The present investigation was conducted in the Laboratory of Cell Biology, Department of Zoology, Gauhati University, Guwahati-14, Assam, India. The material used in this investigation, Muga Silkworm, *Antheraea assama* Westwood, belongs to the

- **Phylum** - Arthropoda
- **Class** - Insecta
- **Sub-Class** - Pterygota
- **Order** - Lepidoptera
- **Family** - Saturniidae
- **Genus** - Antheraea

To be familiar with the nature and culture of muga silkworm and to have the experimental sample from the same environment, the worm was reared continuously during the study period. Details of the rearing procedure was as follows:

**Seed Collection:**

Disease free seeds or layings (DFLs) i.e. eggs of muga silkworm were collected from two sources viz. - Regional Muga Research Station, Boko, Assam and Govt. Basic Muga Seed Farm, Khanapara, Assam.

**Rearing Procedure:**

The collected seed were reared using two rearing methods, 1) Outdoor rearing with the traditional
technique and 11) Indoor rearing in the Laboratory using indoor rearing technique. Only four seasonal crops viz. Jethua (Late Spring) Bhodia (Summer), Katia (Autumn) and Jarua (Winter) were considered for observation. Other two crops, Chotua (Spring) and Aherua (Early Summer) were avoided as it was not possible to maintain all the stock during the experimental period. Nature and period of rearing are Shown in the table 1.

<table>
<thead>
<tr>
<th>Name of the Crop</th>
<th>Rearing Temperature (°C)</th>
<th>Relative Humidity (%)</th>
<th>Average Larval Rainfall (MM)</th>
<th>Remark</th>
<th>Span (days)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Jethua</td>
<td>May-June 30.64 25.04</td>
<td>84.56</td>
<td>1299.80</td>
<td>Second best commercial crop</td>
<td>23-33</td>
</tr>
<tr>
<td></td>
<td>(Late +3.12 +1.92 +3.21)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Bhodia</td>
<td>August 31.14 26.7</td>
<td>86.05</td>
<td>844.20</td>
<td>Most important Seed crop</td>
<td>25-31</td>
</tr>
<tr>
<td></td>
<td>September +2.49 +1.23</td>
<td>+4.43</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Katia</td>
<td>October 27.47 21.04</td>
<td>81.91</td>
<td>275.15</td>
<td>Best commercial crop</td>
<td>26-32</td>
</tr>
<tr>
<td></td>
<td>November +5.23 +3.42</td>
<td>+4.91</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Jarua</td>
<td>December 23.36 12.86</td>
<td>78.02</td>
<td>37.6</td>
<td>Important Seed Crop</td>
<td>50.55</td>
</tr>
<tr>
<td></td>
<td>January, February</td>
<td>+1.42 +0.45 +5.04</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Table - 1. Rearing nature of Muga Silkworm with different climatic factors. (Reared during the years 1988-1991).
1. Outdoor rearing:

Disease free seeds were allowed to hatch and brushed in the medium sized Som trees at the Botanical Garden, Department of Biotechnology, Gauhati University. The plant on which the larvae were reared, was protected by a covering net. Thus the larvae were reared up to the last larval instar under natural condition at the rearing season mentioned above.

ii. Indoor rearing:

The muga worm were reared indoor in the Cell Biology Laboratory. The worms were reared in insect cages prepared with wooden support, covered by iron net on three sides and one side having a full glass door. Adjacent to the hatching area, where healthy seeds were allowed to hatch, leaves of Som were kept. The leaves along with small branches were kept dipped in water in conical flasks to keep the leaves fresh and to maintain a moisture rich environment in which the larvae could grow. It was noticed that the newly hatched larva preferred to consume tender leaves. Thus the larvae were allowed to complete their larval period.

Morphological Observation:

Behavioral and morphological changes were observed and recorded at different seasons throughout the study.
Plate-II Rearing of *A. assama* Ww.

a) Outdoor.

b) Indoor.
period. A comparison was made between the larvae reared outdoor and indoor and an insignificant variation was observed in terms of weight and other biochemical parameters. Since these differences were negligible, larvae from both the groups (indoor and outdoor) were used in different experiments without making any reference to the rearing method employed.

Larval Period:

The time period between hatching of the eggs and initiation of pupation was calculated as the larval period. According to larval growth and moult, the larval period of Muga silkworm could be divided into 5 stages or instars viz. 1st, 2nd, 3rd, 4th and 5th. The larval period in different seasons were recorded as shown in table 1.

Body Weight:

Before weighing, healthy premoult larvae of each instar were allowed to defecate normally. These larvae were then wrapped with blotting paper to remove excess fluid adhering to the body surface and weighed on an electric balance (Mettler, CH-8606, Switzerland) having sensitivity to 0.01 mg. Larval body weight, were also determined on a dry weight basis by drying the larvae at 80°C to constant weights and were expressed in percentage of the total weight.
Table 2. Larval weight (Live, in gm) in 5 different developmental stages of *A. assama* of four seasonal crops with the result of 'T' test, values are mean ± S.E. of 10 observations.

<table>
<thead>
<tr>
<th>Seasons</th>
<th>1st instar</th>
<th>2nd instar</th>
<th>3rd instar</th>
<th>4th instar</th>
<th>5th instar</th>
</tr>
</thead>
<tbody>
<tr>
<td>Late Spring (Jethua)</td>
<td>0.007a</td>
<td>0.266a</td>
<td>3.021a</td>
<td>8.466a</td>
<td>21.876a</td>
</tr>
<tr>
<td></td>
<td>±0.0005</td>
<td>±0.001</td>
<td>±0.001</td>
<td>±0.002</td>
<td>±0.093</td>
</tr>
<tr>
<td>Summer (Bhodia)</td>
<td>0.009b</td>
<td>0.260ab</td>
<td>3.425b</td>
<td>9.594b</td>
<td>22.425b</td>
</tr>
<tr>
<td></td>
<td>±0.0005</td>
<td>±0.003</td>
<td>±0.002</td>
<td>±0.003</td>
<td>±0.045</td>
</tr>
<tr>
<td>Autumn (Katiia)</td>
<td>0.009bc</td>
<td>0.274c</td>
<td>3.942c</td>
<td>10.54c</td>
<td>25.876c</td>
</tr>
<tr>
<td></td>
<td>±0.0005</td>
<td>±0.003</td>
<td>±0.001</td>
<td>±0.009</td>
<td>±0.034</td>
</tr>
<tr>
<td>Winter (Jarua)</td>
<td>0.007ad</td>
<td>0.244d</td>
<td>2.957d</td>
<td>7.425d</td>
<td>19.143d</td>
</tr>
<tr>
<td></td>
<td>±0.0004</td>
<td>±0.003</td>
<td>±0.003</td>
<td>±0.008</td>
<td>±0.065</td>
</tr>
</tbody>
</table>

N.B. Larval weight of each instar in different seasonal crop with at least one superscript in common do not differ significantly (P < 0.05).
Dry body weight and Water Content:

The dry body weight and water content of 5th instar larvae were also determined by drying the larvae at 80°C to constant weight and were expressed in percentages of the total body weight. Data are presented in the table.

