3. MATERIALS AND METHODS

3.1 Comparative Phenology of Both Non-Diapause and Diapause Generations of *A. mylitta.*

3.1.1. Phenology of larvae, pupae and adults.

The insect and rearing condition : All investigations were carried out on the 'Daba' bivoltine brood of *A. mylitta* Drury at Ranchi in the state of Bihar at a longitude, latitude and altitude of 85°18'E, 23°14'N and 708 m MSL respectively. Eggs for both diapause and non-diapause generations were obtained from the Central Tasar Research and Training Institute , Nagri, Ranchi, India. Just after hatching for each generation 40,000 larvae irrespective of sex were maintained in the field on the high bush of Arjun plant (*Terminalia arjuna,* Bedd.), one of the primary food plants of *A. mylitta* under natural environmental conditions. Rearing was done on bushy vegetation of *T. arjuna* made through arboriculture (Fig.6). The rearing was continued in the field from first to fifth larval instars till the formation of cocoon on the food plant after which cocoons were collected from the field and kept at room temperature of insectary. The larval feeding period (phagoperiod) was measured from the day of larval emergence (from egg) to the day of onset of spinning and the non-feeding or non-phagoperiod was counted from the starting of spinning to the onset of pupation. The rearing period in non-diapause generation lasted from middle of July to last week of August and in diapause-destined generation it lasted from first week of October to first week of December.

Body weights and growth indices: The initial body weights for 100 newly hatched larvae (irrespective of sex) were recorded and the average weight was determined. The larval body weight for each instar was recorded immediately before and after each ecdysis to the next instar before the onset of feeding. Sexing could be done only in the 5th (final) instar larvae. Relative growth rate (RGR) of each instar was calculated on green weight following Waldbauer (1968) and Rema Devi et al., (1991) by the formula :

Fig.6 : Photograph of plantation of *Terminalia arjuna*, the primary food plant of *A.mylitta,* raised through arboriculture.

Fig.6

$$\frac{\text{Final larval weight - initial larval weight}}{\text{Mean larval weight x Feeding duration in days}}$$

The larval maximum weight (Lmw) was noted at the fifth instar on larval maturity just after cessation of feeding. In the insectary all cocoons were cut open to record the time of pupation. Cocoons were weighed with and without pupae (empty shell). The shell ratio percentage (SR %) was calculated by

$$\frac{\text{Single shell weight}}{\text{Single cocoon weight (with pupa)}} \times 100$$

Weight of each pupa during the span of pupal life was taken at an interval of 7 day in non-diapause till the emergence of moth (adult). In case of diapause-destined generation carbohydrate profile (Chaudhuri et al., 1993) and anatomical evidence (unpublished observation) suggest that till 150 day of pupal life the development occurs at a very negligible pace. Hence, pupal weight for this generation was initially taken at 30 day interval upto 150 day and thereafter at every 15 day (Table 3). Pupal body become soft on the day before emergence and based on this observation pupal weights were taken on day before emergence. Moth weights were taken just after emergence.

Egg number and hatching percent : This aspect was also studied in the insectary. Generally tropical tasar moths lay eggs continuously spanning over three consecutive days. After proper washing and disinfection the eggs were collected and kept for incubation under natural environmental conditions till hatching which required about 10 days for both the generations (Table 3). Unlaid eggs were counted in each female after completion of oviposition on the third day by dissecting abdomen. Laid and unlaid eggs were counted on the basis of total number of eggs (laid and unlaid) per female. Egg hatchability was calculated by the number of eggs hatched out of total number of eggs laid. For every parameter studied, the data were taken from 100 individuals in each generation.

Environmental records :

Daylength, maximum and minimum temperature and relative humidity(r.h) were recorded daily in the field as well as in the insectary all through the study period for the duration of each larval instar, pupa and adult. Rainfall was also

similarly recorded. Daylength was measured in hours as light : dark cycles of 24 hour periodicity. Relative humidity was measured daily as an average of the records in the morning and in the evening. The data were subjected to student's 't'-test.

