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ESTIMATION OF LEVELS OF BIOCHEMICAL COMPONENTS AND EXCRETORY PRODUCTS OF TROPICAL TASAR SILKWORM *ANTHEREAE MYLITTA DRURY* (SUKINDA) UNDER INDUCED TEMPERATURE ALTERATIONS

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ABSTRACT: Studies on cold acclimation of insects including silkworms have shown significant variations in the levels of various biomolecules to cope with heat-shock. The present study has been carried out on cold-stressed 5th instar *Sukinda T.V* larvae to analyze the mortality rate, variations in the biomolecules and excretory products at varied durations. In this study, various biochemical assays have been performed in haemolymph, and fat body of the larvae. The results revealed that exposure to low temperatures (10°C ± 1°C) for seven days leads to 100% mortality, five days exposure caused 58% mortality and two days exposure to cold stress resulted in 42% mortality after reverting the larvae to normal temperature. The treatment of *Sukinda T.V* larvae with low temperatures resulted in increased aminoacid content in the haemolymph and decreased in fat body of all the three treatments in comparison with control group reared at 28°C ± 2°C. Significant decrease in urea and uric acid was observed in the larvae exposed to cold stress in comparison with control group.
Keywords: Haemolymph, Fat body, Amino acids, Urea, Uric acid, *Sukinda T.V*

INTRODUCTION

The physiological potential of life performance of the insect is always challenged by abundance of food and its quality, various abiotic factors, presence of predators, parasites and diseases. It is well known that temperature plays a major role in the physiological behavior of insects. The insects will get acclimatized to the low temperatures by the production of various cryoprotectants like glycerol, trehalose, sorbitol etc.

In insects, the growth and development is associated with protein metabolism [22]. In silkworms, the protein synthesis activity of the body wall and the midgut decreased when the larvae began to moult and increased from the midstage of the moulting period [13]. Marked increase in protein, pyruvate, total free amino acids, total lipids, phospholipids and triglycerols was observed in response to cold exposure [17]. A recent study has also revealed the involvement of amino acids in interaction with tyranine in *Bombyx mori* [15].
In the silkworm larva, the nitrogenous waste products of metabolism are mainly excreted as urine, together with faecal pellets. The excretory pattern depends upon a number of environmental factors such as temperature and humidity [1,7].

The excretory pattern of silkworm larvae on exposure to F2 alpha increased the nitrogenous end products[4]. Similarly larvae feeding with trace elements like cobalt increased the pattern of excretion[20]. Excretion forms an important factor for the balance of nitrogen in the body. The excretion of nitrogenous waste products has been studied in a number of insects [6,18,25].Uric acid contains comparatively less hydrogen than any other nitrogenous compound excreted by animals and it is therefore well adapted for conservation[26]. Urea is present in small quantities in insects. The excretion in insects, its energetic and functional principles has also been worked out [8].

Although the adaptive strategies exhibited by various insects in response to temperature variations are well known, only a few studies show the change in rate of acclimation in insects in response to cold stress. The present study focused on molecules which were available in the metabolic pool of fifth instar larvae of *Sukinda T.V* ecorace during cold stress conditions.

MATERIALS AND METHODS

Newly hatched larvae of *Sukinda T.V ecorace* (250) were reared on tender fresh leaves of *Terminalia arjuna* in the laboratory Sericulture Unit, Kakatiya University, Warangal, Andhra Pradesh, at 28ºC± 2ºC and humidity 70% to 75%. The 5th instar larvae were divided into 4 treatments; each treatment has 50 larvae and was reared in plastic trays. The larval mortality rate was recorded up to 7 days of treatment duration. Removal of faecal matter, diseased worms and bed cleaning was done at regular intervals.

To study the effect of temperature alterations on the biochemical components, fifth instar larvae were divided into the following four treatments each containing 50 larvae and were reared in plastic trays.

1. Treatment at 28ºC± 2ºC –T1 (Control)
2. Treatment at 10ºC ± 1ºC for 2 days – T2
3. Treatment at 10ºC ± 1ºC for 5 days – T3
4. Treatment at 10ºC ± 1ºC for 7 days – T4

Fifth instar larvae were selected from all the four treatments separately. After 7 days of treatment the haemolymph was collected in the test tubes and stored in the deep freezer. The fat body was isolated in cold condition by using Bodenstein's Ringer solution and weighed in chilled state using "Dhona" electrical balance and was used for subsequent biochemical studies. Free amino acids were measured at µg /100 mg of wet weight of tissue as[12].The excretory pellets of fifth instar larvae of all the four treatments were collected separately and homogenized in precooled mortar and pestle thoroughly. Urea was estimated according to the standard procedure of [14] and uric acid [5,16]estimated as µg /100 mg of wet weight of the pellets. Centrifugation was done by using Remi centrifuge T.8 model (20,000 rpm). The estimations were based on colorimetric principle of Beer- Lambert's law in which the absorbances of coloured complexes are proportional to the concentration of reaction products.

