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Biochemical studies of comparative haemolymph constituents in fourth instar larvae of Daba trivoltine ecorace of topical tasar silkworm Antheraea mylitta D.

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Abstract : *Diapause is endocrine mediated dormancy and the preparatory stages are fourth and fifth instar larvae of tasar silkworm, Antheraea mylitta Drury. Different haemolymph biochemical constituents like total protein, free amino acids, trehalose, glycerol and glycogen were estimated in fourth instar larvae of Daba trivoltine ecorace of tropical tasar silkworm so as to differentiate between non-diapause destined (NDD- the first and second crops) and diapause destined (DD- third crop) generations and also to relate them as preparatory for undergoing diapause. There was significant difference in the presence of haemolymph glycogen in female larvae (1.9361±0.9605mg/ml) than male larvae (1.0859±0.5393) in TV third crop. No significant different was observed in remaining constituents. The concentration of trehalose was significantly higher in female larvae (0.9501±0.3784 mg/ml) than male larvae (0.3811±0.2420) in TV second crop. A comparison made between the female and male larvae of second crop shows the higher level presence of amino acids and proteins in male larvae. Rest of the constituents was higher in female larvae than male in second crop. During third crop, except for total free amino acids all other biochemical constituents were higher in male larvae and the difference was observed to be significant in the concentration of glycerol and amino acids A comparison between the non-diapause destined (2nd crop) and diapause destined (3rd crop) generations indicated that the diapause destined larvae has significantly higher level of amino acids trehalose (6.5873 ± 3.2437 mg/ml in male, 8.1217 ± 3.0710 mg/ml in female) and glycerol (in female). However, a comparison between NDD first crop and third crop indicated that all the haemolymph constituents were significantly higher in diapause destined fourth instar larvae, like amino acids protein (11.3999 ± 2.5290mg/ml in male, 11.3264 ± 2.5672mg/ml in female), trehalose glycogen and glycerol. The findings indicate that fourth instar larvae of Daba trivoltine of tasar silkworm accumulate energy reserves to skip forthcoming unfavorable conditions in pupal stage. SDS PAGE studies did not indicate any major difference in protein profile but a sharp increase in band intensity of 85kDa, probably a major storage protein and a 43kDa diapause specific band in DD larvae.*

Key Words: Haemolymph, Amino acids, Protein, Glycogen,Glycerol, Trehalose, Antheraea mylitta, NDD.

Introduction

Insects undergoing diapause prepare for diapause under adverse conditions. Diapause in insects occurs after activation of endocrine glands. Insects having wide range of geographical variation distinguish adversities of the nature well in advance and thereafter they prepare for undergoing diapause in the preceding stages of development and remain in diapause in pupal stage (Tauber, *et. al.*, 1986; Leather *et al.*, 1993; Denlinger, 1985). For undergoing diapause insects accumulate energy reserves at a specific developmental stage. The most important organ system where changes are seen in the form of its biochemical constituents is the haemolymph of insects. Haemolymph acts as storage reservoir for many materials essential for a variety of insects and its composition tends to vary in response to various conditions or activities (Hirano and Yamashita, 1983; Adedokun and Denlinger, 1985). Carbohydrates, especially, glycogen and trehalose, nitrogenous compounds like proteins and amino acids and glycerol are the main

haemolymph constituents reported to be crucial during growth, development, moulting and metamorphosis and also in maintenance of diapause stage of an insect (Jo and Kim, 2001). Trehalose, glycogen and other haemolymph constituents vary during course of feeding and spinning of silkworms (Horie, 1961; Mishra *et al.,* 2010a) and these constituents are most extensively expressed in the haemolymph of insects which is the only extra-cellular fluid having diverse functions (Pawar and Ramakrishna, 1977; Sowri and Sarangi, 2002; Mishra *et al.,* 2010a). Silkworms conserve sufficient quantity of energy reserves during larval stage to be utilized during pupal and adult stages (Horie, 1961; Mishra et al., 2010b).

The distribution of tropical tasar silkworm *Antheraea mylitta Drury* is very wide ranging between 10° to 32°N latitude and 76° to 93°E longitude, experiencing varied environmental conditions. Under adverse environmental conditions, it undergoes facultative pupal diapause and shows different type of voltinism. At higher latitudes it behaves as univoltine (27 to 30°N). At mid-latitudes (20 - 25°N), it behaves as bivoltine or trivoltine and at low latitude (16 to 19°N) it behaves as trivoltine or multivoltine. However, this voltinism is modified in isolated conditions, depending upon the altitude of place.

In tropical regions of India, commercially exploited ecotypes of *A. mylitta* are either bivoltine (BV) or trivoltine (TV). The Daba trivoltine ecorace of *A. mylitta* completes three generation in an annum. The first crop is reared during June-July, second during August-September and third is October–December. The pupae remain in diapause for about 141-175 days, depending upon the altitude, latitude and location of a specific place. The fourth instar larvae feed voraciously on its food plants so as to build up its energy reserves for undergoing pupal diapause. In the present study an attempt has been made to record and differentiate the presence of different haemolymph biochemical constituents which act as energy reserves in the penultimate instar period of non-diapause destined (NDD) and diapause destined (DD) generations.

Materials and Methods

The experimental Animal : *Antheraea mylitta* Drury [Lepidoptera: Saturniidae] Daba trivoltine stocks were used for the experimental studies which were maintained at Central Tasar Research and Training Institute, Field Laboratory, Piska-Nagri (23.21°N, 85.20°E, 654.16 meters AMSL), Ranchi, India, where it completes three generations in a year. Generations are commonly known as crops. The silkworms were reared on Asan (*Terminalia tomentosa* W and A) in non-diapause destined (NDD- during June-July as first crop and August-September- as second crop) and diapause-destined (DD- during October – December) generations.

