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DETERMINATION OF TEMPERATURE SENSITIVE DIAPAUSE TERMINATION STATE OF DABA TRIVOLTINE ECORACE OF *ANTHERAEA MYLITTA* DRURY

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ABSTRACT

The Daba trivoltine ecorace of tropical tasar silkworm Antheraea mylitta Drury is one of the most commercially exploited non-mulberry silkworms in tropical India. It is generally grown in the latitude range of 20°N to 25°N in Eastern India, especially, in parts of West Bengal, Jharkhand, Orissa, Chhattisgarh and Andhra Pradesh. The pupae of this ecorace remain in facultative pupal diapause state for five to six months (January-June). During this long period of diapause losses occur due to un-seasonal and unsynchronised emergence of male and female moths which is estimated to the tune of 25-30% leading to reduction of seed production in grainages. The losses become more prevalent when dry conditions prevail soon after pre-monsoonal showers. This can be avoided by consigning the pupae of diapause termination state to low temperature. In the present study, the specific age of diapause termination state has been worked out on the basis of the presence of haemolymph biochemical constituents like glycerol, trehalose, glycogen, quantitative total proteins and free amino acids and protein profile comparing them with non-diapausing pupae harvested during first and second crop. Among diapausing pupae, the trehalose concentration always remained at its low level during diapause and an increase in concentration was observed at the fag end of diapause when pupae attained the age of 145 to 150 days. Contrary to this, the level of glycerol and glycogen was always higher through out the diapause period and a down surge in the concentration was noticed when pupae were 145 to 150 days old. The level of protein was higher in non-diapausing generation. However, the level of protein and amino acids showed a fluctuating trend through out diapause development. The haemolymph protein profile of the diapausing pupae showed the presence of a diapause specific protein band of 16kD which remained in its full intensity till the pupae attained the age of 145-150 days, thereafter, its intensity went down and protein profile of diapausing pupae looked similar to non-diapausing pupae. Therefore, it is confirmed that the diapause termination state in Daba pupae occurs when pupae become 145-150 days old. The diapausing pupae of this age can be further exploited by working out low temperature treatment schedule to avoid losses in grainages.

Keywords: Antheraea mylitta, biochemical aspects, temperature sensitive diapause state, haemolymph protein profile

INTRODUCTION

The term diapause suggests a period of arrest in which development comes to a complete halt although it is not regarded as a simple arrest and restart of development (Denlinger *et al.,* 2004). During course of diapause some of the important physiological activities such as maturation of gonads and formation of spermatids progresses at a slow pace hence this diapause period is referred to as period of diapause development (Andrewartha, 1952, Leob, 1982; Friedlander and Reynolds, 1992; Readio *et al.,* 1999). Voltinism among insects varies depending upon the environmental conditions and onset of diapause in an insect population is determined by the sensitive developmental stage and critical environmental cués (Eizaguirre *et al.,* 1994). The distribution of tropical tasar silkworm *Antheraea mylitta* Drury is wide ranging between 10° to 32°N latitude and 76° to 93°E longitude, experiencing varied environmental conditions (Mishra *et al.,* 2011). It undergoes facultative pupal diapause and shows different type of voltinism, at higher latitudes range of 26 to 29°C) it behaves as univoltine, at mid-latitudes (20 - 25°N) it behaves as bivoltine or trivoltine and at low latitude 14 - 17°C it behaves as tri or multivoltine. The voltinism gets modified in isolated conditions, depending upon the altitude of place. The diapause duration of bivoltine ranges in between 205-241 days and for trivoltine from141-175 days. During the course of seed cocoon preservation subsequent proportion of seed stock is lost due to erratic, unseasonal and unsynchronised emergence of adults (Mishra *et al.,* 2010d).

Insect haemolymph contains acts as major energy reserves for the insects to survive adverse environmental conditions during diapause (Mishra *et al.,* 2010a). It contains carbohydrates like glycogen and

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trehalose (Wyatt and Kalf, 1957) required for moulting, metamorphosis and diapause development (Sakamoto and Horie, 1979). Nitrogenous compounds, like proteins and amino acids play major role in different physiological activities of insects (Florkin and Jeuniaux, 1974; Mullin, 1985). Variation in quantity of nitrogenous substances during developmental stages (Wyatt, 1980; Mishra *et al.,* 2010b); sex-specific proteins (Kunkel and Nordin, 1985; Mishra *et al.,* 2010b); precise analysis of haemolymph free amino acids in relation with season, moulting, metamorphosis and diapause have been reported in insects (Mullin, 1985; Mishra *et al.,* 2010c).

The insect species, which enter diapause, have shown the presence of haemolymph glycerol to survive the cold conditions in diapausing state (Harvey, 1962; Mansingh, 1974). The concentration of glycerol increases through out diapause period and a gradual decrease is observed once diapause is set to terminate (Li *et al.,* 2002, Mishra *et al.,* 2010c). Some insects accumulate trehalose instead of glycogen or glycerol in the haemolymph during diapause (Kimura *et al.,* 1992) but *A. mylitta* have low level of trehalose activity through out the diapause period (Mishra *et al.,* 2009). These diapause-associated proteins (DAPs) have been reported from several lepidopteron larvae such as codling moth *Cydia pomonella* (Brown, 1980), pink bollworm *Pectinophora gossypiella* (Salama & Miller, 1992), spruce budworm *Choristoneura fumiferana* (Palli *et al.,* 1998) and wax moth *Galleria mellonella* (Godlewski *et al.,* 2001). These hexameric proteins are generally referred as storage proteins have high content of aromatic amino acids and are classified as arylphorins. These proteins are specific to different states of diapause (pre-diapause, diapause, and diapause termination) and post diapause growth period controlled by their specific gene (Telfer and Pan, 2003).It is reported that in insects these proteins provide amino acids for egg production and rebuilding tissue after diapause (Pan and Telfer, 2001; Lewis, *et al., 2002*, Hahn and Wheeler, 2003; Nagamanju *et al.,* 2003; Chandrashekar *et al.,* 2008).