Selection of host plant varieties:

The muga silkworm is polyphagus feeding on Som, Soalu, Dighalati, Mezankari, Bogori, Kathalua etc. The Som (Machilus bombycina, King) and Soalu (Litsaea polyantha, Juss.) are the two primary host plants and the rest are secondary host plants. It has been reported that larvae reared on Som leaves yield cocoons with more silk content whereas those reared on Soalu leaves stimulate oviposition (Choudhury, 1981; Thangavelu et al., 1984). All the food plants of Muga silkworm belong to the family Lauraceae.

Leaves of Som and Soalu were collected from the field maintained by Applied Botany and Biotechnology Dept. of Gauhati University (Planted by the investigators of the Muga Research Project). The details are as follows:

a) Som (Machilus bombycina, King):

The plant belonging to the family Lauraceae, Sub-class Apetalae or dicotiledons are naturally distributed in
Plate-III Primary Host Plants. (with leaves).

a) Som (Machilus bombycina K)

b) Soalu (Litsaea polyantha)
the lower Himalayas extending from Almora to as far as Nepal. Prevalent throughout North Eastern India upto an altitude of 1,500 meter MSL.

It is a middle sized, erect evergreen species, attaining a height of about 24 meters. The bark is rough and grey, branches spreading rather slender. Young shoots are covered with simple straight hairs. Leaf size and shape are variable in breadth, thickness and are alternate. The upper surface is almost hairless, lower surface slightly silky and hairy along the midrib, exstipulate. Lateral nerves are 6 to 10 pairs. The tip is almost tapering and buds are covered by scales. Flower is hypogynous, bisexual and regular, perianth tube is imperfectly developed, 6 segments in two whorls, slightly united at the base, outer surface silky and hairy, inside villus and persistent.

b) Soalu (Litsaea polyantha, Juss):

The plant belonging to the family Lauraceae, sub-class Apetalae or Dicotyledons is distributed from Punjab in the north upto the salt range along the foot hills of Himalayas ascending to 3000 metres MSL, eastwards to North Eastern India and South Wards to Satpura range. Prevalent throughout Assam, Meghalaya, Mizoram and Nagaland.

'Soalu' is a middle sized, erect, ever green tree attaining a height of about 20 meters. The bark is
brown and rough. Branches are stout, covered with small, simple and straight hair. Leaves are alternate, pubescent and beneath with prominent nerves; size variable, breadth being 2.54 to 22.86 cm, length 7.62 to 15.24 cm, oblong, ovate, apex obtuse rounded and the base in most cases acute rounded. Flower hypogynous, unisexual, perianth tube well shaped, united at the base covered with dense small hairs, 4-6 partitite, membranous.

Preparation of Samples:

Fresh leaves of all age and size from experimental plants were collected separately in late Spring (May-June), Summer (August-Sept.), Autumn (Oct.-Nov.) and Winter (Dec.-Jan.-Feb.). The leaves were properly cleaned, steamed for about one minute, (Dried under) fan to remove surface water and used for different estimation.

Moisture Content:

The moisture content was determined by using the method of AOAC (1970). For this purpose leaf sample (10g) were accurately weighed in aluminium moisture boxes and dried in an oven at 100°C ± 2°C for 16 hrs., cooled in a desiccatior and re-weighed. The experiment was repeated for 5 times and the mean value was recorded for interpretation of results. Results are expressed in (%) of dry weight basis.
Protein Content:

The total water soluble protein of all the leave samples were determined by the method of Lowry et al., (1951). 5% homogenate of fresh leaves were prepared and 1 ml was taken for working sample preparation. Details of the Method is discussed in pages 41-44. Results were expressed in g percent of wet weight basis.

Wet Digestion of leaves for detection of trace elements by Atomic Absorption Spectrophotometer (AAS).

1 gm of ground dried plant sample were taken in a small beaker. 10 ml of Conc. \( \text{H}_2\text{SO}_4 \) was added to it and allowed to stand overnight. The samples were then heated carefully on a hot plate until the production of red \( \text{CO}_2 \) fumes had cleared. The beaker was then cooled down and a small amount of (2-4 ml) 70% \( \text{HClO}_4 \) added. Heated again and allowed to evaporate to a small volume. The samples were then transferred to a 50 ml flask and diluted up to the volume with distilled water.

The elements were detected by Perkin Elmer 3280 AAS against known standard reference lamp and expressed in \( \mu \text{g} \) per gm of dried leaf.

Calculation:

\[
\mu \text{g/gm of sample} = \frac{\text{AAS reading} \times \text{volume taken}}{\text{wt. of sample}}
\]
Biochemical analysis of haemolymph:

Belonging to the holometabola, the muga silkworm undergoes the complex process of metamorphosis. After hatching, they grow by a series of molts, shedding the old cuticular skin (exuvia), expanding into a new and larger ones. Therefore, the chemical components of the body of larvae are affected very much by moulting. Consequently, to avoid the discrepancy of comparison of such chemical components of developing stages, all the experiments were made among those of the premolting larval instar.

During this investigation, the first stage of larval development (1st instar) was generally avoided due to their small size and negligible quantity of obtainable haemolymph. Rest of the larval stages (2nd, 3rd, 4th and 5th instar) were taken for biochemical estimation.

Collection of haemolymph Sample:

Haemolymph, collected from the larvae of different stages by piercing the proleg was taken in small graduated centrifuge tube and centrifuged at 1000 rpm for 5 minutes to remove the haemocytes. The tubes were then kept in an ice bath and the haemolymph samples were used for the following estimations.
Quantitative estimation of total proteins in larval haemolymph

The total concentration of haemolymph protein at different larval stages of A. assama was estimated following the method described by Lowry et al., (1951).