Collection of haemolymph for estimation of haemolymph biochemical constituents : For daily collection of haemolymph samples, synchronized male and female larvae of similar age were segregated during the entire fourth instar larval period and used for collection of haemolymph. The prologs after surface sterilization were punctured with the help of a sterilized needle with 70% ethanol and approximately 500 µl of haemolymph was collected in an eppendorf tube containing a pinch of phenylthiourea which inhibits denaturing or blackening of the haemolymph in a replicated manner. The haemolymph was centrifuged at 10,000 rpm in a refrigerated centrifuge at 4°C for 5 minutes. The clear supernatant or haemolymph plasma was transferred to another eppendorf tube. Thus purified clear plasma samples were deep frozen at - 80°C until biochemical estimation was done.

Quantitative estimation of haemolymph constituents: The estimation of quantitative protein was done following the method of Lowry *et al.* (1951). Quantitative estimation of total free amino acids was done by the modified procedure based on Moore and Stein (1948). The estimation of quantitative trehalose and glycogen

was done following the method of Wyatt and Kalf (1957). An enzymatic method for the estimation of glycerol in haemolymph was followed after Hagen and Hagen (1962).

SDS PAGE study of brain proteins of tropical tasar silkworm : SDS PAGE study was carried out following the method of Laemmli (1970). The haemlymph of synchronized male and female larvae was used for the present study. For running SDS PAGE samples containing 20 big of protein was loaded in each lane.

Data was statistically analysed using student *t* test.

Results and Discussion

Sex-specific presence of haemolymph constituents : Table-1, shows there was significant difference in the presence of haemolymph glycogen in female larvae (1.9361±0.9605mg/ml) than male larvae (1.0859±0.5393) in TV third crop. No significant different was observed in remaining constituents. The concentration of trehalose was significantly higher in female larvae (0.9501±0.3784 mg/ml) than male larvae (0.3811±0.2420) in TV second crop. A comparison made between the female and male larvae of second crop show higher presence of amino acids and proteins in male larvae. Rest of the constituents was higher in female larvae than male in second crop. During third crop, except for total free amino acids all other biochemical constituents were higher in male larvae and the difference was observed to be significant in the concentration of glycerol (males-0.0016±0.0004 µmole/ml and females-0.0006±0.0004 µmole/ml) and amino acids (males- 0.0898±0.0525 mg/ml and females-0.2175±0.11 mg/ml) (Table-1).

Comparative presence of haemolymph constituents in non-diapause and diapause-destined generations : A comparison between the non-diapause destined (2nd crop) and diapause destined (3rd crop) generations indicated that the diapause destined larvae has significantly higher level of amino acids (3.1923 ± 1.2107 mg/ml in male, 3.1948 ± 1.1962 mg/ml in female), trehalose (6.5873 ± 3.2437 mg/ml in male, 8.1217 ± 3.0710 mg/ml in female) and glycerol (0.0358±1.2107µmole/ml) in male 0.0269 ± 0.0269µmole/ml in female). However, a comparison between NDD first crop and third crop indicated that all the haemolymph constituents were significantly higher in diapause destined fourth instar larvae, like amino acids (3.1923 ± 1.2107 mg/ml in male, 3.1948 ± 1.1962 mg/ml in female), protein (11.3999 ± 2.5290mg/ml in male, 11.3264 ± 2.5672mg/ml in female), trehalose (6.5873±3.2437mg/ml in male, 8.1217 ± 3.0710mg/ml in female), glycogen (0.9869 ± 0.5854mg/ml in male, 1.751 5 ± 1.0555mg/ml in female) and glycerol (0.0358 ± 0.0182mg/ml in male, 0.0269 ± 0.0166mg/ml in female) Table-2.

From the above results, it is clear that during penultimate or fourth instar larval period the presence of protein, amino acids, glycogen, trehalose and glycerol in the haemolymph has no sex-specific difference in the same generation barring a few exceptions. However, in the haemolymph of fourth instar larvae of diapause generation there is accumulation of more energy reserves in the form of protein, amino acids, trehalose, glycerol, glycogen and glycogen.

Daily pattern of biochemical constituents : Daily pattern of biochemical constituents were also recorded in the male and female larvae of the two generations. It may be seen from Fig.1 and 2 that in male and female fourth instar larvae the level of amino acids showed an increasing trend during first and second crop. The level of protein also increased with the increase in the age of instar (Fig. 3 and 4). The level of trehalose was higher during mid-instar period and it was lower at the beginning and end of the instar period (Fig.5 and 6). The level of glycogen also showed an increasing trend in both the sexes (Fig. 7 and 8.). The level of Glycerol was always higher at the beginning and end of instar

Table 1. Sex-wise presence of different haemolymph biochemical constituents during fourth instar larval period of different crops of Daba trivoltine ecorace of *A. mylitta*

Crop/Sex	Haemolymph biochemical constituents	Male	Female	t-stat
		Mean ± SD	Mean ± SD	
TV III Crop	Amino acid(mg/ml)	3.3714±1.1599	3.3740±1.1424	NS
	Protein(mg/ml)	12.0928±1.5400	11.3117±2.7440	NS
	Trehalose(mg/ml)	7.3910±2.3196	8.9407±1.9696	NS
	Glycogen(mg/ml)	1.0859±0.5393	1.9361±0.9605	**
	Glycerol(µmole/ml)	0.0368±0.0192	0.0225±0.0107	NS
TV II Crop	Amino acid(mg/ml)	0.7272±0.6314	0.5770±0.1971	NS
	Protein(mg/ml)	10.0087±2.4846	9.6880±1.0106	NS
	Trehalose(mg/ml)	0.3811±0.2420	0.9501±0.3784	**
	Glycogen(mg/ml)	0.8817±0.5301	1.7189±0.6553	NS
	Glycerol(µmole/ml)	0.0019±0.0013	0.0021±0.0008	NS
TV I Crop	Amino acid(mg/ml)	0.0898±0.0525	0.2175±0.1113	**
	Protein(mg/ml)	6.7098±1.8661	6.2010±1.1506	NS
	Trehalose(mg/ml)	4.3483±0.7459	2.8419±1.6818	NS
	Glycogen(mg/ml)	0.5717±0.2057	0.5004±0.0602	NS
	Glycerol(µmole/ml)	0.0016±0.0004	0.0006±0.0004	**

** = significent NS = not significant.

(Fig. 9 and 10). The lower level of Glycerol always conceded with higher level of trehalose.

