

# Study of Haemocyte and Biochemical Changes of *Bombyx mori*L. Infected with *Bacillus thuringiensis* Var. Sotro

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## INTRODUCTION

When the larvae affected with Flacherie (*Bacillus thuringiensis*), the nature of haemolymph and digestive fluid also changes and as a result the cells of the gut wall gets affected. Since haemolymph is a circulatory fluid, with several functions, such as storage and transportation of nutrients and also plays an important role in excretion, defense, moulting and metamorphosis (Mullins, 1985). Nucleated haemocytes number varies with the insects; these haemocytes are classified as *prohaemocytes*, *plasmocytes*, *granular haemocytes*, *oenocytes* or *oenocytoid* and *spherule cells* (Arnold & Hinks, 1976). Major function of haemocytes includes phagocytosis of small particles, encapsulation of large foreign materials, haemolymph coagulation and storage and distribution of nutritive materials. Immune system includes certain types of blood cells. It also includes chemicals, carbohydrates, lipids and proteins. In insects, several types of haemocytes are observed in the haemolymph (Butt & Shields, 1996). Saran *et al.* (2002) classified the blood cells in the silkworm, *B. mori*L. and *A. mylitta* in to six types' viz. *prohaemocytes*, *plasmocytes*, *granulocytes*, *spherulocytes*, *imaginal spherulocytes* and *oenocytes*. Proteins are the derivatives of high molecular weight polypeptides. They play a vital role in the formation of structures in organisms. Like carbohydrates and fats proteins also can be utilized for energy purpose. In silkworm, blood glucose level can be correlated to their level of metabolism, and is comparable with mammalian blood glucose. Lipids play an important role in the biochemical processes delaying growth and development of insects.

To date, reports on haemocyte and biochemical response in *Bacillus thuringiensis*infected silkworm, *B. mori* are scanty. Hence, present investigation was conducted to study haemocyteand biochemical changes of *B. mori*infected with *Bacillus thuringiensis*. The Haemocyte and Biochemical Changes in *Bombyx mori*L. after per oral inoculation of *Bacillus thuringiensis*was observed. The Total Haemocyte Count (THC) increased gradually from day 1 (13650/mm<sup>3</sup>)to 8 (16625/mm<sup>3</sup>) in uninoculated silkworms kept as control. In *Bacillus thuringiensis*treated silkworm larvae of 5<sup>th</sup> instar the THC increased up to day 2 (12700 - 14480/mm<sup>3</sup>)of inoculation. Thereafter, a decrease was noticed from day 3 - 8 (14350 - 3812/mm<sup>3</sup>). The Differential Haemocyte Count (DHC) was different in inoculated silkworms than the control lot of silkworms. In the haemolymph of control larvae the prohaemocyte, plasmocytes and granulocytes were more in number whereas oenocytoids were less in number. The number of degenerated cells increased among treated lot up to 8<sup>th</sup> day of post inoculation.

The total protein content in the haemolymph of treated larvae during the first and second day was similar to that of control. On third day, the protein content increased among treated larvae. But from fourth day onwards, the protein content decreased (20.25 mg/ml) against control (33.73 mg/ml). The total carbohydrate content gradually increased during fifth instar in control lot larvae, whereas, it steadily increased up to 4<sup>th</sup> day and then showed a down ward trend from 5<sup>th</sup> day in treated larvae. The total lipid content in the haemolymph decreased among treated larvae from day 2, whereas in control larvae, an increasing trend was noticed.

## MATERIALS AND METHODS

All the experiments required of the study on determination of Hemocyte and Biochemical Changes of *Bombyx mori*L. Infected with *Bacillus thuringiensis*Var *Sotto* conducted in, Dept of Sericulture, Sri Krishnadevaraya University, Anantapur, Silkworms were reared as per the package practices up to the end of the experiment (Krishnaswami *et al.*, 1973). In this experiment 5<sup>th</sup> instar larvae are taken to study targeted research aspects.

### Purification of Bacteria:

The *Bacillus thuringiensis* is isolated from the worms affected from the regular rearings of the Department of Sericulture, Sri Krishnadevaraya University. The Bacterial spores and crystals are purified with 1.0M NaCl and 0.01percent Triton x-100, vortexed and washed repeatedly with sterile water by centrifugation (Johnson and McGaghey, 1984).

