RESEARCH ARTICLE OPEN ACCESS

Increased Creatine Monohydrate Supplementation Decrease Cold Resistance in *Drosophila Melanogaster*

Sithembiso Sebastian Mamba, Mysore Siddaiah Krishna*
Stress biology lab, Department of Studies in Zoology, University of Mysore, Manasagangotri, Mysore, 560006, Karnataka, India
Mambasebastian0@gmail.com; drosokrishna@gmail.com

Abstract
The survival and dispersion of organisms depend on their capacity to withstand adverse environmental conditions including cold temperatures. The quantity and quality of available nutrients also plays a major role in the ability of an organism to withstand unfavorable situations resulting from unstable environmental conditions.The current study has been undertaken to determine the effect of creatine monohydrate on cold resistance in *Drosophila melanogaster*. The obtained results showed that mated flieshad significantly higher cold resistance compared to unmated flies and female flies were more resistant to cold than their male counterparts in both control media and creatine monohydrate treated media. The only exception was that flies maintained on 2.5% creatine monohydrate supplemented media which were significantly more resistant to cold compared to control media fed flies and creatine monohydrate supplemented flies (5% and 10%). The results suggest that increased creatine monohydrate supplementation have a negative effect on cold resistance in *Drosophila melanogaster.*

Keywords: Creatine monohydrate, *Drosophila melanogaster*, Cold resistance, stress

INTRODUCTION

The quantity and quality of nutrients that organisms ingest have a significant impact on their life histories and characteristics, such as their susceptibility to disease, fertility, ability to reproduce, lifespan, and resilience to stress(Prasad *et al*., 2003; Jenki*et al*., 1997; Bijlsm*et al*., 1996). The ability of an organism to resist stress can be affected by a variety of factors through behavioral and physiological changes. Additionally, it was shown that an organism's exposure to climatic variations might result in physiological changes such the hardening process, coma, the creation of metabolites, and the development of a tolerance for temperature extremes (Sørensen*et al*., 2005; Lalouette and Kostal, 2007). For example,the population dynamics and distribution of overwintering insects are influenced by cold tolerance, which is a crucial need for survival and growth (Mcdonald*et al*., 2000). According to several studies (Bale & Hayward, 2010; Jing and Kang, 2003a, Ren*et al*., 2016, Zhao *et al*., 2008, etc.), insects in temperate and freezing zones adapt their physiological states to low temperatures. They can migrate or enter overwintering habitats to behaviorally avoid the cold. By removing intracorporal ice nucleation materials, lowering water levels, and collecting cryoprotectants, insects can improve their supercooling capacity and keep bodily fluids from freezing (Qiang*et al*., 2008).Life history features like fecundity, fertility, longevity, and stress tolerance are subsequently impacted by these changes.

Stress is often described as any environmental change that reduces an organism's fitness (Sibly and Calow, 1989; Koehn and Bayne,1989). The frequency of an organism's exposure to its environment and the associated physiological costs determines how much genetic variability in stress tolerance led to adaptive change (Hoffmann and Parsons, 1991). From an evolutionary perspective, biological stress is an adaptation that helps organisms to survive and reproduce in challenging environments (McEwen, 2017).

The fruit fly, *Drosophila melanogaster*, is a well-known model organism in nutritional studies. Despite being a rather a relatively simple organism, it is genetically and physiologically quite similar to humans. This makes it the perfect subject for researching how nutrition affects a variety of biological functions, such as metabolism, aging, growth, and development(Kenyon, 2010). The fact that *Drosophila* is reasonably affordable and simple to maintain in the lab is one of the main benefits of employing it in nutritional research. A variety of diets, including intricate solid meals and chemically predetermined diets, can be used to rear flies. The latter are especially helpful for researching the results of certain nutrients or nutrient mixtures (Piper*et al*., 2014). The short lifespan of *Drosophila*, makes it useful for nutritional research.

