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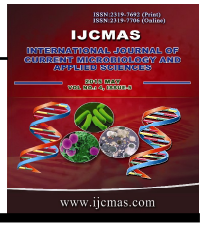

Original Research Article

Studies on Biochemical Aspects of Diapause Preparation in the Tasar Silkworm, *Antheraea mylitta* D. Under Tropical Climate of Vidarbha Region of Maharashtra, India

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Keywords

Tasar Silkworm, DD, NDD, Haemolymph, Fat Body, Protein, Amino Acid

A B S T R A C T

The Indian tasar silkworm, *Antheraea mylitta* Drury, is a wild insect that produces tasar silk of commercial importance having three generations annually under climatic conditions of Vidarbha region of Maharashtra i.e. July-August (rainy crop), September-October (autumn crop) and November-January (winter crop). It is reared by tribal people living adjoining the forest exposed to various environmental factors. The present study was aimed to study the biochemical aspects of diapauses preparation in the tasar silkworm, *A. mylitta* D., under tropical climate of Vidarbha region of Maharashtra. Based on the literature study the effect of environmental factors on the biochemical components of the tasar silkworm has shown significant variations in the level of various biomolecules. The present study was carried out on fifth instar larval and pupal haemolymph and fat body of ecorace Daba Trivoltine (TV) of tasar silkworm *Anthareae mylitta* D., to analyse the concentration of biomolecules during first, second and third rearing cycles. The results of the present study revealed that the concentration of protein and amino acid in haemolymph and fat body was significantly different in fifth instar larvae of diapausing and non diapausing generations and attained a peak during the mid period of fifth instar. Whereas, protein and amino acid in haemolymph and fat body was significantly lower while spinning preparation. In diapauses destined pupae, the concentration of protein and amino acid was first increased up to the age of 15 days and then significantly decreased up to the time of adult emergence.

Introduction

The Indian tasar silkworm, *Antheraea mylitta* Drury, is a wild sericigenous insect, produces silk of commercial importance with its natural inhabitation in the forest areas of Jharkhand, Bihar, Orissa, Madhya Pradesh, Maharashtra and Andhra Pradesh. In India, it is geographically distributed in 43 ecoraces in varied tropical zones. The eco populations are polyphagous. They are univoltine (UV), bivoltine (BV) and

trivoltine (TV) in nature and differ greatly from each other in qualitative and quantitative attributes. Daba TV ecorace is one of the most commercially exploited race of the *A. mylitta* distributed from latitude 16°N to 24°N and longitude 80°E to 89°E (Suryanarayana and Srivastava, 2005). It is reared thrice a year viz. July-August (rainy crop), September-October (autumn crop) and November-January (winter crop) and it is considered as cycle I, cycle II and cycle III respectively. The environmental factors, such as temperature, rainfall and relative humidity influence the growth and development of silkworm. The seasonal fluctuations in environmental factors influence the population density of silkworm, pest, predators and pathogens of diseases. In Vidarbha region of Maharashtra (India), environmental factors are differing greatly during the rearing seasons of all three crops which influence the crop performance in the field. The factors that determine the health and performance of tasar silkworm can contribute to a higher yield of silk (Kumar and Elangovan, 2010; Rahile, 2010).

More than 30% loss in tasar silk production is due to pupal mortality, erratic emergence, poor fecundity, less hatching percentage, depression of egg and diseases (Rahile, 2010). Carbohydrates, especially, glycogen and trehalose, nitrogenous compounds and glycerol are the main haemolymph constituents crucial during growth, development, moulting, and metamorphosis and in maintenance of diapause state of an insect (Adedokun and Denlinger, 1985; Jo Hyun-ii and Kim, 2001).

Proteins are the key factors within the cell influencing growth, development and biosynthesis of silk (Singh and Baquaya, 1971). The process of histolysis and histogenesis operates simultaneously during metamorphosis in insects. These events are accompanied by variation in free amino acid which in turn is associated with proteolysis and protein synthesis. Silkworms conserve sufficient quantity of energy reserves during larval stage to be utilized during pupal and adult stage (Horie, 1961; Williams and Lee, 2005).

