4. MATERIALS AND METHODS

The contemplated work was mostly carried out at the Entomology Research Unit, Department of Zoology, UNB during the period of June, 2011–May, 2014 in a research project funded by National Tea Research Foundation, Tea Board, Kolkata, India.

4.1 COLLECTION OF LOOPERS AND MOTHS

Moth and looper stage of geometrids were collected by sweep nets and hand picking from the tea plantations of Darjeeling Terai and the Dooars (Fig. 4.1). Moths were then allowed to mate and lay eggs in plastic containers, towelled with tissue paper. Looper stages were reared on natural diet (tea leaves) to pupate and emerge as adults for further laboratory culture.

4.2 REARING OF LOOPERS AND MAINTENANCE OF CONTINUOUS CULTURE

Fertilized eggs of geometrid moths were reared on host leaves and on synthetic diet in a BOD incubator at a temperature of 26±2°C, relative humidity (RH) 75±5% and light-dark phase (L:D) 13:11h. During pupal development, RH was carefully maintained below 80% to avoid microbial infection. Laboratory data on moulting, weight of freshly moulted larvae at each instar, pupa and adults along with wing expansion and their structural variability were recorded for preparation of stage-specific key to help in identifying the different stages of geometrid species found in tea plantations area.

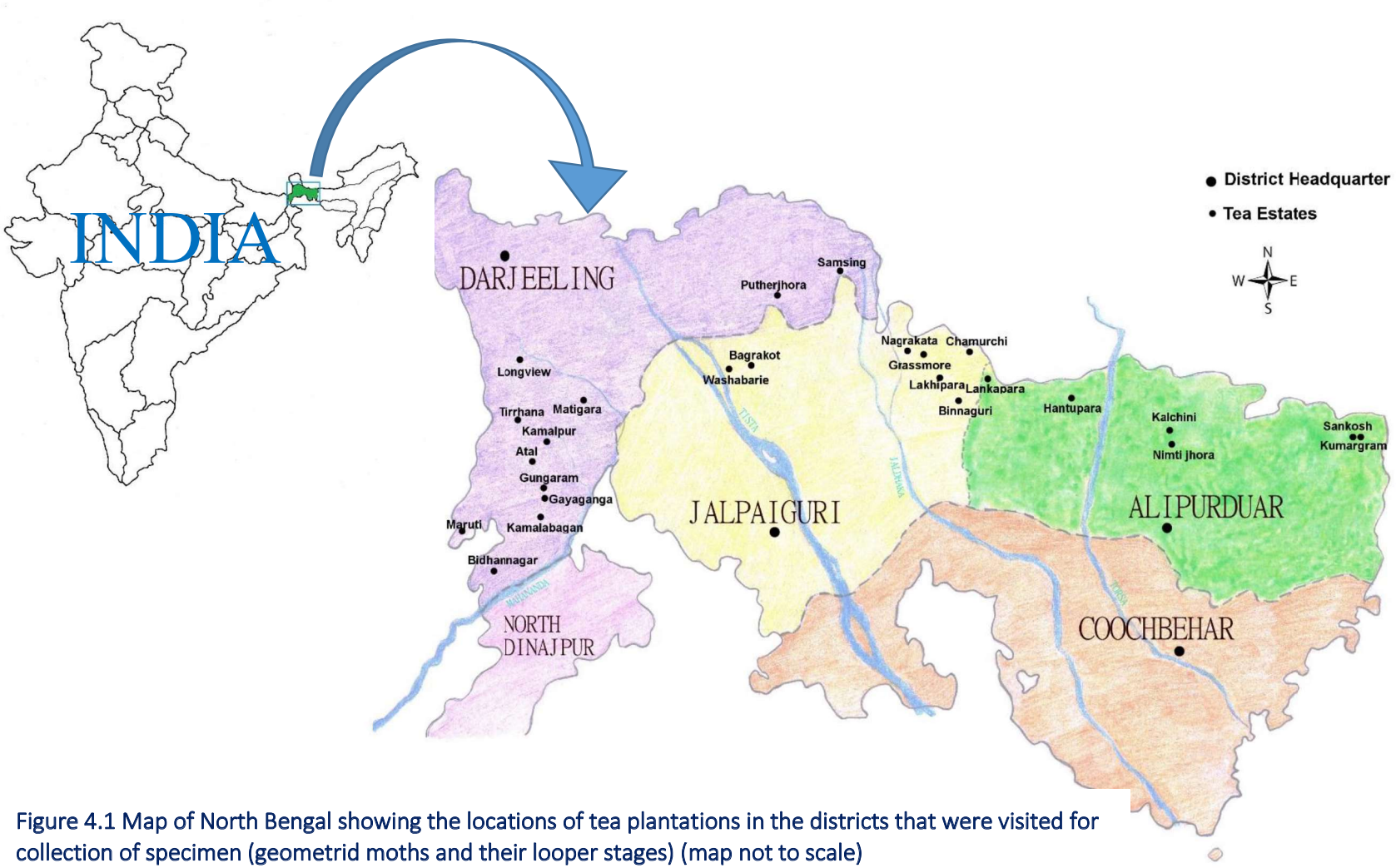

Figure 4.1 Map of North Bengal showing the locations of tea plantations in the districts that were visited for collection of specimen (geometrid moths and their looper stages) (map not to scale)

4.2.1 Natural hosts: Tea and needlewood were selected as natural hosts to rear all the three geometrid species commonly associated with tea plantations of Terai-Dooars.

4.2.1.1 Processing of leaves: Leaves of tea clone TV26 (Tocklai variant) were collected from an experimental plot maintained bio-organically by Entomology Research Unit, Department of Zoology on University of North Bengal Campus (26°42'37.53"E and 88°21'05.60"N) and used for rearing purpose. Leaves of Needlewood was collected from