Currently, one of the most popular supplements and an ergogenic aid with scientific support is creatine. Skeletal muscle has relatively large amounts of the creatine. The ability of creatine to raise muscular phosphocreatine reserves was instantly highlighted in the first research conducted in 1992 (Harris *et al*., 1992) and it was later discovered that these increases were related to improved physical performance (Bembenand Lamont, 2005). The three amino acids arginine, glycine, and methionine are converted into the metabolite creatine by a number of organs, including the liver, pancreas, and kidneys (Arazi*et al*., 2021; Hou*et al*., 2019).As far as the sources of these amino acids are concerned, it is important to keep in mind that these amino acids can be obtained from a variety of sources, including foods like beef, which has substantial amounts of them while vegetables have low concentrations of these amino acids (Hou*et al*., 2019; Valenzano*et al*., 2019). A number of studies on creatine monohydrate has been conducted mainly on its effect on physical performance however there is no available knowledge on the effect of creatine monohydrate on cold resistance in *Drosophila melanogaster*thus the current study has been undertaken.

MATERIALS AND METHODS

Establishment stocks

The *Drosophila* Stock Centre at the Department of Zoology at the University of Mysore in Karnataka, India, provided the Oregon-K strain of *D. melanogaster* flies. For two generations, these flies were bred to create the experimental stock. On culture bottles containing wheat cream agar media, the flies were kept under laboratory conditions. The temperature and relative humidity in the lab where the culture bottles were stored were around $22^0C \pm 1^0C$ degrees Celsius and 70%, respectively.

Wheat cream agar-agar media was used to cultivate the flies that were used as control in the study. The wheat cream Agar-agar media was supplemented with creatine monohydrate at varied percentages (2.5%, 5%, and 10%) for the experimental diets. The Synergy Supplement Store in Mysore, Karnataka, India is where the creatine monohydrate supplement was purchased. Separately, twenty flies were transferred to wheat cream agar media and creatine monohydrate supplemented media. These flies were kept in lab conditions as previously indicated. For this experiment, flies collected from the culture bottles were utilized.

Effect of creatine monohydrate on cold resistance in *D. melanogaster*

Five (5) days old unmated and mated (male and female) fruit flies maintained in control media and creatine monohydrate treated medium were used to test cold resistance. Twenty flies (control/treated) were placed in empty vials to study cold resistance. These vials were refrigerated at 5^0C to maintain continuous coolness. The flies were observed once every 12 hours, until each fly had died. A total of four (4) replicates of each of the control and creatine treated media were utilized, and the cold resistance was measured in hours. Both a male and female trial was conducted separately.

RESULTS AND DISCUSSION

Results

Both control media fed mated male and mated female flies had significantly higher cold resistance compared to 5% and 10% creatine monohydrate treated mated males and females however it had less cold resistance than mated flies maintained on 2.5% creatine monohydrate supplemented media (**Fig 1**). The data subjected to Two-way ANOVA followed by Tukey's post hoc test showed that significance variation in cold resistance of mated male and female raised in creatine monohydrate and control media, between sexes and also interaction between treatment and sex. The mated male was significantly less cold resistant than females in both control and creatine monohydrate treated media. Further, among creatine monohydrate treated flies, 2.5% creatine monohydrate treated flies had significantly high cold resistant compared to 5% and 10%creatine monohydrate treated flies by Tukey's post hoc test.