Several workers have examined the pattern of protein and free amino acid in different insects (Wiliams and Birt, 1972; Mishra *et al.*, 2009; Lakshmi Velide and Purushotham Rao, 2012). But most of the previous studies were not properly focused on crop wise variation. Hence proper understanding of the total protein and total free amino acid concentration in different tissues during larval and pupal development is essential. The present study was undertaken with a dual purpose in which the quantitative pattern of total protein and free amino acid in haemolymph and fat body of fifth instar larva as well as in pupa of tasar silkworm *Antheraea mylitta D.* (Daba T.V.) with respect to the diapause preparation under tropical climate of Vidarbha region of Maharashtra.

Materials and Methods

The fifth instar silkworms and cocoons of *A. mylitta* Drury (Lepidoptera: Saturniidae) Daba TV were collected from different fields of Vidarbha region of Maharashtra. The silkworms were reared on Asan (*Terminalia tomentosa* W and A) and Arjun (*Terminalia tomentosa*). Male and female larvae of the same age group and cocoons of each generation were sexed and collected separately from the field.

Meteorological data of the year 2012–2014 such as temperature, relative humidity and rainfall were collected from the various tasar rearing sites of Vidarbha region of Maharashtra.

Sample collection

The haemolymph sample was collected in an eppendorf tube containing a pinch of phenylthiourea from fifth instar larvae by puncturing the prolegs and from pupae by puncturing rudimentary wing pad after surface sterilization. The haemolymph was centrifuged at 10,000 rpm in a refrigerated centrifuge at 4°C for 5 minutes. The clear supernatant was transferred to another eppendorf tube. Thus purified clear plasma samples were deep frozen at -20°C until biochemical estimations were done.

The Fat body was isolated by dissecting the fifth instar larvae and pupae. The fat body was homogenated using phosphate buffer p^H 7. The homogenate was centrifuged at 10,000 rpm for 30 min at 4°C. The supernatant was collected in a clean eppendorf tube and stored at -20°C until biochemical estimations were done.

Quantitative estimations of biochemical constituents:

The quantitative estimation of total protein was done following the method of Lowry *et al.* (1951) using bovine serum albumin as standard. The concentration of protein in the sample was calculated with the standard curve and results were expressed in mg per ml of haemolymph and mg per 100 mg of fat body.

The quantitative estimation of total free amino acid was done by using the method of Moore and Stein (1954).

Statistical analysis:

Each assay was replicated 3 times. Statistical analysis of the data was done using online Graph Pad Quick calcs Software. Values were expressed as Mean ± SEM of replication and Student's t-test was applied to locate significant differences between different groups.

Results and Discussion

The meteorological data of the year 2012-2014 collected from various tasar rearing sites in the Vidarbha region of Maharashtra showed variations in the temperature, relative humidity and precipitation during different rearing cycles. During the present investigation, the average temperature, relative humidity and precipitation in the first, second and third rearing cycle were found to be 27.5°C, 26.9°C, 21.4°C; 78.5 %, 67.5 %, 55 % and 290.5 mm, 115 mm,17.3 mm respectively (Table 1, Fig. 1 and 2). From the above observations it was cleared that the temperature and relative humidity were decreased gradually from first to third rearing cycle and precipitation was found to be very low in third rearing cycle than first and second.

The protein content in haemolymph of early, mid and late fifth instar larvae was found to be 10.129±0.439 mg/ml, 13.837±0.787 mg/ml and 21.56±8.29 mg/ml respectively. The protein content in fat body of early, mid and late fifth instar larvae was found to be 3.809±0.137 mg/100mg, 4.673±0.189 mg/100mg and 7.15±0.316 mg/100mg respectively (Table 2). This showed that the protein content in haemolymph and fat body was increased gradually during development from early to late fifth instar larvae. The results of the present study are in accordance with the findings of Chen (1966) who reported increase in total protein content in haemolymph during larval development. Similar observations were reported in *A. proylei* Jolly by Sinha and Sinha (1994) and in *Samia cynthia ricini* by Karaki (1969). The protein content in haemolymph of fifth instar larvae of first, second and third

rearing cycle were increased gradually and found to be 9.586±0.38 mg/ml, 14.500±0.93 mg/ml and 21.44±0.94 mg/ml respectively. The protein content in fat body of fifth instar larvae of first, second and third rearing cycles were also increased from first to third rearing cycle and found to be 4.255±0.19 mg/100mg, 4.81±0.21 mg/100mg and 6.565±3.55 mg/100mg respectively (Table 3). From the above observations it is also cleared that the haemolymph protein content was significantly higher in diapausing fifth instar larvae. Similar trend was also found in the protein content in fat body of diapausing and non diapausing fifth instar larvae (Table 4).

