MATERIALS AND METHODS

4. Materials and Methods

4. 1. Survey of tea plantations and collection of lepidopteran pests

Three folivorous lepidopteran pests of tea, *Buzura (Biston) suppressaria* Guen. (Looper Caterpillar), *Eterusia magnifica* Butl. (Red Slug Caterpillar) and lymentrid, *Euproctis latisfascia* Wlk. (Darjeeling Black Hairy Caterpillar) were mainly considered for the present study.

Tea plantations of Terai and tea gardens in and around North Bengal University campus were surveyed for collecting these pests (Plate 1). Names of the tea estates which were frequently surveyed for insect materials during the course of study were Matigara, Maruti Kamalpur, Chandmani, Atal, Gangaram and Dagapur. These gardens had mostly matured tea bushes of Tocklai varieties (Tv_{18},Tv_{25},Tv_{26}) that produced vigorous flush in Terai agro-climate. Usually larval stages of loopers and hairy lymentrid and adults in case of red slug were collected (handpicked) from tea bushes and trunks of shade trees in the morning hours. The collected larvae and adults were brought to the laboratory in polythene packets and containers.

4. 2. Source of natural food (Tea leaves) for laboratory rearing (culture) and experimentation with the lepidopteran folivores

Natural food (tea leaves) which was provided for rearing and for various experiments were collected from the experimental tea plot of Department of Zoology at North Bengal University campus which was about 12 years old. The

clonal varieties Tv_1, Tv_{18}, Tv_{25} and Tv_{26} of the experimental garden were kept under usual cultural practices with organic manure and no pesticidal application.

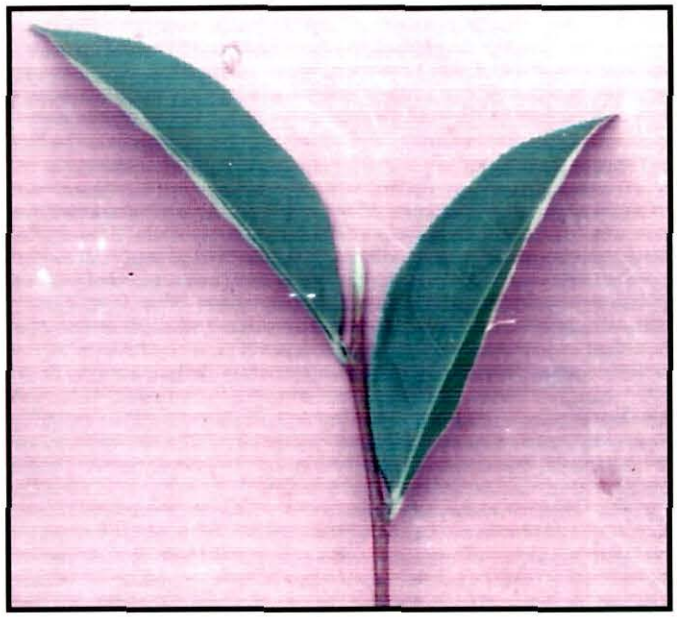
Tv_1: It is one of the earliest clones released by 'Tocklai Experimental Station', Assam (India) in 1949. Tv_1 is a standard clone, having high yield potential and high quality. It has a compact frame with acute branch angle (<50°). Leaves are erect, medium sized with pubescence on lower surface and sunken stomata. Surface matty in nature. Fairly draught tolerant. It is a hybrid of Assam × China origin.

Tv_{18}: It is of Cambod origin. More or less of compact frame with glossy medium sized leaf. Leaf axil with an angle of 50° to 70°. It has a high yield potential but of average quality. Leaf has pinkish pigmentation in the petiole.

Tv_{25} & Tv_{26}: These are high yield clones of Assam × Cambod origin. Morphologically both the clones are similar in nature, having compact frame and glossy medium sized leaf. Both the clones are fairly drought tolerant having high yield potential but of average quality. Leaf axil being >70° (Plate 2).