Protein profile during course of development of non-diapause and diapause destined larvae : The protein bands of 205 to 6.5 kDa were present throughout the development period of fourth instar larvae. However, a sharp Insect haemolymph acts as a storage reservoir for many materials essential for a variety of insects and its composition tends to vary in response to various conditions or activities such as ontogenetic effects (Mullin, 1985). Carbohydrates are important in insects, since they function as major energy source. Insect haemolymph contains trehalose, a non-reducing dimer of glucose, in high concentration (Wyatt and Kalf, 1957) and fluctuation in trehalose level reflects upon the physiological state of insects and is related to moulting and metamorphosis (Sakamoto and Horie, 1979). Nitrogenous compounds, like proteins, amino acids play a increase in thickness 85kDa protein was noticed, may be the major haemolymph as preparatory for diapause. One diapause specific protein band of 43 kDa appeared in diapause destined larvae. Crucial role in maintaining the different physiological state of an insect (Florkin and Jeuniaux, 1974; Mullin, 1985). Variation in quantity of nitrogenous substances during developmental stages (Wyatt and Pan, 1978; Wyatt, 1980); sex-specific proteins (Doira, 1968); precise analysis of haemolymph free amino acids in relation with season and dietary conditions (Mullin, 1985); moulting (Florkin and Jeuniaux, 1974); metamorphosis and diapause (Boctor,

Table 2. Comparative presence of haemolymph biochemical constituents in two sexes of fourth instar larvae in non-diapause (I & II crop) and diapause destined (III crop) generations of Daba trivoltine ecorace of A. mylitta

Haemolymph constituents	Male					Female				
	TV II Crop		TV III Crop		t-Stat	TV II Crop		TV III Crop		t-Stat
	Mean ± SD		Mean ± SD			Mean ± SD		Mean ± SD		
Amino acid(mg/ml)	0.6150	0.6281	3.1923	1.2107	5.39**	0.5288	0.2120	3.1948	1.1962	6.53**
Protein(mg/ml)	9.3737	2.7126	11.3999	2.5290	NS	9.4003	1.1461	11.3264	2.5672	NS
Trehalose(mg/ml)	0.3277	0.2528	6.5873	3.2437	5.76**	0.8563	0.4090	8.1217	3.0710	7.01**
Glycogen(mg/ml)	0.7473	0.5857	0.9868	0.5854	NS	1.4708	0.8442	1.7515	1.0555	NS
Glycerol(µmole/ml)	0.0019	0.0012	0.0358	0.0182	5.57**	0.0020	0.0008	0.0269	0.0166	4.49**
	TV II Crop		TV III Crop			TV II Crop		TV III Crop		
Amino acid(mg/ml)	0.0898	0.0455	3.1923	1.2107	7.68**	0.0330	0.0300	3.1948	1.1962	7.92**
Protein(mg/ml)	6.0976	2.1179	11.3999	2.5290	4.18**	6.1061	1.0189	11.3264	2.5672	5.38**
Trehalose(mg/ml)	0.2865	0.2462	6.5873	3.2437	5.79**	2.8961	1.4615	8.1217	3.0710	4.31**
Glycogen(mg/ml)	0.5110	0.2240	0.9868	0.5854	2.17**	0.4608	0.1027	1.7515	1.0555	3.63**
Glycerol(µmole/ml)	0.0017	0.0004	0.0358	0.0182	5.61**	0.0009	0.0007	0.0269	0.0166	4.67**

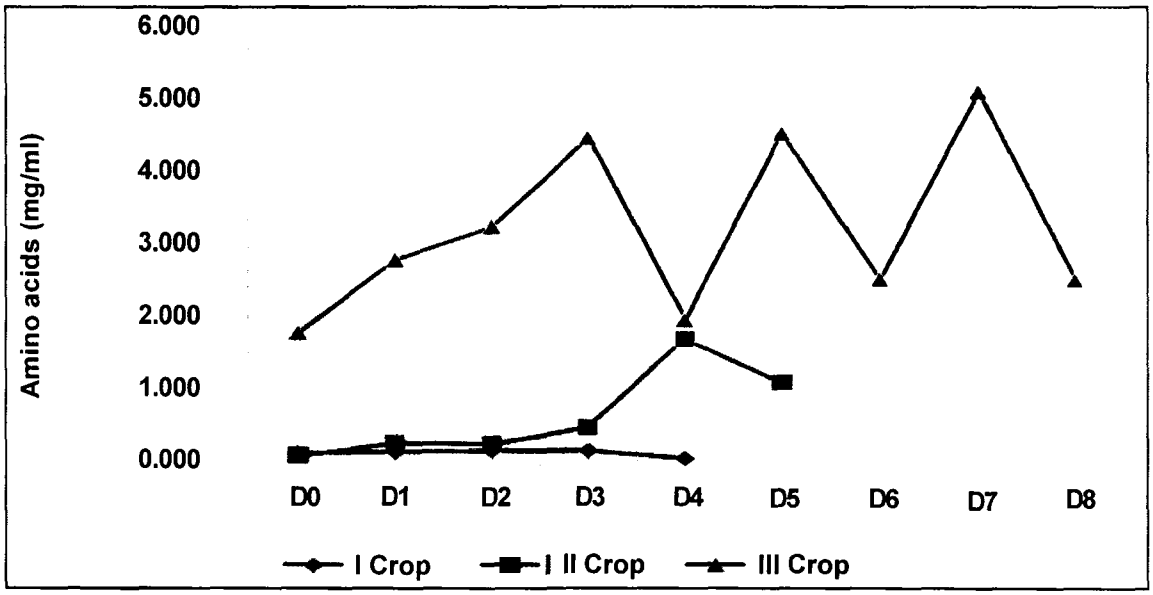

Fig. 1. Comparative presence of amino acids during male fourth instar period of Daba trivoltine ecorace of *A. mylitta* in different crops

