

## Adaptive Significance of Changes in the Biochemical Constituents of Haemolymph to Imposed Thermal Stress on Selected Races of the Silkworm, *Bombyx mori* L.

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Changes in the levels of protein and free amino acids in the haemolymph of three selected races of the silkworm, *Bombyx mori* viz., PM, NB<sub>4</sub>D<sub>2</sub> and CSR<sub>2</sub>, were investigated during 4th moult, 5th instar and pupal period. The levels of total protein in the haemolymph, increased from first day of 5th instar till sixth day. From seventh day till spinning, the protein levels decreased in all the three races. A sustained decrease in the haemolymph proteins was observed during the pupal development in all the three races. The levels of free amino acids, which were high during 4th moult, declined through the 5th age of larval development till spinning. PM showed a relatively higher free amino acid level (3.192 mg/ml) in haemolymph followed by NB<sub>4</sub>D<sub>2</sub> (2.601 mg/ml) and CSR<sub>2</sub> (2.35 mg/ml). The free amino acid levels decreased gradually from prepupal stage but increased again at the end of pupal period. Racial differences in the changes in the levels of protein and free amino acids in the haemolymph were observed in the larvae and pupae when subjected to two high temperature regimes of 30°C and 35°C. The results showed that high temperature induces specific changes in the metabolism (reversible thermal stress) that have different adaptive value in different races of the silkworm. Relatively higher increase in the free amino acid levels in the haemolymph of Pure Mysore presumably provides protective cover to tissues against high temperature by an increase in osmolarity and reduction in evaporative water loss. The absence of such a mechanism may be responsible for tempera-

ture susceptibility of the bivoltine races like NB<sub>4</sub>D<sub>2</sub> and CSR<sub>2</sub>.

**Key words:** *Bombyx mori*, Haemolymph, Protein, Free amino acids

### Introduction

The chemical composition of haemolymph is highly variable among the species and at different developmental stages of the same species (Florkin and Jeuniaux, 1974). Haemolymph, the only extracellular fluid in insects has diverse functions (Pawar and Ramakrishna, 1977) such as immunity, transport and storage (Mullins, 1985) of the products required for cellular metabolism. Thus, the changes in the biochemical composition of haemolymph reflect the morphogenic and biochemical changes in the body of the insect in response to ambient environment.

Species specific variations in the ontogenic pattern of haemolymph free amino acids and haemolymph proteins have been found during the larval development of a number of insect species (Chen, 1971; Mishra, 2005; Ranjini and Mohamed, 2004). Such variations probably reflect the balance between the synthesis, storage, transport and degradation of structural and functional proteins during ontogeny as well as a response to particular ecological and physiological conditions (Florkin and Jeuniaux, 1974). The fat body synthesizes a number of proteins and releases them into the haemolymph during active feeding larval period (Kiran Kumar *et al.*, 1998). Haemolymph proteins undergo radical changes both in quality and quantity during the development in lepidopteron insects (Srivastava and Pareek, 1976). The haemolymph protein concentration in *Bombyx mori* decreases normally by about one-fourth during pharate adult development (Doira and Kawaguchi, 1972).

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Insects have very high free amino acid content in the haemolymph which is considered as a biochemical characteristic of insects (Florkin, 1959). Chen (1966) ascribed several functions to the free amino acids in insect haemolymph such as protein synthesis, osmoregulation and detoxification. The best known case of utilization of haemolymph free amino acids in *Bombyx mori* is silk protein synthesis (Corrigan, 1970). The utilization of free amino acids by silk glands of *Bombyx mori* for silk production could be easily observed by direct measurements of amino acid levels in the haemolymph, because silk is synthesized in the feeding stage, before the beginning of spinning (Lucas *et al.*, 1958). Cook *et al.* (1972) reported that the mobilization of carbohydrates will increase the total free amino acid concentration while that of lipid or protein will result in decrease of them in the haemolymph. The present study was conducted to provide information on the changes in the levels of protein and free amino acids of the haemolymph during larval development and metamorphosis in three selected races of *Bombyx mori* and upon exposure to high temperature of the selected silkworm races.