Sample preparation:

50 μl of haemolymph sample was added to 950 μl of distilled water and then precipitated by 10% and 5% TCA sequentially inside an ice bucket. The content was centrifuged at 4000 rpm for 10 minutes. The supernatant was discarded and the precipitate thus obtained was successively centrifuged by ethanol followed by ether; ethanol (1:3) at the same rpm. The resultant supernatants were discarded and the ultimate amount of precipitate was dissolved in 1 ml of 0.1 N NaOH. 50 μl from this extracted haemolymph was taken in a test tube and was made 1 ml adding redistilled water. This was treated as working unknown sample.

The following reagents were used for the experiment:

Reagent A - 1% Cupric sulphate
Reagent B - 2% Na-K-tarterate
Reagent C - 0.4" NaOH in 2% NaCO₃ solution.
Reagent D - 1 ml A + 1 ml B+98 ml C(Prepared immediately before use).
Reagent E - 1 N Folin Ciocalteau reagent.
Moreover, 0.1% Bovine serum albumin (BSA, from sigma, USA) was used as standard.

Five solutions of different strengths (50, 100, 150, 200, 250 μl) from standard BSA were prepared and each made to 1 ml by adding double distilled water (Table 3). To each tube of standard, working unknown sample and blank (1 ml water), 5 ml of reagent-D was added and mixed thoroughly on a vortex mixture. After 15 minutes 0.5 ml Folin's Ciocalteau reagent was added to each tube. After 30 minutes, the optical density (OD) of both standard and unknown haemolymph samples were taken against the water blank. The OD was determined in a systronic Spectro Colorimeter using a red filter.

Calculation:

A calibration curve was prepared with the values of OD against various concentrations of standard proteins (Table 4). From this curve, (Fig.3) the actual concentration of protein was calculated using the experimental OD values. The results were expressed in mg/ml of haemolymph.
### Table 3 - Preparation of working standard solution of Protein.

<table>
<thead>
<tr>
<th>Tube no.</th>
<th>Volume of Standard stock pollution (µl)</th>
<th>Volume of Water (µl)</th>
<th>Total Volume (ml)</th>
<th>Concentration of standard (µg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>50</td>
<td>950</td>
<td>1</td>
<td>50</td>
</tr>
<tr>
<td>2</td>
<td>100</td>
<td>900</td>
<td>1</td>
<td>100</td>
</tr>
<tr>
<td>3</td>
<td>150</td>
<td>850</td>
<td>1</td>
<td>150</td>
</tr>
<tr>
<td>4</td>
<td>200</td>
<td>800</td>
<td>1</td>
<td>200</td>
</tr>
<tr>
<td>5</td>
<td>250</td>
<td>750</td>
<td>1</td>
<td>250</td>
</tr>
<tr>
<td>6</td>
<td>300</td>
<td>700</td>
<td>1</td>
<td>300</td>
</tr>
</tbody>
</table>

### Table 4 - OD of standard protein.

<table>
<thead>
<tr>
<th>Tube no.</th>
<th>1</th>
<th>2</th>
<th>3</th>
<th>4</th>
<th>5</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>0.250</td>
<td>0.450</td>
<td>0.680</td>
<td>0.812</td>
<td>0.960</td>
</tr>
<tr>
<td>2</td>
<td>0.245</td>
<td>0.450</td>
<td>0.760</td>
<td>0.830</td>
<td>0.960</td>
</tr>
<tr>
<td>3</td>
<td>0.260</td>
<td>0.440</td>
<td>0.680</td>
<td>0.810</td>
<td>0.950</td>
</tr>
<tr>
<td>4</td>
<td>0.250</td>
<td>0.450</td>
<td>0.690</td>
<td>0.805</td>
<td>0.970</td>
</tr>
<tr>
<td>5</td>
<td>0.245</td>
<td>0.460</td>
<td>0.680</td>
<td>0.805</td>
<td>0.960</td>
</tr>
<tr>
<td>Mean</td>
<td>0.250</td>
<td>0.450</td>
<td>0.680</td>
<td>0.812</td>
<td>0.960</td>
</tr>
<tr>
<td>S.D.</td>
<td>±0.006</td>
<td>±0.006</td>
<td>±0.007</td>
<td>±0.010</td>
<td>±0.007</td>
</tr>
</tbody>
</table>

*Table 4 - OD of standard protein.*
Fig. 3. Calibration curve for protein
Electrophoretic (Qualitative) analysis of haemolymph Protein:

Separation of haemolymph protein fractions of the larva of developing insects of all species have been conducted adopting Disc electrophoretic technique. A standard model polyacrylamide gel electrophoresis (PAGE) apparatus (Quick fit-inc) was used and the original method of Davis (1964) was followed. Disc electrophoresis method was slightly modified as described below.

The 6 cm long and 7 mm diameter gel tubes were prepared in 0.2 ml glass tubes. A 7% acrylamide gel (as running gel) and a 3% spacer gel were used. The tris-glycine buffer (tank buffer) was used with a pH value of 8.3.

Ten μl of haemolymph sample was diluted with 990 μl of distilled water and centrifuged at 1000 rpm to remove haemocytes. Before loading to the gel tubes, the already prepared haemolymph sample was mixed with 40% sucrose solution and 0.2% solution of bromophenol blue, as a tracking dye. A pre-electrophoretic run was made applying 1.5 mA current to each tube for 15 minutes to remove ions from the gel tubes. After loading the samples, power supply was maintained at 4 mA per tube at 5°C. Power supply was put off when the tracking dye was about 1 cm from the end of the gel. (approximately 50 minutes in this experiment). The proteins in the gels were then first fixed in 12.5% TCA solution for 1-2 hours followed by a rapid wash with distilled water and
stained with 0.25\% commassie brilliant blue for 2 hours. Stained gels were destained with a mixture of methanol : glacial acetic acid : distilled water (5:1:5). After 24 hours of destaining with several changes, all the excess stain from the gels were removed. The gels were then preserved in 7\% acetic acid solution.

The stored gels were scanned at 650 nm in a microdensitometer attached to a Backman Spectrophotometer Acta III. For better comparison, the detail band pattern obtained from the observation was prepared diagramatically (by hand drawing) and photographically.