Statistical analysis

Each assay was replicated 3 times. Values were expressed as mean ± SE of replication and Student's *t*-test was applied to locate significant ($P < 0.05$) differences between treated and control groups.

RESULTS AND DISCUSSION

The exposure of the insect larvae to low temperature is expected to lead some changes in its biochemical constituents. Hence, the present experiment was conducted to estimate the levels of amino acids in haemolymph and fat body and also excretory products at low temperature for different durations by using standard procedures as described in the materials and methods section. Table 1 explains the mortality of *Sukinda T.V* larvae which were incubated at low temperature (10°C± 1°C) for varying treatment durations. Low temperature exposure of *Sukinda* larvae for 7 days resulted in a high rate of mortality.

Forty six percent of the larvae died on the 8th day. At the end of the 10th day total larvae were found dead.[2] have reported the similar results in *Philosamia ricini* .When the larvae subjected to 5 days cold stress were kept at normal temperature for 6 days has shown 58% mortality rate. Low temperature exposure of *Sukinda T.V* larvae for 2 days has shown 42 % mortality in fourth, fifth and sixth days after reverting the larvae to normal temperature.

The results shown in Table 2 indicate that cold stress profoundly affects the levels of amino acids in different parts of the insect ($p <= 0.05$). When compared with the control, the levels of free amino acids (2.3% – 4.5%) were found to be increased in the haemolymph of cold-stressed larvae after the treatment durations (Fig 1).[21] have reported the increase in amino acid content in the haemolymph of *Anthereae mylitta(Andhra local ecorace)* from first crop to the third crop was mainly because of the environmental factors and qualitative changes in the leaf and photoperiodism. [2] have reported a drastic increase in the amino acid content in the haemolymph of *Philosamia ricini* larvae exposed to cold stress. Climatic changes during the rearing seasons will results in variations in the concentrations of aminoacids in the haemolymph of larva and pupa of *A.mylitta* [23]. The noticeable variation in aminoacid content in the haemolymph was recorded in the larvae exposed to cold stress and it was found to be high in larvae under seven days exposure (Fig 1). The data suggest that the impact of the cold stress on the free amino acids of the insect larvae was dependent on the duration of treatment.

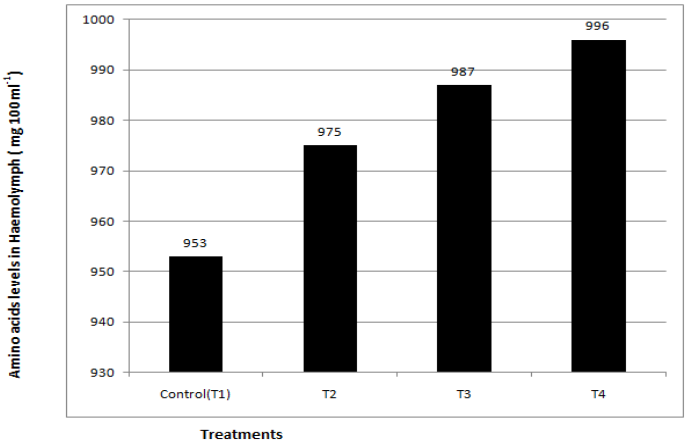

Fig 1. The *Sukinda T.V* 5th instar larvae were acclimated for different durations at 10°C ± 1°C for the experimental set, whereas the control group insects were reared at 28°C ± 2°C. The aminoacids in the haemolymph were estimated after reverting the larvae to normal temperature (28°C ± 2°C) Each value represents the mean ± SEM of 3 different observations.