## Materials and Methods

Disease free layings (DFLs) of the silkworm races viz., PM, NB<sub>4</sub>D<sub>2</sub> and CSR<sub>2</sub> were brushed and reared on fresh mulberry varieties as recommended by Dandin *et al.* (2003). High temperature treatment was given to larvae from 4th molt up to emergence. The chambers with different temperature zones such as control (25 ± 1°C), 30°C

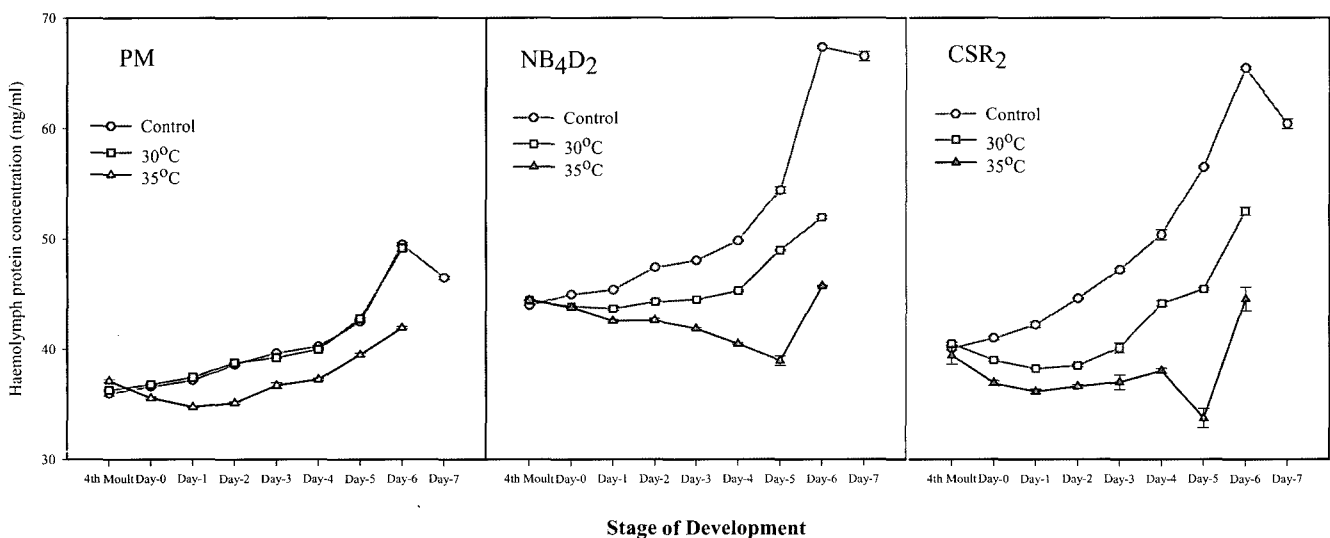
and 35°C were controlled with different thermostats. The silkworms were held in a single constant temperature regime. The humidity in all the chambers was maintained at 70 – 80%. The larvae were transferred one day before 4th moult to the above chambers and reared at selected temperature regimes till emergence.

For determination of total protein and free amino acid levels, haemolymph was collected in a pre-chilled test tube containing a few crystals of thiourea, by cutting the first proleg of larva. Haemolymph from Pupae was obtained by piercing a sharp sterilized syringe needle into the first abdominal segment and applying gentle pressure on the thorax and abdomen. Total protein content was estimated by using Lowry *et al.* (1951) method using crystalline bovine serum albumin as standard. The total free amino acid was estimated by using the method of Moore and Stein (1954) using leucine as standard.

Analysis of variance was used to test the significance of differences in the mean levels of protein and free amino acids in the haemolymph of silkworm larvae and pupae in control and treated batches of silkworm larvae and pupae. Tukey's (1953) multiple comparison tests were used to find significance of differences between the races, treatments and day wise changes.

## Results

The levels of proteins and free amino acids in the haemolymph were significantly different at different stages of larval and pupal development in all the three races of the silkworm (Fig. 1-4).



**Fig. 1.** Total soluble protein levels in the haemolymph on different days of larval development at two selected high temperatures in three different races of the silkworm, *Bombyx mori* L. (Each value is the mean + SEM of 6 separate determinations).