the trees maintained on the same university campus as well as from the nearby forest area adjacent to Kamalpur tea estate, Bagdogra. Leaves were surface sterilized by 1% of sodium hypochlorite (NaOCl) solution and then thoroughly rinsed with distilled water. Petioles of young leaves were submerged in water filled in micro centrifuge tube (2ml) for rearing the early instars on natural diet. This setup was kept in rearing container made of transparent plastic (11cm height x 12cm diameter). Neonates ($n \leq 30$) were released in each container for early development, and at 3rd instar stage they were transferred to transparent buckets (45cm height x 30cm diameter at brim) provided with young leaves as food (i.e., young tea/needlewood twigs inserted in a water-filled conical flask) for rearing. Leaves were changed daily or on alternate days depending on their moisture content and turgidity.

4.2.1.2 Sterilization of the insect culture equipment and set up: The plastic containers were properly sterilized before using for rearing purpose. Initially, the containers were kept submerged in liquid soap for 12 hrs and then thoroughly washed with water. After drying they were again washed with 5% sodium hydroxide (NaOH) solution, followed by thorough washing with distilled water. If any infection was observed in laboratory in any brood, the laboratory and all equipment were

subjected to appropriate sterilization method. Any container having infected loopers was washed with KOH solution followed by NaOCl solution. After drying the containers, they were exposed to UV-rays inside the Laminar flow hood for 10-15 minutes. The glass wares were sterilized in the same method except that they were steam-autoclaved instead of UV-sterilization. The culture room was at times fumigated by 5% formaldehyde solution. The fumigation was performed in absence of any culture. Working tables and the floor were cleaned either with 5% KOH, followed by Lysol solution. Pieces of clean cloth, toothpicks, blotting papers, spatulas and brushes were steam-autoclaved.

