



## TESTICULAR ENZYMES IN SILKWORMS EXPOSED TO HIGH TEMPERATURE AND RM VALUES OF BANDS IN MOTHS OF *BOMBYX MORI*

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### ABSTRACT

The present experiment was conducted in order to find out the effect of testicular enzymes like alanine amino transferase, amino acid transferase, Glutamate dehydrogenase, Malate dehydrogenase, Succinate dehydrogenase, Glucose-6-phosphate dehydrogenase, sorbitol dehydrogenase on sperm production. The result showed that succinate and Glutamate dehydrogenase increases the sperm production where as malate dehydrogenase reduces the sperm production and other enzyme shows normal effects. Since alpha esterases and beta esterases were the prime enzymes involved in the copulation. This was evident from the results obtained from PAGE the amount was maximum before mating in male in significant in virgin male. Initially pure Mysore local variety is selected as the male moth and NB<sub>4</sub>D<sub>2</sub> variety of moth was selected as the female. The normal sequence processes like, sperm production, factors responsible for sperm production and hormones responsible for sperm production were experimented. It was found that, the January month is more favorable for male and female moth to produced  $800 \times 10^2$  /ml of sperm which is counted by haemocytometer and 450 eggs in female at 27°C. In the same way during the same month at 75 to 80 RH maximum production of sperm and egg was observed. Bivoltine race NB<sub>4</sub>D<sub>2</sub> the zymogram pattern for alpha esterase of both virgin female and male showed three bands namely one dark, a moderate and a faint type with slight variability in the degree of activity as well in electrophoretic mobility's. In the material there were three bands of which the cathodal moderate bands found in virgin male was very much reduced though the other two bands remained the same. Slightly reduced activity was evidence the beta esterase activity manifested their bands slightly towards, anodal direction than alpha esterase, where in both the virgin female and male had three bands of which one was moderate followed by a dark and a faint bank. In the mated female also, there were three bands with similar moderate and faint bands but for the dark band which was highly intense with highest activity. In mated male, there is a clear cut reduction from three to two bands, the anodal one of which was a dark banded located behind a reduced moderate band. In the egg - laid female, the moderate band was the same followed by a dark bank with different electrophoretic mobility.

**KEYWORDS:** Rm values, Testicular enzymes, bands, esterases, haemocytometer, etc.

### INTRODUCTION

The male reproductive system functions in the production, storage and delivery of spermatozoa; in the production of seminal fluid, which nourishes and provides an appropriate environment for the sperm; and in providing the female with either chemical or physical signals to tell her that she has mated and that she should commence laying eggs or depositing larvae. In some cases the male may even transfer chemicals that aid the development of the embryo (Engelmann, 1970). The reproductive organs differ from all the other organs of the body in that their function do not contribute primarily to the welfare of the individual of which they are a part, their chief concern lies with the succeeding generation (Snodyorass, 1935). The male may define mating behaviour broadly as the events surrounding the insemination of the female. Typically this involves a whole and sequence of events, which are not always clearly separate from each other, but which it is convenient to treat separately courtship involves a variety of different mechanisms and senses in different insects. Chapman (1982) reported that the readiness to ovipositor

was influenced by mating whereas a virgin female retains most of her eggs for some days. An essentially similar change of behavior occurs in insects belonging to many different species of insects. In some cases the increased tendency to oviposition following mating may occur indirectly as a result of the stimulus provided to oocyte maturation, but certainly in some causes oviposition is affected directly. This is true in moths is which the oocytes are mature at the time of adult emergence. Mating may produce this effect through male accessory gland secretions. In *Bombyx mori* they're diffuse from the bursa copulatrix through the haemolymph of the female and promote spontaneous activity is the motoneurons to the muscles which regulate the extrusion and positioning of the egg. The activity persists for about 24 hrs. and spends the time during the oviposition was completed (Yamaoka and Hirao, 1973). Apart from the obvious function of sperm transfer mating in insects is known to have some very important additional effect including enhancement of fecundity and modification of female receptivity (Gillot and Friedal, 1975). Mated females generally will lay more

eggs than virgins in which, usually egg maturation is slower and oviposition delayed or completely prevented unfertilized egg are usually not viable; one a female has been inseminated, it normally has enough sperms to fertilize a large proportion of the eggs. A broadly linear relationship exists between number of mating and number of eggs produced in species whose receptivity doesn't appear to change markedly after mating. In Bombycidae copulation occurs only once (Richard, 1974). Mating results in an increase in egg production and rate of oviposition in various insect species. This response to mating may be due to changes in hormonal milieu (Engleman, 1970). In silkworms natural copulation continues for 6 to 10 hours (Tanaka, 1964) and some time because of such prolonged copulation a female dies without laying any eggs. Narayanan *et al.*, (1964) have suggested that the copulation of silkworm moths need not go beyond 1 to 2 hours to get maximum number and percentage of viable eggs. The optimum duration of pairing observed was 3 to 4 hrs. Ejaculation of sperm takes place about minutes after copulation and second ejaculation between 60 to 90 minutes only. Machida, and Watanabe, (1964).

Wigglesworth *et al.* (1980) reported similar results for the house cricket, *Gryllos sigillatus*. According to Nayar (1973) and Roth (1974), Maturation and oviposition of eggs in insects are under the control of corpora allata. Mating is said to actuate corpora allata which intern controls the maturation process. It was also enunciated that the male insects secrete a substance, which enhances the fecundity, and increase the egg output (Gillot and Friedel, 1975, Gorden and Bandal, 1967), reported that additional males copulating a female increased the total egg output in weed bug *Oncopeltus fasciatus* was similar results, Benthur and Mothod, 1975 were also reported same result for cricket, *plebeioeryllus quttiventris*.

