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IMPACT OF NUTRIENT-SPECIFIC MODULATION ON COLD STRESS TOLERANCE: INSIGHTS FROM *Drosophila ananassae* AND *D. bipectinata*

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ABSTRACT

***Drosophila* species (fruit flies) have long served as valuable model organisms in evolutionary research. Among the diverse traits exhibited by fruit flies, cold tolerance stands out as a critical factor influencing their ecological distribution and survival in varied environments. The majority of research on cold tolerance concentrates on subjecting flies to a single, stressful temperature without any modulation in the diet. The present study aimed to understand the impact of nutrient-specific modulation on cold tolerance of *Drosophila ananassae* and *D. bipectinata* across a broad range of lower temperatures. Adult flies were transferred to the media composition enriched with sucrose and tryptophan in three replicates and maintained at 22ºC. Twenty flies of particular stages *viz*., adults, eggs, larvae and pupa were exposed to 18, 14, and 10ºC for 5 days. The impact of cold stress on their growth and survival was noted for 5 following days. The impact of dietary modifications on cold stress tolerance and life history traits was positive and consistent across both species. In contrast, *D. bipectinata* had greater survival rates than *D. ananassae* across all temperatures and dietary regimes. Tryptophan (100 mg) diet performed best to improve life traits on cold stress treatment.**

Keywords: Cold tolerance, sucrose, survival frequency, tryptophan, wings deformation

INTRODUCTION

Drosophila (fruit fly) have long served as valuable model organisms in biological research, contributing significantly to our understanding of various physiological processes, genetics, and evolutionary adaptations (Aggarwal *et al*., 2023). The use of fruit flies as a model organism for nutrition research has gained recognition in recent years (Perenon *et al*., 2021). The responses of fruit flies to dietary factors can be systematically determined, including feed intake, developmental stages, locomotor activity, aging, and lifespan (Bäuerlein *et al*., 2012). Among the diverse traits exhibited by these fruit flies, cold tolerance is a critical factor that influences their ecological distribution and survival in various environments. The ability of *Drosophila* to withstand cold temperatures is essential for their persistence in habitats characterized by seasonal fluctuations or harsh winter conditions. Cold tolerance involves a complex interplay of genetic, physiological, and environmental factors, which leads to directed selection favouring stress tolerance (Tarapacki *et al*., 2021).

Recent studies have highlighted the impact of dietary composition on the physiological performance and stress resistance of various organisms, including *Drosophila* (Balyak *et al.,* 2018; De Ro *et al.*, 2021;). Nutrient availability, particularly carbohydrates and amino acids, has been shown to influence metabolic pathways, energy reserves, and stress response mechanisms. Sucrose, as a solute, may play a role in osmoregulation and cellular protection under cold stress (Hurza *et al*., 2021).

Sucrose supplementation affects the cellular water balance and contributes to cryoprotective mechanisms can enhance our understanding of how dietary factors influence the physiological responses to cold in *Drosophila* (Strilbytska *et al.*, 2022). Tryptophan supplementation can influence metabolic pathways, including those associated with energy production. Tryptophan is a precursor for serotonin, a neurotransmitter with known regulatory functions in temperature homeostasis, modulating behavioural and physiological responses to temperature changes in insects (Koštál *et al.*, 2016). However, the specific effects of different dietary components on cold tolerance in *Drosophila*, particularly in species like *D. ananassae* and *D. bipectinata*, remain underexplored. In this study, we aimed to explore the role of diets enriched with carbohydrates and amino acids in shaping the cold tolerance of *D. ananassae* and *D. bipectinata* life-stages.

MATERIALS AND METHODS

Fly stocks and experimental design

Drosophila ananassae and *D. bipectinata* were collected from the *Drosophila* Stock Center Manasa Gangotri University of Mysore, Mysore, Karnataka (India). The stock flies were maintained in a 22°C incubator on 12:12 h light: dark cycle at 60% humidity. A culture medium comprising of 15 g *Triticum durum*, 15 g *Saccharum officinarum*, 1.5 mL propionic acid, and 1.5 g agar dissolved in 150 mL distilled water was used to maintain flies of both the species. The controlled density of both species was attained by allowing 2 females and 2 males to oviposit for 48 h in each vial. The emerging flies were collected and placed in holding bottles having bioassay conditions.