The amino acid content in haemolymph of fifth instar larvae of first, second and third rearing cycle was increased from 3.07±0.24 mg/ml in first cycle and 3.59±0.26 mg/ml in second cycle to the level of 4.577±0.368 mg/ml in third cycle in increasing order. In fat body the pattern of amino acid content was in decreasing order and were found to be 2.96±0.27 µg/100mg, 2.54±0.26 µg/100mg and 1.946±0.189 µg/100mg in fifth instar larvae of first, second and third rearing cycle respectively (Table 3). From the above observations, it is also cleared that the value of amino acid content in haemolymph of diapausing fifth instar larvae was significantly higher than non diapausing fifth instar larvae. But in fat body the diapausing fifth instar larvae showed significantly less value of amino acid content than non diapausing fifth instar larvae (Table 4).

In the present study, variation in protein content during first to third rearing cycle was possibly due to the variations in environmental conditions like temperature, relative humidity and precipitation for rearing in Vidarbha region. The above findings of quantitative differences in the protein and amino acid content between first, second and third rearing cycle are supported by the study of Shamita and Purushotham Rao (2008) who reported, the changes in the protein content during different rearing cycles is mainly because of the environmental factors and qualitative changes in the leaf. Sinha *et al.* (1988) have also reported that variations in the concentration of amino acids and protein content during rearing seasons in the larval and pupal haemolymph of *A. mylitta* was due to the variation in climatic changes during rearing cycles which supports the findings of our study. In the present study, the other possible reason for increase in protein content during larvae of diapausing generation was the haemolymph of fifth instar larvae of diapausing generation accumulate more energy reserves in the form of trehalose, glycerol, glycogen, proteins and amino acids. Our findings are in accordance with the findings of Mishra *et al.* (2010) who reported the increase in protein concentration during diapausing generation. Similar trend of increase was also reported in *Antheraea proylei* Jolly by Sinha and Sinha (1994); and in *Phormia regina* by Chen and Levenbook (1966a, b).

The 15 days old non diapausing pupae showed the protein content 73.42±4.76 mg/ml in the haemolymph and 9.25±0.84 mg/100 mg in fat body during cycle I and 76.54±5.18 mg/ml in the haemolymph and 9.87±0.89 mg/100 mg in fat body during cycle II respectively. In third rearing cycle, the protein content in haemolymph of 15 days old pupae was found to be 151.13±10.5 mg/ml and in fat body 21.90±1.314 mg/100mg, this value of protein content gradually depleted in about 60 days old pupae and further slight increased in about 105 days old pupae until adult emergence (Table 5).

Table.1 Meteorological data recorded during the period of three rearing cycles of *A. mylitta* for the year 2012–2014

Cycle	Cycle I (July-Aug)		Cycle II (Sept-Oct)		Cycle III (Nov-Jan)	
Temperature (^{o}C)	27.5		26.9		21.4	
	Max. 31.5	Min. 23.2	Max. 32.8	Min. 21.8	Max. 29.2	Min. 14.7
Relative Humidity (%)	78.5		67.5		55	
	Max. 96.7	Min. 73.5	Max. 91.3	Min. 55.7	Max. 85.3	Min. 41.3
Precipitation (mm)	290.5		115		17.33	

Table.2 Protein content in haemolymph and fat body of fifth instar larvae of tasar silkworm *A. mylitta*

Stage	Haemolymph Protein (mg/ml)		Stage	Fat body protein (mg/100mg)	
	Mean±SEM	% difference		Mean±SEM	% difference
Early	10.129±0.4393	-	Early	3.809±0.137	-
Mid	13.837±0.787	+30.944 %	Mid	4.673±0.189	+20.37 %
Late	21.56±8.29	+43.64 %	Late	7.15±0.316	+41.90 %