3.1.2 Grainage Performance : This was studied on the basis of percentage of moth emergence, sex ratio, mating, fecundity, egg incubation time and hatching percentage. In a year, the moths (adults) emerged from the first week of June to the second week of August) after breaking the overwintering pupal diapause were considered as the first brood, and the second moth emergence (from the first week of September to the third week of October) was regarded as the second brood for this study.

Depending on the time of availability of tasar seed cocoons during the study seasons, selected healthy pupae (a number of 11,000 in first brood and 14,000 in the second brood) were preserved in the model grainage room of the insectary as improvised by the Central Tasar Research Station, Ranchi, India (Kapila *et al.*, 1992) in order to observe their emergence and post-emergence behaviour.

Daily moth emergence (sex wise) , mating percentage and fecundity of each mated female were recorded in every generation. Following oviposition the eggs were incubated in the insectary for observing the time required for incubation together with their hatching performances. Temperature and r.h. in the insectary were recorded daily for every brood.

These daily recorded data were computed as average of every three consecutive days spanning the entire grainage periods. All these average values were subjected to student's 't' test wherever necessary.

3.2 Starvation and Neck Ligation Experiments for Ascertaining the Timings of PTTH Release by the Fifth Instar Larvae :

3.2.1 Starvation: The experiments were conducted on the same population and on the same location as stated under phenology studies (3.1.1) but on separate arjuna plants for both non-diapause and diapause generations. From the very large population only 10,000 healthy , 0-day-old fifth instar larvae were randomly collected, 5000 males and 5000 females for each generation. The larvae of non-

diapause brood attained the fifth stage during August and those of diapause-destined brood attained during October-November under field conditions. Sexing were done by observing the Herold's gland in females and Ishiwata's spot in males. An equal number of same aged larvae of each sex and each generation was also kept in the field from the same population as control. The control larvae were weighed daily from '0'-day to larval maturity when larval maximum weight (Lmw) was recorded just after cessation of feeding. For the purpose of assessment of larval critical weight (Lcw) 60 male and 60 female larvae of each generation were withdrawn from the host plants daily starting with '0'-day old onwards upto the day of gut purge. Such larvae under starvation were kept on leaf-less branches encircled by nets. The larvae were weighed and observed daily for their morphological and/or behavioural changes associated with larval-pupal transformation. The experiment was conducted in two consecutive years.

3.2.2 Neck ligation : The larvae of each sex were ligated behind the head (neck ligation) with a strong fine cotton thread in both non-diapause and diapause generations separately. Neck ligation was performed daily on 25 males and 25 females of each generation from the '0'-day age onwards till the day before larval-pupal transformation for testing the timings of PTTH release. All larvae (control and experimental) in both generations were individually weighed every 24 hr. interval. Morphological and behavioural changes associated with pupal syndrome such as body shrinkage, decremental mobility of anal proleg and gut purge and pupal ecdysis were recorded daily in both control and experimental batches.

Larval critical weight (Lcw), larval maximum weight (Lmw), latent feeding period, pupal critical weight (Pcw) and adult critical weight (Acw) were determined from the starvation experiment following the procedure of Ochieng'-odero (1990a, 1990b). Lcw was regarded as the weight at which 50% of the silkworms were capable of pupation thereby producing functional adults. Pcw and Acw were calculated as follows :

$$Pcw = Lcw - (Lcw \times D_P)$$

and $$Acw = Lcw - (Lcw \times D_A)$$

where, D_P = Constant weight decrease from Lmw to Pw (Pupal weight)
and D_A = Constant weight decrease from Lmw to Aw (adult weight).

Relative silk conversion efficiency were calculated by

$$\frac{\text{Single shell weight (without pupa)}}{\text{Larval weight after gut purge}} \times 100$$

All weights were taken on green weight basis and gated release of PTTH during the period of larval-pupal development was resolved. The different biological performances such as gut purging and wandering behaviour, silk cocoon spinning, larval duration in respect of phagoperiod and non-phagoperiod, formation of pupal cuticle, time required for pupation, pupal characters, adult emergence and number of egg production (laid + unlaid) were recorded from time to time. The ambient environmental data were also recorded all through the experiment.