Bioassay conditions

D. ananassae adult flies (2 males and 2 females) were transferred to the modified diet media enriched with 1) carbohydrate {sucrose 30 and 70 g}, and 2) amino acid {tryptophan 100 and 400 mg} in five replicates. Similarly, *D. bipectinata* 2 males and 2 females were transferred to the media enriched with (i) carbohydrate (sucrose 30 and 70 g) and (ii) amino acid (tryptophan 100 and 500 mg). The flies were incubated in an incubator maintained at 22°C, considering it a control temperature. Eggs, larvae, pupa and adults generated by the mating of these flies were exposed to different temperature regimes *viz*., 10, 14 and 18°C for 5 days. The impact of cold stress on their survival was noted.

Hatching of eggs: Twenty eggs in three replicates were exposed to different temperatures 22, 10, 14 and18°C for 5 days. The number of larvae hatched was counted under a binocular microscope (100X) [Motic Asia, BA210]. The survival frequency was calculated by using the formula:

$$\text{Percent survival} = \frac{\text{The number of larvae seen x 100}}{\text{Total number of eggs exposed to cold stress}}$$

Effect on pupation: *D. ananassae* and *D. bipectinata* larvae (20 each in three replicates) were exposed to cold stress at 10, 14 and 18°C for 5 days after four days of hatching. The individual larvae were observed daily for attaining pupa stage or death up to 5 days after treatment.

Effect on adult eclosion: Pupa from raised media at 22°C were exposed to cold at 10, 14 and 18°C for 5 days. The insect pupa was observed daily for eclosion of adults and death up to 5 days after treatment.

Deformation in new adult wings: Immediately after attaining the adult stage, adults from control condition (22°C) were exposed to cold at 10, 14 and 18°C for five days. Males and females both were observed for deformation of wings and death for 5 consecutive days.

Impact on fecundity: Adult flies maintained at experimental conditions were exposed to 10, 14 and 18°C for 5 days. The number of eggs laid was counted and recorded.

Statistical analysis

All the experiments conducted in a completely randomized design. The experimental data were compared with respective control data (22°C as control temperature) and the cold stress conditions were exposure to 10, 14 and 18°C for five days. Mean data were calculated and ANOVA used to find significant impact of laboratory diet on cold tolerance of both genotypes of *Drosophilla*.

RESULTS AND DISCUSSION

Genes for cold tolerance in *Drosophila* were highly, but there is less research on diets to improve cold tolerance investigated (Garcia *et al.*, 2020; Malkeyeva *et al.*, 2020). Diet can affect multiple molecular, physiological, tissue, and organ systems, which are involved in cold tolerance. Additionally, these mechanisms operate at different times during and after exposure to cold stress (Overgaard and MacMillan, 2017). The results of the present study revealed that the impact of dietary modifications on life history traits was consistent across both the species. It might be because both the species belong to *ananassae* species subgroup and are genetically similar (Signor *et al.*, 2013). Contrarily, Kim *et al.* (2020) found that dietary composition differentially impacted the longevity, development, and fecundity across six genetically distinct strains of *Drosophila melanogaster*. Staats *et al.* (2020) found no role of diet reservoir on lifespan and stress response in genetic studies.

Egg hatching

Drosophilla species fitness was decreased by cold stress, hatching of eggs took a longer time (28-30 h) at 14 and 10°C as compared to the control temperature (22-24 h). Egg hatching was not affected by the composition of diet. The survival of larvae hatched from the eggs at lower temperatures was decreased but affected by dietary regimes (Fig. 1). Eggs hatched on tryptophan (100 mg) enriched medium had higher survival rates than the flies developed on sucrose-enriched medium and control medium ($P < 0.01$). *D. bipectinata* larvae survival was 87.5, 59.0, 35.0 and 15.0% at 22, 18, 14, and