### Haemolymph protein concentration

The larvae of NB<sub>4</sub>D<sub>2</sub> showed a relatively higher mean level of protein (47.48 mg/ml) followed by CSR<sub>2</sub> (44.82 mg/ml) and PM (40.74 mg/ml) in control batches (Fig. 1). The protein levels showed a significant linear increase from 1st day to 6th day and a significant decrease on 7th day in the of 5th instar larval stage in all the three races. When the larvae were exposed to selected high temperature regimes of 30°C and 35°C, a significant decrease in the protein levels of haemolymph was noticed on all days of development in 5th instar larvae. At 30°C, the decrease in haemolymph protein levels was found to be not statistically significant in PM, whereas, in the two bivoltine races of NB<sub>4</sub>D<sub>2</sub> and CSR<sub>2</sub>, a consistent decrease which is found to be statistically significant was observed. Between the two bivoltine races, the quantum of decrease in the protein level of CSR<sub>2</sub> was more than NB<sub>4</sub>D<sub>2</sub>. At 35°C, a significant decrease in the haemolymph protein levels was observed in all the three races. The order of decrease in haemolymph protein levels in bivoltine races increases by each day and maximum decrease was found on 5th day after which haemolymph protein levels are improved on 6th day. The larval duration of all the races is reduced by one day in all the high temperature treated batches.

Protein levels were estimated on alternate days of pupal development in male and female pupae of the three races. The protein levels of haemolymph were found to be significantly lower on the first day of pupal development when compared to haemolymph protein levels of spinning larvae. The protein levels increased till 7th day in all the races and on 9th day, a significant decrease in protein lev-

els was noticed (Fig. 2). When exposed to high temperature, a decrease in haemolymph protein levels was observed consistently on all days and the order of decrease was found to be more at 35°C than at 30°C in both the sexes in all the three races. Females in general have higher haemolymph protein levels than males in all the three races.

### Haemolymph free amino acid concentration

The mean free amino acid level for the entire 5th instar larval stage is relatively high in PM followed by NB<sub>4</sub>D<sub>2</sub> and CSR<sub>2</sub>. The free amino acid levels on 1st day were also in the same order. The free amino acid levels showed general decrease through the 5th age larval development and rate of decrease was found to be greater in bivoltine races than the multivoltine PM race (Fig. 3). When exposed to higher temperature, the free amino acid levels showed increase over the respective control in all the three races. The increase observed was found to be higher at higher temperature of 35°C than at 30°C. Also, the levels of increase in the free amino acid were found to be higher in PM than in two bivoltine races.

In pupae, the free amino acid levels were found to be relatively higher in NB<sub>4</sub>D<sub>2</sub> in both the sexes than CSR<sub>2</sub> and PM. The estimations were made on alternate days till the date of emergence. The females have showed generally higher free amino acid levels than the males. The free amino acid level increased consistently till 7th day in both the sexes in all the three races. A significant decrease in the free amino acid levels was observed in both the sexes in all the three races on the 9th day of pupal development. When exposed to higher temperature, the free amino acid