Data were statistically analysed by student's 't' test wherever necessary.

3.3 Quantitative Status of Cholesterol, Protein, DNA and RNA of Haemolymph, Fat body and Gonads of Pre-pupae, Pupae and Adults of both the Generations

3.3.1 Chemicals and reagents used for estimation:

3.3.1.1 For Cholesterol : Ferric Chloride - Acetic Acid reagent : 50 mg of ferric chloride (E.Merck, AG Darmstadt) was dissolved in 100 ml glacial acetic acid (GR, Sarabhai M. Chemicals, India). The solution was kept in a refrigerator.

Standard Cholesterol solution : 10 mg of cholesterol (E.Merck AG Darmstadt) was dissolved in 100 ml of aldehyde free glacial acetic acid with slight warming and thus a solution having 100 µg of cholesterol per ml. was prepared. This was used as standard solution and kept in the refrigerator.

3.3.1.2 For Protein :

Reagent A. 2% Sodium carbonate (BDH, Glaxo Laboratories, India) in 0.10 (N) sodium hydroxide (E.Merck, India).

Reagent B. A solution of 0.5% cupper sulphate (BDH, Glaxo Laboratories, India) in 1% Potassium Sodium Tartarate (BDH, Glaxo Laboratories, India).

Reagent C: Alkaline cupper sulphate solution . This solution was prepared

by mixing 50 ml. of reagent A with 1 ml of reagent B just before use.

Reagent D :

Diluted Folin Reagent : The Folin reagent was prepared by refluxing gently for 10 hours a mixture consisting of 100 g. of Sodium tungstate (E. Merck, AG., Darmstadt), 25g. of Sodium molybdate (E.marck, AG., Darmstadt), 700 ml.of distilled water, 50 ml. of 85% Phosphoric acid ('Analar', BDH, India)and 100 ml of concentrated Hydrochloric acid (Analar, BDH, India) in a 1.5 litre flask. After boiling , 150g of lithium sulphate (Analar, BDH, India), 50 ml. of distilled water and a few drops of bromine water were added. The mixture was boiled for 15 minutes without condensor to remove excess bromine. It was cooled and diluted to 1 litre and filtered. The acid concentration of the reagent was determined by titration with 1(N) Sodium hydroxide to a phenolpthalein end point. Just before use the reagent was diluted accordingly so as to prepare a diluted Folin reagent of 1(N) of acid strength.

Reagent E. Standard protein solution : Bovine serum albumin Cohn fraction V (Sigma, USA) was used for the preparation of a standard protein solution. A standard solution containing 200 µg of protein per ml. was prepared by dissolving 20 mg of the protein in 100 ml. of distilled water.

3.3.1.3 For DNA : Standard DNA solution: 5 mg of calf thymus DNA (Sigma, U.S.A.) was dissolved in 10 ml of 5% Perchloric acid (GR, E.Merck, AG Darmstadt) at 70° C for half an hour. 5 ml. of this solution was taken and volume was made upto 50 ml with 5% Perchloric acid and thus a solution having 50 µg of DNA per ml was prepared. This was used as standard solution and was kept in the refrigerator at 4°C.

3.3.1.4 For RNA : Standard RNA solution: Yeast RNA (Sigma, U.S.A.) was purified in the laboratory. 10 mg of purified RNA was dissolved in 10 ml of 5% Perchloric acid (GR, E.Merck, AG, Darmstadt) by heating the mixture at 70°C for 30 minutes in a water bath. 5 ml of this solution was taken and the volume was made upto 50 ml with 5% perchloric acid so as to prepare a solution containing 100 µg of RNA per ml. This was used as standard solution and was kept in the refrigerator at 4°C.

3.3.2 Procedure and methods of analysis

Stages of the insect and sample size : Every time 10 individuals of pre-pupae, pupae and moths of each sex and of both the generatioins were sacrificed on different days as required during the experimental period for all the quantitative estimations. In the non-diapause generation pupae were sacrificed on 0, 7, 14 and 21 day while in diapause generation pupae of 0, 40 , 105, 150, 170 and and 200 day old were sacrificed for the estimation.