**Estimation of Enzyme Activity:**

Two transaminases namely Alanine amino transferase (ALAT) and Aspartate aminotransferase (AAT) were estimated following the method of Reitman and Frankel (1957). The activity of Glucose-6-phosphatase (G-6-pase) was determined following the method of Hers (1964, 1966) by estimating the liberated inorganic phosphate by the method of Fiske & Subba Row (1925). The Alkaline phosphatase (ALP) activity was estimated by the method of Bodansky (1933). Substrates and other conditions followed to estimate those enzymes activity are shown in the Table 6.
<table>
<thead>
<tr>
<th>Enzymes</th>
<th>Substrates</th>
<th>Incubation time (Min.)</th>
<th>Temp°C</th>
<th>pH</th>
<th>Methods</th>
<th>Unit</th>
</tr>
</thead>
<tbody>
<tr>
<td>Aspartate aminotransferase (AAT)</td>
<td>dl-aspartate + L-Ketoglutarate</td>
<td>60</td>
<td>37</td>
<td>7.4</td>
<td>Reitman &amp; Frankel (1957)</td>
<td>Units/ml</td>
</tr>
<tr>
<td>Alanine aminotransferase (ALAT)</td>
<td>dl-alanine + L-Ketoglutarate</td>
<td>30</td>
<td>37</td>
<td>7.4</td>
<td>Do</td>
<td>Units/ml</td>
</tr>
<tr>
<td>Alkaline phosphatase (ALP)</td>
<td>Sodium - B-glycerophosphate</td>
<td>60</td>
<td>37</td>
<td>8.6</td>
<td>Bodansky's Unit (B.U.)</td>
<td>Bodansky (1933)</td>
</tr>
<tr>
<td>Glucose-6-Phosphatase (G-6 pase)</td>
<td>Glucose-6-phosphate disodium salt + EDTA</td>
<td>60</td>
<td>37</td>
<td>6.5</td>
<td>Hers method ml/h (1964)</td>
<td>mg phosphate ml/h</td>
</tr>
</tbody>
</table>

Table 5. Substrates and other conditions of determination of enzyme activity.

The methods used in this experiment are as follows -

Reagents Preparation for Transaminase (AAT and ALAT)

1. 0.1 M Phosphate buffer, pH 7.4.
a) 420 ml of 0.1 M disodium hydrogen phosphate was mixed (Na₂HPO₄ 7.1 gm was dissolved in 500 ml Dist. water) with 80 ml of 0.1 M potassium dihydrogen phosphate (6.8 potassium dihydrogen phosphate was dissolved in 500 ml dist. water).

2. Pyruvate, 2 mM/L (for standard curve).
   a) 22 mg sodium pyruvate was dissolved in 100 ml phosphate buffer.

3. L-Ketoglutarate, 2 mM/L dl-aspartate 200 mM/L (for AAT substrate).
   a) 29.2 mg L-Ketoglutaric acid and 2.66 gm dl-aspartic acid was placed in a small beaker.
   b) 1 N sodium hydroxide was added until solution complete.
   c) The pH was adjusted at 7.4 with sodium hydroxide and transferred quantitatively with buffer to a 100 ml volumetric flask and diluted to the mark with buffer solution.

4. L-Ketoglutarate, 2 mM/L dl-alanine, 200 mM/L (for ALAT substrate).
   a) 29.2 mg L-Ketoglutaric acid and 1.78 gm dl-alanine were kept in a small beaker.
   b) 1 N sodium hydroxide was added until the solution is complete.
   c) The pH was adjusted at 7.4 with sodium hydroxide and transferred quantitatively with buffer solution to
5. 2.4 Dinitrophenylhydrazine, 1 m M/L.

a) 19.8 mg 2.4 dinitrophenylhydrazine was dissolved in 100 ml 1 N hydrochloric acid.

6. 0.4 N sodium hydroxide solution.

a) 16 gm NaOH was dissolved in 1000 ml dist. water.

Working Procedure:

(1) 1 ml desired substrate was pippeted into a test tube and placed in 37°-40°C water bath for 5 mins. one extra tube was prepared similarly for the blank.

(2) Pippeted into the tubes 0.2 ml haemolymph, mixed by swirling, replaced in water bath and begun timing. 0.2 ml water was taken as blank.

(3) After exactly 60 min for AAT or 30 Min for ALAT, the tube was removed from the water bath and immediately added 1 ml of the 2.4 dinitrophenylhydrazine and mixed thereby stopping the reaction.

(4) As both transaminases were being run simultaneously, the ALAT specimens after the addition of hydrazine were allowed to stand at room temperature until the AAT samples reached the same stage.
(5) After a minimum of 20 min. in the presence of the hydrazine, 10 ml of 0.4 N NaOH was added & mixed by inversion.

(6) After a minimum of 5 min. all tubes were read including the blank against water at 505 nm.

The final colour developed does not obey Beer's law, hence a calibration curve is plotted using pyruvate standard and the O.D. value obtained from the sample is directly read on the calibration curve.

**Calibration curve for Aspartate amino transferase (AAT).**

5 clean test tube were taken and different concentration of standard pyruvate solution were added and followed the working procedure as shown in Table 6.

<table>
<thead>
<tr>
<th>Tube No.</th>
<th>1</th>
<th>2</th>
<th>3</th>
<th>4</th>
<th>5</th>
</tr>
</thead>
<tbody>
<tr>
<td>Enzyme Activity (units/ml)</td>
<td>0</td>
<td>24</td>
<td>61</td>
<td>114</td>
<td>190</td>
</tr>
<tr>
<td>Buffered substrate (ml)</td>
<td>0.5</td>
<td>0.45</td>
<td>0.40</td>
<td>0.35</td>
<td>0.30</td>
</tr>
<tr>
<td>Pyruvate Standard (ml)</td>
<td>-</td>
<td>0.05</td>
<td>0.10</td>
<td>0.15</td>
<td>0.20</td>
</tr>
<tr>
<td>Dist. water (ml)</td>
<td>0.10</td>
<td>0.10</td>
<td>0.10</td>
<td>0.10</td>
<td>0.10</td>
</tr>
<tr>
<td>DNPH colour Reagent (ml)</td>
<td>0.50</td>
<td>0.50</td>
<td>0.50</td>
<td>0.50</td>
<td>0.50</td>
</tr>
</tbody>
</table>

Mixed well and allowed to stand at RT for 20 mins.

| Working Sod. Hydroxide (ml) | 5.00 | 5.00 | 5.00 | 5.00 | 5.00 |

**Table - 6:** Preparation of standard solution and working procedure for calibration curve of AAT.
Mixed well and allowed to stand at room temperature for 10 minutes then the OD value of tubes 2 to 5 were taken respectively against tube 1 (Reagent blank) on photocolorimeter using green filter. Same procedure were repeated numbers of time and mean ± SD of OD calculated out as shown in Table 7.