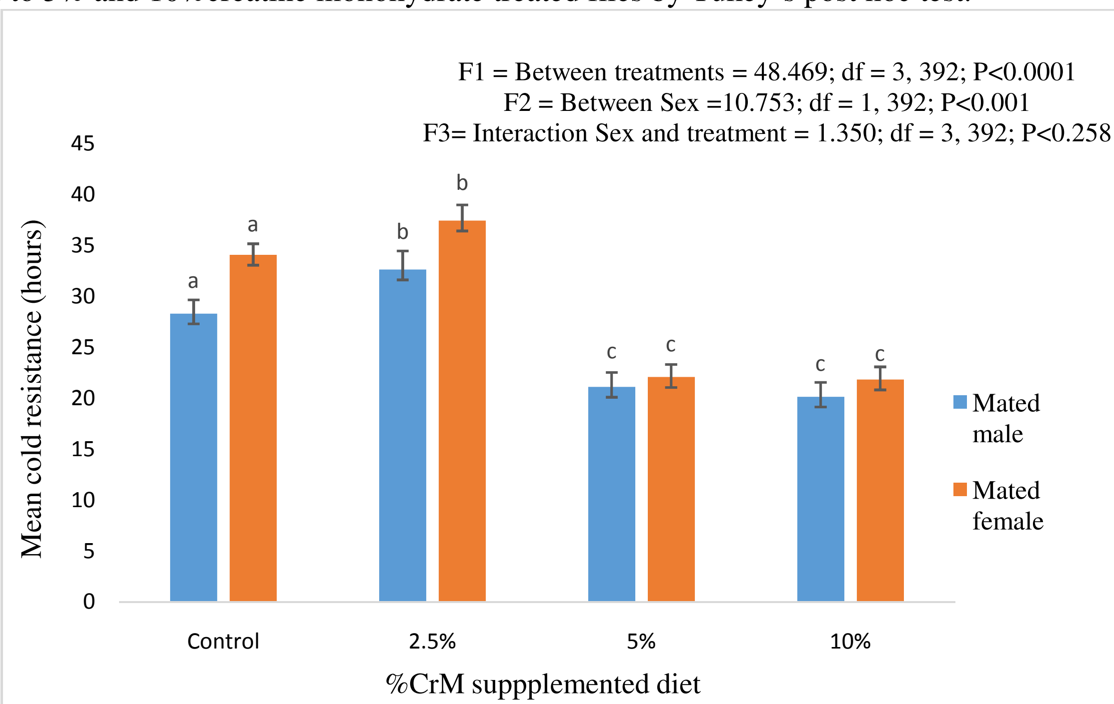

Figure 1: Effect of creatine monohydrate supplemented diet on cold resistance of mated Drosophila melanogaster (male and female).Different letters on the bar graph indicates significance at 0.05 levels by Tukey's Post Hoc test.

Creatine monohydrate treated unmated males and unmated females were significantly less cold resistance compared to unmated males and unmated females of control media except 2.5% creatine monohydrate supplemented media (Fig 2) which had significantly higher cold resistance compared to all treatments and control diet fed unmated flies. Further, there was no significant difference in cold resistance of flies maintained on 5% and 10% creatine monohydrate supplemented diet both unmated males and females. The data subjected to Two-way ANOVA followed by Tukey's post hoc test showed significance variation in cold resistant between treatments, between sexes and also interaction between treatment and sex. The unmated male had less cold resistant than females in both control and creatine monohydrate treated media. Further among creatine monohydrate treated flies, 2.5% creatine monohydrate treated flies had taken significantly high cold resistance compared to 5% and 10% creatine monohydrate treated flies.