The biochemical analysis carried out on fat body of cold-stressed 5th instar larvae showed a remarkable decrease ($p<= 0.05$) in the level of amino acids (40 - 93%) after the treatment duration (Fig 2). Thus the aminoacid content of haemolymph and fat body were found to be in the reverse order. In the fat body of *Sukinda T.V* larvae the amino acid content reduced drastically for seven days exposure. The high amino acid content in the haemolymph can be attributed to high proteolytic activity. [24] have reported that the low transaminase activity or high proteolytic activity results in high amino acid content.[2] working on *Philosamia ricini* have reported the decrease in amino acid content in the fat body of the larvae under cold stress conditions. Decrease in the free amino acid content in fat body may indicate the possibility of active feeding of amino acid in Kreb's cycle and glycolytic pathway to meet the emergent energy needs as well as their utilization in the production of some new proteins synthesized to cope with the low temperature stress [9,28].

Table 1. Mortality (in number) of *Sukinda T.V* larvae exposed to low temperature for different duration s after reverting back to normal temperature 28ºC±2ºC)

Day of rearing at Normal Temp after treatment duration	No. of larvae died on 2 days exposure (T2) (50 larvae)	No. of larvae died on 5 days exposure (T3) (50 larvae)	No. of larvae died on 7 days exposure (T4) (50 larvae)
First	-	-	23
Second	5	10	13
Third	8	8	14
Fourth	8	5	-
Fifth	-	6	-
Sixth	-	-	-
Seventh	-	-	-
Eigth	-	-	-

Table 2. Impact of low temperature exposure on amino acids in the haemolymph and fat body of the 5th instar larvae of *Sukinda T.V* ecorace for 2, 5, and 7 days (in µg /100 mg of fat body, mg/ 100 ml of hemolymph).

Location	Control(T1)	T2	T3	T4
Haemolymph	953.0±4.0	975.0±4.0 (+2.3)	987.0 ±4.0 (+3.6)	996.0 ±3.7* (+4.5)
Fat body	2.45± 0.17	1.48± 0.04 (-39.6)	1.16± 0.05 (-52.7)	0.17± 0.05* (-93.1)

Each value represents the mean ± SEM of 3 different observations. The values presented in parentheses indicate the percentage increase (+) or decrease (-) over control. *Significantly different at $P <= 0.05$ (Students' *t*-test).

The results shown in Table 3 depicts that cold stress greatly affects the levels of urea and uric acid in the fifth instar larvae ($p <= 0.05$). When compared with the control, the levels of urea (65% – 71%) and uric acid (50% - 58%) were found to be decreased in the cold-stressed larvae after the treatment durations (Fig 3, 4). Thus in cold stressed larvae, the urea content found to be high when compared to uric acid. In comparison with the uric acid, high level of urea was noted in the outdoor worms of *A.mylitta(Andhra local ecorace)* reared in the winter season than the worms reared at 25°C - 30°C[21]. The decrease in urea and uric acid increases as the duration of exposure increases. [27] have reported that the variability in the excretion of insects depends on their habitat. In the control group during fifth instar the growth rate is less but silk synthesis in the glands takes place rapidly which finally increases the excretory products. [21] have reported that the outdoor worms are more active than indoor worms and growth rate has been higher in outdoor worms than indoor which finally results in high level of excretory products in indoor worms reared at 25-30°C. A negative correlation between the growth rate and excretory pattern of silkworm has also been reported [19].The excretory products of the genus *Anthereae* were reported by [10-11]. The excretory metabolism of lepidopteran larvae at different stages of larval life was studied and concluded that the end products fluctuate enormously from day to day[3].

Table 3. Impact of low temperature on Urea and Uric acid of the 5th instar larvae of *Sukinda T.V* ecorace for 2, 5, and 7 days (in µg/100 mg excretory pellet)

Excretory Product	Control(T1)	T2	T3	T4
Urea	9.5 ±0.086	3.31± 0.03 (-65.2)	3.13 ± 0.03 (-67.1)	2.75 ± 0.04* (-71.1)
Uric acid	6.31 ± 0.053	3.18± 0.048* (-49.6)	3.02 ± 0.02 (-52.2)	2.66 ± 0.04 (-57.9)

Each value represents the mean ± SEM of 3 different observations. The values presented in parentheses indicate the percentage increase (+) or decrease (-) over control. *Significantly different at $P <= 0.05$ (Students't-test).