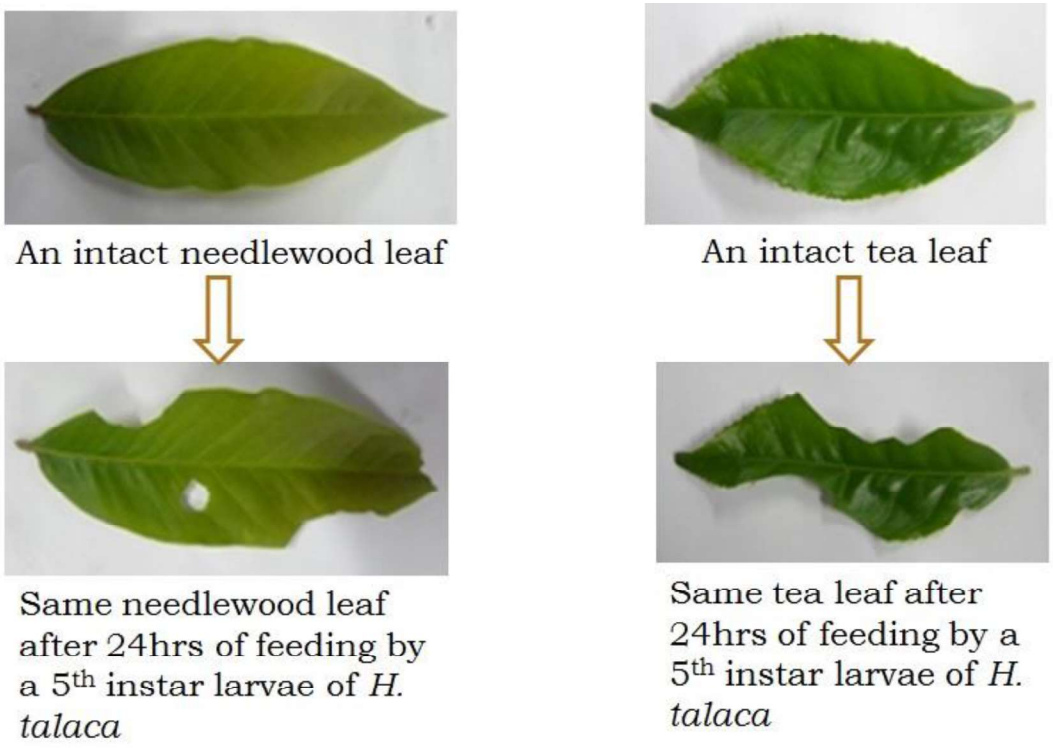

Figure 4.2 Feeding by geometrid loopers on natural diet (leaves)

4.2.1.3 Maintenance of culture: Sterilization of hand and use of hand gloves were obligatory before handling any insect culture. Thorough washing of hands with sanitizers, followed by washing it with rectified spirit was done before handling cultures. A regular observation of each and every culture was done to avoid any

mortality either by moisture or infection. Containers were discarded if any mortality was observed. For mass culture, containers were changed every alternate day, whereas in individual culture the same container was used for whole life-cycle. Only excreta were removed if get deposited excessively. The leaves were changed if they get dried or were consumed completely and care was taken that leaf petioles get continuous supply of water from micro centrifuge tubes or conical flask. Advanced instar larvae were capable of consuming whole of a medium sized tea leaf in 24 hrs (Fig. 4.2). So, for rearing and maintaining a culture, minimum of 15 man-days was required for *Hyposidra* spp. and 30 man-days for *B. suppressaria*.

4.2.2 Synthetic diets: Formulation of the diet for species of geometrid pests of tea was done initially after the essence of the composition prescribed by Lyon and Brown (1970) who developed a successful diet for fall cankerworm, *Alsophila pometaria* (Lepidoptera: Geometridae). Diet was modified after adopting some ideas suggested by Cohen (2004) and others.

4.2.2.1 Preparation of synthetic diet: Basic ingredients used in diets were divided into two groups: Group A (Wheat Germ, Cellulose powder, Casein, Potassium sorbate, Dextrose, Wesson's salt mixture, Choline chloride, Cholesterol) and Group B (Sodium alginate, Sucrose, Vitamin mixture, Linoleic acid and Wheat germ oil). Ingredients of Group A were mixed with autoclaved solution of agar dissolved in distilled water with the help of a blender (make: Philips) for 30-40sec. After blending it smoothly, the mixture was allowed to cool down between 60-70°C and then other ingredients (Group B) were mixed and blended. The slurry of fresh hot diet was poured into sterilized plastic containers and allowed to cool to room temperature and stored in the refrigerator at 4°C for future use.