<table>
<thead>
<tr>
<th>Tube No.</th>
<th>2</th>
<th>3</th>
<th>4</th>
<th>5</th>
</tr>
</thead>
<tbody>
<tr>
<td>O.D.</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>0.120</td>
<td>0.181</td>
<td>0.271</td>
<td>0.385</td>
</tr>
<tr>
<td>2</td>
<td>0.110</td>
<td>0.182</td>
<td>0.275</td>
<td>0.381</td>
</tr>
<tr>
<td>3</td>
<td>0.140</td>
<td>0.183</td>
<td>0.273</td>
<td>0.379</td>
</tr>
<tr>
<td>4</td>
<td>0.120</td>
<td>0.180</td>
<td>0.274</td>
<td>0.382</td>
</tr>
<tr>
<td>5</td>
<td>0.100</td>
<td>0.180</td>
<td>0.277</td>
<td>0.385</td>
</tr>
<tr>
<td>Mean</td>
<td>0.118</td>
<td>0.181</td>
<td>0.274</td>
<td>0.382</td>
</tr>
<tr>
<td>S.D.</td>
<td>0.014</td>
<td>0.002</td>
<td>0.002</td>
<td>0.002</td>
</tr>
</tbody>
</table>

Table 7. O.D. of standard solution (AAT).

A calibration curve was obtained putting the mean ± S.D. values of O.D. of tube no. 2, 3, 4 and 5 on y axis versus corresponding enzyme activity on x axis. Fig. 4.
Fig. 4 AAT calibration curve
Calibration curve for Alanine amino transferase (ALAT).

Working procedure was same as described for AAT. Being the substrate of ALAT was used here the enzyme activities were as shown in Table 8. The O.D. value was recorded and presented in the Table 9. Likewise, the calibration curve was prepared from the O.D. value putting against the standard concentration. Fig. 5.

<table>
<thead>
<tr>
<th>Tube No.</th>
<th>1</th>
<th>2</th>
<th>3</th>
<th>4</th>
<th>5</th>
</tr>
</thead>
<tbody>
<tr>
<td>Enzyme Activity (Units/ml)</td>
<td>0</td>
<td>28</td>
<td>57</td>
<td>97</td>
<td>150</td>
</tr>
<tr>
<td>Buffered substrate (ml)</td>
<td>0.5</td>
<td>0.45</td>
<td>0.40</td>
<td>0.35</td>
<td>0.30</td>
</tr>
<tr>
<td>Pyruvate standard (ml)</td>
<td>-</td>
<td>0.05</td>
<td>0.10</td>
<td>0.15</td>
<td>0.20</td>
</tr>
<tr>
<td>Dist. Water (ml)</td>
<td>0.10</td>
<td>0.10</td>
<td>0.10</td>
<td>0.10</td>
<td>0.10</td>
</tr>
<tr>
<td>DNPH colour Reagent (ml)</td>
<td>0.50</td>
<td>0.50</td>
<td>0.50</td>
<td>0.50</td>
<td>0.50</td>
</tr>
</tbody>
</table>

Mixed well and allowed to stand at RT for 20 mins.

| Working NaOH (ml) | 5.00 | 5.00 | 5.00 | 5.00 | 5.00 |

Table 8. Preparation of standard solution and working procedure for calibration curve of ALAT.
Table 9. O.D. of standard solution (ALAT).

Reagents Preparation for Alkaline Phosphatase:

1. Substrate - In a 100 ml volumetric flask 3 ml of petroleum ether (B.P. 20° to 40°C), 80 ml of redistilled water, 0.5 gm of sodium - glycerophosphate, 0.424 gm of sodium diethyl barbiturate and water to volume were added successively. The solution was transferred to a 100 ml of glass stoppered bottle containing an inch Layer of petroleum ether and kept in a freeze at 4-5°C.

2. 30% Trichloroacetic acid solution (TCA).
Fig. 5 ALAT calibration curve
3. Standard phosphate solution - 0.315 gm of analar grade dry KH$_2$PO$_4$ (E/M) was dissolved in a small volume of redistilled water in a 1 lit. volumetric flask. To this solution 10 ml of 10N H$_2$SO$_4$ was added and diluted with redistilled water to the mark and mixed well. This solution contains 0.4 mg of P/5 ml which was stable indefinitely.

4. Working standard solution - 6.25 ml of the stock standard phosphate solution (No.3) containing 0.5 mg of P was taken into 100 ml volumetric flask. To this 16.7 ml of 30% TCA solution was added and diluted to the mark with redistilled water and mixed perfectly. This solution contains 0.04 mg of P/8 ml in 5% TCA. It was kept in freeze and is stable indefinitely.

5. Molybdate II - 25 gm of analar grade ammonium molybdate (E/M) was dissolved in 200 ml redistilled water in a beaker. This solution was added to 300 ml of 10N H$_2$SO$_4$ in a 1 lit. volumetric flask and diluted to the mark. This solution is also stable at freeze for indefinite period.

6. 1-Amino-2-naphthol-4 sulphonic acid solution - 1.95 ml of 15% NaHS$_3$ solution was taken in a stoppered flask. To this solution 0.5 gm of 1,2,4 amino naphthol sulphonic acid was added and 5 ml of 20% Na$_2$SO$_3$ solution was added and shaked by adding 1 ml of Na$_2$SO$_3$ at a time till the powder was dissolved. The solution was transferred to an another flask and kept in freeze. This solution was used for 4 weeks only.

7. 10N H$_2$SO$_4$ - 450 ml of concentrated H$_2$SO$_4$ was dissolved in 1620 ml of redistilled water.
8. 5% TCA - 5 gm TCA was dissolved in 100 ml redistilled water.

Working Procedure:

Haemolymph of the larvae was collected separately and the enzyme activity was estimated according to the following procedure.

(a) Incubated sample - 9 ml of the alkaline phosphate substrate was taken in a glass stoppered cylinder and placed in the incubator kept at 37°C to attain the incubated temperature by the substrate. After few minutes of attaining the temperature 1 ml of the haemolymph was added and mixed well and incubated exactly for 1 hour. At the end of one hour the cylinder was removed and cooled in ice water for several minutes and then 2 ml of 30% TCA was added. Mixed and allowed to stand for few minutes and finally centrifuged to collect the supernatant. This supernatant was taken for analysis.