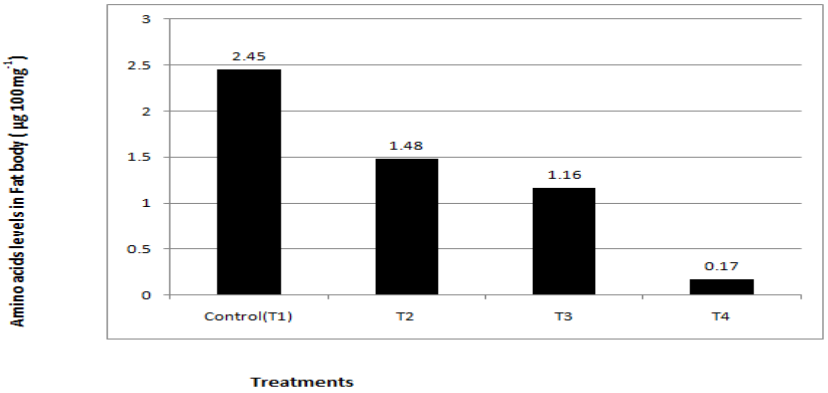

Fig 2. The *Sukinda T.V* 5th instar larvae were acclimated for different durations at 10°C ± 1°C for the experimental set, whereas the control group insects were reared at 28°C ± 2°C. The aminoacids in the fat body were estimated after reverting the larvae to normal temperature (28°C ± 2°C) Each value represents the mean ± SEM of 3 different observations.

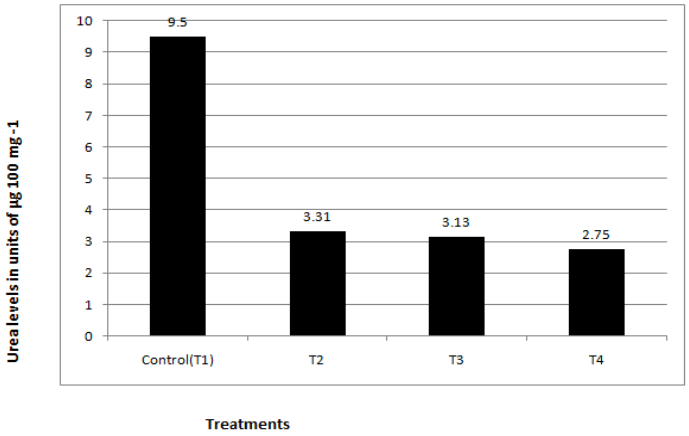

Fig 3. The *Sukinda T.V* 5th instar larvae were acclimated for different durations at 10°C ± 1°C for the experimental set, whereas the control group insects were reared at 28°C ± 2°C. The Urea content was estimated after reverting the larvae to normal temperature (28°C ± 2°C). Each value represents the mean ± SEM of 3 different observations.

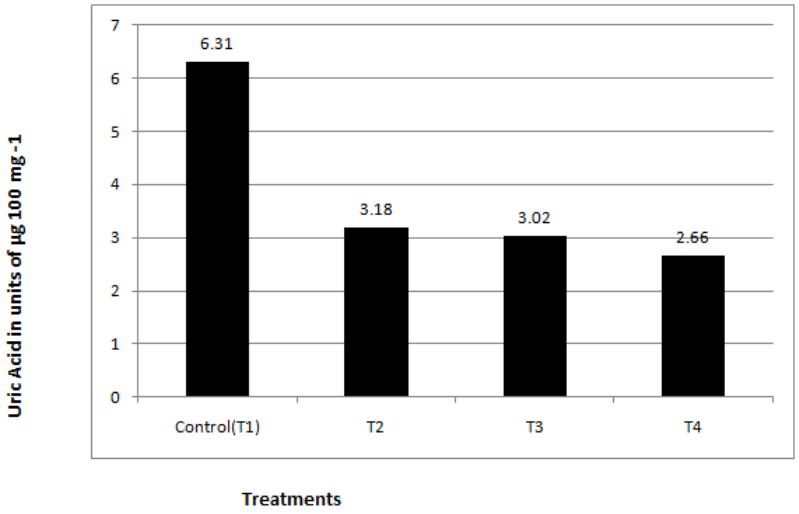

Fig 4. The *Sukinda T.V* 5th instar larvae were acclimated for different durations at 10°C ± 1°C for the experimental set, whereas the control group insects were reared at 28°C ± 2°C. The Uric acid content was estimated after reverting the larvae to normal temperature (28°C ± 2°C). Each value represents the mean ± SEM of 3 different observations.

Thus in conclusion the mortality rate of *Sukinda T.V* larvae increases as the duration of cold stress increases and a complete loss was noted in longer duration even at the expense of reverting the larvae to normal conditions. The mortality rate can be attributed to the decrease in amino acid content which is the main source of energy. The excretory products in the larvae exposed to low temperature decreased a lot which can be correlated with the reduced silk synthesis. Thus the impact of the cold stress on the biochemical components of insect larvae was dependent on the duration of treatment.

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