#### MATERIALS AND METHODS

The seed of bivoltine race of silkworm was obtained from silkworm breeding centre, Vaniyambadi. It was multiplied and utilized for the present studies. The work was undertaken in the laboratory of the Department. The cocoons were separated and kept isolated in specimen glass tubes (3" X 3/4") for the studies on mating duration. They were arranged side by side in an enamel tray and kept in the insect rearing cage. Early in the morning, newly emerged moths were taken out and paired for various durations of matings. The male was separated from the female after completion of specific duration of mating. Every time a fresh male was copulated with the fresh female. The copulated females were kept for egg laying on ordinary white paper sheets (3" X 2") covered with plastic cylinders (2.5" X 3") in insect rearing cages. The females were removed after their death and egg laid was allowed to hatch at room temperature. Observations on the total number of eggs laid and percentage of hatching were recorded. This experiment was conducted twice to confirm the results. In order to study the mating capacity of males, at the optimal minimum mating duration, a fresh female was mated with the same male at every 90 minutes interval of copulation, till the time it dies away or becomes functionless. The females thus served

were allowed to lay eggs as given above. The observations in respect of total number of eggs laid by each served female and the percentage of hatching were recorded.

#### Estimation of reproductive senescence in female moth of *Bombyx mori*

The experiment was conducted at Vellore where the conditions are most ideal during the month of July. The prevailing conditions of temperature 32.4°C and 75 RH respectively. *Bombyx mori* female moths were collected during night after 2-3 hrs of eclosion. 0, 1, 2, 3 and 4 days old females were allowed to mate with newly emerged male moths (0 day) in nylon nets of definite size (the ratios of female to male was kept 1:1.5 which was found to be optimum). The mating duration was 4 hours in each case. The mating success was recorded and after the required mating period, the female was decoupled. Oviposition was allowed for 24 hour in earthen cups which were the most ideal egg laying device for Mulberry silkworm *Bombyx mori*. The female moths were provided with new sets of earthen cups at the interval of 5 hrs up to 24 hrs were record egg laid in each day. A parallel set of virgin females was maintained to study the egg laying behavior in different days. There were 5 replications for every age groups each having a sample size of 20 moths. Data on mating success (mating %) coefficient of egg laying (% egg laid), egg hatchability and mortality in virgin and mated females were noted and analysed through correlation coefficient, students 't' test and ANOVA.

#### Estimation of effect of temperature, humidity, on reproductive activity in *Bombyx mori*

Effect of Temperature, Humidity, and Reproductive activity of *Bombyx mori* under natural condition was studied. For each month, 5 male and 5 female *Bombyx mori* were taken for studying sperm counting and production of eggs. The average atmospheric Temperature and Relative humidity were recorded in each month of a year.

#### Sperm counting

Only newly emerged male and female were used in the present study. They were collected from Vaniyambadi. The 5 male and 5 female was mated for 4 hrs. After 4 hrs the pairs are detached. Two females were dissected for sperm counting and 3 females are allowed for egg laying. The sacculus spermathecae situated at the junction of the ovarian limb, were dissected out and washed in distilled water for about wash off the haemolymph. It was ensured that such washing did not give any osmotic stress to the sperm judged by microscopic examination. After removing the outer epithelium, the spermathecal content was collected in a micropipette and transferred to microcentrifuge tubes for further analysis. The spermathecal content released by puncturing the spermathecal wall; was diluted with phosphate buffer (pH 7.1), a medium that gave no osmotic stress to the sperm cells as revealed by microscopic observation. The diluted spermathecal content was centrifuged for five minutes at 3,000 rpm at 4<sup>0</sup> C, in order to separate out the spermatozoa. The washing was repeated twice to ensure complete separation of the spermatozoa to be used for sperm counting. The portion of the suspension was further diluted with a known volume of phosphate buffer and the sperm density was estimated using a Haemocytometer.

(Barnes, 1962). The spermathecal content is placed in the counting chamber of the Haemocytometer. The slide is gently transferred under the microscope without disturbing the settled spermatazoas and counting is started. The values are expressed by 102x1ml. After the oviposition the number counts the eggs.

#### Estimation of $\alpha$ and $\beta$ esterases

Two races of silkworm *Bombyx mori* namely, local variety X Bivoltine. The two races were drawn from silkworm breeding centre, Vaniyambadi. Before using the races for the experiment, they were reared under standard laboratory conditions (25±1°C and 75 to 90% RH). Virgin moths and mated moths (3h mating after emergence) both the sexes along with oviposited female moths (24 h after mating of each of the two races were used. Individual samples for each stage identified was prepared by homogenizing whole individual moth in double distilled water (1:10 w/v) which centrifuged for 3000 rpm for 3 minutes and the supernatant was subjected to electrophoresis on 7.5% poly acrylamide gel electrophoresis (Davis, 1964).

#### Poly Acrylamide Gel Electrophoresis (PAGE)

The method developed by Davis (1964) was adopted.

#### Reagents

##### 1. Stacking gel buffer

6 g Tris was dissolved in 40 ml water adjusted to pH 6.8 using 1 M HCl and the volume was made upto 100 ml with water.

##### 2. Resolving gel buffer

36.3 g Tris and 48 ml 1M HCl were mixed and the volume was made up to 100 ml with water.

##### 3. Reservoir buffer

3 g Tris and 14.4 g Glycine were dissolved in 1 litre water (pH adjusted to 8.3 with 1 M HCl).

##### 4. TEMED (N, N', N' – tetra methylethylene diamine)

##### 5. Staining solution

0.25% (W/V) of Coomassie brilliant blue R-250 was dissolved in 10% (W/V) methanol, and acetic acid (7% V/V) in water.

##### 6. Destaining solution

10% (V/V) methanol and 7% (V/V) acetic acid in water.

##### 7. Acrylamide and Bisacrylamide solution

Cyanogum-41 (a mixture of 28 g acrylamide and 0.735 g of N, N'-methylene bisacrylamide in 100 ml water).