3.3.2.2 Collection of haemolymph : The haemolymph from pre-pupae was collected by inserting the needle of a microlitre syringe through the second abdominal leg. But the haemolymph of pupal and adult stages was collected by a microsyringe piercing through the anal margin of the wings by applying a gentle pressure on the abdomen. After collection of haemolymph, a few granules of phenylthiourea was added to it in order to prevent blasckening of the haemolymph. The haemolymph was then subjected to centrifuge in cool condition at 3000 rpm for 5 minutes for precipitating the blood cells. The separated plasma was taken for the extraction and estimation of protein and cholesterol.

3.3.2.3 Collection of tissue : The fat body and gonads were quickly and carefully dissected out of the individuals surrounded by ice for making a cold condition. The haemolymph was perfectly soaked. These separated fat body and gonads of males and females were weighed and used for preparation of tissue hamogenate and extraction of cholesterol, protein, RNA and DNA.

3.3.2.4 Preparation of tissue homogenates for

A. Cholesterol : A 5% tissue homogenates of fat body and gonads were

prepared in each case in ferric chloride - acetic acid reagent by using a Potter-Elvehjem all glass homogenizer and kept overnight for precipitation to extract the cholesterol. The tissue samples were then centrifuged for 15 minutes at 3000 rpm and the supernatants were collected for estimation of cholesterol.

B. RNA : A 5% homogenates of the tissues from fat body and gonads of both the sexes were prepared separately in 0.65% cold saline (NaCl) using a Potter-Elvehjem all galss homogenizer. 1 ml. of homogenate was transferred to a centrifuge tube and 1 ml. of 0.6 (N) and 1 ml. of 0.3 (N) Perchloric acid (PCA) were added and mixed thoroughly and kept standing for 10-15 minites. The mixture was then centrifuges at 3000 rpm for 5 minutes. The entire procedure was maintained under cold condition. The supernatant was discarded. The precipitate was washed with 5 ml. of cold 0.3 (N) PCA, mixed thoroughly and again centrifuged. The supernatant was discarded again. The precipitate was then treated with 1 ml. of 0.3(N) KOH and was kept on a water bath at 37°C for 2 hours with occasional stirring and cooled to the room temperature, 2 ml. of cold 0.6 (N) PCA and 2 ml. of cold 0.3 (N) PCA were added to the mixture, kept standing for 15 minites and centrifuged under cool situation. The supernatant was collected for RNA estimation.

C. DNA : The precipitate thus obtained was once again washed with 5 ml of cold 0.3(N) PCA and centrifuged. The supernatant was discarded. The precipitate was then treated with 4 ml. of 0.6(N) PCA, heated at 70°C for about half an hour, cooled to room temperature and then kept at cold (4°C) for 10 minutes after removing the red washing with 1 ml. of 0.6(N) PCA. It was then centrifuged and supernatant was collected for estimation of DNA.

D. Protein : The precipitation left after extraction of RNA and DNA was treated with 5 ml; of alcohol-chloroform - ether mixture (2:1:1), stirred and centrifuged . The supernatant was discarded. The precipitate was then washed by suspension and sedimentation with 5 ml. of absolute alcohol once and finally with 4 ml. of solvent ether. The precipitate was allowed to dry at room temperature. The dried protein thus obtained was dissolved in 5 ml. of 0.3(N) sodium hydroxide and kept overnight at room temperature. The solution thus obtained after thorough mixing was suitably diluted and used for protein estimation.

Calculations : The results of cholesterol, protein, RNA and DNA contents of fat body and gonads of male and female individuals were expressed per 100 mg tissue. The haemolymph (plasma) protein and cholesterol contents were expressed

per ml. of plasma.

Results were statistically analysed using student's 't' test.

3.3.3 Procedure of Estimation :

3.3.3.1 Estimation of Cholesterol - Haemolymph : 50 ml. plasma (from previously isolated stock) was taken in a centrifuge tube and washed with 5 ml. of ferric chloride - acetic acid reagent and kept overnight for precipitation. It was then centfifuged for 15 minites at 3000 rpm. The supernatant was collected for the estimation of cholesterol.