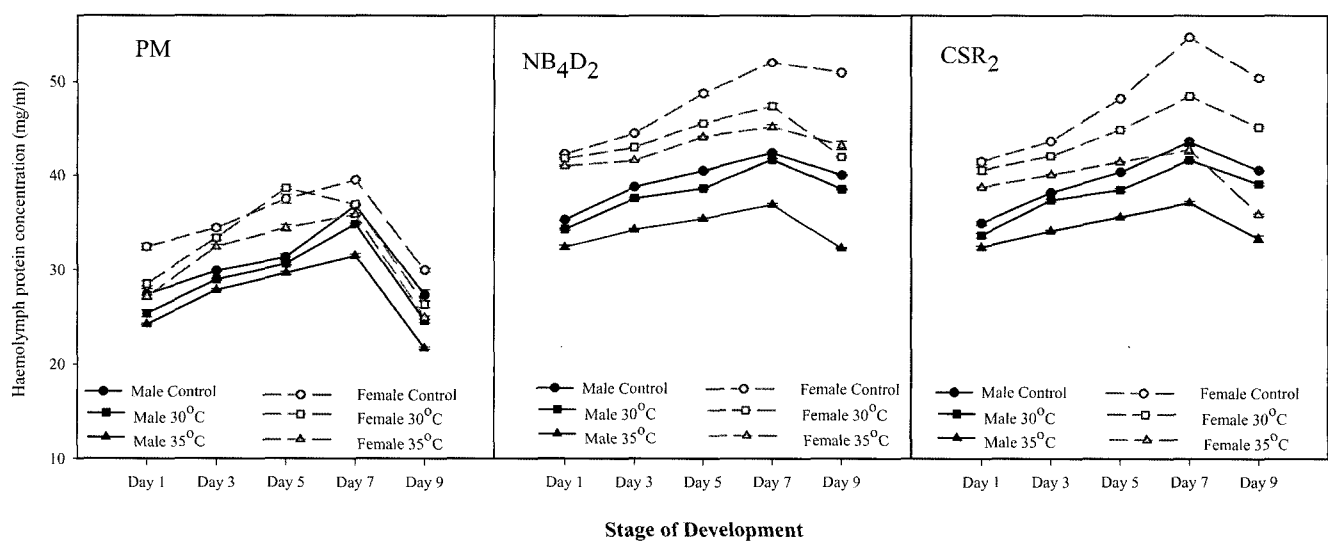
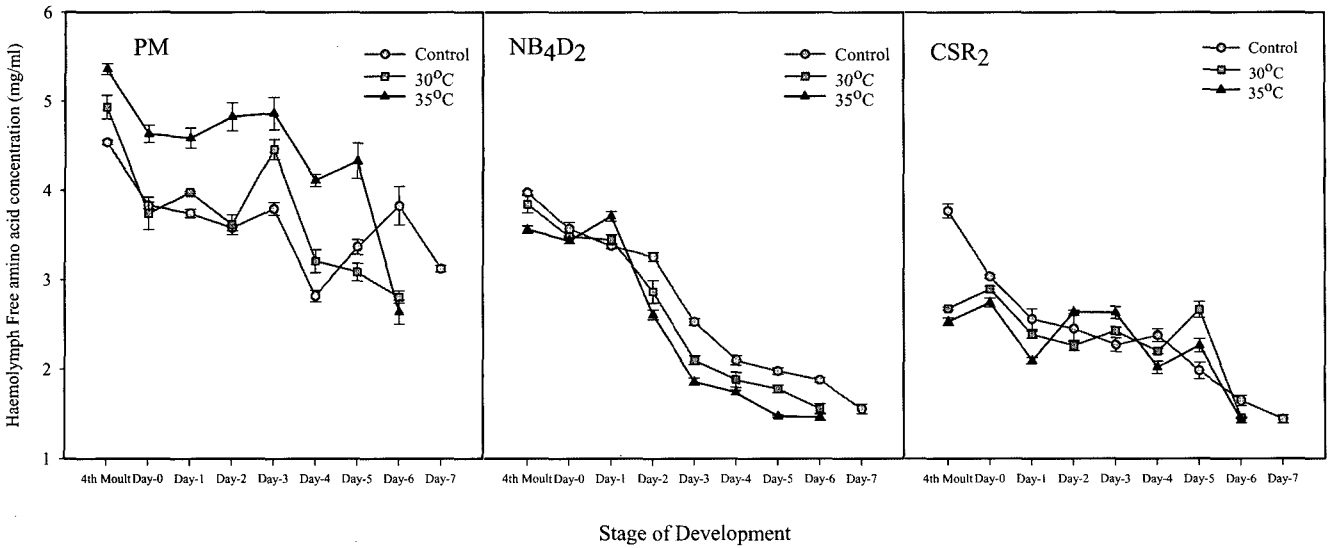
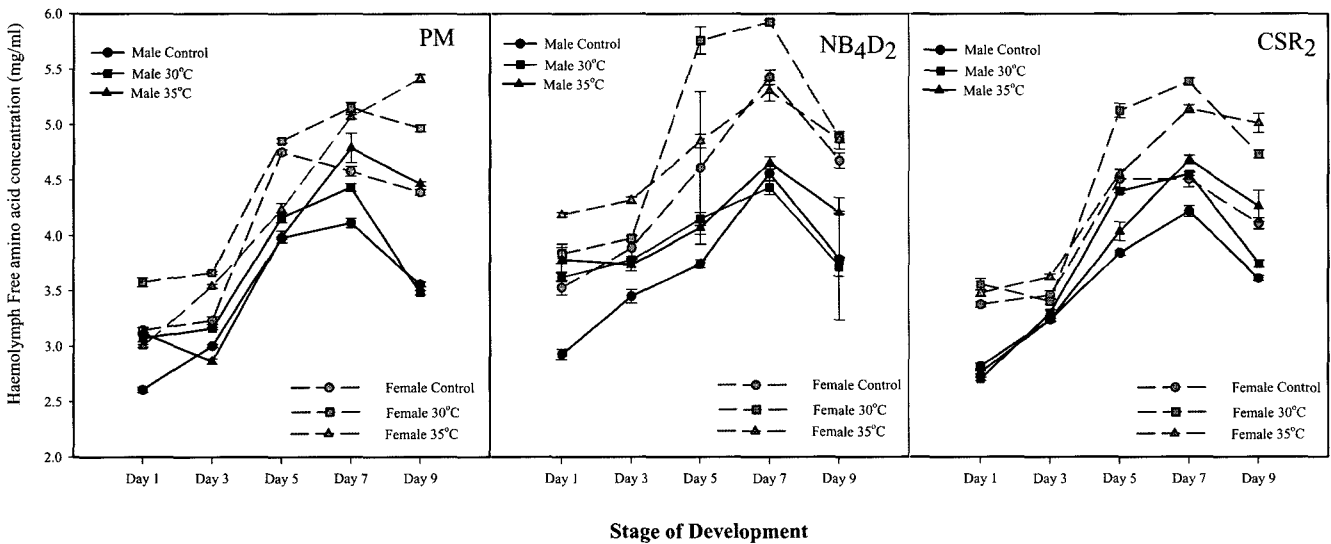