#### Procedure

7.5 ml of Acrylamide and Bisacrylamide solution, 3.8 ml of separating or resolving gel buffer, water 15.3 ml, TEMED 20 $\mu$ l and ammonium persulphate were taken. The contents were mixed well and digested using an aspirator and poured in to clean dry Perspex tubes and allowed polymerizing. 5.15 $\mu$ l of purified enzyme samples (1mg/ml) dissolved in glycerol (1:1 V/V dilution in water) was carefully layered on the gel surface along with bromophenol blue as the tracking dye. The electrophoresis was conducted at 4°C. A starting current of 2 mA per tube for 10 minutes followed by 4 mA per tube for 120 minutes was supplied across the electrodes. After the electrophoretic run, the gels were removed and stained for protein with the staining solution for 1 hour. Destaining was carried out with destaining solution till the band appeared distinct.

#### Staining of $\alpha$ -esterase and $\beta$ -esterase

Borate buffer (0.3 M, pH 8.65) was used as electrode buffer and electrophoresis was carried out at 4°C at 2 mamps/sample for 2 to 2 ½ hours until the tracking dye touched the 8 cm mark. The staining method of Ayala *et al.* (1972) was employed with slight modification by using fast blue RR salt as dye coupler and 1-naphthyl acetate for alpha esterase and 2-naphthyl acetate for beta esterase as respective substrates in 0.1 m phosphate buffer. After the enzyme bands appeared, the gels were stored in 7% acetic acid. Based on the Rm values and intensity of bands the zymograms were plotted to correlate the isozyme activity in different stages.

#### Estimation on effect of high temperature on testicular enzymes

##### Induction of male sterility

Healthy and uniform size matured V instar larvae of univoltine, Pure Mysore (local) (4.5 ± 0.1g body weight) races, hundred each, were used in the present study. The larvae were transferred to the incubators at 32 ± 1°C and 70 – 80% relative humidity (RH) after the initiation of spinning and kept at laboratory conditions (25 ± 1°C and 70 – 80% RH) till 7<sup>th</sup> day. The pupae of 7 days old cocoons were killed and testes were dissected out in cold insect rings with minimal mechanical stress or injury. The adherence blood and damp were removed, washed in physiological saline (0.9% NaCl) and used for the estimation of various biochemical parameters.

##### Biochemical Assay

The total anthrone positive substance (TAPs) was estimated by the method of (Roe 1955). Glycogen and Glucose was estimated by the method of Benedict's. Pyruvic acid content was measured by the method of Friedman *et al.*, 2000.

##### Enzyme Assay

The tissue homogenates (5% w/v) in required media were prepared, centrifuged and the cell free extracts were used for enzyme assays. The enzyme assay was made under the conditions following zero order kinetics after preliminary standardization regarding linearity with respect to time of incubation and enzyme concentration.

Alanine (EC 2.6.1.1) and aspartate (2.6.1.2) amino transferases (ALAT and AAT) were assayed by the method of (Bergmeyer and Bernt, 1977). The activity levels of succinate dehydrogenase (EC 1.3.99), malate dehydrogenase (EC 1.1.1.37) were assayed by the method of Gnanam and Francis (1976), glutamate dehydrogenase (EC 1.4.1.14) and sorbitol dehydrogenase (EC 1.1.1.14) and glucose –6- phosphate dehydrogenase (EC 1.1.1.49) were assayed by the method of Delma Doherty (1970).

##### Total Anthrone Positive Substance (TAPS)

The TAPS or otherwise called carbohydrate content of the tissue extracts were estimated by the method of (Roe, 1955) Sulphuric acid hydrolyses the di and oligosaccharides into monosaccharides and converts the monosaccharides into furfural or furfural derivatives which react with anthrone and development of a complex blue colour is proportional to the concentration of carbohydrates.

**Reagents**

**1. Anthrone reagent**

50 mg anthrone powder was dissolved in 100 ml of 66% sulphuric acid, to this 1 g of thiourea was added to stabilize the colour.

**2. Standard**

1 mg of glucose was dissolved in 10 ml saturated benzoic acid to prepare the standard solution.

**Procedure**

From the extracts prepared for protein and carbohydrates analysis, the supernatants were used to find out the amount of carbohydrates present in each sample. 0.5 ml of supernatant was taken and 5 ml of anthrone reagent was added to it. This mixture was kept in a boiling water bath for 15 minutes. Then it was cooled to room temperature in dark, to prevent exposure to light. The colour developed was read at 620 nm against a reagent blank in spectronic-21 (Bausch and Lomb, USA). The amount of carbohydrate present in the sample was calculated by using the formula.

Od of sample

————— X concentration of standard

Od of standard

The quality of carbohydrate was expressed as mg/100 g tissue and mg/100 ml of haemolymph.

**Estimation of Glucose**

**Estimation of Glucose by the method of Benedict's**

All sugars that contain a free potential aldehyde or carboxyl group undergo enolisation and act as strong reducing agent. The alkali in the Benedict's serves to dissolve that cuprous oxide that is formed while the cyanate helps to convert red cuprous oxide to white cuprous thiocyanate which gives a clear end point

**Reagent A Benedict's quantitative solution:**

Dissolve 200gm of sodium citrate 100gm of anhydrous sodium carbonate and 125gm of potassium thiocyanate in 600ml of water with acid of gentle heating. Filter, cool and add 10gm of copper

Sulfate dissolved in about 100ml of distilled water. Pour this slowly by stirring continuously adds 5ml of 5% potassium ferrocyanide solution and made up to 1 lit with distilled water.

**Reagent B standard glucose solution:**

Dissolved 1gm of glucose in distilled water and made up to 100ml.

**Reagent C: working standard solution**

10ml of the stock standard glucose is made upto 100ml with distilled water.

**Procedure:**

To 5ml of the benedict's Reagent 3gms of anhydrous sodium carbonate was added to which a few porcelain bits as added and heated to boil with a moderate flame and this is titrate it against the glucose solution in the burette till the blue colour disappears. The volume of glucose run down from the burette was noted. The titration is repeated to concordant volume. Then the given solution is made up to 100 ml in standard flask. This unknown glucose was takes in the burette and filtrated flask. End print is disappearance of blue color and appearance of white colour. Volume of glucose in down from the burette is

noted and filtration is repeated for concordant values. The result was expressed by gms.