No attempt has been made to identify the biotic conditions which may indicate that the diapause has reached a stage of termination in Daba trivoltine ecorace of *A. mylitta* on the basis of biochemical markers and protein profile although such study is essential to work out a temperature sensitive diapause termination state of pupae so as to exploit this period for regulated and delayed moth emergence during extreme summer conditions during first crop grainage. Hence, this study has been undertaken to estimate the presence of overall quantitative proteins, free amino acids, trehalose, glycogen, glycerol and qualitative protein profile during entire pupal period of both non-diapausing and diapausing generations to work out the possible period of diapause termination.

MATERIALS AND METHODS

The experimental Animal: Daba trivoltine stock of *A. mylitta* used in this experiment were maintained at Central Tasar Research and Training Institute, Ranchi, India. First crop was raised during last week of June to last week of July, second crop in the months of August to September. Pupae of first and second crops behave as non-diapausing. Third crop was raised during October to January. The pupae of third crop remain in diapause from January to June. For biochemical and the protein profile studies the haemolymph of synchronised male and female pupae of similar age and weight were used.

Collection of haemolymph, estimation of biochemical

constituents and protein profile studies of pupae: Approximately 500 µl of haemolymph was collected from the pupae and centrifuged at 10,000 rpm in a refrigerated centrifuge at 4°C for 5 minutes. The clear supernatant was deep frozen at -80°C until biochemical estimation was done. The estimation of quantitative total protein was done after Lowry *et. al.,* 1951, total free amino acids after Moore and Stein (1948), trehalose & glycogen after Wyatt and Kalf (1957) and glycerol after Hagen and Hagen (1962). Data was statistically analysed using student t- test. The qualitative protein profile studies were carried out following the methods of Laemmli(1970) and the specific lanes of SDS PAGE were further analyzed with the help of GE Healthcare Software IMAGEQUANTTL for densitometry and annotated molecular weight of proteins.

RESULTS AND DISCUSSION

Sex-specific presence of haemolymph constituents: Sex-wise presence of haemolymph biochemical constituents were subjected for statistical analysis. It was observed that there was no significant difference in the level of glycerol during first and second crop. During third crop female pupae had significantly higher level of glycerol in females (300.885±109.214 micromole/ml) than male pupae (184.689±106.099 micromole/ml). Contrary to this the level of trehalose was significantly higher in male pupae (10.282±3.207 mg/ml) than females (5.76± 2.40mg/ml). Glycogen content was significantly higher in female pupae than males in TV first crop (4.375±1.357 mg/ml) and third crop (1.879±0.724 mg/ml). Male pupae of second crop contained more amino acids than females. However, females of TV I and III crop had significantly higher level of protein concentration in the haemolymph (Table-1).

Comparative haemolymph biochemical constituents in NDD and DD generation: The level of glycerol was always higher in diapausing pupae than non-diapausing pupae of both the sexes. Difference in haemolymph trehalose level was not significant in between TV I & TV III crop male and female pupae although level of trehalose was higher in TV II crop than TV III crop. Total protein was also significantly higher in diapausing pupae. Total amino acids quantity was significantly higher in TV III crop than TV II crop although the difference was non-significant in between TV I crop and TV III crop. Haemolymph glycogen content was significantly higher in non-diapausing pupae (Table 2).

Periodical changes observed through out the developmental period of pupae in non-diapause and diapausing generations: Presence of amino acids showed a fluctuating trend in both male and female pupae of TV I and II crops. Three major peaks were observed on day 1, 5 in I crop and 12-14 in II crop. The concentration of amino acids went down prior to emergence of adults (Fig.1 & 2). In diapausing generation too the amino acid concentration showed a fluctuating trend and the two prominent peaks were observed when pupae were 90-100 and 160-164 days old (Fig. 3). In non-diapausing generation the concentration of protein was initially high in male pupae on day 1, thereafter it remained at low level. In case of females a peak of protein was observe on day 8. The level of protein went down prior to adult emergence (Fig.4 & 5). In diapausing generation peak of protein peak of protein concentration was observed when pupae were 140-144 days old. Another peak was in between 160-164 days when adults emerged (Fig. 6). The concentration of glycogen was observed to be higher in just formed pupae or when pupae or when pupae were 0-3 days old in non-diapausing generations. Again a rise in its level was observed when adult emergence was nearing (Fig. 7 & 8). In diapausing generation glycogen was high up to

Table 1: Sex-wise and crop specific comparative presence of haemolymph biochemical constituents of pupae in different crops in Daba trivoltine ecorace of *A. mylitta.*

Haemolymph constituents	Crops	Male Mean ±SD	Female Mean ± SD	t Stat
Glycerol (micro mole/ml)	TV I Crop	58.125±28.593	51.406±22.952	NS
	TV II crop	64.050±24.718	80.900±72.637	NS
	TV III crop	184.689±106.099	300.885±109.214	4.641**
Trehalose (mg/ml)	TV I Crop	14.565±3.816	12.979±3.603	NS
	TV II crop	15.101±3.886	10.630±3.260	NS
	TV III crop	10.282±3.207	5.768±2.402	3.749**
Glycogen(mg/ml)	TV I Crop	2.307±1.203	4.375±1.357	4.561**
	TV II crop	2.422±1.198	3.286±1.516	NS
	TV III crop	1.261±0.538	1.879±0.724	4.495**
Amino acids(mg/ml)	TV I Crop	2.047±1.533	1.707±1.125	NS
	TV II crop	0.771±0.724	0.296±0.221	2.802**
	TV III crop	1.526±0.477	1.398±0.622	NS
Protein(mg/ml)	TV I Crop	15.800±8.242	26.865±14.538	2.729**
	TV II crop	34.284±7.629	32.411±12.883	NS
	TV III crop	35.470±12.363	60.364±8.445	11.029**

Note: ** Values are significant at 99% level

Fig. 1. Haemolymph amino acids in the firstc rop pupae and adult of daba trivoltine ecorace of *A. mylitta*