Table.3 Total protein and total free amino acid content in the haemolymph and fat body of fifth instar larvae of tasar silkworm *A. mylitta*

Source	Constituents	Cycle I	Cycle II	Cycle III
		Mean±SEM	Mean±SEM	Mean±SEM
Haemolymph	Protein (mg/ml)	9.586±0.38	14.500±0.93 (+40.804%)	21.44±0.94 (+38.62%)
	Amino Acid (mg/ml)	3.07±0.24	3.59±0.26 (+15.62%)	4.577±0.368 (+24.17%)
Fat body	Protein (mg/100mg)	4.255±0.193	4.81±0.21 (+12.245%)	6.565±0.355 (+30.86%)
	Amino Acid (µg/100mg)	2.96±0.27	2.54±0.26 (-15.27%)	1.946±0.189 (-26.48%)

Table.4 Total protein and total free amino acid content in the haemolymph and fat body of non diapause destined and diapause destined fifth instar larvae of tasar silkworm *A. mylitta*

Source	Constituents	NDD	DD	t- test
		Mean±SEM	Mean±SEM	
Haemolymph	Protein (mg/ml)	12.043±0.549	21.44±0.945	9.177*** (p<0.0001)
	Amino Acid (mg/ml)	3.329±0.18	4.577±0.368	3.436** (p=0.0011)
Fat body	Protein (mg/100mg)	4.534±0.144	6.565±0.355	6.283*** (p<0.0001)
	Amino Acid (µg/100mg)	2.75±0.19	1.946±0.189	2.67** (p=0.0097)

Table.5 Total protein and total free amino acid content in the pupae of tasar silkworm *A. mylitta*

Source	Constituents	Cycle I	Cycle II	Cycle III		
		15 days	15 days	15 days	60 days	105 days
		Mean±SEM	Mean±SEM	Mean±SEM	Mean±SEM	Mean±SEM
Haemo lymph	Protein (mg/ml)	73.42±4.76	76.54±518 (+4.16%)	151.13±10.5 (+65.524%)	69.25±4.87 (-74.31%)	76.95±5.69 (+10.53%)
	Amino Acid (mg/ml)	0.705±0.14	0.947±0.205 (+29.30%)	1.95±0.33 (+69.24%)	3.51±0.416 (+57.14%)	2.92±0.267 (-18.35%)
Fat body	Protein (mg/100mg)	9.25±0.84	9.87±0.89 (+6.48%)	21.90±1.314 (+75.73%)	12.13±1.25 (-57.42%)	13.51±1.44 (+10.76%)
	AminoAcid (µg/100mg)	0.68±0.176	0.778±0.153 (+13.44%)	1.238±0.18 (+45.64%)	2.347±0.319 (+61.87%)	2.21±0.31 (-6.01%)

Table.6 Total protein and total free amino acid content in the non diapause destined and diapause destined pupae of tasar silkworm *A. mylitta*

Source	Constituents	NDD	DD	t-test
		Mean±SEM	Mean±SEM	
Haemolymph	Protein (mg/ml)	74.98±3.44	99.279±7.99	2.3802* (p=0.0213)
	Amino Acid (mg/ml)	0.826±0.125	2.79±0.225	6.667*** (P<0.001)
Fat body	Protein (mg/100mg)	9.559±0.60	15.846±1.09	4.3975*** (P<0.001)
	Amino Acid (µg/100mg)	0.729±0.114	1.93±0.18	5.01*** (P<0.001)

Fig.1 Daily Mean Temperature (°C) recorded during the period of three rearing cycles of *A. mylitta* for the year 2012–2014