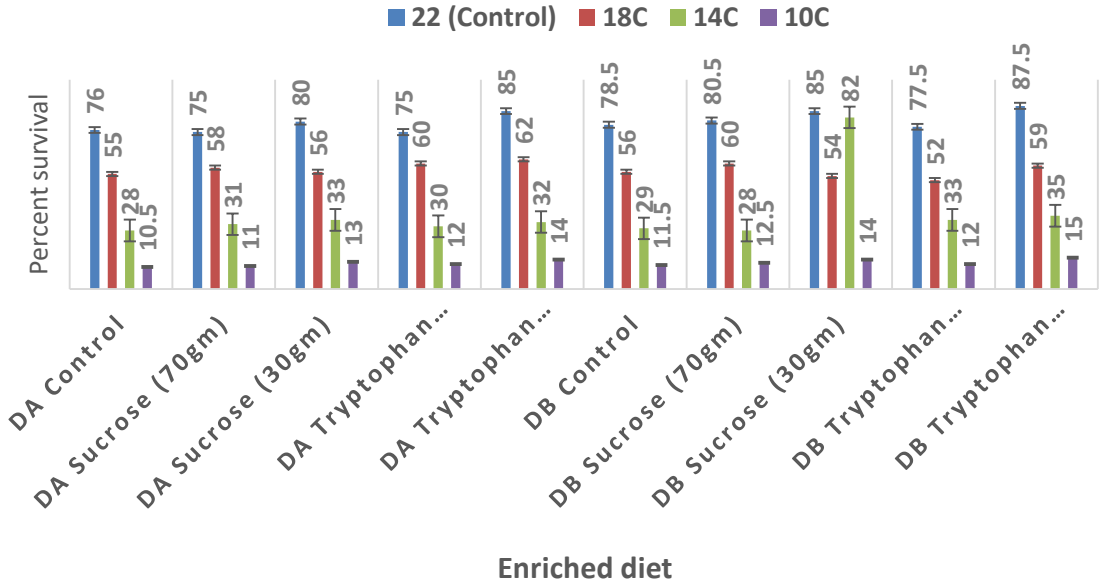

Fig. 1: Impact of dietary supplements on egg hatching under cold stress; DA refers to *D. ananassae* culture medium and DB to *D. bipectinata* culture medium; sucrose (70 g): culture medium enriched with 70 g sucrose; sucrose (30 g): culture medium enriched with 30 g sucrose; Tryptophan (400 mg): culture medium enriched with 400 mg tryptophan; Tryptophan (100 mg): culture medium enriched with 100 mg tryptophan**.**