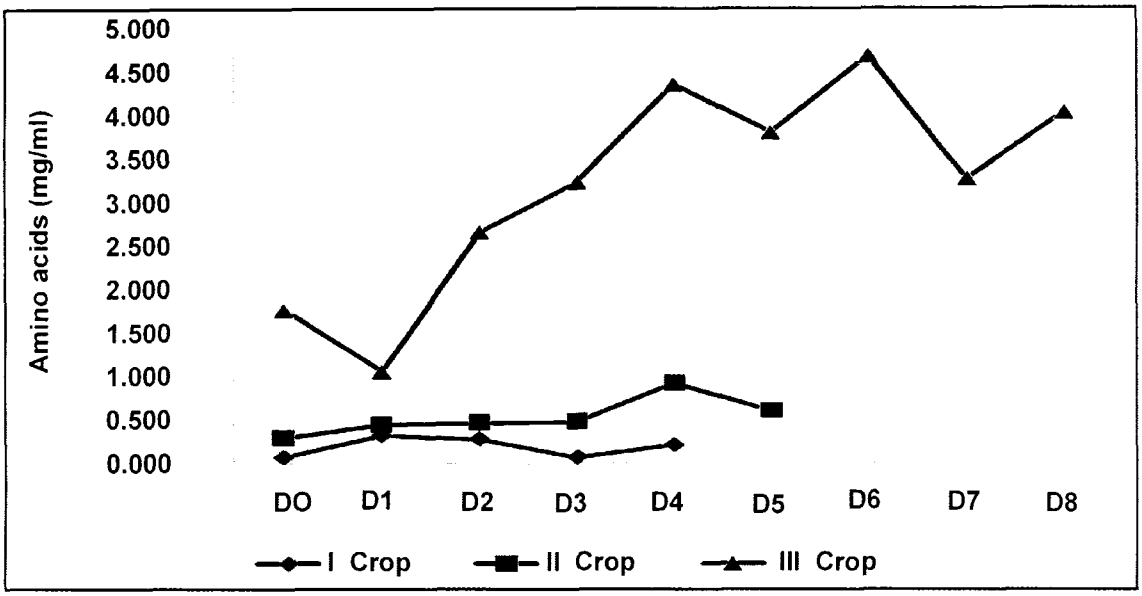

Fig. 2. Comparative presence of amino acids during female fourth instar period of Daba trivoltine ecorace of A. mylitta in different crops

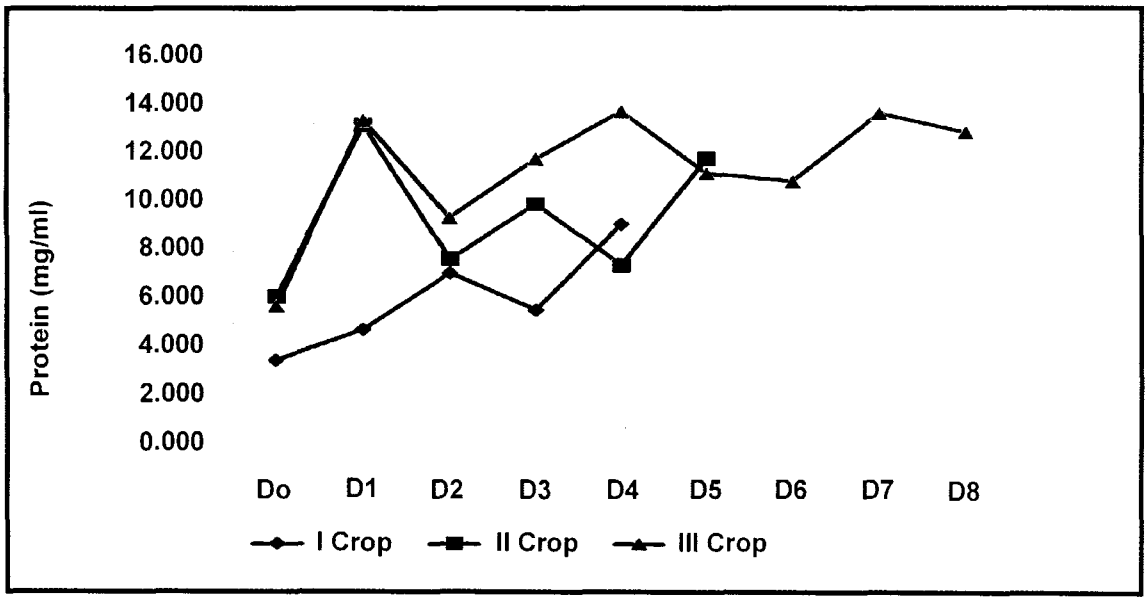

Fig. 3. Comparative presence of total proteins during male fourth instar period of Daba trivoltine ecorace of A. mylitta in different crops