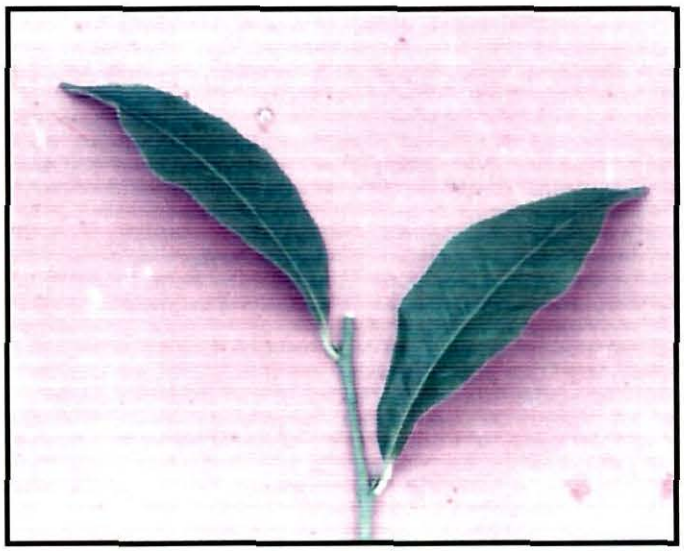
4. 3. Mass Rearing on Natural and Artificial Diets.

Laboratory rearing of immature stages were conducted in transparent buckets (27.5 cm.x27cm.) containing several twigs with leaves of host plant immersed in water in a conical flask (250ml), plugged by cotton and covered by muslin cloth. The larvae were transferred daily into sterilized and clean buckets along with fresh clean food. Mass rearing was continued till the emergence of adults in aseptic conditions to avoid contamination.



Tv_1

Tv_{18}



Tv_{26}

Plate 2. Clonal Varieties of Tea.

Freshly emerged adults and collected adults were sexed, paired and allowed to mate in glass chimneys (19.5 cm x 8.5 cm), containing a twig with leaves of the host plant immersed in water in a conical flask to elicit oviposition. Two strips of brown paper were also stretched inside the chimneys as egg laying surface. A cotton swab moistened with diluted honey with water (1:1) was hung inside the chimney as food for the adults. The chimneys were covered with muslin cloth. The eggs were transferred onto towels (tissue paper) and placed in plastic containers (9.5 cm.x5 cm.) covered by lid with fine holes. The eggs were kept in BOD incubator till the emergence of neonates.

The larvae from IIIrd instar onwards in case of *B. suppressaria* and *Et. magnifica* and neonates of *E. Latisfascia* were mass reared on artificial diets in plastic containers (9.5cm x 5cm) in batches of five per container. The larvae were transferred into freshly disinfected containers on alternate days and fresh food (artificial diet) were supplied. Rearing was continued till the adults emerged which were then transferred to glass chimneys (with similar conditions as in the case of mass rearing of adults on natural diet) for oviposition (Plate 7) .

In order to reduce contamination of insect diets with pathogens or microbial contaminants, sterilization of eggs was accomplished by surface sterilization with 0.3% AI (NaOCl) solution for 5 min. (Leppla et al., 1984) and thorough rinsing with distilled water which was followed by drying under laminar air flow hood prior to infestation of the diets with the freshly laid eggs. Aseptic condition was maintained as far as possible to avoid contamination.

4. 4. Preparation of Artificial diets:

The lepidopteran tea pests considered in the present investigation were reared for the first time on artificial diets. As no defined diet of any related species of the above pest species were known, several combinations of diet components (formulations) had to be tried. After several trials, the artificial diet used for geometridae: *Alsophila pometaria* (Lyon, 1970) was found suitable for rearing immature stages of *Buzura suppressaria* and *Eterusia magnifica*. For *Euproctis latisfascia*, a diet for *Lymantria dispar* (Magnoler, 1970) was found suitable. Details of diet composition are given in Table I and Table II.

Glassware used for preparation of diets were autoclaved. Preparation and dispensing of diets were done under the laminar flow hood to avoid contamination. Glucose was the carbohydrate used. Ascorbic acid was incorporated because it is widely accepted that it is required for normal development of phytophagous insect species (Farinos et al., 1999). Minerals were supplied by Wesson's salt mixture (Sigma) and wheat Germ (Sigma), the latter also being the source of cellulose. The Brewer's yeast powder (Sigma) was used to supply vital nutritional factors because it is a good source of micro - and macronutrients needed for some insect species (Tsitsipis, 1989). Sorbic acid, methyal p-hydroxybenzoate (Sigma) were incorporated to inhibit microbial growth of yeast, bacteria and filamentous fungi in diets. Linoleic acid was incorporated in the diet as it is considered useful for successful pupal eclosion (Fraenkel and Blewett, 1946a ; Sivapalan and Gnanpragasam, 1979b). Tea infusion was used in the diet because host plant material as fresh or ground whole plants, plant

Table 1. Components of the Artificial Diet (Lyon, 1970) used for rearing of *Buzura suppressaria* and *Eterusia magnifica.*