### Inoculation of *Bacillus thuringiensis*:

*Bacillus thuringiensis*( $1 \times 10^5$  Cells/ml.) was smeared on to the mulberry leaves and fed to 5<sup>th</sup> instar of silkworm after 24<sup>th</sup> hr of 4<sup>th</sup> moult. The treated and controlled batches were reared at optimum conditions.

### Samples collection:

Every day from 0 to 8<sup>th</sup> day 6 larvae/day were collected from each replication, the haemolymph from all the 6 larvae was collected in to three Eppendorf tubes (2 larvae haemolymph/tube) on ice and stored at 4°C. A total of 6 tubes represented 3 replication collections.

### Estimation of haemocytes count:

Every day total haemocyte count (THC) in the haemolymph of treated and control batches were estimated following the method described by Tauber and Yeager (1935) using haemocytometer. The THC per mm<sup>3</sup> of haemolymph was estimated according to the formula suggested by Jonesh (1962). Different haemocytes were identified (DHC) based on the morphological features as described by Nittono (1960). The observations were made on THC and DHC counts.

### Analysis of data:

Data recorded for THC and DHC counts, total protein, total carbohydrate and total lipid content were statistically analyzed using Completely Randomized Design (Snedecor and Cockron, 1971).

## RESULTS AND DISCUSSION

Nucleated haemocytes number varies with the insects; these haemocytes are classified as *prohaemocytes*, *plasmocytes*, *granular haemocytes*, *oenocytes* or *oenocytoid* and *spherule cells* (Arnold & Hinks, 1976). Major function of haemocytes includes phagocytosis of small particles, encapsulation of large foreign materials, haemolymph coagulation and storage and distribution of nutritive materials. Haemolymph being a circulatory fluid performs several functions (Mullins, 1985). The most prominent haemocytes in *Bombyx mori* are *prohaemocytes*, *granular haemocytes*, *plasmocytes*, *oenocytoids* and *spherule cells* (Akai, 1976). Hence it is of interest to know the role of haemocytes and their participation in immune response.

### Total Haemocyte Counts (THC)

The total hemocyte count in the control silkworm increased from 1<sup>st</sup> day to 6<sup>th</sup> day and decreased on 7<sup>th</sup> and 8<sup>th</sup> day. In the control, total hemocyte counts was 13650/mm<sup>3</sup> and increased to 17702/mm<sup>3</sup> by 6<sup>th</sup> day (Table 1). On 7<sup>th</sup> day total hemocyte count was 16878/mm<sup>3</sup> and 8<sup>th</sup> day the count was decreased to 16625/mm<sup>3</sup>. While in *Bacillus thuringiensis* treated silkworm the count was increased after inoculation up to 2<sup>nd</sup> day of infection and then there was a decrease for a period ranging from 3-8 days.

The present observations are in agreement with the earlier investigation that the number of haemocytes may increase (Balavenkatasubaiah *et al*, 2001) and decrease (Gillium and Shimanuki, 1967) to counter foreign body when infected. The cellular responses to infection have been worked out in many insects by earlier workers (Horohove and Dunn, 1983). On the basis of the above findings of the earlier workers it is evident that *Bacillus thuringiensis* induce the defense response through multiplication of haemocytes as is indicated by the increase in total haemocyte counts of the hemolymph of the worms.

### Differential Haemocyte Counts (DHC):

In the present study, in control batches haemocytes showed a gradual increasing trend during developmental period. The number of *prohaemocyte*, *plasmatocytes*, *granulocytes* and *spherulocytes* viz. 22-35, 32- 38, 35-38 and 17-28, respectively in control (Table 2). While during progressive infection, the gradual decrease in number of *prohemocyte*, *spherulocytes* and *oenocytoids* viz. 28-12, 24-9 and 13-3 respectively was noticed in *Bacillus thuringienesis* treated batch. The plasmatocytes and granulocytes increased up to 3<sup>rd</sup> day and decreasing trend was observed from 5<sup>th</sup> day onwards. The number of oenocytoid was less in both the treatments and ranged from 3 to 15. Few vermiform cells, synonyms of plasmatocyte were recorded in both treatment and control. The number of degenerated cells was comparatively less in control than the treated batch.