4.2.2.2 Dispensing of diet in culture container: Before dispensing the diet to insects, the water droplets accumulated on the diet surface was soaked by autoclaved blotting paper. To provide freshly prepared diet to neonates, more care was taken while removing the accumulated water droplets. The neonates cannot move on a wet surface and stick to diet and consequently die. Initially rearing was done inside the containers shallowly laid with diet at their bottom. As the mortality of early stages was very high due to water droplets accumulated on the surface of the diet, some toothpicks were provided inside the containers for clinging and easy movement of the looper caterpillars. Further, to minimise the problem of mortality of early instars, cubes of diets pierced-across with toothpicks were kept suspended inside the containers. This new method of food dispensing reduced the accumulation of water droplets inside the container as well as roused in the caterpillars their natural instinct of moving on tea twigs (=sticks).

4.2.2.3 Maintenance of culture: A regular observation was taken to note the level of mortality on synthetic diet as was done for natural diet. Special care was taken to avoid accumulation of water droplets and fast drying of diet. Containers were used in respect to the size of looper stage to be reared which provide easy access to the diet at the same time prevented wastage of diet. Since, the early instar larvae were unable to finish even a small cubes within 3-4days and same usually got dry by that time, a small container (6cm x 4cm diameter) with 3-4 diet cube pegged on toothpicks were used for 20 caterpillars. In this stage, container's mouth was covered with autoclaved cotton cloths and the actual lid were just kept loosely to cover the mouth. This setup allowed proper aeration, therefore no accumulation of water droplets vis-a-vis the lid of the container prevented fast drying of the diet cubes. As the larvae entered 3rd instar, the number of caterpillars per container was

reduced to 10 and then to 5 for 4th instar. At this stage, cloth covering was no more required, as accumulation of water droplets inside the container had no deleterious effect on larval movement. However, for pupation an excess humidity was detrimental. So tactical covering the mouth of the container with lid helped in management of the humidity level. As a 5th instar larvae alone can consume a single cube (0.75 cm^3) in 1-2 days, there was no chance of drying of the diet. Hence, for maintaining the setup only 10 man-days for *Hyposidra* spp and 15 days for *B. suppressaria* were required as no need of checking water level and change of food were necessary in 2-3 days interval. The same setup and dispensing system helped in successful formation of pupa and emergence of adult. Male and female when kept in the ratio of 2:3 was found to have successful mating. The containers used for mating of *Hyposidra* spp. were comparatively smaller (6cm x 4cm) than the one used for *B. suppressaria* (10cm x 8cm diameter).

4.3 DEVELOPMENTAL TRAITS

4.3.1 Developmental period: Egg-hatching, moulting, pupation and adult emergence were observed and recorded throughout the rearing process to determine total developmental period (eggs to adult emergence) and other life-cycle parameters.

4.3.2 Survival: An account of stage specific survivorship (Edillo et al. 2004) of all the three species were prepared both on synthetic as well as on natural diet (tea) using the formula:

$$S_i = \frac{N_i}{N_{i-1}}$$

Where, S_i is stage specific survivorship, N_i is the total number of immature form entering the life stages 'i' (i.e. instar) and (N_{i-1}) is the number of alive in the previous instar.

$$\% \, Survival \, (S) = 100 \times S_i$$

4.3.3 **Morphometrics:** A record of weight and length of each stage was kept for morphometric analysis. A fine electronic balance (BSA 2245 CW, d=0.1mg, Sartorius made) was used for weighing different developmental stages.

Percentage of adult emergence was calculated using the following formula:

$$\%of \, Adult \, emergence = \frac{number \, of \, adult \, emerged}{number \, of \, pupa \, harvested} \times 100$$

4.4 KEY PREPARATION

Morphology and morphometric data were used to prepare the keys for all the stages of the three major species of geometrid moths. Such keys are expected to help in easy determination of the species and its immature stages both in field and in laboratory.