Components (g)	
Vitamin – Free Casein	98.00
Glucose	98.00
Wesson's Salt Mixture	28.00
Cholesterol	8.40
Potassium Sorbate	3.36
Choline Chloride	2.80
Wheat Germ	140.00
Alphacel	84.00
Wheat Germ Oil	7.50
Agar	70.00
Sodium Alginate	14.00
Potassium Sorbate	14.00
Vanderzant's fortification Mixture	40.00
Linoleic Acid	6.00 ml
Tea Infusion with Distilled Water	2100.00 ml

Table 2. Components of the Artificial Diet (Magnoler, 1970) used for rearing of *Euproctis latisfascia.*

Components (g)	
Vitamin Free Casein	2.80
Wesson's Salt Mixture	1.00
Glucose	1.98
Cholesterol	0.02
Wheat Germ	5.70
Sodium Alginate	0.50
Alphacel	1.40
Choline Chloride	0.10
Sorbic Acid	0.08
Methyl p-hydroxybenzoate	0.08
Agar	2.28
Ascorbic Acid	0.50
Aureomycin	0.01
4M KOH	0.47 ml
Linoleic Acid	0.50 ml
Sunflower Oil	0.25 ml
Vitamin Solution	1.00 ml
Distilled Water	20.00 ml
Tea Infusion with Distilled Water	62.00 ml

parts or extracts have been, and are still used to induce feeding and increase vigor in laboratory-reared fastidious insects (Doss, 1980; Schoonhoven et al., 1998).

Hot infusion of tea was prepared by thoroughly washing fresh tea leaves, cutting into small pieces and simmered in distilled water for 5 minutes. Wheat germ was roasted for a while to improve the taste. To prepare the diet, gelling agent agar was dissolved in hot tea infusion and mixed thoroughly in a beaker, and the mixture was cooled to 50° C, then the rest of the ingredients were added gradually when the medium was still hot and mixed thoroughly into a fine paste till there were no lumps. The preparations (diets) were then allowed to cool to room temperature after which diets were transferred to plastic containers with lids and kept in a refrigerator for future use. Maximum precautions were taken during diet preparation to avoid contamination and the working areas as well as the containers used for rearing were disinfected by 0.4 % sodium hypochlorite solution on a daily basis.

4. 5. Host plant selection:

The laboratory eclosed larvae of *Buzura suppressaria*, *Eterusia magnifica,* and *Euproctis latisfascia*, were used for host plant preference experiments. Fifteen replicates each, of the two different categories (specimens) considered for the study were:

i. the early instars represented by IIIrd stage larva, and

ii. advanced instars represented by Vth or VIth stage larva.

Each category of larval stage used were starved for twelve hours only; further starvation was not done to avoid emaciation and death out of starvation.

In the first set of experiments, three tocklai clones (varieties) TV_1, TV_{18} and TV_{26} were selected. Leaf discs of each variety were cut from the same portion of tea leaves of same maturity and were placed at reasonable distances in petridishes (15 cm. in diameter) on the moistened filter paper with wet cotton balls to prevent wilting of leaf disc. In the center of each petridish, a single larva of each category of the experimental insects was released and allowed to feed for 12 hours (Jermy,1961 ; Jermy et al., 1968). After 12 hours of feeding, leftover leaf discs were oven dried and weights were recorded.

Dry weights of food consumed were determined by subtracting dry weights of leaf discs after consumption from the dry weights of an intact leaf disc of equivalence. For two different categories of three lepidopteran insect pest species, similar experiments were conducted to ascertain preference for the host variety. The values obtained based on feeding were converted into percentage and was converted into arc sin for statistical analysis. The mean values are based on fifteen replicates.

4. 6. Morphometric & Allometric growth study:

Measurements of the life cycle from egg to adult stage of each of the lepidopteran tea pests were done based on ten specimens of each stage. The body length, interocular distance and head length of various stages were

measured using oculometer fitted in Wild M_3 binocular wherever needed. The structures and colour of the various stages were also noted.

Post embryonic development in insects is punctuated by a series of moults and ecdyses, each preceded by a period of active growth (Wigglesworth, 1965). The extent of growth at each moult can be predicted using certain empirical laws. Huxley (1924) and Huxley and Tessier (1936) proposed the allometric or heterogenic growth pattern. They suggested the term allometry for denoting the growth of a part that is growing at different rate from that of the body as a whole (or any stable part). They further suggested that the growth relation could be calculated using the equation $y=bx^k$ (where, y is the allometrically growing segment or part; 'b' is a constant, denoting initial growth index, and is the theoretical value of 'y' when standard measurement x equals unity; k is another constant, denoting growth ratio and indicates the value at which 'y' grows in relation to the standard measurement 'x' (any stable part or the whole body).