The number of prohaemocyte decreased due to the conversion of prohaemocyte to other types of haemocyte during course of infection and number of plasmatocytes and granulocytes increased as both are involved in defense mechanism against entry of pathogens.

**Table 1. Total haemocyte count in *Bacillus thuringienesis* (B.t) treated and healthy silkworm, *Bombyx mori*L. (mm<sup>3</sup>)**

Treatment	Days post inoculation							
	1	2	3	4	5	6	7	8
<b>BT treated</b>	12700	14480	14350	12671	9851	6203	5009	3812
<b>control</b>	13650	12530	12982	14455	16670	17702	16878	16625
<b>S.E. ±</b>	131.26	141.51	126.07	105.82	155.98	1823.12	191.76	191.23
<b>C. D. at</b>	137.18	152.26	177.43	124.28	256.75	308.45	289.45	331.87

**Table 2. Differential Haemocyte Count (DHC) in *Bacillus thuringienesis* (Bt) treated and healthy silkworm, *Bombyx mori*L.**

Treatment	Haemocyte	Days post inoculation							
		1	2	3	4	5	6	7	8

BT treated	PR	28	25	24	23	18	17	15	12
	PL	33	39	44	42	38	35	32	25
	GR	35	45	48	45	44	41	32	27
	SP	24	20	18	17	15	14	12	9
	OE	13	11	10	8	8	5	4	3
	VER	1	-	-	-	-	-	-	-
	DEG	22	24	25	26	30	37	39	42
Control	PR	22	25	27	29	30	33	34	35
	PL	32	33	35	37	39	41	37	38
	GR	35	36	38	39	42	40	38	38
	SP	17	18	20	22	25	26	27	28
	OE	5	6	8	10	10	12	13	15
	VER	-	-	-	-	4	1	-	-
	DEG	10	12	13	14	16	9	7	8
PR = Prohaemocyte		GR = Granulocyte		OE = Oenocytoid		DEG = Degenerated cell			
PL = Plasmacyte		SP = Spherulocyte		VER = Vermiform cell					

### INFERENCE

The total haemocyte count (THC) increases gradually in healthy silkworm where as in *Bacillus thuringiensis* infected silkworm larvae at initial stages THC increases there after decreases. In the healthy larvae the prohaemocyte, plasmacytes and granulocytes will be more in number whereas oenocytoids were less in number. The number of degenerated cells increases as the intensity of the disease increases. At the initial infection of *Bacillus thuringiensis*, total protein, carbohydrate and lipid contents will be improved as like healthy silkworm but, when disease enhances it shows down ward trend.

#### Biochemical changes in *Bacillus thuringiensis* treated and control batches:

##### Collection of samples

100 samples of Silkworm larvae of 5<sup>th</sup> instar were collected from the 2<sup>nd</sup> crop rearing of Silkworm, Haemolymph, fat body and mid gut from larvae were collected separately in Eppendorf tubes on ice cubes and preserved at -20°C for further use.

## Hemolymph protein sample preparation

Hemolymph taken from flachery infected and healthy larvae then phenyl-thiourea *was* added to avoid coagulation/*degradation*. Appropriate volume of hemolymph sample is taken in a 1.5 ml centrifuge tube and utilized for present study.

## Biochemical estimation

**Estimation of Protein by using Bradford's method:** The protein content present in haemolymph of silkworm larvae *Bombyx mori* was estimated according to Bradford protein assay method (Bradford, 1976) with slight modification and absorbance was measured at 595 nm. Concentration of protein sample were determined from a standard curve drawn using BSA (Bovine Serum Albumin). Protein determination method which involves the binding of Coomassie Brilliant Blue G-250 *with* protein *molecules*. The binding of the dye to protein causes a shift in the absorption maximum of the dye from 465 to 595 nm, and it is the increase in absorption at 595 nm which is monitored. Bradford reagent (1l): prepared by adding 100mg Coomassie brilliant blue G250 in 50 ml ethanol. Then 100ml 85% orthophosphoric was added and mixed properly. Volume was made 1l by adding 850ml of distilled water. This solution was then filtered through Whatman filter paper.