**Estimation of Glycogen**

The glycogen is an insoluble form that converts into glucose by added NaOH (Horobin 1982). Then this was estimated by Benedict's method.

**Estimation of Malate dehydrogenase**

**Principle**

Malate dehydrogenase was estimated by the method of Gnanam and Francis (1976). Since it is an oxidoreductase involving nicotinamide adenine dinucleotide the decrease in absorbance is due to the oxidation of NADH is followed.

**Procedure**

Oxaloacetic acid, magnesium chloride, Tris HCl buffer, enzyme extract, all these are taken in a test tube each solution was contain in 0.5 ml and mixed well. The spectrophotometer was settled to a zero absorbance at 340 nm without added NADH in the test against the blank in the reference cuvette. Added NADH was quickly and mixed well. Recorded the initial absorbance and absorbance every 30 seconds for at least 5 minutes. Calculate the enzyme activity as follows with decreased in absorbance for one minute.

**Estimation of Glutamate dehydrogenase**

Glutamate dehydrogenase was estimated by the method of Delma Doherty (1990). GDH is like other dehydrogenase assayed by following the oxidation of the reduced coenzyme, NADH or NADPH. These reduced coenzymes absorb light at 340 nm, which are most biological systems is uniquely uncluttered with interfering absorption by other compounds. Thus even in crude extracts the absorption of NADH at 340 nm is easily detected.

**Reagents**

**1. Potassium phosphate buffers 1.0 M.**

**Stock solution: -**

A. 0.2 M solution of monobasic sodium phosphate (27.8 g in 1000 ml).

B. 0.2 M solution of dibasic sodium phosphate (53.65 g of  $\text{NH}_2\text{PO}_4 \cdot 7\text{H}_2\text{O}$  or 71.7 g of  $\text{Na}_2\text{HPO}_4 \cdot 12 \text{H}_2\text{O}$  in 100 ml).

8.5 ml of A, 91.5 ml of B was diluted to a total of 200 ml of distilled water.

**2. 2-Oxoglutarate 0.1 M: -**

14.6 g 2-oxo-glutarate was dissolved in one litre of distilled water.

**3.  $\text{NH}_4\text{Cl}$  (6.0 M): -**

53.5 g of  $\text{NH}_4\text{Cl}$  was dissolved in one litre of distilled water.

**4. NADH: -**

10 mg of NADPH was dissolved in 10 ml of water.

**5. NADPH: -**

10 mg of NADPH was dissolved in 10 ml of water.

**Procedure**

1 ml of potassium phosphate buffer 7.0 and 7.8 are taken into the test tube. Added 0.31 of 2-oxoglutarate, 0.5 ml of  $\text{NH}_4\text{Cl}$  and 0.12 ml of NADH solution. The 0.2-ml of enzyme extract and 8 ml of water was added and mixed well. Added 3 ml of water in black instead of 2-oxoglutarate. This solution was incubated at 37°C for 15-

30 minutes. The values are recorded the changes in absorbance at 340 nm.

#### Calculation

The amount of NADH or NADPH oxidized was calculated from the molar extinction coefficient. Activities are expressed as n mole NAD (P) H oxidized per minute per mg protein.

$$\text{GDH n mole of NAD (P) H oxidized/min/mg protein} = \frac{A_{340} \times \text{volume of assay solution} \times 1000}{\text{X time of incubation (min)} \times \text{mg protein in enzyme extract used}}$$

#### Estimation of Alanine Aspartate Amino transferase

##### Reagent A Phosphate buffer, pH 7.4

Taken 11.3g dry anhydrous disodium hydrogen phosphate mixed with 2.7g dry anhydrous potassium dihydrogen phosphate in one litre volumetric flask checked the pH and stored at 4°C.

##### Reagent B substrate solution

13.3 g DL – aspartic acid dissolved with minimum amount of 1N sodium hydroxide and the pH of the solution was 7.4 (about 90 ml is required). 0.146 of 2- oxalo glutarate was dissolved by a little more sodium hydroxide solution. The pH was adjusted to 7.4 then this solution was made to 500 ml. with phosphate buffer. This solution was divided into 10-ml portions and stored frozen at 15°C.

##### Reagent C Pyruvate standard

22 mg sodium pyruvate was dissolved in 100 ml water in a standard flask.

##### Reagent D 2, 4 Dinitropheny Hydrazine (DNPH)

19.8 mg dinitrophenyl hydrazine was dissolved in 10-ml conc. HCl acid and made to 100 ml with water. It was stored in an amber bottle at room temperature.

##### Regent E

Sodium Hydroxide 0.4 N

16-g sodium hydroxide was dissolved in one litre water.

##### Reagent f: Enzyme extract

The crude extract was prepared by grinding the tissue in 0.2m potassium phosphate pH 7.5 in a homogenizer for 2 minutes. The slurry was passed through eight layers of cheesecloth and then centrifuged at 25, 00 g for 15 min to get the enzyme fraction.

#### Estimation of Leanne Amino Transferees

##### Reagent A Substrate solution

Phosphate buffer, pyruvate standard, DNPH and sodium hydroxide, 0.4N –same as in Glutamate oxaloacetate aminotransferase.

##### Reagents B Substrate solution

9.0 g alanine as dissolved with 90-ml water with addition of about 2.5-ml sodium hydroxide, IN pH was adjusted in 7.4-0.146 g 2-oxo glutarate added and dissolved it by adding a little more sodium hydroxide solution. The pH was adjusted 7.4. The solution was made by 500 ml with phosphate buffer. This was divided into 10 ml portions and stored frozen at – 15°C

##### Estimation of protein

Fifty mg of the wet tissue was homogenized with 5 ml of 10% trichloroacetic acid (TCA) and centrifuged for 5 minutes at 4000 rpm. The supernatant was used for the estimation of carbohydrates. The precipitate was dissolved in 5 ml of 1N sodium hydroxide for the estimation of protein. 3 ml of haemolymph collected was diluted with 5

ml of 10% TCA and treated in the same was as described for tissue extraction.