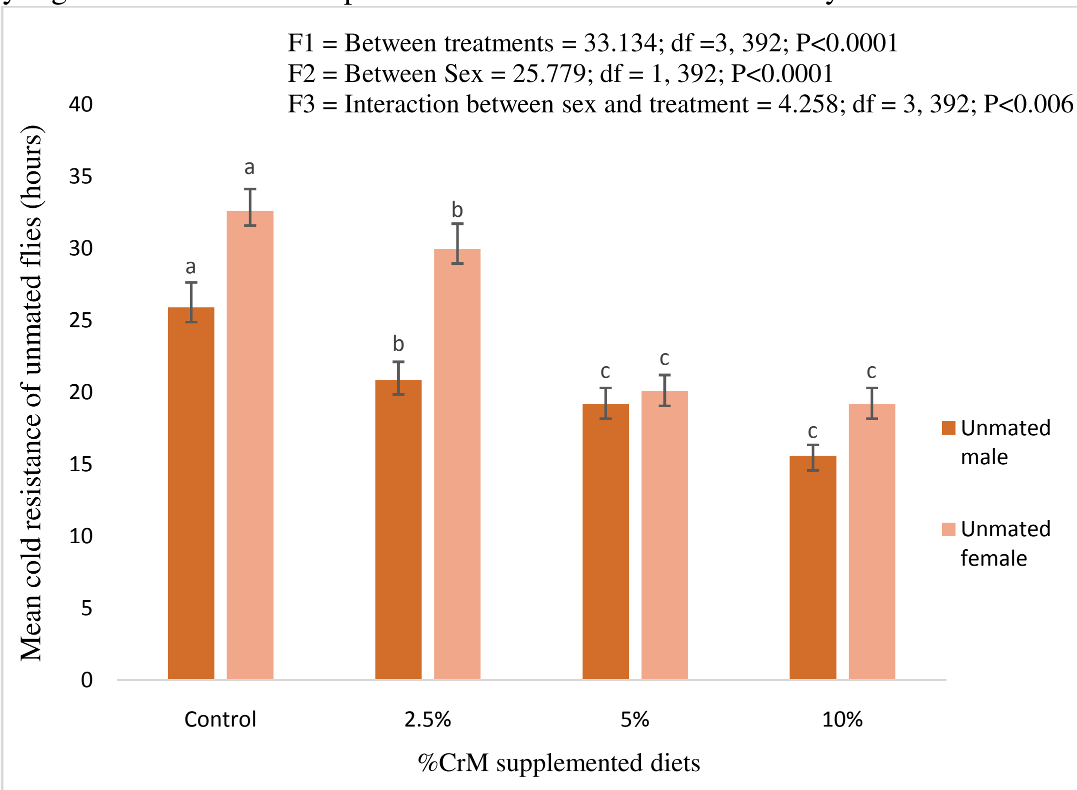

Figure 2: Effect of creatine monohydrate supplemented diet on cold resistance of unmated flies (male and female) *Drosophila melanogaster*.Different letters on the bar graph indicates significance at 0.05 levels by Tukey's Post Hoc test.

Fig. 3. Shows cold resistance mean ± standard error of control and creatine monohydrate treated flies (2.5%, 5%, 10%). From the data obtained it was noticed mated male flies had higher cold resistance compared to unmated male flies in all treatments including the control. Male flies maintained on 2.5% creatine monohydrate supplemented media had higher cold resistance compared to all treatments. Both mated and unmated male flies on 5% and 10% creatine monohydrate supplemented diet had lowest cold resistance. The data subjected to two-way ANOVA followed by Tukey's Post hoc test showed that significance variation in male cold resistant between control and creatine monohydrate treated flies, between conditions (mated v/s unmated) and also interaction between treatment and sex. The Tukey's post hoc test between control and 10% creatine monohydrate treated flies was found to be significant. Further among creatine monohydrate treated flies 5% and 10% flies had significantly less cold resistance compared to 2.5% creatine monohydrate treated flies.