- 50µl of hemolymph was extracted from larvae in centrifuge tubes and these protein samples were kept in -80°C.
- As per requirement of experiment Centrifuge tubes were taken out from -80°C and it is kept in ice so that it is converted into liquid form.
- Two centrifuge tubes were taken and 10µl of each flachery infected larvae and healthy larvae protein sample were taken.
- 190µl of double distilled water was added to each centrifuge tube so that quantity becomes 200µl each.
- Another 4 centrifuge tubes were taken 2 for flachery infected larvae protein sample and 2 for healthy larvae protein sample.
- It was then filled with protein samples at the rate of 10µl and 20µl. Samples were dissolved in double distilled water at the rate of 90µl and 80µl respectively so that the content becomes 100µl each.
- Two cuvettes were taken and filled with 1ml Bradford reagent. 100µl of double distilled water was added to it. Blank sample is taken at auto zero as blank.
- Absorbance was determined at 595 (visible lamp).

## Protocol for Acid phosphatase assay:

**Materials required:** PNPP (para-nitro phenyl phosphate), 0.05M acetate buffer pH=4, flachery infected larvae and healthy larvae hemolymph sample (little amount of phenylthiourea was added to it to stop coagulation), centrifuge tubes, spectrophotometer for taking absorbance. In this experiment an artificial substrate, para-nitro phenyl phosphate was used which will be hydrolyzed by acid phosphatase, releasing p-nitrophenol and inorganic phosphate (pi). Both para-nitrophenylphosphate and p-nitrophenol are colorless at acidic pH values. Addition of 0.05M NaOH after 30 min. Incubation at 35-37°C stops the reaction due to an increase in the pH of the reaction medium. Para-nitrophenol is yellow at alkaline pH and its concentration can be measured at 405nm (Akio et. al., 1973)

### Solution preparation:

- 0.05M acetate buffer was prepared (pH4.0) from 5M stock.
- 1ml stock solution was prepared by dissolving 0.32mg PNP in 0.05M acetate buffer (pH 4.0).

### Procedure:



- The reaction mixture for the assay consisted of p-nitrophenyl-phosphate per ml and hemolymph in 0.05M acetate buffer (pH 4.0). The reaction was carried out at 35°C for 10 minutes.
- 50µl of 0.2N NaOH is added to the mixture. The reaction mixture turns yellowish in colour.
- Blank sample was taken at auto zero.
- O.D of the sample is taken at 405nm.

#### Protocol for Ascorbic acid assay:

##### Material required:

- 1 µl sodium molybdate.
- 0.5ml Na<sub>2</sub>HPO<sub>4</sub>.
- 0.5ml H<sub>2</sub>SO<sub>4</sub>.

##### Sample preparation:

- 100 µl samples were taken in a tube.
- Added 1 µl sodium molybdate, 0.5ml Na<sub>2</sub>HPO<sub>4</sub> and 0.5ml H<sub>2</sub>SO<sub>4</sub>
- Prepared blank by using above mentioned chemical
- O.D. Taken at 660nm.

**Protocol for Carbohydrate estimation:** Estimation of carbohydrate was done using Anthrone method. Hydrolysis of carbohydrates with H<sub>2</sub>SO<sub>4</sub> produces furfural, which reacts with Anthrone to form green colored complex that can be measured calorimetrically or spectrophotometer. The Anthrone reaction is the basis of a rapid and convenient method for the determination of carbohydrates either free or present in polysaccharides.

##### Material required:

- Anthrone reagent (0.2% in conc. H<sub>2</sub>SO<sub>4</sub>).
- Sample (1g/10ml).
- Spectrophotometer

##### Sample preparation:

- Taken 1g of sample in a tube and added 10ml 80% ethanol after that homogenized it properly and centrifuged it at 5000 rpm for 5 min.
- Taken the supernatant in another tube.
- From that supernatant taken 0.5ml. Supernatant and added 3ml Anthrone reagent.
- After that mixed the solution properly and given water bath for 10 min.
- The colour of the solution turns in green color.
- Blank sample was taken at auto zero.
- Take O.D at 620nm.