The protein content of the tissue extracts was estimated by the method of Lowry *et al.*, 1951. The amino acids containing phenolic hydroxyl group *viz.*, tyrosine and tryptophan reacts with Folin-ciocalteu phenol reagent to give a blue colour due to the reduction of phosphomolybdate; the intensity of the colour is proportional to the concentration of proteins.

#### Reagents

##### 1. 0.1 N sodium hydroxide

0.4 g of sodium hydroxide dissolved in 100 ml of distilled water (w/v).

##### 2. Reagent A

Sodium carbonates 2% (w/v), 2 g of sodium carbonate dissolved in 100 ml of 0.1 N NaOH.

##### 3. Reagent B

Copper sulphate 0.5% (w/v), 500 g of copper sulphate dissolved in 100 ml of 1.35-% sodium potassium tartarate solution (1.35-g sodium potassium tartarate dissolved in 100 ml of distilled water (w/v). This was prepared just before use.

##### 4. Reagent C

Alkaline copper reagent. This was prepared just before use by mixing 50 ml of reagent use.

##### 5. Folin-ciocalteu phenol reagent (1N)

Commercially available 3N reagent diluted to 1N solution with distilled water.

##### 6. Standard

A standard solution of bovine serum albumin (BSA) containing 250 mg/ml was prepared in 0.1N sodium hydroxide.

#### Procedure

The dissolved precipitate was made upto 10 ml with 1N sodium hydroxide. From this, 1 ml was taken and treated with 5 ml of alkaline copper reagent and allowed to stand for 10 minutes at room temperature then 0.5 ml of Folin-ciocalteu reagent was added to each test tube and shaken well. The colour developed in each test tube was read at 720 m against a reagent blank in spectronic 21 (Bausch & Lomb, USA). The protein content of the sample was calculated using the formula

$$\frac{\text{OD of sample}}{\text{OD of standard}} \times \text{X concentration of standard}$$

The protein concentration was expressed as mg/100 g of tissue and mg/100 ml of haemolymph.

#### Estimation of Aspartate Amino Transferase (AAT)

Estimation of aspartate Amino Transferase by the method of (Bergmeyer and Bernt, (1977). Glutamate oxaloacetate amino transferase (GOT) catalyses the reversible interconversions between glutamate and aspartate and their 2-oxo analogues. The oxaloacetic acid is measured colorimetrically by a reaction with 2,4-dinitrophenyl hydrazine giving a brown-coloured hydrazine after the addition of 0.4 N sodium hydroxide.

##### Reagent A Phosphate buffer, pH 7.4

Taken 11.3g dry anhydrous disodium hydrogen phosphate mixed with 2.7g dry anhydrous potassium dihydrogen phosphate in one litre volumetric flask checked the pH and stored at 4°C.

**Reagent B substrate solution**

13.3 g DL – aspartic acid dissolved with minimum amount of 1N sodium hydroxide and the pH of the solution was 7.4 (about 90 ml is required). 0.146 of 2- oxalo glutarate was dissolved by a little more sodium hydroxide solution. The pH was adjusted to 7.4 then this solution was made to 500 ml. with phosphate buffer. This solution was divided into 10-ml portions and stored frozen at 15°C.

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The crude extract was prepared by grinding the tissue in 0.2m potassium phosphate pH 7.5 in a homogenize for 2 minutes. The slurry was panned through eight layers of cheesecloth and then centrifuged at 25, 00 g for 15 min to get the enzyme fraction.

**Procedure**

0.5 ml of substrate solution was warmed in a water bath at 37°C for 3 minutes and 0.2-ml enzyme extract was added and mixed gently. This solution was incubated for 60 min. at 37°C after the tubule was removed from the bath 0.5-ml dinitrophenyl hydrazine solution was added immediately and mixed. 0.5 ml substrate was mixed with 0.5 ml DNPH solution and 0.1-ml enzyme extract was added for control. DNPH was allowed to react for 20 min at 200-m temperature, 0.5 ml of 0.4 N sodium hydroxide was added and well. This solution was left for 10 more min. The absorbance at 510 nm was recorded 0.05 to 0.20 ml. Pyruvate standard was pipetted out and made up to 0.2 ml. 0.5 ml substrate and 0.5-ml substrate, 0.2-ml water was added for blank 0.5 ml substrate, 0.2 ml water and 0.5 ml DNPH solution were mixed. Then this was preceded. The pyruvate in standard produces the difference between standard and blank. Express the enzyme activity as micromole of pyruvate formed per hr. per mg protein.

**Estimation of Alanine Aminotransferase**

Estimation of Alanine Aminotransferase by the method of (Bergmeyer and Bernt, 1977). Glutamate pyruvate aminotransferase catalyses the reversible inter conversions between glutamate and alanine and their 2 oxo analogues. The pyruvate formed after 30 minutes incubation period was measured colorimetrically by reaction with 2-4 dinitrophenylthiohydrazine giving a brown-coloured hydrazine after the addition of 0.4 N sodium hydroxide.

**Reagent A Substrate solution**

Phosphate buffer, pyruvate standard, DNPH and sodium hydroxide, 0.4N –same as in Glutamate oxaloacetate aminotransferase.

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phosphate buffer. This was divided into 10 ml portions and stored frozen at – 15°C

**Procedure**

Similar to aspartate aminotransferase produce e alanine as substrate and incubate for 30 min. Express the enzyme activity as micromole of pyruvate formed per hr. per mg protein.

**Estimation of Alpha esterase and Beta esterase**

**Reagents;**

**1. Stacking gel buffer**

6 g Tris was dissolved in 40-ml water adjusted to pH 6.8 using 1 M HCl and the volume was made upto 100 ml with water.