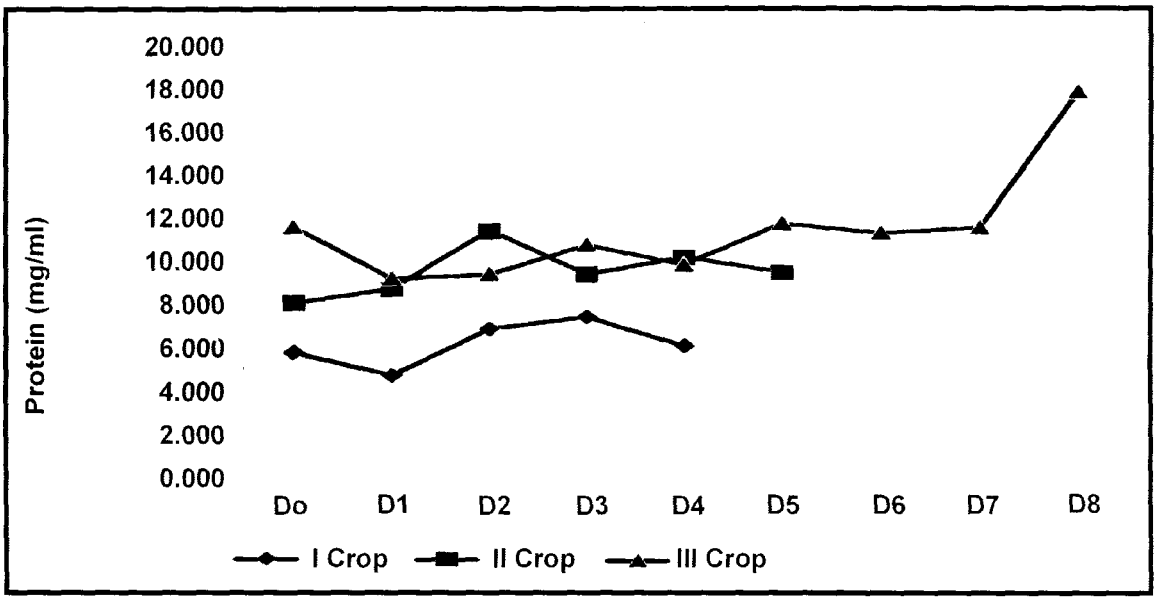

Fig. 4. Comparative presence of proteins during female fourth instar period of Daba trivoltine ecorace of A. mylitta in different crops

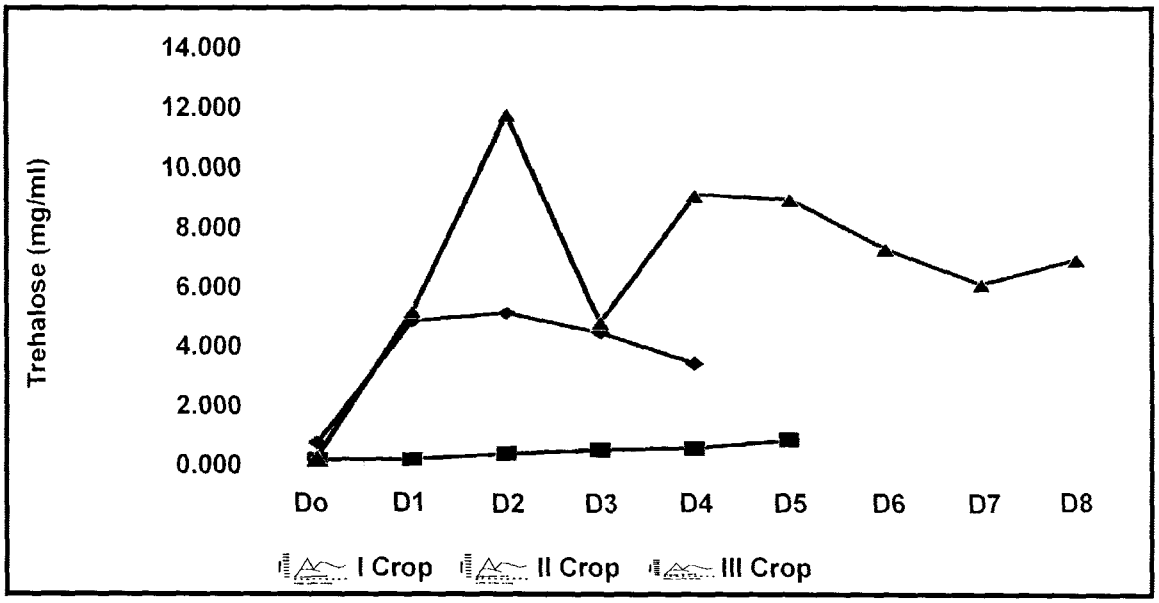

Fig. 5. Comparative presence of trehalose during male fourth instar period of Daba trivoltine ecorace of A. mylitta in different crops

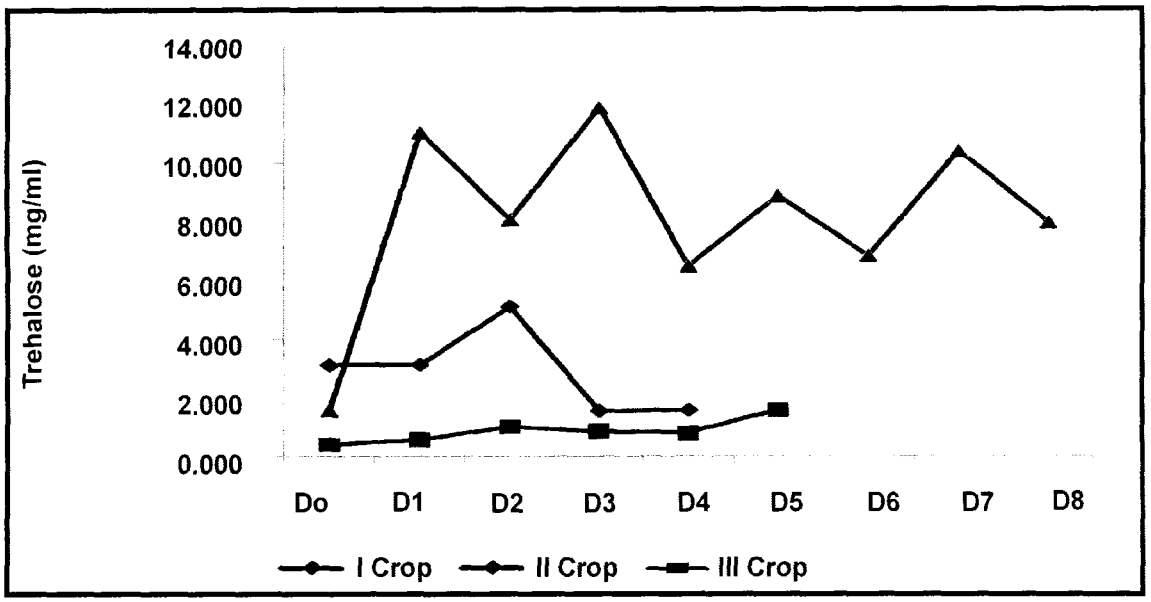

Fig. 6. Comparative presence of proteins during female fourth instar period of Daba trivoltine ecorace of A. mylitta in different crops