The value of 'b' and 'k' are calculated by the method of least squares. When k=1, 'y' grows at the same rate as 'x' i.e. isometry, when k<1, 'y' grows slower than 'x' i.e. negative allometry. When k>1, 'y' grows faster than 'x' i.e. positive allometry. The other form of the equation $y=b\ x^k$ is: $\log y=\log b + k \log x$, means that if the logarithms of the size are plotted, a straight line is expected and from the slope of which the value of 'k' can also be determined.

In the present study the width of head was chosen as the standard measurement because it is the most stable part of the body and least subjected to change in size owing to changes in physiological state of the individual. By computing the mean of ten measurements of ten individuals of each stage for all three folivores, based on different body parts the values of 'k' and 'b' were calculated. By using value of k (equal to gradient value of the straight line equation) and b (intercept on ordinate i.e. log b) the regression equations could be formulated and the regression lines for all the three variables could be polled for three species. Matsuda (1960) after his extensive study of relative growth in Gerridae hypothesized that when growth ratio (k) increases, the initial growth co-efficient (b) decreases and vice versa.

4. 7. Post-embryonic Development period on Natural and Artificial Diets:

The development and life cycle of three species, *Buzura suppressaria*, *Eterusia magnifica* and *Euproctis latisfascia* were studied by rearing in the laboratory conditions of 28 ± 2°C, 75 ± 5% Relative humidity and 12 hours L:D. To study stadial periods, twenty neonates were separated and reared in batches of four in plastic containers (26 cm×8.5 cm.). Twigs of host plant with leaves were offered as food everyday. Daily observations were made to find out change in the instar by detecting the head capsule and exuvum. Duration of pupal and adult stages was recorded. Similar study was conducted for all the three folivores on artificial diets (with IIIrd instar onwards for *B. Suppressaria* and *Et. magnifica* and neonates of *E. latisfascia*). Individual rearing on artificial diet was conducted in (9.5cm×5cm) plastic containers toweled with tissue paper. Artificial diets meant

for each folivore was provided everyday. Daily observations were made to find out changes. Duration of all the stages was recorded.

4. 8. Survivorship study:

Survivorship studies were conducted with immature stages of three lepidopteran tea pests reared on natural (specific host plant) and artificial diets under laboratory condition as mentioned earlier. Rearing on natural diet was conducted in (26 cm.x8.5 cm) plastic containers, 100 newly hatched neonates were separated in small batches of ten for each observation. Observations were made on the preferred host plant of each species at the time interval of 24 hours (x). The number of individuals alive during a particular age interval (lx) and the number of larvae dying within the age interval (dx) were recorded. The rate of mortality (qx) was also calculated.

A survivorship study was conducted in case of Darjeeling black hairy caterpillar on artificial diet with 100 newly hatched larvae separated in small batches of five and reared in (9.5cm.x5cm.) plastic containers. However, in case of red slug and looper caterpillars studies were conducted from IIIrd instar onwards on artificial diets. This rearing technique had to be adopted for two of three species as several attempts to rear the Ist and IInd instars on artificial diets met with high mortality and partial success. Rearing on artificial diets was conducted in (9.5 cm.x5 cm.) plastic containers. For comparison of the observed survivorship curves, a figure with four hypothetical situations was adopted from Pearl (1928) and Dash (1995).

4. 9. Daily food consumption and larval weight :

In order to find out the daily food consumed and weight changes in late instars of the caterpillars of the three species in question on two different diets (tea leaves and artificial diet), freshly ecdysed IV^{th} and V^{th} instar stages (10 replicates each) of looper and red slug and V^{th} and VI^{th} stages of black hairy caterpillars were kept throughout the stadial period under observation. Experiment was conducted inside the BOD incubator under conditions mentioned earlier.

Daily preweighed fresh food (tea leaves with twig) was offered to each individual kept in (26cm×8.5cm) plastic containers. After 24 hours of feeding, leftover food and excrement were removed, oven dried and weighed. Dry weight of the actual food consumed was calculated by subtracting the dry weight of the leftover food from the dry weight of an equivalent amount of the food offered. In a similar manner, daily consumption of food and larval weight change (based on dry weight) was recorded for folivores reared on artificial diet. Larval weight change (based on dry weight) was calculated by drying a larva of similar weight (reared on two different diets) in the oven at 50° C for 72 hours.