**2. Resolving gel buffer**

36.3 g Tris and 48 ml 1M HCl were mixed and the volume was made up to 100 ml with water.

**3. Reservoir buffer**

3 g Tris and 14.4 g Glycine was dissolved in 1 litre water (pH adjusted to 8.3 with 1 M HCl).

**4. TEMED (N, N', N' – tetra methyl ethylene diamine)**

**5. Staining solution**

0.25% (W/V) of Coomassie brilliant blue R-250 was dissolved in 10% (W/V) methanol, and acetic acid (7% V/V) in water.

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**7. Acrylamide and Bisacrylamide solution**

Cyanogum-41 (a mixture of 28 g acrylamide and 0.735 g of N, N'-methylene bisacrylamide in 100 ml water.

**Estimation of Glucose and Glycogen**

**Reagent A Benedicts quantitative solution:**

Dissolve 200gm of sodium citrate 100gm of anhydrous sodium carbonate and 125gm of potassium thiocyanate in 600ml of water with acid of gentle heating. Filter, cool and add 10gm of copper

Sulphate dissolved in about 100ml of distilled water. Pour this slowly by stirring continuously adds 5ml of 5% potassium ferrocyanide solution and made up to 1 lit with distilled water.

**Reagent B standard glucose solution:**

Dissolved 1gm of glucose in distilled water and made up to 100ml.

**Reagentc: working standard solution**

10ml of the stock standard glucose is made upto 100ml with distilled water.

**Reagent A Benedicts quantitative solution:**

Dissolve 200gm of sodium citrate 100gm of anhydrous sodium carbonate and 125gm of potassium thiocyanate in 600ml of water with acid of gentle heating. Filter, cool and add 10gm of copper Sulphate dissolved in about 100ml of distilled water. Pour this slowly by stirring continuously add 5ml of 5% potassium ferrocyanide solution and made up to 1 lit with distilled water.

**Estimation of Malate dehydrogenase**

**Reagent B standard glucose solution**

Dissolved 1gm of glucose in distilled water and made up to 100ml.

**Reagentc: working standard solution**

10ml of the stock standard glucose is made upto 100ml with distilled water.

**Estimation of Glutamate dehydrogenase**

**Reagents****2. Potassium phosphate buffer 1.0 M.****Stock solution**

C. 0.2 M solution of monobasic sodium phosphate (27.8 g in 1000 ml).

D. 0.2 M solution of dibasic sodium phosphate (53.65 g of  $\text{NH}_2\text{PO}_4 \cdot 7\text{H}_2\text{O}$  or 71.7 g of  $\text{Na}_2\text{HPO}_4 \cdot 12 \text{H}_2\text{O}$  in 100 ml).

8.5 ml of A, 91.5 ml of B was diluted to a total of 200 ml of distilled water.

2. 2-Oxoglutarate 0.1 M:-

14.6 g 2-oxo-glutarate was dissolved in one litre of distilled water.

3.  $\text{NH}_4\text{Cl}$  (6.0 M): -

53.5 g of  $\text{NH}_4\text{Cl}$  was dissolved in one litre of distilled water.

4. NADH: -

10 mg of NADPH was dissolved in 10 ml of water.

5. NADPH: -

10 mg of NADPH was dissolved in 10 ml of water.

**Statistical Procedures**

The mean and the standard deviations were calculated from the determined values by using the standard procedures (Bailey, 1984). The standard deviations were calculated by using the formula.

$$S = \frac{(X_1 - \bar{X})^2}{n - 1}$$

where  $X_1$  = value of individuals

$\bar{X}$  = mean value of the sample

n = number of samples

In order to examine whether the difference in results obtained was significance or not the following formula (Student's 't' test) was employed.

$$t = \frac{\bar{X}_1 - \bar{X}_2}{\sqrt{\frac{S_1^2}{n_1} + \frac{S_2^2}{n_2}}}$$

Where  $\bar{X}_1$  = mean value of one sample

$\bar{X}_2$  = mean value of other sample

$S_1$  and  $S_2$  = are corresponding standard deviations

$n_1$  and  $n_2$  are the number of tests for each sample

The level of significance (P-value) between  $X_1$  and  $X_2$  was determined by using the student's 't' distribution table of Fractiles and critical values (Radhakrishna Rao *et al.*, 1985).

**RESULTS**

The present investigation has been launched in order to find out the various factors which influence reproductive physiology of mulberry silkworm *Bombyx mori*. The factors *viz.*, influence of mating tenures, impact abiotic factors such as temperature, humidity, analysis of effect of high temperature on testicular hormone and assessment on the influence of vertebrate hormones, like ovine prolactin, thyroxin and insulin on the reproduction were observed.

**TABLE 1.** Testicular enzymes in silkworms exposed to high temperature

Parameter	L (PM)			NB <sub>4</sub> D <sub>2</sub>		
	Control	Experimental	% change	Control	Experimental	% change
ALAT	0.192	0.286	48.95	0.201	0.262	30.34
AAT	0.264	0.298	12.87	0.297	0.336	13.13
GDH	0.348	0.363	4.31	0.198	0.268	35.35
MDH	0.127	0.962	65.74	0.128	0.116	-93.75
SDH	0.146	0.121	-17.12	0.138	0.112	-18.84
SDH/GDH	1.01	0.760	-24.75	0.796	0.462	-41.95
MDH/GDH	0.896	0.726	-18.97	0.692	0.442	-36.12
Sorbitol dehydrogenase	0.197	0.176	-10.65	0.262	0.212	-19.08
G-6-PDH	0.196	0.146	-25.51	0.199	0.159	-20.10

ALAT -Alanine & aspartate amino transferase (EC.No.2.6.1.1.), AAT – Aminoacid transferase (EC.No.2.6.1.2)