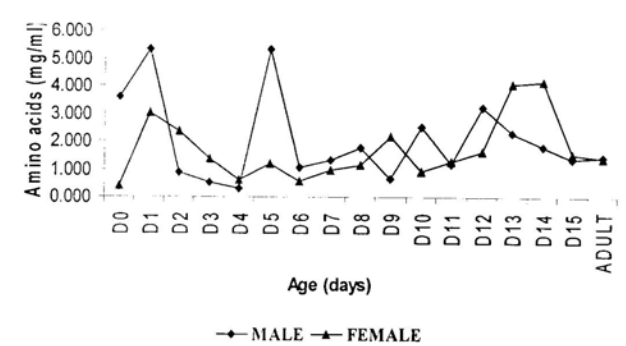

Fig. 2. Haemolymph amino acids in second crop pupae and adult of daba trivoltine ecorace of A. mylitta

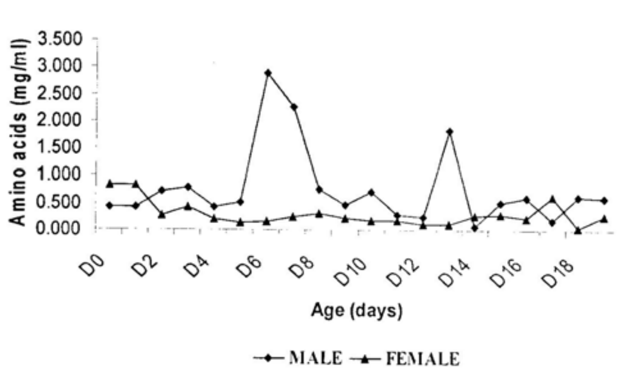

Fig.3. Haemolymph amino acids in the third crop pupae and adults of daba trivoltine ecorace of *A. mylitta*

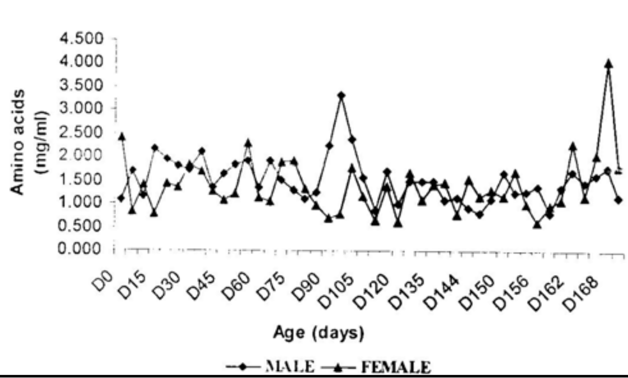

Fig. 4. Haemolymph protein in first crop pupae and adult of Daba trivoltine ecorace of *A. mylitta*

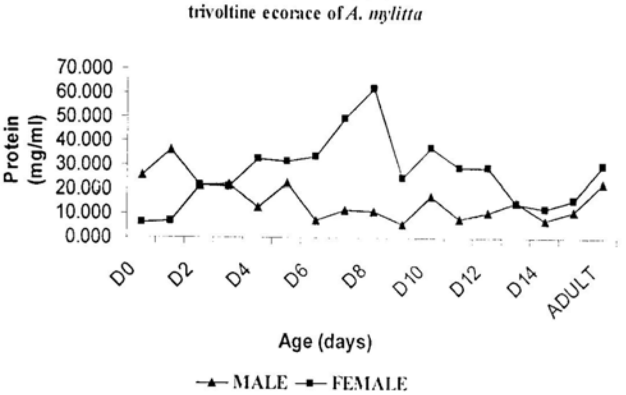

Fig. 5. Haemolymph protein in second crop pupae and adult of Daba trivoltine ecorace of *A. mylitta*

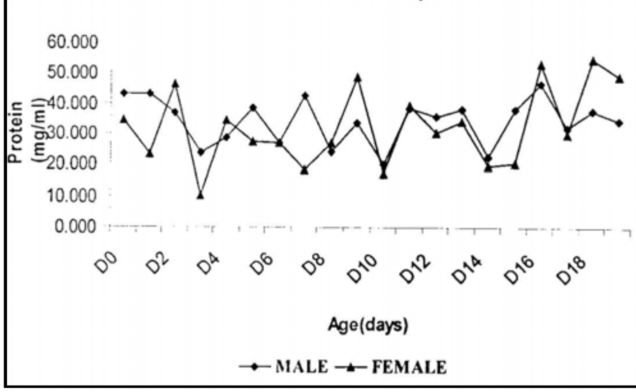

Fig.6. Haemolymph protein in third crop pupae and adult of Daba trivoltine ecorace of *A. mylita*

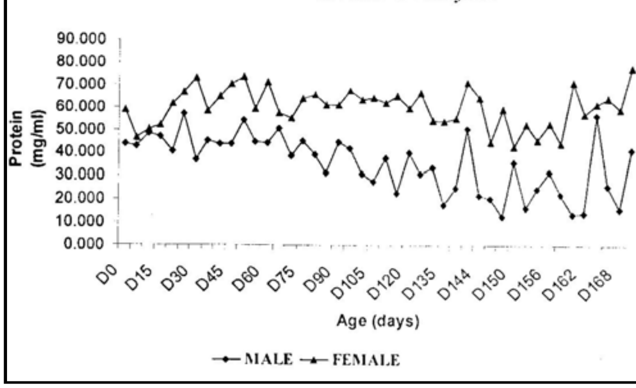

Fig.7. Haemolymph Glycogen in first crop pupae and adult of Daba TV ecorace of *A. mylitta* in first crop

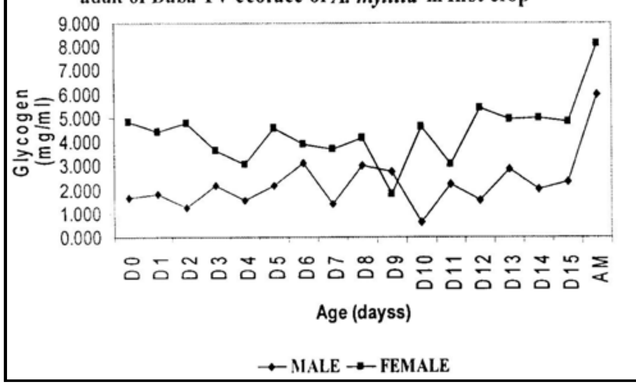

Fig. 8. Haemolymph glycogen in second crop pupae and adults of Daba trivoltine ecorace of *A. mylitta*