**Data analysis:** Data analysis was conducted using Student's t test statistical tools.

## RESULTS

### Impact of flachery infection on various morphological, behavioral and physiological parameters of silkworm *Bombyx mori* L.

Marked change in morphology, behaviour and physiology of flachery infected silkworm *Bombyx mori L.* has been observed and listed below.

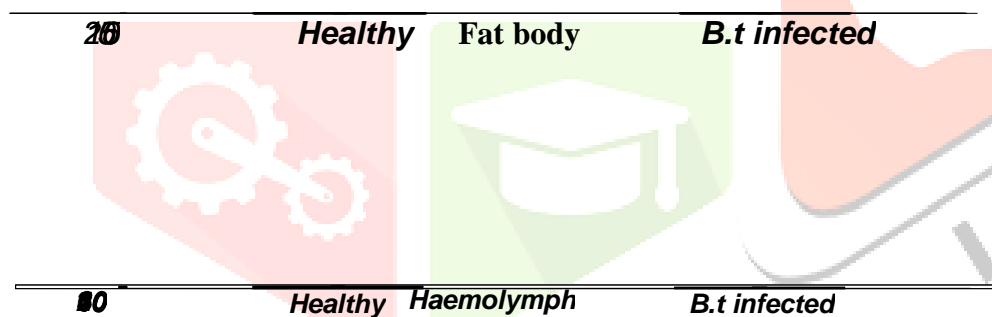
- Diseased worm loses their appetite.
- Worm show disparity in growth leading to unequal in size.
- The worm becomes sluggish and slow.
- Irregular moulting with increased moult duration in many.
- Where the infection occurs in later instar, the larvae may spin good or flimsy cocoons and die inside the cocoon, decompose and oozes black fluid with bad odour causing stains on cocoons.

**Impact of flachery infection on Protein concentration:** Results of protein concentration in flachery infected larvae are presented in Fig. 3. It is found that protein concentration in healthy larvae of silkworm fat body is less than that of larvae infected with flachery. The haemolymph protein concentration was more in flachery infected larvae than the healthy larvae. In gut protein concentration of flachery infected larvae was less than that of healthy one. *i.e.* 0.489 abs. in flachery infected larvae and 0.507 abs. in controlled larvae.

**Impact of flachery infection on Carbohydrate concentration:** The carbohydrate in gut was more in control larvae of silkworm but it is found less in flachery infected larvae *i.e.* 0.463 abs in flachery infected larvae and 0.783 abs in healthy larvae. (Fig.4)

**Impact of flachery infection on Ascorbic acid concentration:** Ascorbic acid level was more in flachery infected larvae than that of healthy larvae *i.e.* 7.938 µg/ml in flachery infected larvae and 4.704 µg/ml in control larvae. (Fig. 1 &2).