4. 10. Reproductive performance:

In order to find out the reproductive performance of all the three species on natural and artificial diets, pupae from the mass culture were separated and kept individually in plastic containers (9.5cm. x 5 cm.) till the emergence of the adults. Freshly emerged adult moths of the three pest species were sexed and

reared in glass chimneys containing a twig of host plant dipped in water for rearing and oviposition. A cotton ball soaked with diluted honey (1:1) was hung from the wall of the chimney as food. Two strips of brown paper were stretched in the chimney as an alternate surface for laying of eggs. Fecundity alongwith pre and post oviposition and egg laying periods were observed for six pairs of each species. Pupae and freshly emerged adults of the three lepidopteran tea pests from mass culture (natural and artificial diets) were sexed, oven dried and weighed (n=6).

4. 11. Dry Mass budget:

Freshly eclosed IVth and Vth instars of looper and red slug and Vth and VIth instars of black hairy caterpillars (15 replicates for each species) were used for assessment of dry mass budget. Experiment was conducted in the environmental chamber at 28±2°C, 75±5% R.H. and 12 hours L:D. For experiment with natural food, each larva was kept / reared singly in (26cm.x8.5cm) plastic containers and provided with known quantity of food (tea leaves with twig).

Daily record of weight of larva, food offered, food not consumed and excrement produced were taken. Control was run concurrently by keeping the tea leaves with twig immersed in water in conical flask plugged by cotton ball. The food not consumed, the faecal matter and the control leaves were then dried to constant weight at 50°C. Dry weight of food consumed was determined by subtracting dry weight of the eaten twig with leaves of host plant from the dry weight of an equivalent intact twig with leaves of the same age.

Similar methodology was adopted for mass budget assessment for all the three pest species on artificial diets. Experiment was conducted in (9.5cm×5cm) plastic containers under similar conditions. Dry weight of food consumed in case of a larva reared on artificial diet was determined in a similar way by subtracting the dry weight of the left over food from an equivalent food (initially offered to the larva). Gravimetric technique was used to determine food consumption, growth ratio and post ingestive food utilization efficiencies (all based on dry weight) after Waldbaurer (1968), Slansky (1985), Slansky & Scriber(1985), Petrusewicz and Macfadyen (1970), Muthukrishnan & Pandian (1987) and Farrar et al.(1989).

Units used: weights in mg; time in days; and efficiencies in percentage.

a. Assimilation (As) = Food consumed (C) – Faeces (Fu)

b. Production (P) = Final body weight (W_2) – Initial body weight (W_1)

c. Respiration (R) = As – P

d. Maintenance Cost = R / P

e. Production Index = P/As

f. Approximate Digestibility (AD)

$$= \frac{\text{Wt.of food ingested} - \text{Wt. of faeces}}{\text{Wt. of food ingested}} \times 100$$

g. Efficiency of Conversion of Ingested food (ECI)

$$= \frac{\text{Wt. gained by the insect}}{\text{Wt.of food ingested}} \times 100$$

h. Efficiency of Conversion of Digested food (ECD)

$$= \frac{\text{Wt. gained by the insect}}{\text{Wt. of food digested}} \times 100$$

i. Relative Consumption Rate (RCR) $= \frac{C}{\overline{BA} \times T}$

j. Relative Growth Rate (RGR) $= \frac{P}{\overline{BA} \times T}$

Where $\overline{BA}$ = Arithmetic Mean of Body Weight of a stage and T = Feeding Period in Days.

4. 12. Biochemical analysis:

Biochemical analysis of the basic dietary components of three clonal varieties and final larval stages of the three tea pests under study were conducted. In all cases, an equal amount of dry weights of natural (tea leaf) and artificial diets and larval body mass were used for the analysis of the biochemical components which were subsequently converted into percentage for convenience of comparison. The methods adopted are described below:

4. 12. 1. Preparation of dry leaf powder: (Banerjee and Haque, 1985)

Young and matured leaves of Tv_{26} (preferred by Looper and Red Slug Caterpillars respectively) and senescent leaves of Tv_{18} (preferred by Hairy Caterpillar) were collected from the tea experimental plantation of Zoology Department, at North Bengal University campus. These leaves were cleaned, and oven dried at 50° C for 48 hours. Later each category of leaves were

crushed to fine powder in a grinder and kept in sealed polythene packets in desiccator over silica gel for further analysis.