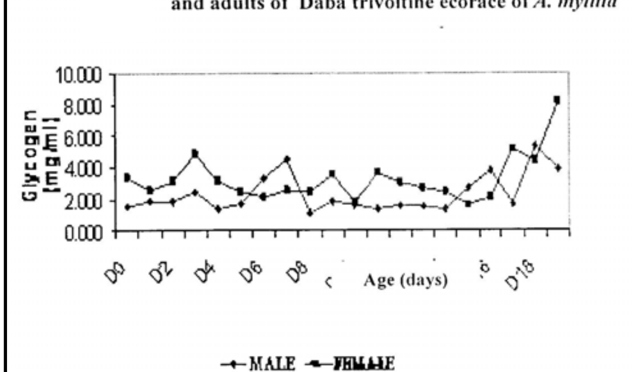

Fig. 9. Haemolymph glycogen in third crop pupae and adults of Daba trivoltine ecorace of *A. mylitta*

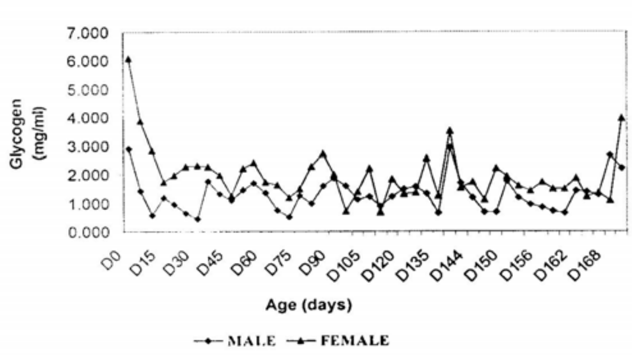

Fig. 10. Haemolymph Glycerol in first crop pupae and adult of daba trivoltine ecorace of *A. mylitta*

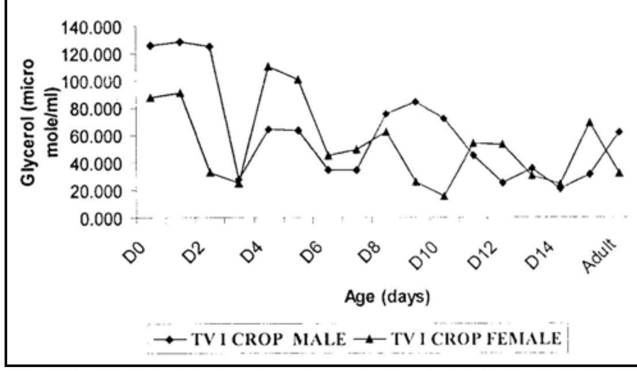

Fig. 11. Haemolymph glycerol in second crop pupae and adult of daba trivoltine ecorace of *A. mylitta*

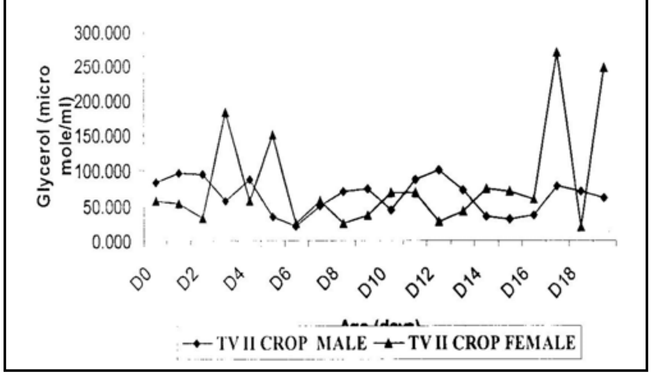

Fig.12. Haemolymph Glycerol in third crop pupae and adult of Daba trivoltine ecorace of *A. mylita*

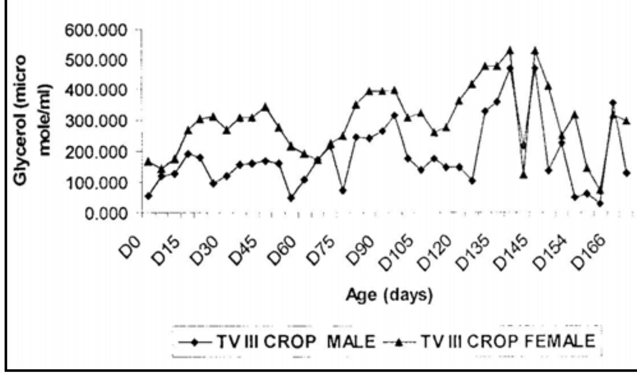

Fig.13. Haemolymph trehalose in first crop pupae and adult of daba trivoltine eocrace of *A. mylitta*

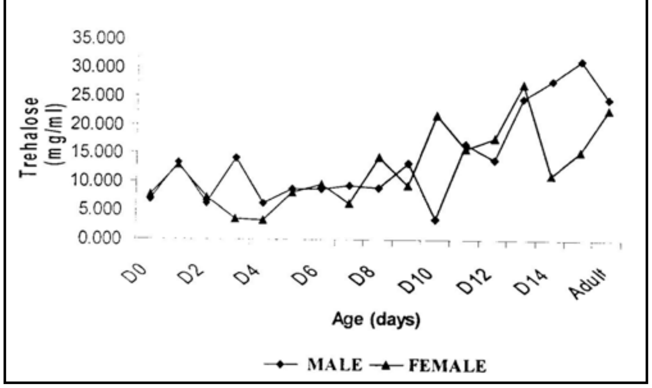

Fig. 14. Haemolymph trehalose in second crop pupae and adults of Daba trivoltine ecorcae of *A. mylitta*