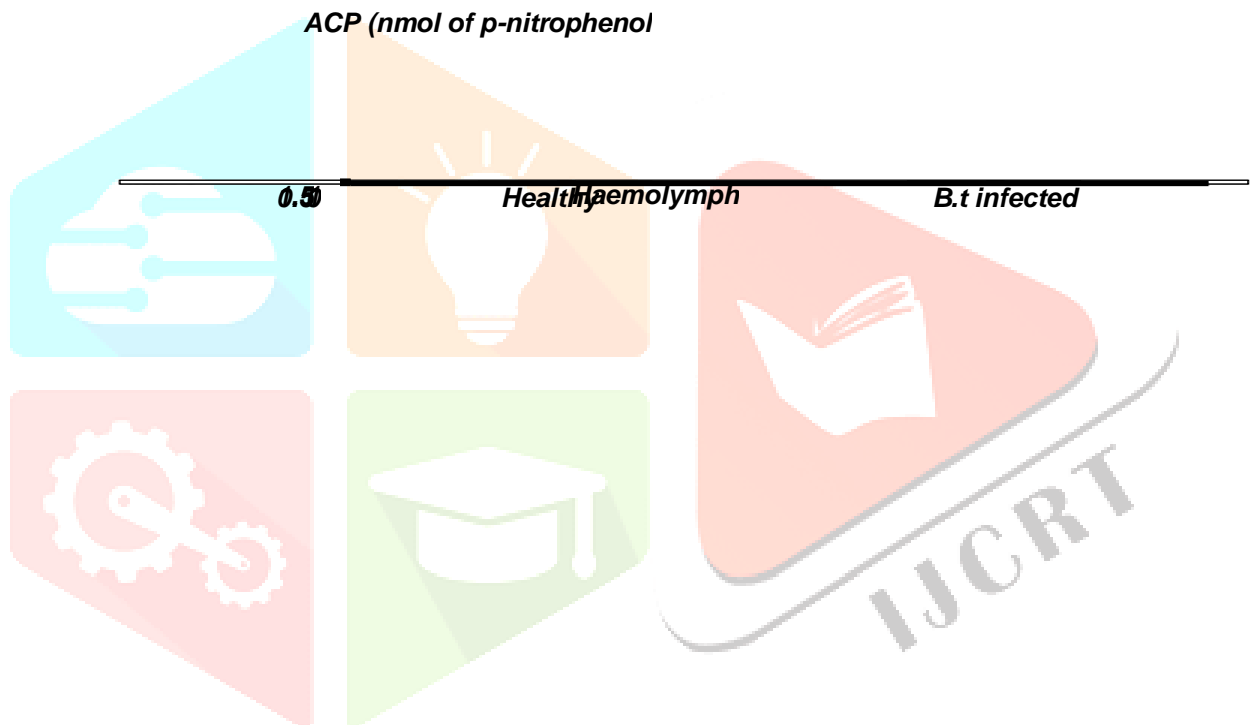
**Impact of flachery infection on Acid phosphatase:** Acid phosphatase is more in the haemolymph of flachery infected larvae than control larvae. (Fig.5)



0.0

Healthy Haemolymph

B.t infected



**Total protein content:**

The haemolymph protein in control silkworm increased gradually from 16.31 mg/ml on 1<sup>st</sup> day to 33.73 mg/ml on day 8<sup>th</sup> (Table 3). In treated silkworm, the total haemolymph protein have shown increasing trend from 1<sup>st</sup> (16.26 mg/ml) to 3<sup>rd</sup> day (24.22 mg/ml) and decreasing trend from 4<sup>th</sup> day onwards and reached 20.25 mg/ml by 8<sup>th</sup> day from the inoculation.

The results indicated that changes occurred in the haemolymph protein, during the course of Bacillus thuringiensis infection. The difference in the haemolymph protein healthy silkworm and treated silkworm becomes more pronounced as the diseases progresses. This would probably indicate that during infection the synthesis and release of proteins from fat bodies are greatly increased. There are reports of production of antimicrobial substances such as lectin, defensin and attacin with the entry of foreign bodies (Wago, 1995).



## Total carbohydrate and total lipid contents:

The haemolymph carbohydrate in control silkworm increased gradually from 6.75mg/ml on 1<sup>st</sup>day to 11.12 mg/ml on day 8<sup>th</sup> (Table 4). In inoculated silkworm, the total haemolymph carbohydrate have shown increasing trend from 1<sup>st</sup> (6.73 mg/ml) to 6<sup>th</sup> day (8.05 mg/ml) and decreasing trend from 7<sup>th</sup> day onwards and reached 7.56 mg/ml by 8<sup>th</sup> day from the treatment.

The hemolymph lipid in control silkworm increased gradually from 10.22 mg/ml on 1<sup>st</sup>day to 12.85 mg/ml on day 8<sup>th</sup> (Table 5). In inoculated silkworm, the total haemolymph lipid have shown increasing trend from 1<sup>st</sup> (10.18 mg/ml) to 4<sup>th</sup> day (10.12 mg/ml) and decreasing trend from 5<sup>th</sup> day onwards and reached 9.20 mg/ml by 8<sup>th</sup> day from the treatment.