The cholesterol content of haemolymph plasma was determined by the method of Kabara (1962). An aliquot of 4 ml. of the extracted cholesterol was taken in a test tube. The final volume was made to 5 ml. by addition of 1 ml. of ferric chloride - acetic acid reagent. 3 ml. of concentrated suiphuric acid was then added and mixed thoroughly in a cyclomixer. For standard curve, five different concentrations of cholesterol (10 µg, 20 µg, 30 µg, 40 µg, and 50 µg) were used and treated as above. For the reagent blank 5 ml. of ferric chloride - acetic acid reagent was used instead of cholesterol solution and treated as above. The standards and the reagent blank were run simultaneously along with the test samples each time. After mixing the samples the tubes were kept in a cold dark place for 30 minutes. The readings were taken at 560 nm with 'Turner' spectrophotometer (USA).

Fat body and gonad : The cholesterol content of fat body and gonads were determined separately by the same method of Kabara (1962) at 560 nm with 'Turner' spectrophotometer (USA).

3.3.3.2 Estimation of RNA : RNA content of fat body and gonads was estimated by the method of Munro and Fleck (1966) modified by Abalain et al., (1980). Aliquote of 2 ml. for fat body (male and female) and 1 ml each for ovary and testis were taken all through the investigation relating to pre-pupae, pupae of different days/age and freshly emerged adults (moths). Each aliquot was diluted to 3 ml. by 0.3(N) PCA. The samples were read at 260 nm using ultraviolet unit of 'Turner' spectrophotometer (USA).

For standard curve, five different concentrations of purified yeast RNA (10 µg, 20 µg, 30 µg, 40 µg, and 50 µg) were prepared from the standard solution and the volume of each concentration was made to 3 ml with 0.3 (N) PCA. 3 ml. of 0.3(N) PCA was used as reagent blank.

3.3.3.3 Estimation of DNA : DNA content of fat body and gonads was estimated by the method of Munro and Fleck (1966) modified by Abalain et al. (1980). Aliquot of 1 ml. for fat body, ovary and testis of extracted nucleic acid solution was diluted upto 2 ml. with 0.6 (N) PCA and were read at 260 nm using ultraviolet unit of 'Turner' spectrophotometer (USA).

For standard curve, five different concentrations of calf thymus DNA (5 µg, 10 µg, 15 µg, , 20 µg, and 25 µg) were prepared from the stock concentration (standard solution) and the volume of each concentration was made to 2 ml. with 0.6 (N) PCA in each case. 2 ml. of 0.6(N) PCA was used as reagent blank.

3.3.3.4 Estimation of protein:

Haemolymph - From the previously isolated plasma stock 50 µl of plasma was taken in a centrifuge tube and 5 ml. of 0.65% NaCl, 1 ml. of 2/3 (N) H_2SO4 and 1 ml. of 10% sodium tungstate were added. After mixing thoroughly it was centrifuged. The supernatant was discarded. The precipitate was treated with 5 ml. of absolute alcohol, stirred and centrifuged. Again the supernatant was discarded. The precipitate was washed with 4 ml. of solvent ether. The precipitate was allowed to dry at room temperature. The dried protein thus obtained after thorough mixing was suitably diluted and used for protein estimation.

Protein content of haemolymph plasma was determined by the method of Lowry *et al.*, (1951). An aliquot of 0.1 ml diluted solution was taken in a test tube and mixed with 0.9 ml of distilled water. 5 ml. of alkaline copper reagent was added, mixed and kept standing for 15 minutes at room temperature. To this mixture 0.5 ml of diluted Folin reagent was added, mixed well and left for 30 minutes at room temperature for colour development.

For standard curve, five different concentrations of bovine serum albumin viz. 20 µg, 40 µg, 60 µg, 80 µg, and 100 µg were used and for reagent blank, distilled water was used instead of protein solution. The standards and the reagent

blank were run simultaneously with test samples each time. All the samples were read at 750 nm in a 'Turner' spectrophotometer (U.S.A.).