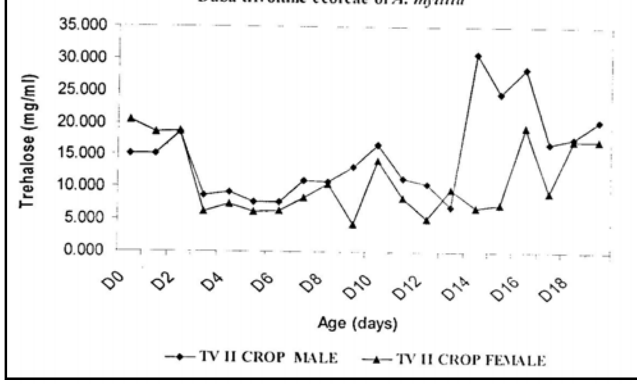

Fig.15. Haemolymph trehalsoe in third crop pupae and adult of Daba trivoltine ecorace of *A. mylitta*

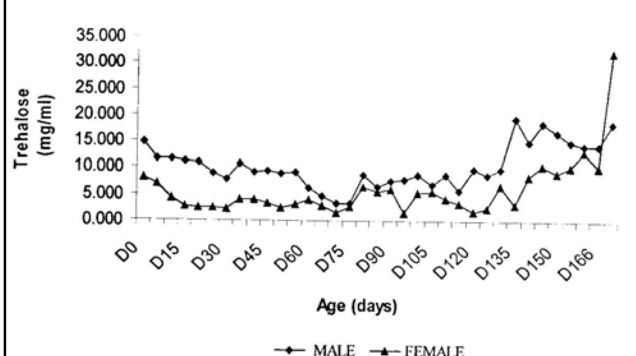

Table 2: Comparative presence of haemolymph biochemical constituents among pupae of Daba trivoltine ecorace of *A. mylitta* in diapausing and non-diapausing generations

Haemolymph constituent	Crops	Male (Mean ± SD)		t Stat	Female (Mean ± SD)		t Stat
		NDD	DD		NDD	DD	
Glycerol (micromole/ml)	TV I & TV III crops	62.14±29.27	181.329±109.95	6.13**	53.556±29.27	297.316±109.95	12.697**
	TV II & TV III crops	64.05±29.27	181.329±109.95	6.45**	53.556±80.90	297.316±109.95	8.97**
Trehalose (mg/ml)	TV I & TV III crops	14.11±8.33	10.409±4.15	NS	12.677±6.86	5.835±5.49	NS
	TV II & TV III crop	15.10±6.84	10.409±4.15	2.78**	11.117±5.50	5.835±5.49	3.42**
Protein (mg/ml)	TV I &TV III crops	15.80±8.24	35.470±12.36	7.19**	26.865±14.53	60.364±8.44	8.93**
	TV II & TV III crops	34.28±7.62	35.470±12.36	NS	32.411±12.88	60.364±8.44	8.87**
Amino acid (mg/ml)	TV I & TV III crops	2.08±1.57	1.526±0.48	NS	1.707±1.125	1.399±0.62	NS
	TV II & TV III crop	0.77±0.72	1.526±0.48	4.26**	0.296±0.22	1.399±0.62	10.39**
Glycogen (mg/ml)	TV I & TV III crops	2.27±1.17	1.298±0.59	3.25**	4.402±1.31	1.974±0.95	6.92**
	TV II & TV III Crops	2.38±1.29	1.298±1.14	3.87**	3.293±1.47	1.974±0.95	3.663**

Note: ** Values significant at 99% level

day 10, thereafter, the concentration remained at low level till pupae attained the age of 140-144 days and this higher level of glycogen was maintained till emergence of adults (Fig. 9).In non-diapausing generation concentration of glycerol was higher in just formed pupae and at he time of adult emergence (Fig.10 & 11). In non-diapausing generation a consistent increase in the level of glycerol was observed which attained a peak once pupae were 145-150 days old, thereafter, adown surge in its concentration was recorded till emergence of adults (Fig.12). The concentration of trehalose consistently increased till emergence of adults in non-diapausing generation (Fig. 13 & 14). In case of diapausing pupae the trehalose level remained at low level till pupae were 140-145 days old thereafter its level gradually increased till emergence of adults (Fig.15).

The high concentration of trehalose (Wyatt and Kalf, 1957) and fluctuation in trehalose levels are related to moulting, metamorphosis and diapause (Sakamoto and Horie, 1979) which was also observed in the pupae of tasar silkworm during course of diapause development. The concentration of trehalose increased when adult development was initiated in diapausing pupae of 140-145 days old in *A. mylitta*. Nitrogenous compounds, like proteins, amino acids *etc.* are important for different physiological activities of *A. mylitta* and such variation in their concentration confirms the reports of other workers in insects (Florkin and Jeuniaux, 1974; Mullin, 1985). Variation in quantity of nitrogenous substances during developmental stages (Wyatt, 1980), metamorphosis (Wirtz and Hopkins, 1974) and diapause (Boctor, 1981) has already been evidenced in insects. In the haemolymph of *A. mylitta* biochemical changes in concentrations of total proteins (Poonia and Mishra, 1975) and soluble carbohydrates, cholesterol and free ascorbic acid (Mohanty and Mitra, 1985) have been correlated with developmental events. An increase in the level of glycerol through out the diapause period and its downward trend at the age when pupae attain the age of 140-145 days indicates that in Daba trivoltine diapause termination starts at this age. It is in conformity with the views of earlier authors that the accumulation of glycerol takes place during diapause period and it goes down when diapause terminates (Jo and Kim, 2001 and Li *et al.,* 2002). At the same time low level of glycogen and trehalose show an increasing trend when pupae attain the age of 145-150 days. The level of protein also shows a downward trend at the same age. Thus pupae of this age group can be used for giving temperature treatment so as to regulate the emergence of moths in grainages in order to get more couplings thereby enhanced egg production.