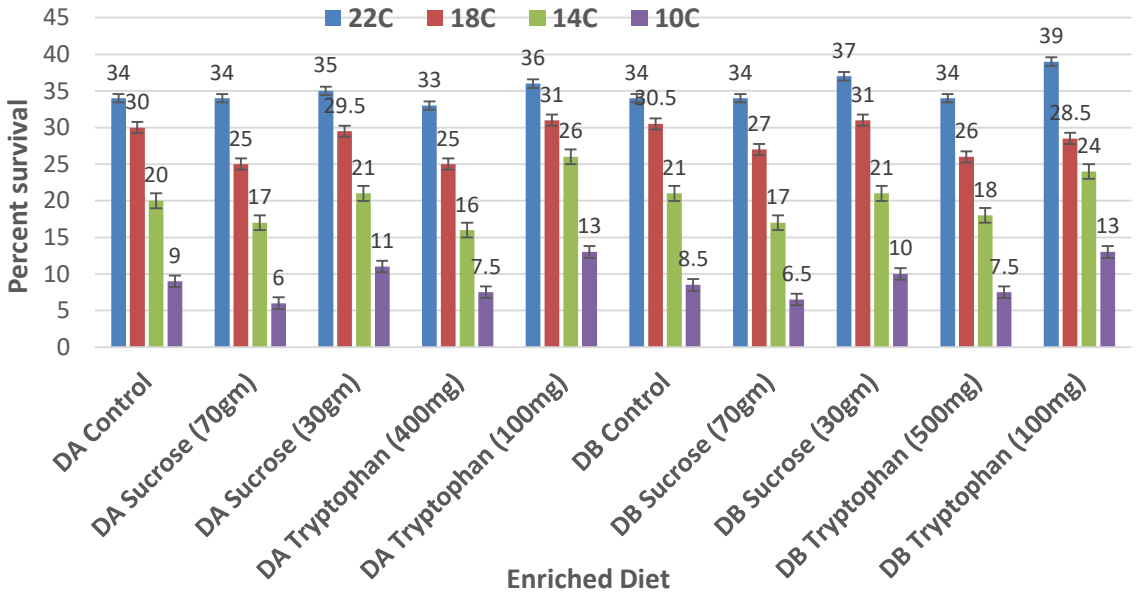

Fig. 2: Impact of dietary supplements on pupation under cold stress; DA refer to *D. ananassae* culture medium and DB to *D. bipectinata* culture medium; sucrose (70 g): culture medium enriched with 70 g sucrose; sucrose (30 g): culture medium enriched with 30 g sucrose; Tryptophan (400 mg): culture medium enriched with 400 mg tryptophan; tryptophan (100 mg): culture medium enriched with 100 mg tryptophan

10ºC at tryptophan (100 mg) whereas standard media eggs/ larvae survival was 78.5, 56, 29 and 11.5% (Fig. 1). Similar survival frequency (85, 62, 32 and 14% was noted for *D. ananassae* on providing low tryptophan (100 mg) dose. Hence, a significant improvement in survival was noted for both species at cold stress conditions, when supplemented with a low dose of tryptophan. Our results are supported by Koštál *et al.* (2019) who found the diet of *D. melanogaster* larvae supplemented with proline and arginine had greatest potential to increase freeze survival from 42.1 and 50.6%, respectively.

Pupation

A positive correlation (p = < 0.01) between lower supplementary amounts of carbohydrates and tryptophan with high freeze tolerance was noted during pupation studies. However, higher concentrations of sucrose and tryptophan showed detrimental effects on pupation (Fig. 2). The survival of *D. ananassae* and *D. bipectinate* pupa at 10˚C was 9 and 8.5% without any supplement. It improved up to 13% by adding tryptophan (100 mg) for both the species. Similar effects of sucrose and amino acid-rich diet were noted by Colinet *et al,* (2012) and Bayliak *et al*. (2020), respectively.

Eclosion of adults

When *D. ananassae* and *D. bipectinata* pupa were exposed to cold stress for 5 days, a range of sublethal consequences were seen. These effects included death of pupa that happened gradually following the cold stress, delayed development to the adult stage, and lesser survival of adult males and females. A maximum of 27 and 28% survival rates of adults was noted after eclosion at 10ºC on tryptophan (100 mg) diet in case of *D. ananassae* and *D. bipectinata*, respectively, which was significantly high (p = <0.05) as compared to the control medium which were 19 and 16%, respectively (Fig. 3). It appears that extremely cold conditions induced physiological stresses, hence lowered survival, which could be improved by a directional selection for stress tolerance by the positive impact of the metabolism of amino acids (Enriquez and Colinet, 2019).