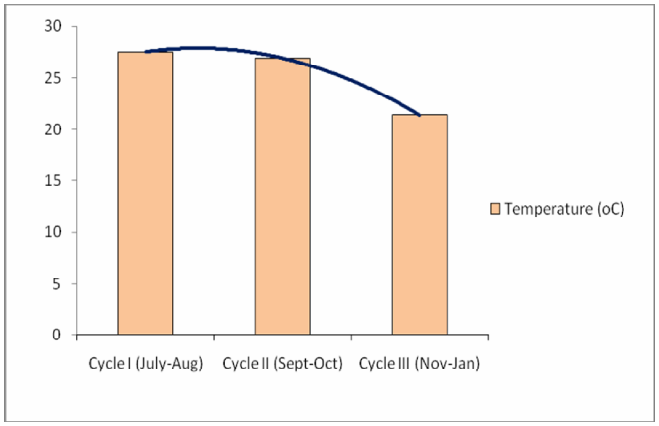
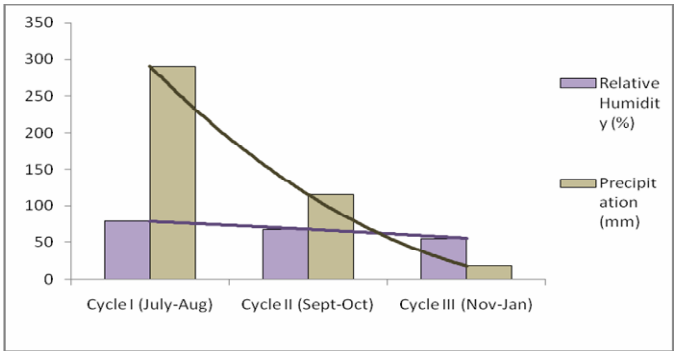


Fig.2 Precipitation (mm) and Relative Humidity (%) recorded during the period of three rearing cycles of *A. mylitta* for the year 2012–2014



From the above observations, it is also cleared that the haemolymph protein content was significantly higher in diapausing pupae (99.279±7.99 mg/ml) than non diapausing pupae (74.98±3.44 mg/ml). Similar trend was also found in the protein content in fat body of diapausing and non diapausing pupae (Table 6).

The 15 days old non diapausing pupae showed the total amino acid content 0.705±0.14 mg/ml in the haemolymph and 0.68±0.176 µg/100 mg in fat body during cycle I and 0.947±0.205 mg/ml in the haemolymph and 0.778±0.153 µg/100 mg in fat body during cycle II respectively. In third rearing cycle, the amino acid content in haemolymph of 15 days old pupae was found to be 1.95±0.33 mg/ml and in fat body 1.238±0.18 µg/100mg, this value gradually depleted in about 60 days old and further slight increased during 105 days old pupae until adult emergence (Table 5). From the above observations, it is also cleared that the value of amino acid content in haemolymph of diapausing pupae was significantly higher than non diapausing pupae. But in fat body the diapausing pupae showed significantly less value of amino acid content than non diapausing pupae (Table 6).

The protein content in haemolymph and fat body of the non diapausing pupae during cycle I was found to be comparatively less than cycle II. Whereas in diapausing pupae the protein content in haemolymph and fat was found to be more than non diapausing pupae. There was gradual decline in the total protein content from 15 to 60 days and further slight increased after 60 days in diapausing pupae until adult emergence. Simultaneous increase in the amino acid content of the same pupae has been observed in the haemolymph and fat body. The present findings are supported by Radhapant and Geeta Jaiswal (1981) who reported that the increase in protein and amino acid content in haemolymph and fat body of diapausing pupae of *A. mylitta* is possibly due to the effect of environmental factors and Mishra *et al.* (2009) reported the increase in protein and amino acid during diapause in *A. mylitta*. Similar observations were also recorded by Agrell (1964b) in *Calliphora*.

Proteins and amino acids play a crucial role in maintaining the physiology of insect (Florkin and Jeuniaux, 1974 and Mullin, 1985). The amino acid pool in *A. mylitta* might be useful for osmo-regulation (Beadle and Shaw, 1950), protein synthesis (Buck, 1953), energy production (Sactor, 1965) and also in regulation of diapause. Haemolymph proteins contribute in cuticle formation and as they are taken up by epidermal cells, and

utilized in epidermis (Koeppe and Gilbert, 1973).

On the basis of present study, it can be concluded that the biochemical components like proteins and amino acids accumulate more in the diapausing than the non diapausing larvae and pupae of tasar silkworm *A. mylitta* and are used for the survival in adverse climatic conditions during the period of diapause.

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