GDH – Glutamate dehydrogenase (EC.No.1.4.1.30), MDH – Malate dehydrogenase (EC.No.1.1.1.37), SDH – Succinate dehydrogenase (EC.No.1.3.99.1), G-6-PDH – Glucose –6 -phosphate dehydrogenase (EC.No.1.1.1.49), Sorbitol dehydrogenase (EC.No.1.1.1.14)

Values given are Mean ( $\bar{x}$ ) of six individual experiment, df = (n-1) observations, Values given are the mean value ( $\bar{X}$ ) of 4 data's, d.f. = degrees of freedom = n-1, Significance ++ = p < 0.001, + = p < 0.05 NS = Not significant

Table 1 expressed the testicular enzyme in silkworm exposed to high temperature. These are about nine testicular enzymes namely, ALAT – Alanine and Aspartate amino transferase, (EC 2.6.1.1), AAT – Amino acid transferases (EC 2.6.1.2), GDH – Glutamate dehydrogenase (EC 1.1.1.37), SDH - Succinate dehydrogenase (EC 1.3.99.1), G-6-pDH – Glucose, 6-phosphate dehydrogenase (EC 1.1.1.49) and Sorbitol dehydrogenase (EC 1.1.1.14).The Alanine and aspartate amino transferase was increased from 0.192 to 0.286 in the

local variety Pure mysore with the increase of 48.95%. In the bivoltine variety ALAT is increased from 0.20% to 0.262 with the increase of 30.34%.The Amino acid transferase is increased from 0.264 to 0.298 in the local variety (Pure mysore) with the increase of 12.87%. In the bivoltine variety AAT is increased from 0.297 to 0.336 with the increase of 13.13%. The Glutamate dehydrogenase was increased from 0.348 to 0.363 in the local variety with the increase of 4.31%. In the bivoltine variety GDH increased from 0.198 to 0.268 with the

increase of 35.35. The malate dehydrogenase is increase from 0.127 to 0.962 in the local variety (PM) with increase of 65.74%. In the bivoltine variety there is a significant reduction from 0.128 to 0.116 with the -93.75 % of reduction. The Succinate dehydrogenase was decrease from 0.146 to 0.121 in the local variety with the decrease of -17.12. In the bivoltine variety there is a significant reduction from 0.138 to 0.112 with the -18.84% of reduction. The SDH / GDH dehydrogenase was decrease from 1.01 to 0.76 in the local variety with the decrease of -24.75. In the bivoltine variety there is a significant reduction from 0.796 to 0.462 with the -41.95% of reduction. The MDH / GDH dehydrogenase was decrease

from 0.896 to 0.726 in the local variety with the decrease of -18.97. In the bivoltine variety there is a significant reduction from 0.692 to 0.442 with the -36.12% of reduction. The Sorbitol dehydrogenase was decrease from 0.197 to 0.176 in the local variety with the decrease of -10.65. In the bivoltine variety there is a significant reduction from 0.262 to 0.212 with the -19.08% of reduction. The Glucose - 6 - Phosphate dehydrogenase was decrease from 0.196 to 0.146 in the local variety with the decrease of -25.51. In the bivoltine variety there is a significant reduction from 0.199 to 0.159 with the -20.10% of reduction.

**TABLE 2:** Rm values of bands in moths of silkworm *Bombyx mori*

Race	Stage*	Alpha esterase bands				Beta esterase bands			
		1	2	3	4	1	2	3	4
Local (L)	1	3.9	3.0	1.6	-	2.0	1.8	-	-
	2	3.1	1.9	1.3	-	3.1	1.9	1.3	-
	3	4.1	3.1	2.0	1.1	3.4	2.2	1.8	-
	4	3.0	2.2	1.1	-	3.1	1.6	1.3	-
	5	4.1	2.9	1.9	-	3.0	2.1	1.6	-
NB <sub>4</sub> D <sub>2</sub>	1	3.1	2.4	1.1	-	4.5	3.6	2.4	-
	2	4.3	3.6	2.8	-	4.7	3.4	2.3	-
	3	3.4	2.9	2.1	0.8	4.2	3.2	2.0	-
	4	4.0	3.4	2.2	-	3.0	2.0	-	-
	5	3.2	2.0	0.6	-	3.1	2.1	-	-

Values given are the mean value (  $\bar{X}$  ) of 4 data's

d.f. = degrees of freedom = n-1

Significance ++ = p < 0.001

+ = p < 0.05

NS = Not significant

\*1. virgin female: \*2- virgin male \*3- mated female \*4- mated male 5- egg laid female

Table 2. Indicates RM values of bands in months of silkworm *Bombyx mori* based on the intensity of bands they are classified into three namely, Dark bands, moderate bands faint bands. From Rm values of the bands alpha esterase shows 3 bands for each of the virgin female and mated moths. These three bands included a cathedral band and a dark and followed by an anodal faint band in the virgin female. In the virgin male these molecular species different electrophoretically in that they were more cathedral than its female counterpart. In the mated female the number of bands increased to four with one moderate cathodal band, two dark and faint bands. In mated male, there was reduction of esterase activity as evidenced by narrowing of bands, which were quite predominant in virgin male. In the oviposited female, the activity declined since there were three bands (one moderate, one dark and a faint one). For beta esterase the virgin female had only two bands one of which was dark and the other is faint but in the virgin male, there were three bands which are electrophoretically different from virgin female represented by a moderate cathedral and an anodal moderate band followed by a dark and a faint band while in the egg laid female, there were again three bands starting from cathedral end and represented by a dark, a moderate and a faint band.

In the bivoltine race NB<sub>4</sub>D<sub>2</sub> the zymogram pattern for alpha esterase of both virgin female and male showed three bands namely one dark, a moderate and a faint type with

slight variability in the degree of activity as well in electrophoretic mobilities. In the material there were three bands of which the cathodal moderate bands found in virgin male was very much reduced though the other two bands remained the same. In the egg laid female though were three bands. Slightly reduced activity was evidence the beta esterase activity manifested their bands slightly towards, anodal direction than alpha esterase, where in both the virgin female and male had three bands of which one was moderate followed by a dark and a faint band. In the mated female also, there were three bands with similar moderate and faint bands but for the dark band which was highly intense with highest activity. In mated male, there is a clear cut reduction from three to two bands, the anodal one of which was a dark banded located behind a reduced moderate band. In the egg - laid female, the moderate band was the same followed by a dark band with different electrophoretic mobility.