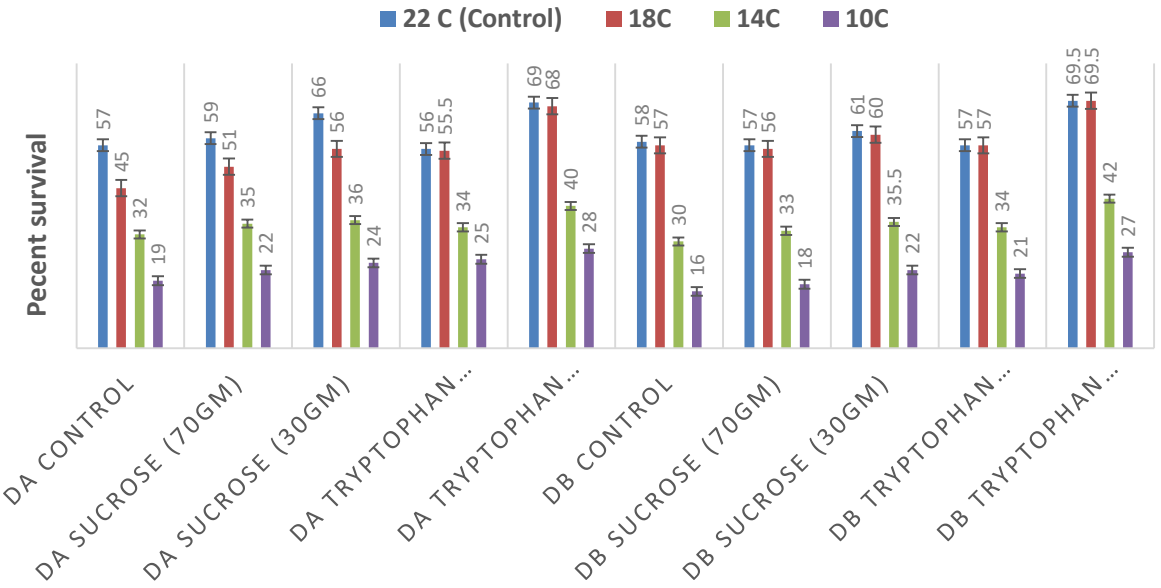

Fig. 3: Impact of dietary supplements on eclosion under cold stress; DA refers to *D. ananassae* culture medium and DB to *D. bipectinata* culture medium; sucrose (70 g): culture medium enriched with 70 g sucrose; sucrose (30 g): culture medium enriched with 30 g sucrose; tryptophan (400 mg): culture medium enriched with 400 mg tryptophan; tryptophan (100 mg): culture medium enriched with 100 mg tryptophan

Fecundity

Our results revealed that low doses of tryptophan (100 mg) maximized oviposition rate, hence better fecundity at normal and cold temperatures for both the species studied. *D. ananassae* and *D. bipectinata* at tryptophan (100 mg) diet laid 12 and 14.5 eggs per female/day at 10°C and 13.5 and 17.5 eggs female^{-1} day^{-1} at 14°C, which are significantly higher (p= <0.05) than the control and other conditions tested (Fig. 4). Several physiological models demonstrated that alterations in membrane function, such as cell membrane depolarization and ion homeostasis, resulting from cold exposure, are

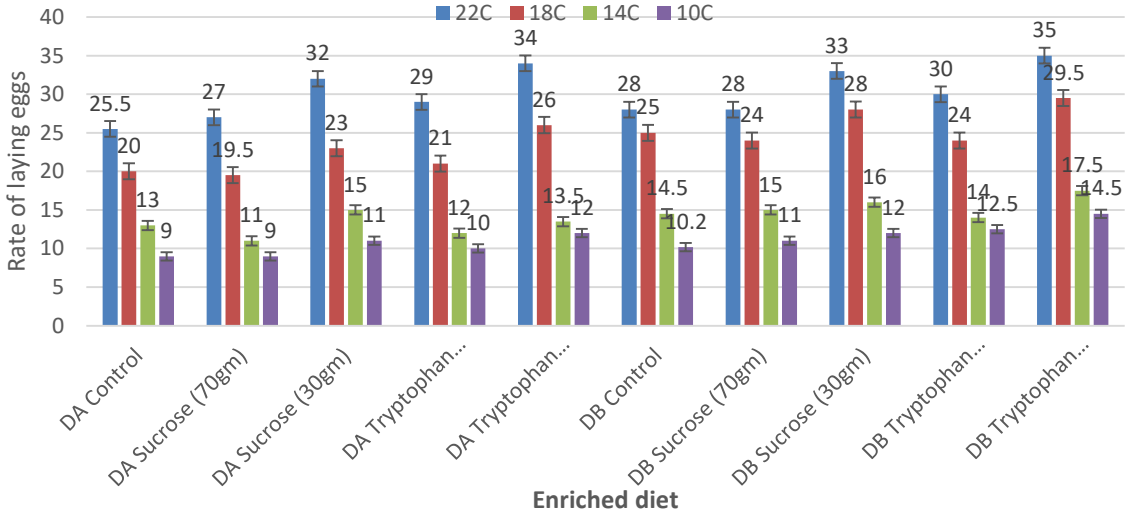

Fig. 4: Impact of the dietary supplements on oviposition under cold stress; DA refers to *D. ananassae* culture medium and DB to *D. bipectinata* culture medium; sucrose (70 g): culture medium enriched with 70 g sucrose; sucrose (30 g): culture medium enriched with 30 g sucrose; Tryptophan (400 mg): culture medium enriched with 400 mg tryptophan; tryptophan (100 mg): culture medium enriched with 100 mg tryptophan