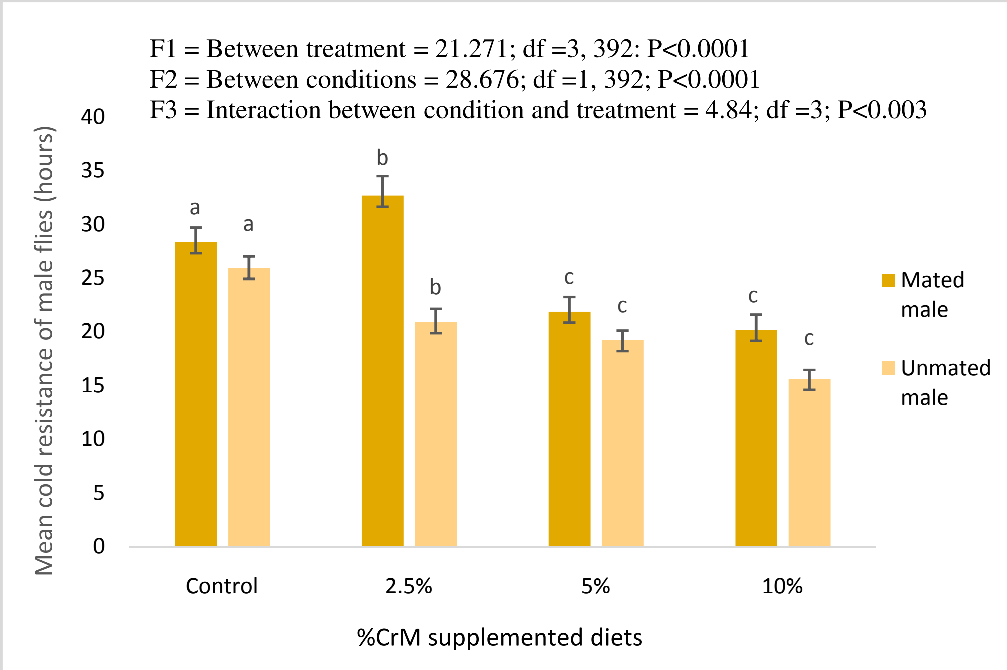

Figure 3: Effect of creatine monohydrate supplemented diet on cold resistance of male (mated and unmated) *Drosophila melanogaster*.Different letters on the bar graph indicates significance at 0.05 levels by Tukey's Post Hoc test.

Cold resistance data of control and creatine monohydrate treated flies (2.5%, 5%, and 10%) are provided in Fig. 4. From the data obtained it was noticed that in bothmated and unmated female the lowest cold resistance was noticed in 5% and 10% flies and highest cold resistance was observed in 2.5% creatine monohydrate treated flies. Mated female flies proved to be more resistant to cold compared to unmated females in both control and creatine monohydrate treated flies. The data subjected to two-way ANOVA followed by Tukey's Post hoc test showed significance variation in cold resistance between treated flies of mated and unmated condition and also in the interaction between treatment and control.

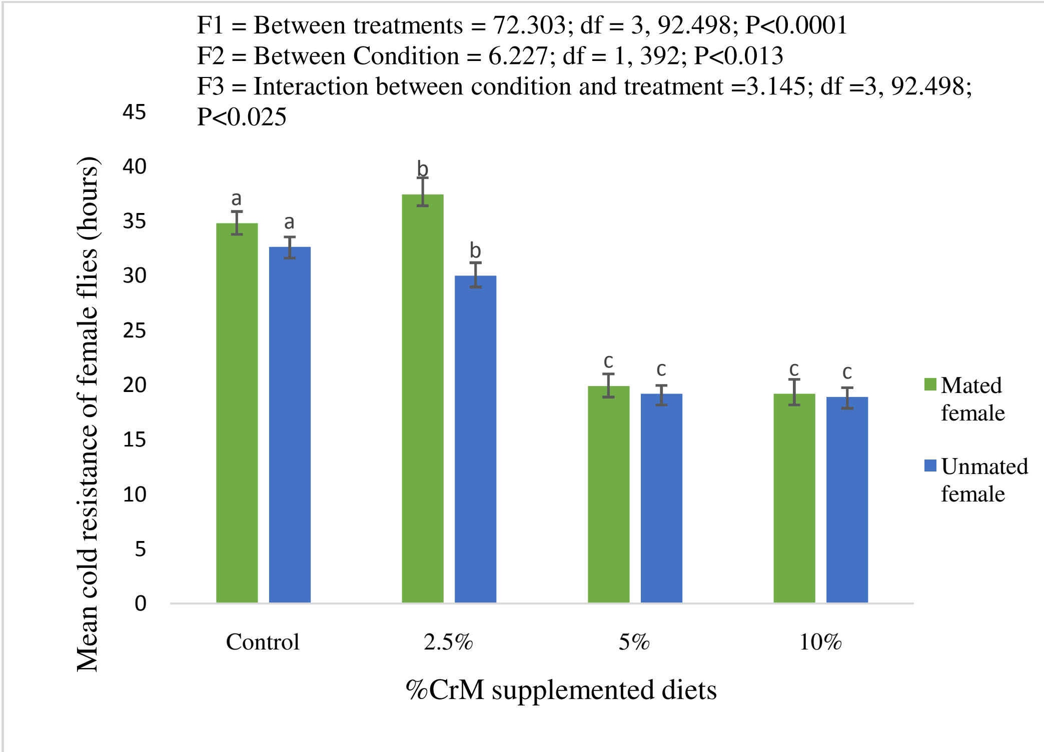

Figure 4: Effect of creatine monohydrate supplemented on cold resistance of female (mated and unmated) *Drosophila melanogaster*.Different letters on the bar graph indicates significance at 0.05 levels by Tukey's Post Hoc test.