4.5 FOOD UTILIZATION STUDY

Food consumption and utilization study was conducted on freshly moulted larvae. The gravimetric (dry mass) technique was used to determine the efficiency of conversion of ingested food (ECI), efficiency of conversion of digested food (ECD), approximate digestibility (AD), relative consumption rate (RCR), relative growth rate (RGR), maintenance cost (MC) and production index (PI) (n=20) following the basic methodologies of Waldbauer (1968), subsequently modified by Kogan and Cope (1974), Slansky and Scriber (1985) and Parra et al. (2012). Weight of larvae, quantity of diet consumed in weight and leaf-area were taken after and before the experiment. Caterpillars of similar weight, diet cubes/leaves (of same age) and their faecal pellets were dried for gravimetric studies at 50°C until a constant weight was attained. A control of both synthetic diet and natural diet was kept to calculate the normal loss of moisture.

Nutritional indices for two host plants were compared for 4th instar onwards as they consume substantial amount of leaves. However, while comparing with synthetic diet, only 5th instar in case of *Hyposidra* spp. and 5th and 6th instar for *B. suppressaria* were taken into consideration. As the tiny faecal pellets stuck to the diet cubes, therefore chances of error in calculating nutritional indices were expected to be high in early instars reared on synthetic diet.

The indices were calculated using the following formulae:

$$Production\ (P) = Final\ body\ weight - initial\ body\ weight$$

$$Assimilation\ (As) = Food\ consumed\ (C) - Faecal\ matter\ (F)$$

$$Respiration\ (R) = As - P$$

$$MC = R/P$$

$$PI = P/As$$

$$AD = \frac{As}{C} \times 100$$

$$ECI = \frac{P}{C} \times 100$$

$$ECD = \frac{P}{As} \times 100$$

$$RCR = \frac{C}{mean\ of\ the\ body\ weight\ of\ a\ stage\ \times feeding\ period\ (in\ h)}$$

$$RGR = \frac{P}{mean\ of\ the\ body\ weight\ of\ a\ stage\ \times feeding\ period\ (in\ h)}$$

4.6 MID-GUT ENZYME ANALYSES

Hydrolases and defence enzyme activities of final instar were compared as this is the stage on which larva consumed most and utilized maximum to gain critical body mass before pupation.

4.6.1 Enzyme isolation: Extraction of enzymes was done from laboratory-reared 5th instar larvae of *Hyposidra* spp. and 6th instar larvae of *B. suppressaria* starved for 8h to clear gut (n=10). Each larva was dissected in ice-cold 0.1M sodium phosphate buffer (pH 7.0) and its midgut was removed. The excised midguts were homogenized separately in fresh sodium phosphate buffer (0.1M). The homogenate was centrifuged (Sigma 3K30) at 10,000g for 15min at 4°C. The supernatant of this preparation was stored at -20°C for future use.

4.6.2 Enzyme quantification

4.6.2.1 Amylase: Amylase was assayed by measuring the increase in reducing ability of buffered starch solution with 3,5- dinitro salicylic acid (DNS) (Bernfeld 1955). The enzyme sample (40µl) were incubated with 1% starch solution (40µl) (pH 7.0) at room temperature for 3mins and the reaction was stopped by adding DNS (80 µl).The mixture was then kept inside the boiling water for 5 mins followed by immediate cooling. Distilled water (800 µl) was added before taking final reading. Amylase activity was expressed in micromoles of maltose liberated per minute per milligram of protein based on absorbance value (OD) taken on microplate reader (Opsys MR, DYNEX Technologies, Chantilly, VA, USA) at 540nm.

4.6.2.2 Invertase: Invertase activity was estimated using 1% sucrose solution and DNS solution as a reaction stopper using same procedure as given in amylase assay. The standard curve was prepared with a mixture of 0.001M glucose and fructose (1:1) in 0.1% benzoic acid. The absorbance (OD) was observed on microplate reader at 540nm.

4.6.2.3 Protease: Proteolytic activity was assayed after the method of Walter (1984). 2 ml of 1% (w/v) casein in Tris-HCl buffer (0.1M, pH 7.8) was incubated with the enzyme (100µl) for 2h. Reaction was stopped by adding 2ml of 10% TCA

(Trichloroacetic acid). The reaction mixture was then filtered and the absorbance (OD) was noted spectrometrically [UV-Visual spectrophotometer (Rayleigh UV-2601, China)] at 273nm. The total protease activity was expressed as nanomoles of tyrosine liberated per hour per milligram of protein.