The age specific protein profile of protein profile of non-diapausing (NDD) and diapausing pupae (DD) has been shown in details in Fig, 16 to 23 A &B. From the protein profile studies it was observed that a protein band ranging in between 70-73kD was highly unregulated through out diapause period in the haemolymph of both male and female pupae (Fig. 16 A & B). A protein band of 16 -17kD appeared in the haemolymph of diapausing pupae up to day 145 in male and female pupae (Fig.20 A & B). Its intensity decreased when pupae became older than 145-150 days and thereafter this band completely disappeared from the haemolymph indicating thereby that the diapause termination process has started at this age of pupae. The number of protein bands became less when pupae became older and the minimum number of protein bands was seen at the time of adult emergence (Fig. 21, 22, & 23). The diapause specific protein bands have also been reported in other insects (Salama & Miller, 1992, Palli *et al.,* 1998, Godlewski *et al.,* 2001). A group of storage proteins are also up regulated during diapause and they quickly disappear from the haemolymph when diapause is set to terminate (Chandrashekar *et al.,* 2008). These proteins are specific to different states of diapause (pre-

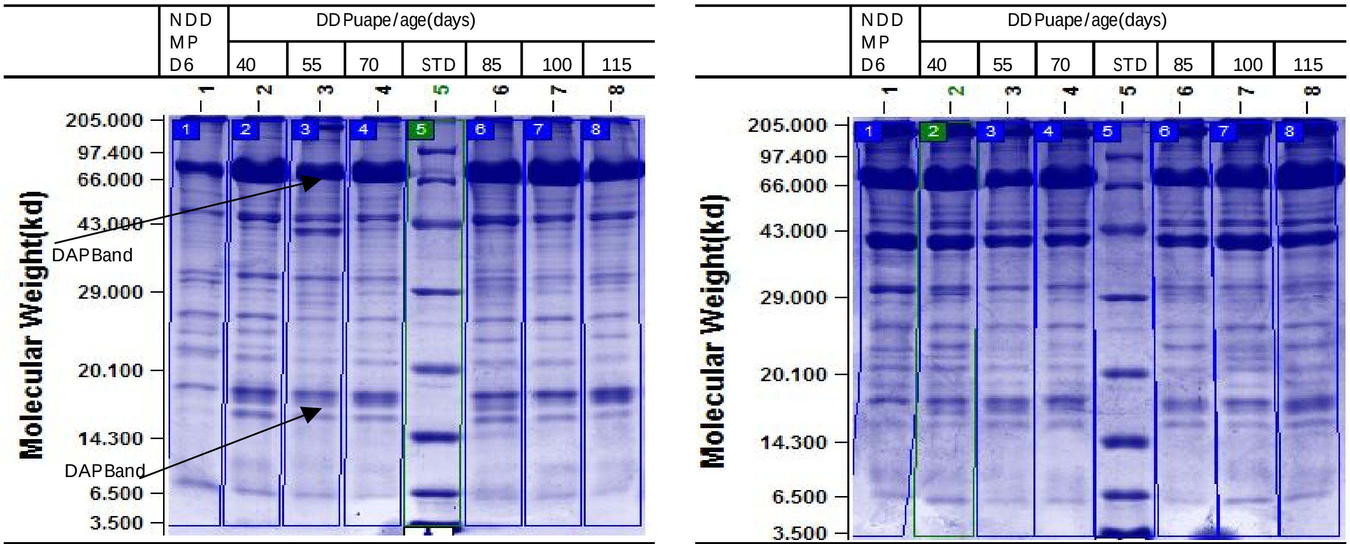

Fig.16 A & B. SDS - PAGE analysis showing comparative haemolymph protein profile of Daba trivoltine NDD & DD male (A) and female (B) Daba Tv puape.

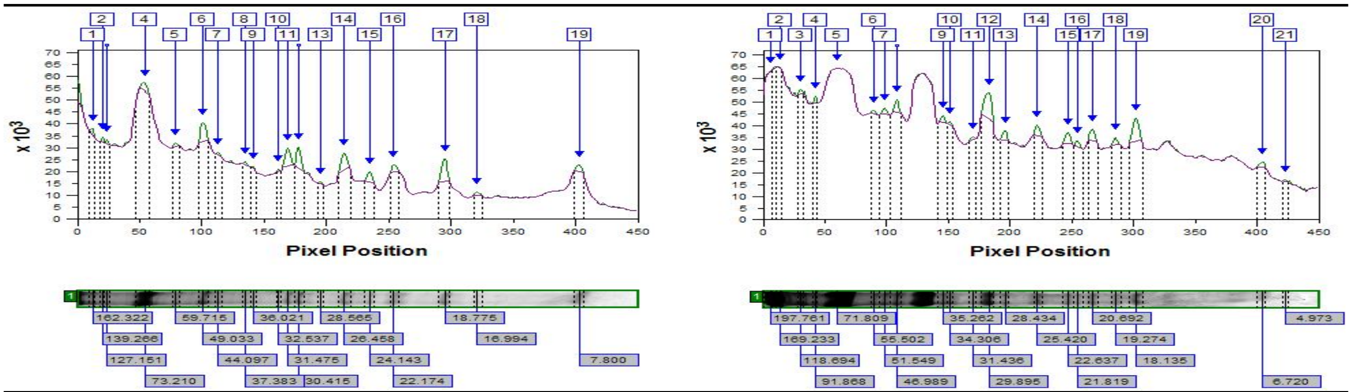

Fig.17 A & B. Densitogram and annotated protein profile and molecular weight of D6 NDD male (A) and female (B)Daba TV puape.

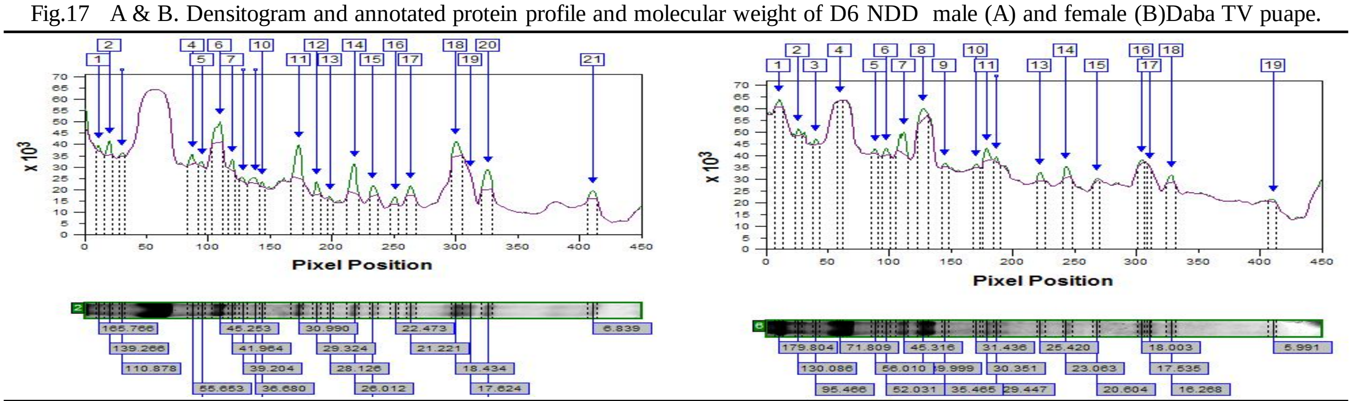

Fig. 18 A & B. Densitogram and annotated protein profile and molecular weight of D40 DD male (A) and D40 female (B) Daba TV diapausing pupae.