4. 12. 2. Preparation of dry powder of late instars of *B. suppressaria, Et. magnifica and E. latisfascia*

From the mass rearing on natural (tea) diets a good number of healthy V^{th} instar stages of Looper Caterpillar and Red Slug, and VI^{th} instar stage of hairy Caterpillars were selected and kept hungry for 12 hours to completely empty their gut content. Subsequently these were cold narcotized and oven dried at 50° C for 72 hours. Fine powder of dried larvae of each species was made separately by grinding and kept in sealed polythene packets over silica gel in a desiccator. Similarly, healthy samples of final larval instars of lepidopteran pests reared on artificial diets were processed, powdered and kept sealed in polythene packets.

4. 12. 3. Analysis of Biochemical components

4. 12. 3. 1. Total Nitrogen.

The method as described in AOAC (1990) was followed. Approximately 2g of sample was accurately weighed, placed in a digestion flask and was added with 7.0 g catalyst ($CuSO_4$: K_2SO_4, 1:9) and 25ml concentrated H_2SO_4. The flask was gently heated until frothing ceased, the content was boiled briskly until the solution became clear, and then the boiling was continued for about 1 hour. The solution was transferred quantitatively to a round bottomed flask and the volume was made up to 100 ml. with distilled water. A layer of 40 % W / V aqueous NaOH (approx. 100 ml.) was added carefully till the solution of flask turned black.

The flask was immediately connected to a distillation apparatus and the tip of the condenser was immersed in standard 20 ml. 0.1 (N) H_2SO_4 containing about 2 drops of methyl indicator (0.5 % W / V methyl red in ethanol). The flask was rotated to mix the contents thoroughly and heated until all the ammonia had distilled. The receiver was removed and the tip of the condenser was washed with distilled water. The remaining acid in the receiver was titrated with standard 0.1 N NaOH solution. The value of blank was determined (Water + Catalyst + 25ml. H_2SO_4 = digested) in order to make correction factor.

Total Nitrogen (%) = {[ml.of standard acid ×.1(N) of standard acid] – [(ml. of standard NaOH – correction factor) ×.1(N) of standard NaOH]} × 1.4007 / Wt. of sample (g)

4. 12. 3. 2. Fat:

Fat content was extracted using a glass soxhlet (AOAC, 1990). Round bottomed flask was oven dried and kept in a desiccator for cooling. The weight (W_1) of the round bottomed flask was taken. A cellulose thimble (dry and fat free) was taken in which ~ 2 g of sample was placed and put in the soxhlet. Fat was extracted by using petroleum ether (with boiling range 40 - 60° C) in the round bottomed flask fitted with the soxhlet containing the thimble with sample on a heating mantle at 60° C for 5 h. The round bottomed flask with the extract was dried for 1 h at 100° C to evaporate ether and moisture, cooled in desiccator and weighed (W_2). Fat was calculated in percentage.

Fat (%) = (W_2– W_1/sample weight) × 100

4. 12. 3. 3. Total ash:

2 g of sample was accurately weighed into a previously dried and weighed crucible. This was placed in a Muffle furnace and heated to 550° C for 3 h. The crucible was transferred directly to a desiccator, allowed to cool to room temperature and weighed immediately (AOAC, 1990).

$$\text{Ash (\%)} = W_2 - W_0 \,/\, W_1 - W_0 \times 100$$

Where W_0 = Wt. of crucible

W_1 = Wt. of crucible + sample (before heating)

W_2 = Wt. of crucible + sample (after heating)

4. 12. 3. 4. Moisture

Moisture of natural (tea leaf) and artificial diets and larval body mass were calculated by subtracting the final weights from the initial weights. Drying was done at 50°C for 72 hours or till the constant weight was attained. The moisture is expressed in percentage of the fresh weights of natural (tea leaf) diet, artificial diet and larval body mass.

The Biochemical estimates were based on five replicates and the values presented are the percentage of mean.

4. 13. Statistical Analysis

Studies on Host Plant Selection, Allometric Growth, Post – embryonic development period on Natural & Artificial Diets, Daily Food Consumption and Larval Body Weight, Reproductive Performance and Dry Mass Budget were

subjected to statistical treatment as per requirements for computation of Mean, Standard Deviation, Standard Error, Regression, Student's t – test, LSD and ANOVA from the Systat Package and Excel was used for Graphics.