Discussion

Food is an essential component that all organisms require for growth,development, physiology, famine resistance, and survival. The quantity and quality ofnutrients consumed by an organism influence life history process such as reproductionand stress tolerance (Sisodia and Singh, 1988). Furthermore, the quantity and qualityof nutrients available in different diets vary. Insects' ability to adapt to and mitigate theconsequences of changing climatic circumstances is dependent on differences instress-related characteristics (Chownand Terblanche, 2006). In *Drosophila*, for example, high desiccation resistanceis associated with adaptation to arid environments, whereas high cold resistance isassociated with adaptation to high latitudes.Creatine monohydrate' influence on *D. melanogaster's* ability to withstand coldwas therefore the subject of the current investigation. The cold resistance of creatinemonohydrate-treated flies was found to be substantially higher in 2.5% CrMsupplemented diet flies than that of control flies. This indicates that supplementing diet with creatine monohydrate at a certain extent may improve *D. melanogaster's* ability towithstandcold. This confirms the earlier studies of dietary effect, cold resistance in*Drosophila*(Chown and Nicolson, 2004; McCue, 2010; Laparie*et al*., 2012; Ribeiro*et al*., 2010; Burger *et al*., 2017). They also demonstrated that the amount and quality of nutrients consumedhad a significant impact on the ability to withstand cold. Physiological changes arerequired for increased cold tolerance, which are likely to impair other fitness-relatedtraits. By reducing the amount of food, notably protein (yeast), available to adult flies,caloric restriction increases their resilience to cold and cold, with up to a twofolddifference between females previously fed ad labium yeast and those given no yeast (Chown and Nicolson, 2004; McCue, 2010; Laparie*et al*., 2012; Ribeiro*et al.,* 2010;Burger *et al*., 2017). Additionally, Sisodia and Singh (2012) found that south Indian *D.ananassae*groups that eat fruits high in carbohydrates were less susceptible to a foodcrisis than north Indian tribes that eat fruits high in protein.In the present study also found that females were more resistant to cold thanmales in both control and treated flies (Fig 1-2). This is because females in speciesof *Drosophila*are larger and heavier than male flies in contrast to them. In manyorganisms, males and females were likely to respond to resistance in different ways,mostly because their nutritional requirements and methods of usage differ (Hoyenga*et al.,* 1982). It has been shown that strains, even within *D. melanogaster*, considerably affect the pattern of sexual dimorphism in cold and cold resistance.In the present study, the cold and cold resistance of mated and unmated malesand females of *D. melanogaster*was also examined. It was found that unmated malesconsiderably outperformed mated males in both the control and creatine monohydrate treated fliesin terms of cold and cold resistance. The most likely reason for this is that matedfemales consumed more food and built up more lipids than male flies (Lee *et al*., 2013). It has also been demonstrated in more recent research that female *D. melanogaster*can dramatically increase their midgut size during mating. Mated females are able to meet their heightened energy requirements for egg production byincreasing their post-ingestive nutrient consumption (Winkler and Smith, 2008). They further explained that this increase in nutrient consumption is achieved through a combination of increased foraging activity and increased efficiency of nutrient digestion and absorption.

CONCLUSION

Increased creatine monohydrate supplementation has a negative effect on cold resistance in *Drosophila melanogaster*. Female flies have higher cold resistance than male flies and mated flies more resistant to cold in both control media and creatine monohydrate treated media. The present study has showed that creatine monohydrate has an effect on cold resistance in *Drosophila melanogaster.*

ACKNOWLEDGEMENTS

We appreciate the Chairperson,National *Drosophila* Stock Center and Department of studies in Zoology, University of Mysore for the provision of working equipment. We value the cooperation and help provided by the *Drosophila* stock center and Stress biology lab team during the course of the study.