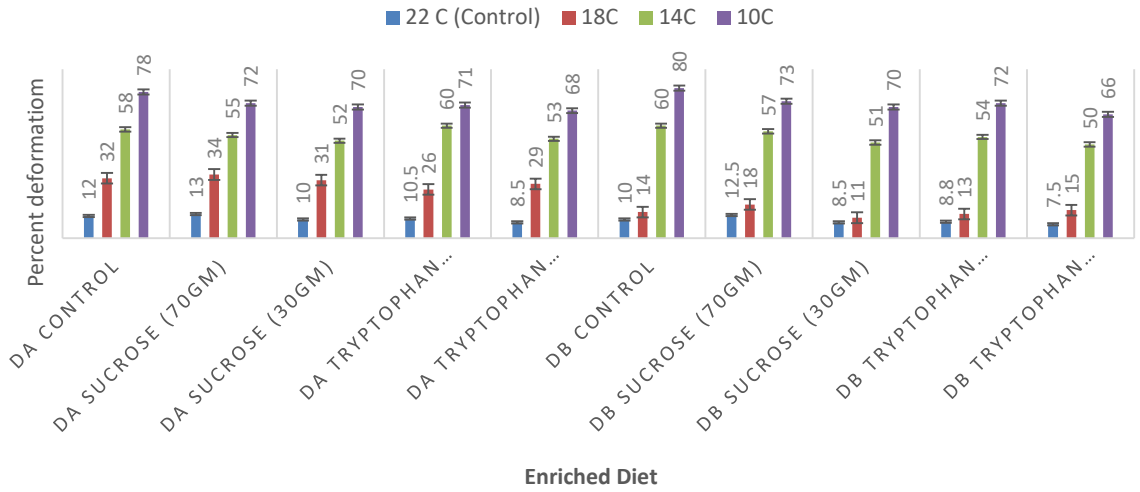

Fig. 5: Impact of the dietary supplements on deformation of wings under cold stress; DA refers to *D. ananassae* culture medium and DB to *D. bipectinata* culture medium; sucrose (70 g): culture medium enriched with 70 g sucrose; sucrose (30 g): culture medium enriched with 30 g sucrose; tryptophan (400 mg): culture medium enriched with 400 mg tryptophan; Tryptophan (100 mg): culture medium enriched with 100 mg tryptophan

responsible for several traits related to cold tolerance and survival during short-term cold stress (Freda *et al.*, 2017). Protein-rich diets typically improve reproductive outputs relative to carbohydrate-rich diets, likely by increasing protein pools for egg production and vitellogenesis in insects (Littlefair *et al.*, 2017; Goane *et al.*, 2019). The hormone signals needed for oogenesis were also noted highly dependent upon dietary proteins in *Drosophila* (Mirth *et al.*, 2019).

Deformation of wings

In present study *Drosophila* populations typically exhibited greatest cold tolerance when fed with tryptophan (100 mg). *D. ananassae* and *D. bipectinata* in control diet showed 78 and 80% deformation of wings which was significantly reduced to 68 and 66%, respectively, at tryptophan (100 mg) diet. Adult flies of both the species showed comparatively less wing deformation at 14 and 18°C as compared to 10°C at all diet ranges (Fig. 5). It might be because, sometimes when they are brought back to comfortable temperatures, flies regain their ability, if not significantly harmed (Hoffmann, 2010). Adult flies are chill-sensitive insects so lose their ion and water balance when exposed to cold, which eventually causes cell death, tissue damage, or impairments in physical performance (Andersen *et al.*, 2015). A modified protein-rich diet causes physiological changes and reduces these effects (MacMillan *et al.*, 2016).

Conclusions: The impact of dietary modifications on cold tolerance and life history traits was consistent across *D. ananassae* and *D. bipectinata* species. Tryptophan-rich (100 mg) diet improved life traits better relative to control and sucrose-rich diets on cold stress treatment.

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