we also noticed the heat resistance. Also varies in the mated and unmated conditions and sexual differences also influences the thermal stress of the flies. In the present study, according to the results (Figure 3), among the mated male and unmated male, the unmated males shown the greater heat resistance than the mated male flies, as the earlier studies mating may not be a harmful process but could be not beneficial to males with reference to resistance to heat. And mating rate and activity may be is not advantageous for the females in *D.melanogaster*. So, we can explain that like females, the post mating may be results the large food intake by increasing the gut size in males this may results the greater heat resistance than the mated flies. However, in our study we don't measure amount of food intake thus results the utilization of more energy to enhance the thermal resistance in the *D. melanogaster*. As well as another possible explanation is that the mated male flies may be continuously expose to the pheromones released by females during the copulation may alters the male physiology that may be results the lesser resistance to the heat than the virgin males. In contrast our study, several studies shown that mated males are shown that sexual activity in mated male transfer the sperms and accessory gland proteins (Acp's) during mating and loses its energy results the sensitive to the heat than the virgins' flies. Studies have shown that HSP70 plays a vital role in thermotolerance and recovery from heat shock in *D.melanogaster*. Flies lacking HSP70 exhibit reduced survival rates following heat exposure, indicating the importance of this protein in heat stress responses, while direct evidence of caffeine's effect on HSPs in *D.melanogaster* is limited, existing research in related species and the known role of HSP70 in heat stress responses suggest that caffeine may influence the heat shock response in *D.melanogaster* Further studies are needed to elucidate the specific mechanisms through which caffeine modulates HSP expression and to determine its potential as a modulator of heat tolerance in *D.melanogaster*.

Among the mated and unmated females, according to the Figure 4, the unmated females are had the greater resistant to the heat resistance than the mated females. Our study result also confirms the works of Goenaga *et al*., (2012) [4], who while works on *D. melanogaster* have also demonstrated that mated females exhibit an increased tolerance to stress in comparison to virgins. As well as, the food intake has been shown to increase in female *D.melanogaster* after mating (Ravi Ram and Wolfner 2007, Carvalho *et al*., 2006) [20, 8], but due to the effect of caffeine but due to the antagonistic effects of caffeine which works as neurotoxic agent it has decreased the resistance in case of mated females. In *Caenorhabditis elegans*, caffeine has been demonstrated to induce the heat shock response in an HSF-1-dependent manner. This induction leads to the upregulation of HSPs, such as HSP70, which are crucial for protein homeostasis and protection against stress-induced damage. The protective effects observed in *C. elegans* suggest that caffeine may have similar effects in other organisms, including *D.melanogaster*. Further, may be due to the mating process gives reduces the energy levels of the females, i.e., Males transfer accessory gland proteins to females during the mating process, and females transfer seminal gland proteins during copulation. The seminal fluid, which is transported to the female along with sperm during copulation, is made up of a complex protein mixture that is produced and secreted by accessory glands (Wolfner, 2002) [30]. Accessory gland proteins (Acps) cause behavioural and physiological changes in mated females (Gillott, 2003), additionally, the cystoid kinase-like (Eckel) gene family in *D.melanogaster* has been implicated in the detoxification of caffeine, suggesting that these genes play a role in mitigating the effects of caffeine exposure. While direct evidence linking caffeine consumption to enhanced heat tolerance *D.melanogaster* is limited, its effects on heat shock proteins and metabolic pathways suggest that caffeine could influence an organism's response to heat stress. This may result the greater heat resistance in unmated females compared to mated So, from our results we can explains that the mating condition is advantageous to influencing the physiological behaviour, which helps to cope up and withstand the heat resistance in the both sexes of the *D.melanogaster*. The current experiment focuses on the effect of caffeine in case of heat resistance in *D.melanogaster*. here the heat tolerance is more in case of males when compared to females that is due to the more activity of heat shock proteins in case of males which gets enhanced by the caffeine whereas the females show less tolerance because of the fluctuating body temperature because of physiological and hormonal differences females have higher body fat and less muscle mass fact as an insulator which make it harder to dissipate heat resist the heat externally, males generally produce more sweat than females so less sweating in females leads to lower ability to cool down. Compared to mated one and unmated ones mated ones die fast and the unmated or the virgins live longer because of physiological and hormonal activity while mating. When compared between unmated females and unmated males the males live long due to the muscle weight and more availability of heat shock proteins.

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