## DISCUSSION

Out present investigation observed that the activity levels of alanine and aspartate amino transferase (ALAT and AAT) have been tested in this regard. Since, the activity level of ALAT forms general index of amino acid oxidation's and AAT marks the mobilization of amino acid into gluconeogenesis (Adibi, 1966; Davison and Langslow, 1975). The observed elevation in experimental tissue many indicate the possible mobilization of amino



acid for carbohydrate formation. Since glutamate dehydrogenase activity marks deamination of aminoacids (Harper, 1977). The increased activity level of this enzyme in the experimental tissue suggests acceleration of oxidative deamination of aminoacids into citric acid through and  $\alpha$ -ketoglutarate. In view of inhibited activity of glucose-6-phosphate dehydrogenase (G-6-PDH). Impaired operation of pentose phosphate path way was expected. This pathway plays an important role in the supply of NAPH for lipogenesis and provision of ribose-50 phosphate for the formation of nucleotides which are necessary for cell replication in the testis (Almeida *et al.*, 1989) it is tempting to speculate that the low activity of G-6-PDH in experimental testis may be indicated of reduced cell proliferate capacity. Since sorbital dehydrogenase forms a marker towards sperm metabolism (Samuels, Harding and Mann, 1962). It is inhibition under high temperature condition is suggestive of impaired sperm metabolism. Under high temperature environment the testicular tissue seems to orient towards decreased aerobic phase of sperm metabolism as evident from the lower level of NAD- SDH and NAD-MDH activities. The ratios of SDH/GDH and MDH/GDH were lower than the control. Suggesting that the extent of elevation in the citric acid cycle enzyme was not to the tune of addition of keto acids. Hence, it appears that the testicular biochemical changes in the silkworm under high temperature may be correlated to abnormal spermatogenesis, which results in impaired utilization of metabolites, by the spermatozoa. As printed out by wigglesworth (1974), insects represents a highly specialized group where mating is a prerequisite to facilitate fertilization. Mating and mating behavior as such has been studied in several insects such as drosophila and certain Lepidoptera namely *Cimex* and *priplaneter* (Davey, 1985). Among the Lepidoptorans, silkworm *Bombyx mori* has been given special attention since multiple mating have been made by several workers (Sidhu, *et al.*, 1967; Vijayan *et al.*, 1994), the biochemical aspects of mating has been analyzed only by a few workers. (Osanai *et al.*, 1986; Kosuga *et al.*, 1987; Aigaki *et al.*, 1988). One of the aspects of great biochemical and physiological interest in the transfer of materials from male to female insects at the time of mating (Copulation), which according to (Richmond and Alayne, 1981) is to provide opportunity for males to influence both the physiology and behavior of their mates. A variety of chemical substances have been identified in seminal fluids of insects which are known to send appropriate signals to stimulate or trigger the process of fertilization. As early as 1969, Butterworth had pointed out that in drosophila, the ejaculatory bulbs is a sources of lipid which is transformed into material known as an acetate ester of cis-11-octodeconoic acid and this has also been reported from the hair pencil of a male butterfly as demonstrated by (Brieger and Butter worth, 1970). It has been shown by Richmond *et al.* (1980) that the seminal fluid of drosophila melanogaster contains, in the anterior ejaculatory duct a non-specific high active carboxyl-esterase called esterase-6. It is unknown whether this esterase acts on the lipid (Davey, 1985) but this active esterase is shown to be contributed to females where higher number of fertilizations occurs followed by more progeny, as

opposed to females inseminated by males with low or nil esterase activity in their seminal fluid. It has been pointed out that, this indirectly reflects not only on higher fertilization rate but also on the ability for multiple matings to produce higher progeny.

Our result suggested that the role of esterases and their contributed by the male into female genital tract during mating in silkworm *Bombyx mori* is highlighted (Kosuga *et al.*, 1987; Osanai, 1987b). The present studies have provided some interesting information, perusal of zymogram patterns for alpha and beta esterases in pure mysore reveals that the alpha esterase activity which was lower in virgin female has increase by the addition of a dark band at the locus 3 which gets reduced to a moderate band later since this specific commonest of alpha esterase probably gets utilized often mating in the female which the beta esterase which in the virgin female at locus 2 is presented by a faint band is transformed into a dark band indicating that this added contributed has increased the activity of isozyme at locus 2. Following the oviposition, the band at locus 2 which was dark with high activity gets transformed to a moderate band thus authenticity the utilization of the beta esterase component at this locus during mating.

However in the Bivoltine race NB<sub>4</sub> D<sub>2</sub> the additions of alpha esterase component from the male to the female s a dark band at locus 2 during mating is quite obvious. In the egg laid female this particular band is transformed into a moderate band thus suggesting the utility of certain alpha esterase component immediately after mating. Quite is the feature that in virgin and mated females and males and egg-laid females of NB<sub>4</sub> D<sub>2</sub>, the zymogram pattern for alpha esterase. Here the moderate band at locus 4 found in the male is the main component contributed during mating. Further, the oviposited female showed moderate band at locus 4 indicating the partial use of this beta esterase component. The present finding clearly revealed higher alpha esterase activity NB<sub>4</sub> D<sub>2</sub> under study. The higher esterase activity in terms of both number of bands as well intensity of activity as visualized in the mated female than virgin female of both pure Mysore race over bivoltine NB<sub>4</sub> D<sub>2</sub> indicate that there was higher metabolic efficiency in female genital tract of pure Mysore. This is authenticated by the fact that oviposited female shows reduced activity as evidenced by disappearance of contributed bands and /or reduction in the degree of esterase activity in terms of differential intensity of bands.

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