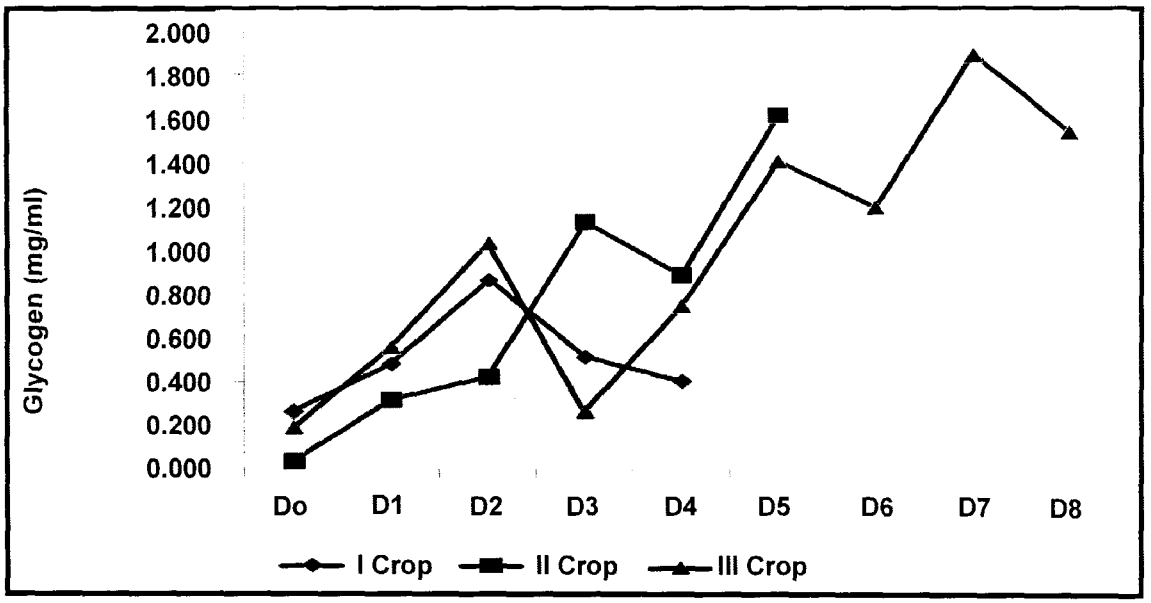

Fig. 7. Comparative presence of glycogen during male fourth instar period of Daba trivoltine ecorace of A. mylitta in different crops.

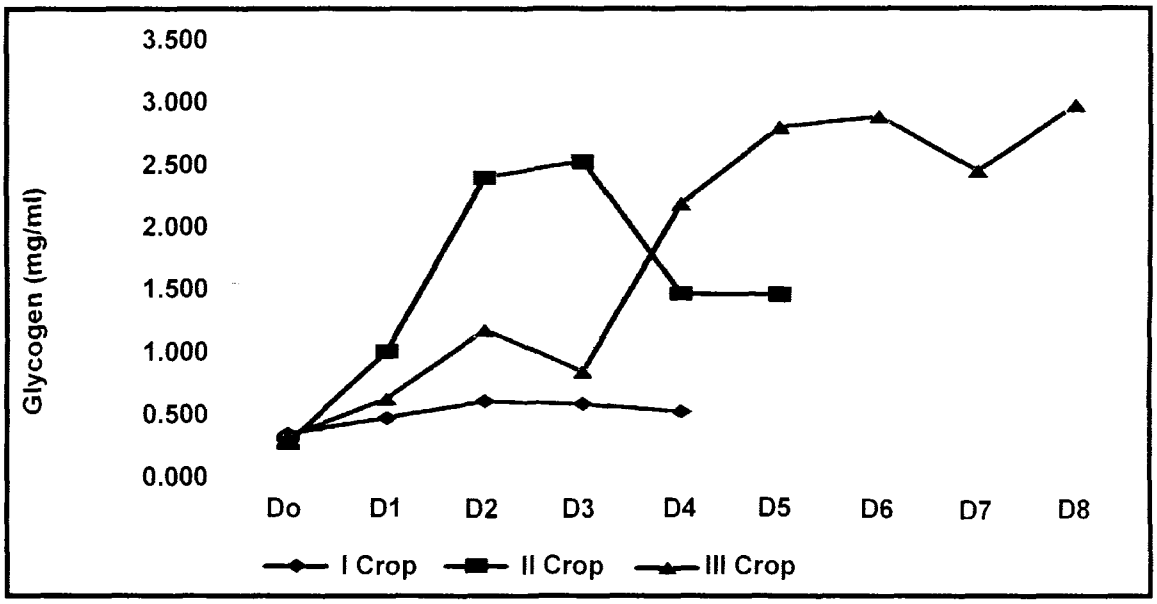

Fig. 8. Comparative presence of glycogen during female fourth instar period of Daba trivoltine ecorace of A. mylitta in different crops.