Fig. 2. Total soluble protein levels in the pupal haemolymph on different days of development at two selected high temperatures in three different races of the silkworm, *Bombyx mori* L. (Each value is the mean + SEM of 6 separate determinations).



**Fig. 3.** Total free amino acid levels in the haemolymph on different days of larval development at two selected high temperatures in three different races of the silkworm, *Bombyx mori* L. (Each value is the mean + SEM of 6 separate determinations).



**Fig. 4.** Total free amino acid levels in the pupal haemolymph on different days of development at two selected high temperatures in three different races of the silkworm, *Bombyx mori* L. (Each value is the mean + SEM of 6 separate determinations).

levels increased on all days of pupal development in all the three races in both the sexes. The increase in the levels of free amino acid was found to be relatively higher at 30°C than at 35°C.

**Discussion**

The changes in the chemical composition of haemolymph reflect tissue specific changes in different developmental stages and in response to the imposed thermal stress in the three selected races of the silkworm, *Bombyx mori*. The

total soluble protein levels in bivoltine races at 4th moult are higher than multivoltine PM. The protein levels in the haemolymph increased significantly during ontogenic development of the 5th instar larvae. The relative increase in protein levels were more in NB<sub>4</sub>D<sub>2</sub> followed by CSR<sub>2</sub> and PM. The increases in protein levels of haemolymph were due to synthesis of new proteins by the tissues and release into haemolymph. Martin *et al.* (1969) observed that the increase in soluble proteins during early stages of last instar larvae in *Calliphora stygia* was due to high rate of protein synthesis by the fat body. Nagata and Kobayashi (1990) have reported an increase in protein synthe-

sis during feeding stage in *Bombyx mori*. The protein levels showed a significant drop on the day of spinning (7th day) in control batches of all the three races which could be correlated to their sequestration and retention in the larval fat body or due to reduced rate of synthesis as observed by Kinnear *et al.* (1971). The decrease in the quantity of haemolymph protein is too small to account for silk protein synthesis. On exposure to higher ambient temperatures, haemolymph proteins decreased significantly at 35°C in all the three races and also at 30°C in the two bivoltine races. Several tissues including the fat body synthesize haemolymph proteins and the rates of synthesis and their export are impaired at higher ambient temperatures. The larval period is reduced by one day at both temperatures in all the three races and the cocoon characters are not severely impaired at 30°C which suggest that haemolymph proteins do not play a significant role in supporting silk protein biosynthesis. A small reduction in cocoon weight, shell weight and shell % observed in exposure to 30°C (data not shown) could be attributed to reduction in larval weight in 5th instar.