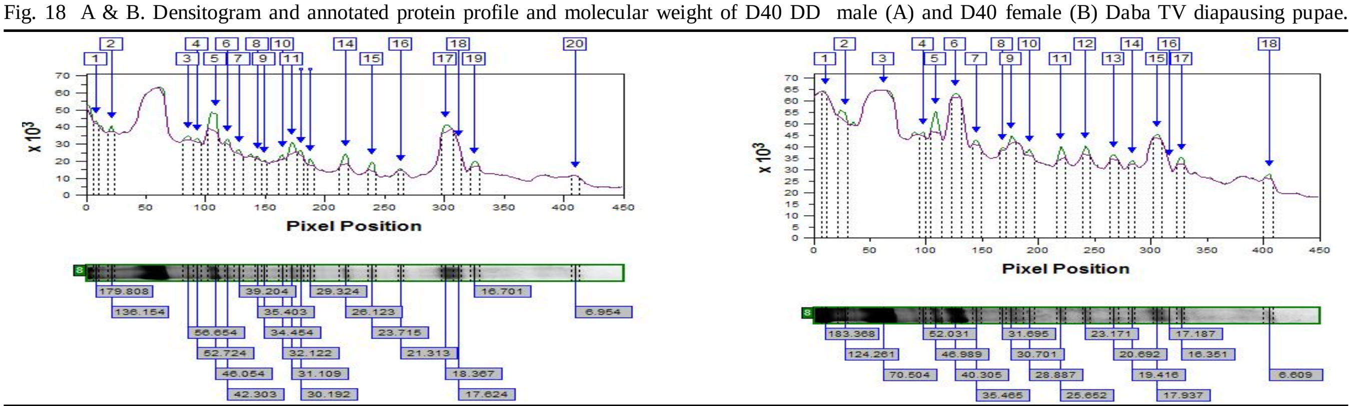

Fig. 19 A & B. Densitogram and annotated protein profile and molecular weight of D115 DD male (A) and D40 female (B)Daba TV diapausing pupae.

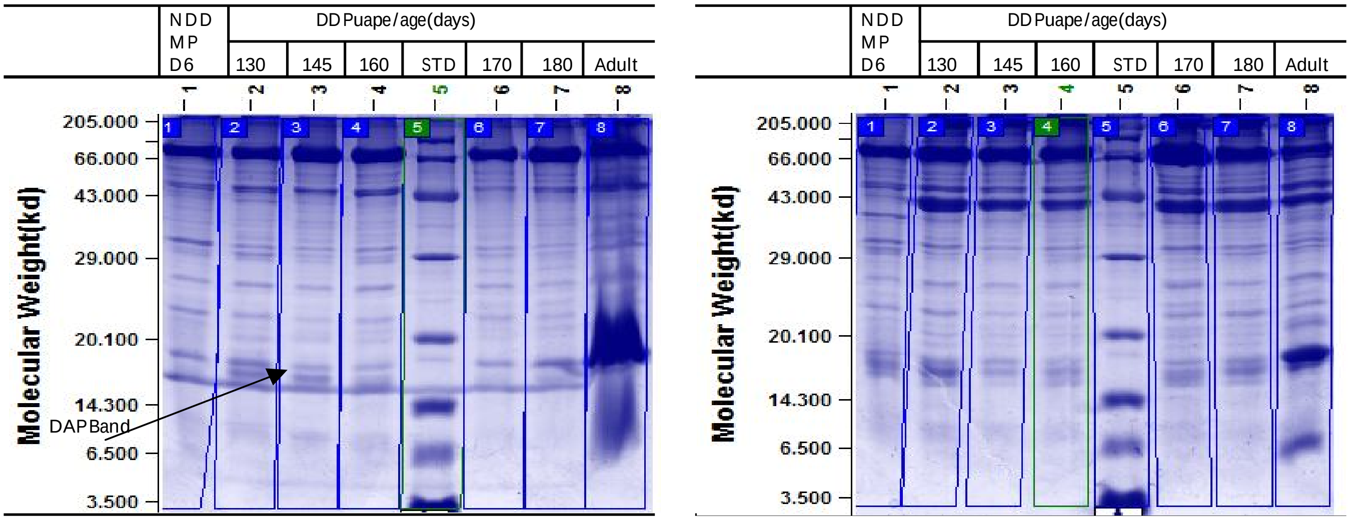

Fig. 20. A & B. SDS - PAGE analysis showing comparative haemolymph protein profile of Daba trivoltine NDD & DD male (A) and female (B) Daba TV puape