(b) Control sample - To 9 ml of the substrate in a glass stoppered cylinder added 2 ml of 30% TCA and shaked well. To this solution 1 ml of haemolymph was added, stoppered, shaked and after few minutes centrifuged to collect the supernatent and this supernatant was used for analysis.

8 ml each of the incubated and control supernatant as prepared above were taken in previously dried test
tubes. In a third test tube 8 ml of standard phosphate solution was taken containing 0.04 mg of phosphate. In a fourth similar test tube 8 ml of 5% TCA was taken and treated as blank.

The phosphate activity was then calculated from the difference between the inorganic phosphate of the incubated and control sample.

To each of these tubes, then 1 ml of molybdate II reagent was added and mixed well. Then 0.4 ml of 1,2,4 amino naphthol sulphonic acid reagent was added and immediately the solution was diluted to 10 ml with redistilled water and mixed. They were then kept for 5 minutes in order to develop the colour. The colorimetric reading was taken at 680 nm (red filter).

Calculation:

After colorimetric reading the following equation has been used to find out the mg of inorganic P/100 ml of haemolymph/hour.

\[
\frac{\text{Unknown} - \text{Blank}}{\text{Standard} - \text{Blank}} \times 0.04 \times \frac{3}{2} \times 100 = \text{mg of inorganic P/100 ml haemolymph/hour or Bodanky 's Unit (B.U.) (incubated or, control).}
\]

Preparation of standard calibration curve:

Different volumes of working phosphate solution were taken in 5 numbers of tube and volume made upto 10
ml with distilled water as shown in table 10. 8 ml of solution was taken from each tube and treated as control or incubated sample. The experiment was repeated for numbers of times and the O.D. values were recorded Table 11. A calibration curve was prepared taking the concentration of standard on X axis and the mean O.D. on Y axis and presented in Fig. 6.

<table>
<thead>
<tr>
<th>Tube No.</th>
<th>1</th>
<th>2</th>
<th>3</th>
<th>4</th>
<th>5</th>
</tr>
</thead>
<tbody>
<tr>
<td>Dilution Factors</td>
<td>2</td>
<td>4</td>
<td>6</td>
<td>8</td>
<td>10</td>
</tr>
<tr>
<td>Working Phosphate standard solution (ml)</td>
<td>8</td>
<td>6</td>
<td>4</td>
<td>2</td>
<td>0</td>
</tr>
<tr>
<td>Distilled water (ml)</td>
<td>10</td>
<td>10</td>
<td>10</td>
<td>10</td>
<td>10</td>
</tr>
<tr>
<td>Total volume</td>
<td>0.016</td>
<td>0.032</td>
<td>0.048</td>
<td>0.064</td>
<td>0.08</td>
</tr>
<tr>
<td>Concentration of 'p' present mg/10ml</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Table 10. Preparation of standard phosphate solution of different concentration.

Reagent preparation for Glucose-6-phosphatase

1. Substrate:

(1) 0.1 M Glucose-6-phosphate - pH 6.5:
G-6-phosphate disodium salt was obtained from sigma corporation,
<table>
<thead>
<tr>
<th>Concentration (P/8 ml)</th>
<th>0.013</th>
<th>0.026</th>
<th><strong>0.038</strong></th>
<th>0.051</th>
<th>0.064</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mean S.D</td>
<td>0.192</td>
<td>0.360</td>
<td>0.544</td>
<td>0.706</td>
<td>0.96</td>
</tr>
</tbody>
</table>

Table-11: Optical density of standard phosphate.
Fig. 6. Calibration curve for ALP
U.S.A. 0.188 gm was measured and dissolved in 5 ml of redistilled water. pH adjusted to 6.5.

(ii) 0.002 M EDTA solution in 0.1 M glucose-6-phosphate.

0.004 gm of EDTA (BDH analar) was dissolved in 5 ml of 0.1 M glucose-6-phosphate solution.

The solution was stored at 4°C.

2. 10% TCA solution:

10 gm of TCA was dissolved in 100 ml of distilled water.

3. Standard phosphate solution:

0.351 gm of pure monopotassium phosphate was quantitatively transferred to 1 lit. Volumetric flask and dissolved in a minimum amount of distilled water. 10 ml of 10 N H₂SO₄ was added and volume was made up to the mark with distilled H₂O. This solution contained 0.4 mg of phosphorus/5 ml and was stable indefinitely.

4. 10 N Sulphuric Acid:

450 ml conc. H₂SO₄ was carefully added to 1,300 ml of distilled H₂O and mixed.
The normality was estimated by titration against standard NaOH using phenolphthalein as indicator and adjusted accordingly to 10 N.

5. Molybdate II reagent:

25 gm of ammonium molybdate was transferred to 1 lit. volumetric flask and dissolved in about 200 ml of distilled water, 300 ml of 10 N \( \text{H}_2\text{SO}_4 \) was added and the volume was made upto the mark with distilled \( \text{H}_2\text{O} \).

6. 15% sodium bisulphite solution:

30 gm of sodium bisulphite was taken in a beaker and dissolved by stirring in 200 ml of distilled \( \text{H}_2\text{O} \). The solution was kept in a well stoppered bottle.

7. 20% sodium sulphite solution:

20 gm of sodium sulphite was dissolved in 100 ml of distilled \( \text{H}_2\text{O} \) and stored in a well stoppered bottle.

8. Amino-nepthol sulphanonic acid:

195 ml of 15% sodium bisulphite solution was taken in a glass stoppered cylinder. 0.5 gm of 1,2,4 amino-nepthol sulphanonic acid was added followed by 5 ml of 20%
sodium sulphite solution. The cylinder was stoppered and shaken till the powder dissolved. 1 or 2 ml of sodium sulphite could be added in order to facilitate solution. The solution was stored in a dark glass bottle in cold. It was stable for about 4 weeks.

9. Working phosphate standard solution :

5 ml of standard phosphate solution containing 0.4 mg of phosphorus was transferred to a 50 ml volumetric flask and made up to the mark with 10% TCA. 5 ml of this diluted standard contained 0.04 mg of phosphorus.