4.6.2.4 Lipase: Lipase was assayed according to the method of Winkler and Stuckman (1979), following the release of *p*-nitrophenol (*p*NP) from *p*-nitrophenyl palmitate (*p*NPP). The substrate was prepared by dissolving 30mg pNPP in 10ml of isopropanol mixed with 90 ml of 0.05M Phosphate buffer (pH 8.0) containing 207mg of sodium deoxycholate and 100mg of gum Arabic. A 2.4ml of freshly prepared substrate was pre-warmed at 37°C and incubated with enzyme extracted for 15mins. The absorbance was recorded on microplate reader at 410nm. The total lipases was expressed as nanomoles of *p*-nitrophenol liberated per min per milligram of protein.

4.6.2.5 General Esterase: Activity levels of esterase were estimated using 30mM of α-Napthyl acetate as substrate, following the method of van Asperen (1962). The enzyme supernatant (20µl) was incubated with substrate (200µl) (1:100) for 15mins at 25°C. Reaction was stopped by 50µl of a staining solution [0.1% fast blue BB salt: 5% sodium dodecyl sulphate (SDS) = 2:5] and kept for 5mins. The absorbance was recorded at 570nm on microplate reader. General esterase activity was expressed as micromoles of the α-napthol per minute per milligram of protein (van Asperen 1962).

4.6.2.6 Glutathione *S*-transferase: Glutathione *S*-transferase activity was measured using 1-chloro-2, 4- dinitrobenzene (CDNB) as the substrate (Habig et al. 1974). A reaction mixture of 50µl of CDNB (50mM in ethanol) and 150µl of reduced glutathione (50mM in 0.1M PBS, pH 6.5) were mixed with 2.78ml of

Sodium phosphate buffer (0.1M, pH 6.5 containing 1mM EDTA). 20µl of enzyme supernatant was then added and shaken well. Absorbance at 340 nm was recorded spectrophotometrically for 10–12 min employing kinetics (time scan) menu. The GST activity was calculated using the formula CDNB-GSH conjugate (µM/min/ mg protein) = (Absorbance increase in 5 min × 3 × 1000)/ (9.6* × 5 × mg of protein) (*9.6 mM/cm is the extinction coefficient for CDNB-GSH conjugate at 340 nm).

4.6.2.7 Cytochrome P450: As heme protein is a major constitute of the majority of Cytochrome P450, its activity was calculated by estimating heme peroxidase activity (Penilla et al. 2007 and Tiwari at al. 2011). 20µl of enzyme homogenate was incubated with 200µl of TMBZ solution (0.01g of TMBZ in 5ml of methanol+15ml of 0.25M sodium acetate, pH 5.0) and 80µl of 0.0625M PBS (pH 7.2) and 25µl of 3% H_2O_2 for 30 minutes at 25°C. Absorbance was recorded at 630nm on microplate reader. The standard curve of heme peroxidase activity was prepared using Cytochrome C from horse heart type IV. Total Cytochrome P450 was expressed as nmoles of Cytochrome P450 equivalent units (EUs) per mg protein per minute.

4.6.3 Protein Estimation: Protein present in enzyme suspension was measured following the method of Lowry et al. (1951).

4.7 Statistical Analyses: All statistical analyses were performed using computer software *InStat* and *Origin*. The significant difference between means was calculated by unpaired *t*-test ($P \leq 0.05$) and subsequently Welch's correction was applied if standard deviations were found to be unequal. Mann-Whitney test was done whenever data did not passed the normality test. Data was processed by Microsoft Excel for graphical presentation. While comparing more than two mean, one-way analysis of variance (ANOVA) followed by Tukey's multiple comparison

test using Least Significant Difference (LSD) at 5% level of significance was performed using SPSS 20 software (IBM Corp.).

H. talaca, H. infixaria and *B. suppressaria* were selected for the detailed studies as they are found to be the most potential pests of tea, causing substantial crop loss in Terai-Dooars tea plantations.