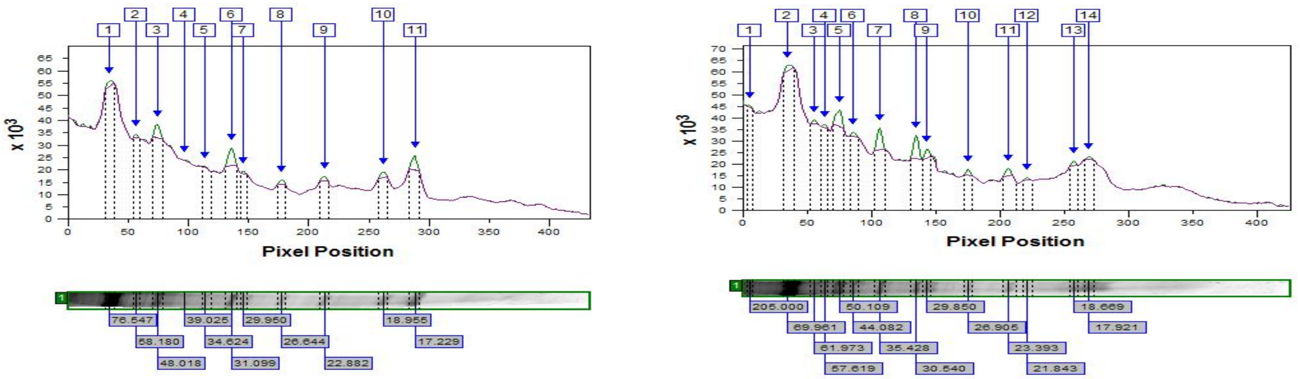

Fig.21 A & B. Densitogram and annotated protein profile and molecular weight of D14 male (A) and D14 female (B) Daba TV non-diapausing pupae.

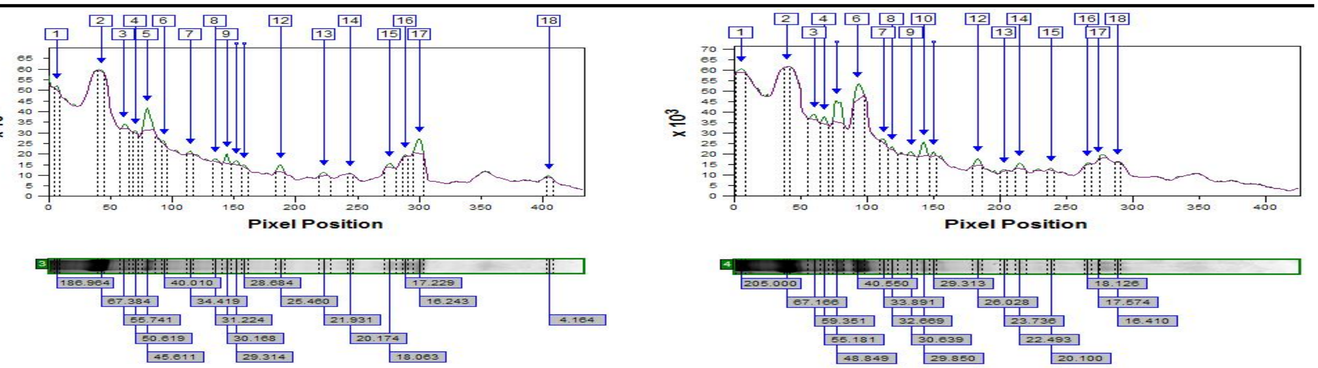

Fig.22. A & B. Densitogram and annotated protein profile and molecular weight of D145 male (A) and D145 female (B) Daba TV diapausing pupae.

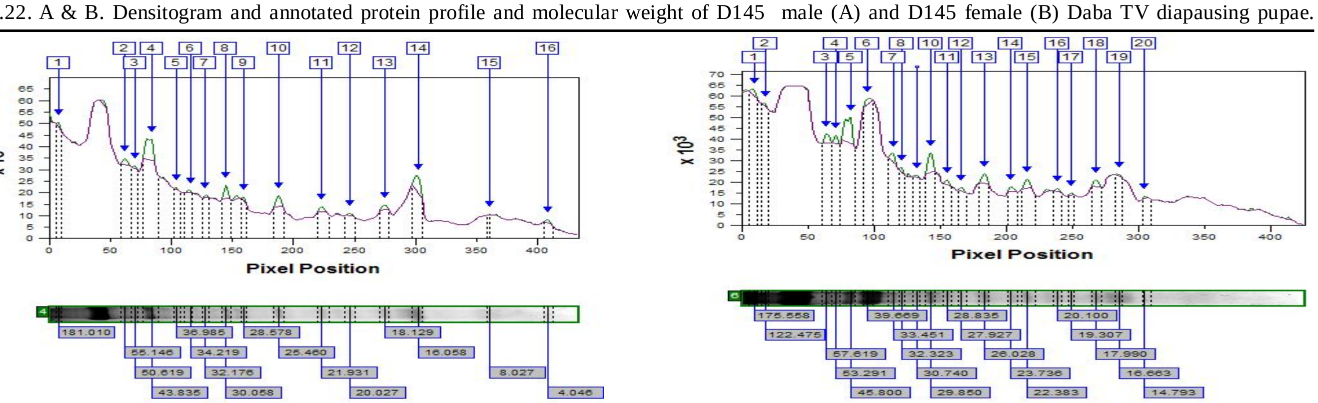

Fig. 23. A & B. Densitogram and annotated protein profile and molecular weight of D160 male (A) and D160 female (B) Daba TV diapausing pupae.

Abbreviations: d=day, NDD=Non-diapause destined, DD: Diapause destined, TV= trivoltine

diapause, diapause, and diapause termination) and post diapause growth period. Such band has also been identified during brain protein profile studies of Daba bivoltine pupae of Daba bivoltine ecorace of *A. mylitta* (Mishra *et al.,* 2009).

From the present study, it is inferred that there is accumulation of glycerol in the haemolymph of diapausing pupae of daba trivoltine ecorace of *A. mylitta*. The trehalose remains at its low level through out diapause period and an upsurge is noticed when diapause is set to terminate when pupae were 140-145 days old. Similarly, the level of glycogen in the haemolymph is lower through out the diapause period and shows an upward trend at the 145-150 days of age of pupae. The concentration of protein is always higher in diapausing pupae. Fluctuating pattern of amino acids is noticed through out the development period may be due to its time to time requirement for developmental processes involved during diapause development. The appearance of diapause specific band of 16-17kD protein through out diapause period and its disappearance when pupae are older than 145 days is indicative of the period of initiation of diapause termination process among Daba TV pupae.The pupae of this age can be utilized for developing a technology of low temperature treatment schedule so that loss of seed stock is avoided once extreme summer conditions prevail just after pre-monsoonal showers during first crop grainage to produce disease free layings matching with delayed cropping schedule.

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