The total carbohydrate and lipid contents increased to certain level and decreased steadily as the disease developed and it is reasonable to assume that as the disease progresses the number of pathogens increases and carbohydrates and lipids were utilized as a source of energy required for the growth and development of the pathogen *Bacillus thuringiensis*

**Table 3: Total protein content in *Bacillus thuringiensis* (B.t) treated and healthy silkworm, *Bombyx mori*L.**

Treatment	Total protein content mg/ml							
<b>Table 4: Total carbohydrate content in <i>Bacillus thuringiensis</i> (B.t) treated and healthy silkworm, <i>Bombyx mori</i>L.</b>								
Treatment	Total carbohydrate content mg/ml							
	Days post inoculation							
	1	2	3	4	5	6	7	8
BT treated	6.73	6.98	7.83	8.31	8.2	8.05	7.93	7.56
Control	6.75	7.02	7.89	8.42	9.86	10.51	10.69	11.12
S.E. ±	0.19	0.27	0.19	1.36	0.46	0.51	0.42	0.57
C. D. at 5%	1.91	1.26	1.21	1.51	1.64	1.93	2.61	2.14

**Table 5: Total lipid content in *Bacillus thuringiensis* (B.t) treated and healthy silkworm, *Bombyx mori*L.**

Treatment	Total lipid content mg/ml							
Treatment	Days post inoculation							
	1	2	3	4	5	6	7	8
	BT treated	10.18	10.59	10.25	10.12	9.88	9.73	9.51

<b>Control</b>	10.22	10.63	11.15	11.84	12.38	12.65	12.72	12.85
<b>S.E. ±</b>	0.21	0.27	0.19	1.36	0.49	0.52	0.42	0.57
<b>C. D. at 5%</b>	1.93	1.27	1.21	1.52	1.71	1.94	2.62	2.13

## DISCUSSION

With the perusal of result of the experiment, overall it is observed that in all the biochemical aspects taken for the study have shown higher values in the case of healthy samples where as in flachery infected samples lesser values were observed. The observations made in present investigation are more or less similar to the earlier studies on biochemical aspect reported by various workers in pathogen infected silkworm. Pallavi (2009) reported that the bio-chemical values in tasar silkworm were recorded higher in the case of pupal stage and followed by moth and larval stages respectively. It is may be due to stage, importance of purpose of stage and activities render at particular stage. That means, among the four stages of the silkworm only feeding stage is larval stage during this period the digestive track is prominent and it is very active during this period and it needs more energy to perform various activities. So, it may be cases for recording comparatively lesser values than the other stages. In the pupal stage, the energy will be stored in the form of fats. It is said to be resting stage and requires less energy to perform life activities. So, above mentioned points may reasons for recording comparatively higher values.

Changes in the level of carbohydrates in hemolymph and midgut epithelium of *B. mori*, during the CPV infection were observed by Kadoya *et al.* (1984). Jacob (1972) reported that significant decrease in the concentration of glycogen in NPV infected larvae but in the silkworm pupae, decreased glycogen content was observed by Kobayashi and Kawase, (1981). Yaginuma *et al.* (1990) reported changes in the activity of carbohydrates in the midgut epithelium of the silkworm affected with CPV. It is reported that the levels of glycogen decreases in the fat body during starvation in *B. mori*(Horie, 1961; Saito, 1963).

Hypoproteinemia, decreased carbohydrate content unto the 5th day of infection and decreased lipid content are vivid in haemolymph (Sarma *et al.*, 1994). The glycogen content in the tissues of *B. mori* was utilized for viral multiplication along with serving as fuel reserve for the growing energy demand of the host during combating virus infection (Gururaj, 1996). Increased lipid content was observed in 5th day of infected larvae in haemolymph by Sarma *et al.* (1994). Haemocoel volume increase up to 1.5 to 2.0 times and increased in lipid content were reported by Govindan *et al.* (1998).

## CONCLUSIONS

As per the present study impact of flachery infection on morphology and physiology of silkworm *Bombyx mori*L. has been evaluated. Although based on initial information it is too early to conclude much, however, observation taken during the study is being mentioned below:

- Present study indicates that the more protein concentration, carbohydrates and Ascorbic acid in Flachery infected larvae and in contrast to control larvae.
- In addition, marked change in morphology and physiology of Flachery infected silkworm has been observed.
- The noticeable changes in various bio-molecular parameters were observed (Figs. 1-5).
- It is assumed that change in bio-molecular profile of silkworm is responsible for change in morphology and behavior leading to overall impact on fitness of silkworm *Bombyx mori*L.