10. 0.1 M acetate buffer - pH 5.0 :

The buffer was prepared by mixing 3 vols. of 0.1 M acetic acid with 7 vol. of 0.1 M sodium acetate solution.

a) 0.1 M acetic acid was prepared by dissolving 0.57 ml of glacial acetic acid in 100 ml of distilled water.

b) 0.1 M sodium acetate was prepared by dissolving 1.36 gm of sodium acetate in 100 ml distilled water.

Working procedure :

Test - Two clean dry test tubes were taken and .1 ml of 0.002 M EDTA in 0.1 M Glucose-6-phosphate solution was
placed in each tube, 0.1 ml of haemolymph sample was added to one of the tubes, mixed and incubated at 37°C for 1 hour. At the end of this time the reaction was stopped by addition of 9.8 ml of 10% TCA and the tube was kept in ice. In the 2nd tube, 0.1 ml of tissue homogenate was added and placed in the ice bath after immediate addition of 9.8 ml of 10% TCA.

Both the tubes were centrifuged, rpm, and phosphate content of the supernatant fluid was determined following the procedure described below. The difference in phosphate concentration between the two is the amount of phosphate released during 1 hour incubation at 37°C.

Control: A control experiment was run simultaneously. 0.1 ml of the haemolymph sample was mixed with 0.01 ml of 0.1 M acetate buffer and incubated at 37°C for 5 minutes. 0.1 ml of 0.002 M EDTA solution in 0.1 ml Glucose-6-phosphate was added and the mixture was incubated for 1 hour. Reaction was stopped by the addition of 9.8 ml of 10% TCA and the tube was placed in an ice bath. Another tube was similarly prepared and without incubation after addition of 0.002 M EDTA in G-6-phosphate was added and the mixture released during the 1 hour incubation was estimated following the same procedure described below.

The actual phosphate released due to glucose-6-phosphatase activity was the difference between phosphate released in the test and phosphate released in the control.
runs. The Glucose-6-phosphatase activity is calculated in terms of micromols of phosphate released per ... per ml of haemolymph.

Estimation of inorganic phosphate (Fiske & Subba Row, 1925).

5 ml of the supernatant was transferred to a stoppered graduated cylinder, 1 ml of molybdate II reagent was added and mixed. 0.4 ml of amino-nepthol sulphonlic acid was added, mixed again and the solution was diluted to 10 ml with distilled water. The colour was allowed to develop for 5 minutes.

A blank was similarly prepared treating 5 ml of 10% TCA, 5 ml of the working phosphate standard was taken in a 3rd tube and treated in an identical manner.

The tubes were then read in a colorimeter set to zero density with the blank, using red filter.

Calculation: Since 5 ml of the solution taken represent only 0.05 ml of the haemolymph the amount of phosphate present in 1 ml of haemolymph may be calculated as follows -

\[
\text{Optical density of Unknown} \times 0.04 \times 20.
\]

\[
= \text{mg of phosphorus/ml/hour.}
\]

Preparation of Standard Calibration Curve:

Different volumes of the working phosphate
solution were taken in 5 numbers of tubes and volume made upto 10 ml as shown in table 12 with distilled water. 5 ml of solution was taken from each tube and the inorganic phosphorus was estimated as described earlier. The experiment is repeated number of times using same method as shown in the table 13.

<table>
<thead>
<tr>
<th>Tube No.</th>
<th>1</th>
<th>2</th>
<th>3</th>
<th>4</th>
<th>5</th>
</tr>
</thead>
<tbody>
<tr>
<td>Dilution Factors</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Working phosphate standard solution (ml)</td>
<td>2</td>
<td>4</td>
<td>6</td>
<td>8</td>
<td>10</td>
</tr>
<tr>
<td>Distilled water (ml)</td>
<td>8</td>
<td>6</td>
<td>4</td>
<td>2</td>
<td>0</td>
</tr>
<tr>
<td>Total Volume</td>
<td>10</td>
<td>10</td>
<td>10</td>
<td>10</td>
<td>10</td>
</tr>
<tr>
<td>Concentration of 'p' present mg/10ml</td>
<td>0.016</td>
<td>0.032</td>
<td>0.048</td>
<td>0.064</td>
<td>0.08</td>
</tr>
</tbody>
</table>

Table 12. Preparation of Standard Solution of different concentrations.
Table 13. Optical Density of Standard phosphate.

<table>
<thead>
<tr>
<th>Concentration of ml(mg)</th>
<th>0.008</th>
<th>0.016</th>
<th>0.024</th>
<th>0.032</th>
<th>0.04</th>
</tr>
</thead>
<tbody>
<tr>
<td>no. of observation</td>
<td>1</td>
<td>2</td>
<td>3</td>
<td>4</td>
<td>5</td>
</tr>
<tr>
<td></td>
<td>0.11</td>
<td>0.10</td>
<td>0.10</td>
<td>0.09</td>
<td>0.11</td>
</tr>
<tr>
<td></td>
<td>0.20</td>
<td>0.18</td>
<td>0.21</td>
<td>0.19</td>
<td>0.20</td>
</tr>
<tr>
<td></td>
<td>0.30</td>
<td>0.33</td>
<td>0.32</td>
<td>0.30</td>
<td>0.32</td>
</tr>
<tr>
<td></td>
<td>0.42</td>
<td>0.40</td>
<td>0.40</td>
<td>0.38</td>
<td>0.42</td>
</tr>
<tr>
<td></td>
<td>0.54</td>
<td>0.52</td>
<td>0.50</td>
<td>0.50</td>
<td>0.52</td>
</tr>
<tr>
<td>Mean</td>
<td>0.102</td>
<td>0.196</td>
<td>0.314</td>
<td>0.404</td>
<td>0.516</td>
</tr>
<tr>
<td>S.D.</td>
<td>0.007</td>
<td>0.010</td>
<td>0.012</td>
<td>0.015</td>
<td>0.015</td>
</tr>
</tbody>
</table>

A calibration curve is plotted taking mean O.D. values in Y axis against concentration in X axis. (Fig. 7).

Haemolymph sample preparation for Atomic Absorption Spectrophotometry (AAS).

The haemolymph samples were analysed directly without pretreatment. Collected haemolymph centrifuged first in 1000 rpm for 10 minutes to remove the haemocytes. Then it was diluted with 8% butanol. 8% butanol was prepared adding deionised water and checked for its mineral contamination.
Fig. 7. Calibration curve for G-6-P
Haemolymph equivalent solution is prepared by 0.9 gm NaCl and 0.29 gm KCL in 100 ml deionised water and is used as blank.