REFERENCES

1. Arazi H, EghbaliE, Suzuki K. Creatine supplementation, physical exercise and oxidative stress markers: A review of the mechanisms and effectiveness. Nutrients. 2021. 6;13 (3):869.15.
2. Bale, J. S., and Hayward, S. A. L. (2010). Insect overwintering in a changing climate. *The Journal of Experimental Biology*, 213(6), 980–994.
3. Bemben MG, Lamont HS. Creatine supplementation and exercise performance: Recent findings. Sports Med. 2005; 35 (2):107-25
4. Bijlsma, R., van der Horst, G., and Visser, J. (1996). The effects of temperature and photoperiod on feeding behavior and growth of the red flour beetle, *Triboliumcastaneum*. Journal of Insect Physiology, 42(12), 1277-1284.
5. Burger, J. M., van Hulle, M., and Leirs, H. (2017). Coping with cold stress: Thermal tolerance of insects is associated with the expression of cold shock proteins. Journal of Thermal Biology, 66, 13-21.
6. Chowanski, S., Lubawy, J., Spochacz, M., Ewelina, P., Grzegorz, S. ,Rosinski, G. , and Slocinska, M. (2015). Cold induced changes in lipid, protein and carbohydrate levels in the tropical insect *Gromphadorhinacoquereliana*. *Comparative Biochemistry and Physiology Part A: Molecular and Integrative Physiology*, 183, 57–63.

7. Chowanski, S., Mery, F., and Tsubaki, J. (2010). Sex differences in cold tolerance and winter survival of *Drosophila*. Functional Ecology, 24(2), 372-380.
8. Chown SL, Nicolson SW., (2004) Insect Physiological Ecology. Oxford University Press, New York Press, New York.Chown SL, Nicolson SW. Insect Physiological Ecology. Oxford University Press, New York 2004.
9. Chown, S. L., & Terblanche, J. S. (2006). Physiological diversity in insects: Ecological and evolutionary contexts. In S. L. Chown& J. S. Terblanche (Eds.), *Physiological diversity in insects* (pp. 1-39). Springer, Dordrecht.
10. Chown, S. L., and Nicolson, S. W. (2004). The ecological roles of water loss and conservation in insects. Physiological Entomology, 29(1), 1-19.
11. Harris RC, Soderlund K, Hultman E. Elevation of creatine in resting and exercised muscle of normal subjects by cre-atine supplementation. Clin Sci. 1992
12. Hoffmann AA, Parsons PA. Evolutionary genetics and environmental stress. Oxford University Press, Oxford.1991.
13. Hou Y, He W, Hu S, Wu G. Composition of polyamines and amino acids in plant-source foods for human consump-tion. Amino Acids. 2019 Aug; 51 (8):1153-1165.16.
14. Hoyenga*et al.* Gender and energy balance: sex differences in adaptations for feast and famine Physiol. Behav. (1982).
15. Jenki, S., O'Donnell, S., and Reynolds, S. J. (1997). The effect of temperature on the feeding behavior of the German cockroach, Blattellagermanica. Journal of Insect Physiology, 43(12), 1219-1227.
16. Jenkins, B. J., and Sohal, R. S. (2002). Creatine monohydrate protects against age- and stress-induced oxidative damage and loss of function in mitochondria of *Drosophila melanogaster*. Free Radical Biology and Medicine, 33(1), 114-122.
17. Kenyon, C. (2010). The genetics of ageing. Nature, 464(7288), 504-512.
18. Koehn, R. K., and Bayne, B. L. (1989). Towards a physiological and genetical understanding of the latitudinal variation in marine bivalve mollusks. Biological Journal of the Linnean Society, 37(1-2), 1-15.
19. Lalouette, J., and Kostal, L. (2007). The effect of body size and temperature on the thermal performance of the wood mouse (*Apodemussylvaticus*). Journal of Thermal Biology, 32(3), 215-223.
20. Laparie, M., Renault, D., Bonnefoy, X., and Voituron, Y. (2012). Metabolic regulation of insect diapause. Frontiers in Physiology, 3, 350.
21. Lee, K. P., Kim, J. S., and Min, K. J.Sexual dimorphism in nutrient intake and life span is mediated by mating in *Drosophila melanogaster*Anim. Behav. (2013).
22. McCue, M. D. (2010). The role of hormones in insect diapause. Annual Review of Entomology, 55, 353-370.
23. Mcdonald, J., Head, J., Bale, J. S., and Walters, K. A. (2000). Cold tolerance, overwintering and establishment potential of *Thripspalmi*. *Physiological Entomology*, 25, 159–166. 10.1046/j.1365-3032.2000.00179.x
24. McEwen, B. S. (2017). Allostasis and the brain: Steroids and the neural basis of stress and adaptation. Oxford University Press.
25. Miller, B. R., and Shaw, W. J. (2001). Creatine monohydrate supplementation improves flight performance in *Drosophila melanogaster*. Journal of Insect Physiology, 47(3), 359-363.
26. Piper, M. D., Partridge, L., Rauser, C. L., and Simpson, S. J. (2014). Dietary restriction in *Drosophila*. Methods in enzymology, 548, 353-363.