The total free amino acid levels in the haemolymph of the larvae at 4th moult are highest in PM followed by NB<sub>4</sub>D<sub>2</sub> and CSR<sub>2</sub>. Free amino acid level in the haemolymph is a dynamic pool withdrawn by the tissues for various anabolic and catabolic activities. The free amino acid levels in the haemolymph showed a steady decline in the two bivoltine races inspite of increased feeding activity observed in the 5th instar larvae. The haemolymph of multivoltine PM maintained a relatively greater level of free amino acids than bivoltine races NB<sub>4</sub>D<sub>2</sub> and CSR<sub>2</sub>. The differences in the levels of free amino acid pool in the two voltine groups reflect the difference in the rates of their utilization for anabolic functions by the tissues. A higher rate of the withdrawal of free amino acids of the haemolymph provides a stimulus for greater digestive and absorptive functions of midgut. The midgut tissue of bivoltine races processes relatively greater quantities of mulberry leaf than the midgut tissue of multivoltine races. Gokulama and Srinvasa Reddy (2003) reported that 5th instar larvae consume 7.4 g and 6.5 g of mulberry leaf as against 3.16 g larvae of PM. Though there is no significant difference in the digestive and absorptive abilities of the midgut tissue in the two voltine groups, relatively faster movement of throughput leads to higher rates of ingestion in the bivoltine races. Higher ambient temperatures lead to an increase in free amino acid levels in PM and the increases were found to be relatively more at 35°C on different days of development of 5th instar larvae. In the two bivoltine races, the free amino acid levels were lower at higher temperatures. The absence of increase in free amino acid levels at 35°C in the bivoltines suggests

impairment of compensatory metabolic adjustments to counteract the imposed thermal stress. An impaired 5th instar larva exposed to 35°C shows loss of growth and economic characters. Shiva Kumar *et al.* (1997) reported decrease in cocoon characters and larval weight in NB<sub>4</sub>D<sub>2</sub> exposed to 30°C and 32°C. The dichotomy on changes in the free amino acid levels in response to higher ambient temperatures in the two voltine groups could be attributed to the osmolar function of free amino acid levels of the haemolymph. The higher osmolarity, contributed by an increase in the free amino acid levels of haemolymph, reduces water loss in PM. The biochemical composition of haemolymph reflects the biochemical composition of inter and intracellular fluids in the insect body. Higher osmolarity increases elevation of the boiling point, reducing the evaporative water loss from the body. Multivoltine races are known to be more temperature tolerant than bivoltine races, since they are well equipped with such physiological mechanisms which minimize water loss from the body even at higher temperatures. In most cells, amino acids act as intracellular compatible osmolytes (Yancey *et al.*, 1982). Changes in cell hydration and volume can act as important regulators of cell function. Glutamine and proline act as most compatible osmolytes in the regulation of cell volume (Haussinger, 1996). Further, free amino acids in haemolymph are major precursors of silk biosynthesis during 5th instar stage of larval development in silk gland.

The increase in haemolymph soluble protein levels during pupal development could be attributed to compensatory replacement of the proteins which are utilized for the formation of puparium. Higher haemolymph protein levels in female pupae are due to the presence of female specific proteins and vitellogenic proteins in the females. Doira (1968) has shown a vitellogenic female specific protein to the extent of 17 percent of the total haemolymph protein in pupal and pharate adult haemolymph. In females homozygous for the small egg mutant gene *sm/sm*, the concentrations of haemolymph protein remain constant throughout the pupal and pharate adult stages, since the gene does not allow the synthesis of vitellogenic female proteins. The protein levels decreased in the haemolymph of all the races and the decrease was found to be greater at 35°C than at 30°C in both the sexes.

The free amino acid levels on the other hand show an increase at higher ambient temperatures in the haemolymph of all the races. The negative correlation between protein and free amino acid levels in the pupal haemolymph suggest increased proteolysis at higher ambient temperatures. Whether the proteolysis of haemolymph protein occurs in the soluble or cellular components of haemolymph or the haemolymph proteins are

taken up by the tissues like fat body where proteolysis occurs releasing the free amino acids into the haemolymph is obscure. The levels of amino acids are also affected by carbohydrates and products of intermediary metabolism which provide carbon skeleton for the synthesis of non-essential amino acids in insects (Winteringham, 1959; Mansing, 1964). Chino (1958) observed that the free amino acids undergo a significant change during metamorphosis. The increased free amino acid levels might protect the non-feeding pupae against the thermal stress by the protection of cell volume and by preventing the release of stress activated protein kinases (SAPK) (Haussinger, 1996).

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