**Method for light microscopic study of Haemocytes:**

**Blood film preparation** - Heat fixation was used by plunging the insect into water held at approximately 60°C for a short period (10 min.). Then the slide was prepared with this heat fixed blood in a simple process of placing a drop of haemolymph on the slide and touching with the edge of a cover slip, which is then drawn along the slide with the drop trailing behind. The resulting blood film was air-dried (preferably on a slide warmer at about 32°C) before staining.

**Full giemsa staining** - The air dried films then immersed in Giemsa solution (1:1) for 30 minutes. Thereafter rinsed and immersed in distilled water to which a drop of lithium carbonate were added to differentiated red staining structures.

It was rinsed and immersed in dist. water again, and a few drops of Conc. HCL was added to differentiate blue staining structures. After rinsing with distilled water, the slides were allowed to dry and mounted in D.P.X. and observed under light microscope.

**Haemocyte sample preparation for Scanning Electron Microscopy (SEM):**

A drop of haemolymph were fixed in 0.1 M 2%
glutaraldehyde buffered with sodium cacodylate. Fixation was completed in 30 minutes and centrifuged for 5 minutes at 1500 rpm and washed with glass distilled water. Resuspended again in distilled water and centrifuged. The resuspended haemolymph cells were allowed to decant and a thin film of cell was applied on a clean coverslip. The sample was then air dried. A piece of coverslip filmed with cells was secured to brass stub (10 mm in diameter, 32 mm high) with double sided sticky tape. Care was taken to avoid any trapped air inside the adhesive. Conductive coating was applied to the sample using gold as a target metal in a JFC - 1100 (Jeol) ion sputter coater and samples were viewed with JSM - 35 (Jeol) scanning electron microscope (SEM) operated at 15 KV. W.D. selector was used to study the samples both at 15 mm and 39 mm working distance.

Total haemocyte count (THC):

Total haemocyte counts were made adopting the method of (Rosenburger & Jones, 1960). Healthy fifth instar larva of A. assama were put into hot water (56°- 58°C) for 3-4 minutes to heat fix the haemolymph. Some 3 or 4 drops of haemolymph were allowed to flow on a glass slide. A portion of the blood was drowned into a Thoma white blood cell pipette, diluted with physiological saline solution in a ratio of 1:20. Then counted in a haemocytometer.

THC are counted in a standard haemocytometer as follows:
Differential Haemocyte count (DHC):

The procedure of Arnold (1982) was followed for collection of blood samples and for blood film preparation. Measured quantity of blood was drawn out to a micropipette for each blood film preparation on the glass slide. This quantity was kept constant for each slide. A thin film was prepared by gently drawing the blood to the other end of the slide with the help of another clean slide. The slide was then air dried at a temperature of about 25°C for minimum 8 hours. The dried slide was then stained with Wright's stain by following the standard method. The differential counting of haemocytes was made (mm$^3$) under the microscope. The different types of cells with their cell shapes were counted under light microscope.

Haemolymph volume (HV):

The blood volume (B.V) or haemolymph volume (H.V) were determined following the dye method used by Yeager & Munson (1950) and modified by Lee (1961).

Each larva was weighed and injected with a 2% aqueous amaranth solution. The volume injected was equal to 5% of
the insect's body weight. After the amaranth was injected the larva was placed in a shell vial (1.5 X 6.4 cm) and the dye was allowed to circulate within the haemocoel. After 10 min., a proleg on the sixth abdominal segment was cut, and the blood was collected in a capillary tube (Kimax No. 34500) that had been flooded with pure nitrogen to retard melanization. The blunt end of the tube was sealed on an alcohol burner, and the tube was refrigerated (4°C) for several minutes and centrifuged (3,100 rpm for 10 min). The tube was recooled, and the portion of the tube containing sedimented haemocytes was cut off and discarded. The plasma was drawn into a disposable Drummond micropipette (10 ul capacity) and diluted in 1 ml Aronsson's buffer (0.995 M). This solution and the standard (Aronsson's buffer) were placed in separate 1.0 ml cuvettes (10 mm light path) and the relative absorbances were determined with a spectrophotometer at 515 nm, the wavelength at which the maximum absorbance of amaranth occurred.

Previously, the absorbances of known concentrations of amaranth had been determined and, in accordance with Beer's Law, the absorbance was proportional to the concentration. The absorbance value of the test sample was plotted against the concentrations of known samples, and the concentration of the test sample was thus obtained. Once the concentration of amaranth in the test sample had been determined, the haemolymph volume was calculated by the following formula.
Calculation -

\[ V = \frac{d (C^1 - C^{11})}{(C^{11})_U} \]

Where - \( V \) = haemolymph volume in microliter, \( d \) = volume of dye injected in ul ; \( C^1 \) = original concentration in percent ; \( C^{11} \) = concentration of dye after circulation in percent. In order to obtain the haemolymph volume, \( V \) is devided by the body weight of the larva.

Statistical method of analysis:

The experimental data of the biochemical parameters were statistically analysed using Analysis of Variance test (Anova). Since the seasonal variants were 4 (four) and larval stages also being 4(four), replications (9) were also introduced for the efficient composite analysis using the modified version of the two way Anova test. These arrangements enable to calculate/to establish interaction between the seasonal variation and the larval stages. The higher number of df. for the even of variation increased the sensitivity and efficiency of the experimental findings.

The critical differences were calculated by using the following formula for seasonal variation, larval development and the interaction between season and development.
Since the number of seasons and larval stages are 4 (four) each and the replicate for each sample being nine, the degree of freedom for both the factors were 36. Hence, critical difference (C.D) were found to be the same for both season and larval development.

\[ C.D. = \text{Error MSS} \times \left( \frac{1}{n_1} + \frac{1}{n_2} \right) \]

at 5 percent level of probability \((P < 0.05)\)

Where MSS = Mean sum of squares.

\( n = \text{degree of freedom} \).

Complete data from all the biochemical experiments are presented as mean of total replicates \pm the standard error (S.E.) and have been shown graphically (line or bar diagram).

Data obtained from other parameters were tested for significance by student's T test where T is given by

\[ T = \frac{m_1 - m_2}{\sqrt{(SEm_1)^2 + (SEm_2)^2}} \]

at 5 percent level of probability \((P < 0.05)\).