Biochemical studies of comparative haemolymph in silkworm

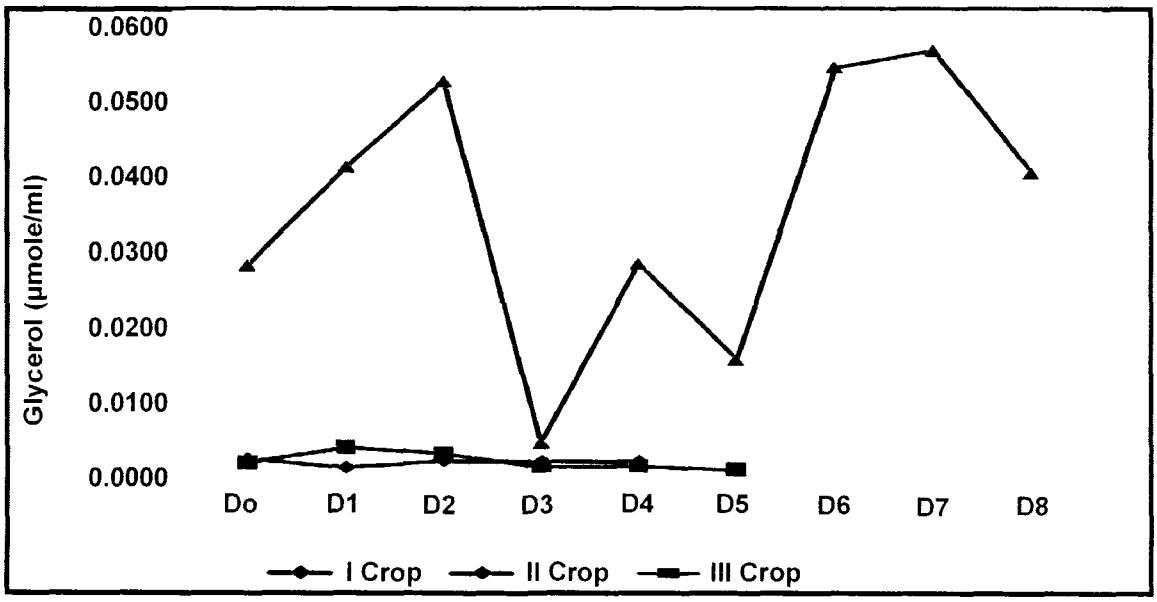

Fig. 9. Comparative presence of glycerol during male fourth instar period of Daba trivoltine ecorace of A. mylitta in different crops

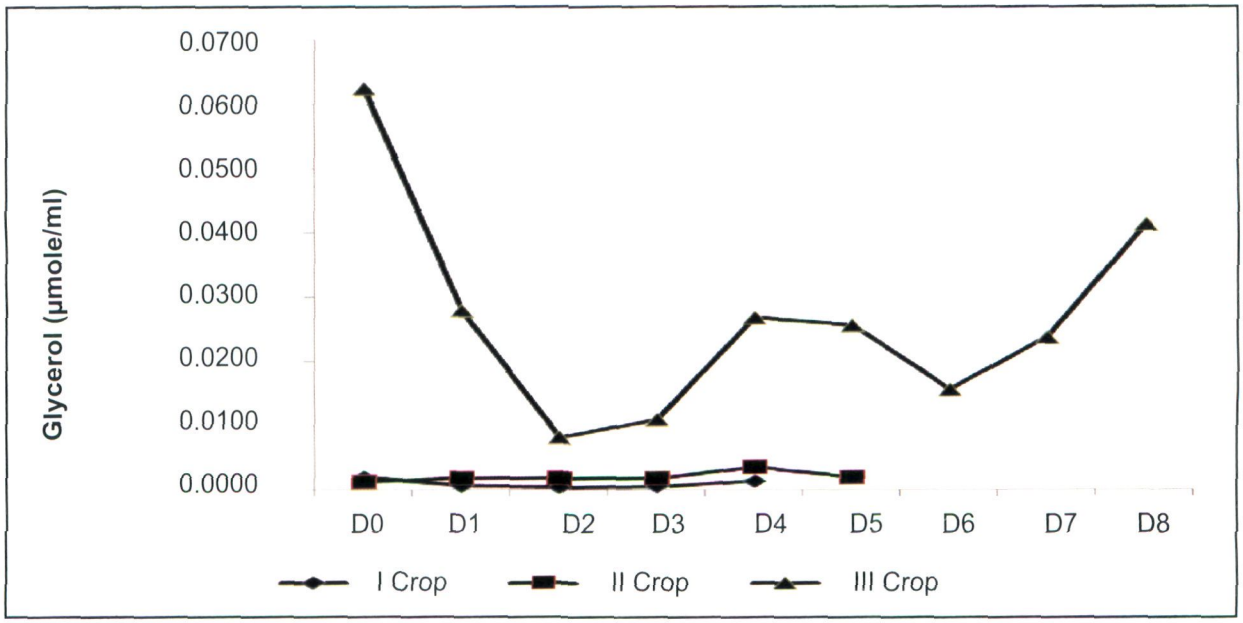

Fig. 10. Comparative presence of glycerol during female fourth instar period of Daba trivoltine ecorace of A. mylitta in different crops

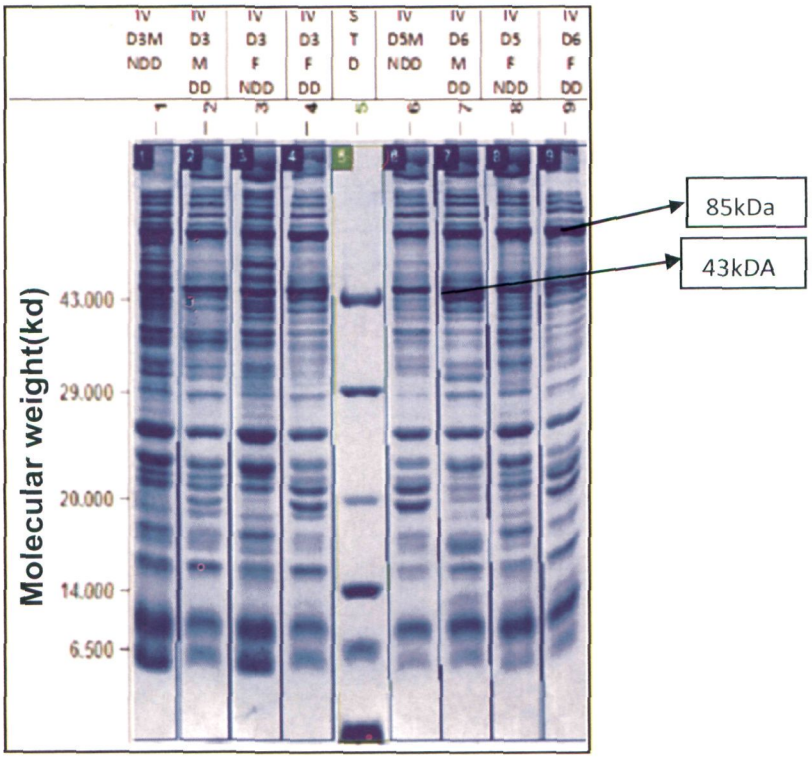

Fig. 11. Haemolymph protein profile of non-diapause and diapause destined larvae of Daba trivoltine of A. mylitta