Fat body and Gonads : Protein content of fat body and gonads was determined by the method of Lowry et al., (1951). An aliquot of 0.1 ml. diluted solution was taken in a test tube and mixed with 0.9 ml of distilled water and the protein was estimated similarly as for haemolymph.

3.4 Treatment of Pre-pupae and Early and Late Pupae of Diapausing Generation of *A. mylitta* with Insulin.

Ox pancreas insulin (40 i.u. per ml. which contains 1.67 mg of crystalline insulin) procured from Boots chemical Company, India Ltd. was used for the experiment. Pre-pupae, 40 day old early pupae and 150 day old late pupae of diapausing generation were treated with insulin. A single injection of insulin was given with the help of a Hamilton microlitre syringe to the individuals of each set of experiment. Pre-pupae received separately three doses of insulin each of 1 µl (0.04 i.u.) 5 µl (0.2 i.u.) and 10 µl (0.4 i.u.) per individual. But only two doses of 5 µl and 10 µl of insulin/individual were applied to the 40-day-old and 150-day-old pupae as 1 µl dose of the hormone was found to be ineffective when treated during pre-pupal stage. The control individuals were treated with 10 µl of 0.65% saline. A number of 100 males and 100 females were used for both experimental and control individuals for each dose except the pre-pupae. In the pre-pupae external visible sexual dimorphic characters were extremely inconspicuous. However, sexing was possible in the pupae resulted from the pre-pupae. Pre-pupae were taken out by removing the anterior cap of the silk cocoon and selected irrespective of sex at random with average body weight of 15.76 $\pm$ 1.05 g. 200 pre-pupae (3-day before pupation) were treated separately with each dose of insulin as well as with saline as control.

Pupal duration, moth weight egg production and hatching percentage were all noted separately for each treatment and control.

Total protein, RNA and DNA and cholesterol contents of haemolymph, fat body and gonads of diapausing pupae and adults after insulin treatment were estimated by the methods stated earlier (3.3). Weights of gonads after insulin treatment were also recorded.

The total protein, DNA, RNA and cholesterol contents of different tissues of normal diapausing pupae of different ages and adults were used as controls for this experiment since there was no differences in the status of these bimolecules in different tissues of Sham-operated animals (injected with 0.65% saline) when compared with those of control individuals without any saline injection i.e. normal animals.

3.5 Treatment of Dipause-destined Pre-pupae with 20-Hydroxyecdysone (20-HE)

0-day-old prepupae, after the completion of spinning were taken out by removing the anterior cap of the silk cocoon and were selected at random irrespective of sex and having average body weight of 15.76 $\pm$ 1.05g. 50 prepupae were treated with each of the dosages of 20-HE.

20-hydroxyecdysone (No. H-5142, Sigma Chemical Company, USA, anhydrous molecular weight 480.60) was used in this experiment. The hormone was first dissolved in 96% ethanol and then diluted with distilled water to yield a solution of 10% ethanol. Because of limited availability of 20-HE and incidence of developmental derangements due to progressively higher doses beyond 5 µg/ individual, only 4 dosages of this hormone were applied at the rate of 1, 2, 5 and 10 µl/pre-pupa. The hormone was injected into the abdominal region with the help of a Hamilton microlitre syringe. Every 10 µl of ethanol solution contained each of the desired doses of the hormone. Control insects were administered with 10 µl of 10% ethanol.

The observation was taken on the pupation time(time required by the prepupae to become pupae), pupal duration, emergence percentage, moth weight, morphological derrangements and pupal mortality.

An additional 50 samples of prepupae were treated with similar doses of 20-HE for each treatment along with the control for recording the morphometry and protein content of gonads on 150 day of diapause development. The weights of testes and ovaries of 8-10 individuals in each case were measured on 150 day of diapause development after dissection. Dimension of gonads were taken on the same day using an ocular micrometer under a dissecting binocular microscope. Fecundity and hatching percentage were also recorded from the moths resulted from 20-HE treatments. Gondal protein contents were also estimated.