27. Prasad, B. S., Kumar, A., and Sharma, R. K. (2003). Effect of temperature on the feeding behavior of the desert locust, Schistocercagregaria. Journal of Insect Physiology, 49(11), 1113-1121.
28. Qiang, C. K. , Du, Y. Z. , Yu, L. Y. , Cui, Y. D. , Lu, M. X. , and Zheng, F. S. (2008). Dynamic changes of cold resistant substances of overwintering *Chilosuppressalis* (Walker) larvae. *Chinese Journal of Applied Ecology*, 19(03), 599–605.
29. Ren, X. Y., Qi, X. Y., An, T., Han, Y. H., Chen, H. Y., and Zhang, L. S. (2016). Research on the accumulation, transformation and regulation of nutrients in diapause insects. Chinese Journal of Applied Entomology, 53(04), 685–695.
30. Ribeiro, J. M. C., Rollo, C. D., and Lopes, J. F. (2010). Physiological adaptations of insects to cold stress. In Physiological Adaptations: Phenotypic Plasticity and Evolution (pp. 143-163). Springer, Berlin, Heidelberg.
31. Sibley, R. M., and Calow, P. (1989). The evolution of life histories: An ecological perspective. Blackwell Scientific Publications.
32. Sisodia S, Singh BN. Experimental evidence for nutrition regulated stress resistance in *Drosophila*ananassae. PLoS One. 2012; 7 (10):e46131.
33. Sisodia, S. S., and Singh, B. P. (1988). Physiological adaptation to high altitude in humans: A review. Journal of Human Ecology, 9(2), 101-110.
34. Sørensen, K., Nielson, P. J., and Andersen, H. J. (2005). The effect of body size and temperature on the thermal performance of a small mammal, the house mouse, Musmusculusdomesticus. Journal of Experimental Biology, 208(16), 2933-2940.
35. Valenzano A, Polito R, Trimigno V *et al*., Effects of very low calorie ketogenic diet on the orexinergic system, visceral adipose tissue, and ROS production. Antioxidants (Basel). 2019 Dec 13; 8 (12):643
36. Walker, D. W., and Dowse, H. B. (2004). Creatine monohydrate supplementation enhances cold stress tolerance in *Drosophila melanogaster*. Journal of Insect Physiology, 50(12), 1021-1029.
37. Winkler, D.W., and Smith, S.M. published in the book *Avian Biology** (5th edition), edited by Donald S. Farner, John R. King, and Kenneth C. Parkes (2008).
38. Zhao, J., Yu, L. Y., Li, M., Zheng, F. Q., Zhang, F., and Xu, Y. Y. (2008). Seasonal variation in cold tolerance of the multicolored ladybeetle, *Harmoniaaxyridis* (Pallas) (Coleoptera: Coccinellidae) adults. *ActaEntomologicaSinica*, 51(12), 1271–1278.