Abbreviations: NDD: Non-diapause destined, DD: Diapause destined, IV: Forth instar, D: Day, M: Male, F: Female

1981) have been reported in insects.

The level of trehalose are also reported to decrease on the final day of fourth instar may be due to its greater utilisation to furnish fuel for the moulting process (Sowri and Sarangi, 2002; Mishra et al, 2010a). Trehalose levels are also related to moulting, metamorphosis and diapause (Wyatt, 1967; Hirano and Yamashita, 1983). The role of sugar and sugar alcohols are reported in diapause as Hayakowa and Chino (1982a, b) maintained that diapausing insects can be categorised into two types in terms of glycogen metabolism, one is sugar alcohol accumulating type, while the other is trehalose accumulating type. Hayakawa and Chino (1982 a,b) have demonstrated a temperature dependent inter-conversion between glycogen and trehalose in diapausing pupae of the saturniid moth *Philosamia Cynthia*. Proteins are among the most complex of all known chemical compounds and the most characteristic of living organisms (Florkin and Jeuniaux, 1974). The haemolymph proteins of insects have been investigated from various viewpoints. These include (i) mapping of protein patterns in various species, (ii) the identification of protein composition at successive developmental stages by both electrophoretic and immunological techniques, (iii) proposal of possible fluctuations of the protein components on the basis of enzymological and histochemical tests, and (iv) the analysis of both site and mechanism of synthetic processes by isotopic labelling (Chen and Levenbook, 1966 a, b and revised after Buck, 1953; Chen, 1966).

A number of studies have shown that total content of haemolymph proteins increased during larval development (Chen, 1966). A similar increase is also reported in *Antheraea proylei* Jolly protein changes during development (Sinha and Sinha, 1994); *Phormia regina* (Chen and Levenbook, 1966, b) and *Samia cynthia ricini* (Karaki, 1969). Haemolymph proteins also contribute in cuticle formation (Koeppe and Gilbert, 1973). An especial characteristic of insects, especially holometabolous insects is the high titre of amino acid present in their haemolymph (Evans and Crossley, 1974; Firling, 1977). In addition to their function as protein constituents, they enter into diverse metabolic pathways and participate in many other physiological activities. The role of amino acids in the development process is also reported in *Bombyx mori* (Wyatt and Kalf, 1956); in *A. mylitta* (Jolly *et al.,* 1972; Agarwal *et al.,* 1981; Sinha *et al.,* 1988). More so, several functions are attributed to free amino acids in insect haemolymph such as (i) osmo-regulation (Beadle and Shaw, 1950), (ii) protein synthesis (Buck, 1953), (iii) energy production for flight (Sactor, 1965), cocoon spinning (Fukuda and Matuda, 1953; Wyatt, 1961). Amino acid pool in *A. mylitta* might be useful for the above activities and also in preparation for diapause.

One event occurring shortly before the onset of diapause is the synthesis of specific proteins that are released into the haemolymph and remain there in abundance throughout diapause. When diapause is terminated, they quickly disappear from the haemolymph. These haemolymph proteins, first noted in the south-western corn borer, *Diatraea grandiosella* (Brown and Chippendale, 1978), are referred to as diapause-associated proteins (DAPs) and have been reported from larvae of several additional Lepidoptera including the codling moth *Cydia pomonella* (Brown, 1980), pink bollworm *Pectinophora gossypiella* (Salman and Miller, 1992), stem borer *Busseola fusca* (Oshir *et al.*, 1989), spruce budworm *Choristoneura fumiferana* (Palli *et al.,* 1998) and wax moth *Galleria mellonella* (Goldewski *et al.*, 2001), as well as adults of the colorado potato beetle *Leptinotarsa decemlineata* (Koopmanschap *et al.*, 1995) and the red fire bug *pyrrochoris apterus* (Sula *et al.*, 1995), among these. These proteins are hexameric proteins generally referred to as storage proteins. Most, but not all, have high content of aromatic amino acids and thus are classified as arylphorins. Based on their appearance, they are of various types: There are haemolymph proteins which are specific to different states of diapause (pre-

diapause, diapause, and diapause termination) and post diapause growth period. All are controlled by their specific genes. It is well examplified in *Sarcophaga crassipalpis* (Denlinger, 2002).

In the present study also the level of trehalose declined sharply at the fag end of larval instar period. Many diapausing species are reported to utilize stores of glycogen to generate cryopotectants such as glycerol, sorbitol and trehalose (Wyatt, 1967; Nordin *et al.,* 1984). Trehalose is reported to play a major role in maintenance of diapause (Jo and Kim, 2001; Mishra *et al.,* 2010b). In the present study the levels of protein, glycogen, trehalose, glycogen and glycerol were also significantly higher in DD larvae than NDD and support the findings of these workers. It is further clear that trend of haemolymph biochemical constituents during fourth instar of *A. mylitta* are generation specific. From the present study, it is now clear that as a preparatory measure for the induction of diapause the fourth instar larvae of *A. mylitta* have higher level of amino acids, trehalose, glycogen and glycerol to survive adverse climatic conditions during diapause.

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