CHAPTER - TWO

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CHAPTER-TWO

MATERIAL AND METHODS

I. MATERIAL:

1. Selection of Material:

The *Mythimna separata* is an economically important pest causing damage to crops like sorghum, rice, wheat, sugarcane etc.This pest is also known to satisfy all requirements like moderate availability, easy breeding and maintainance in the laboratory.The proteins and proteases enzymes in the different developmental stages can be easily studied

2. Classification:

ARMYWORM, *MYTHIMNA SEPARATA* (Walker) :

Systematic position:

Kingdom	:	Animalia
Sub kingdom	:	Metazoa
Phylum	:	Arthropoda
Class	:	Insecta
Sub class	:	Pterigota
Division	:	Endopterigota
Order	:	Lepidoptera
Suborder	:	Ditrysia
Super family	:	Noctuoidea
Family	:	Noctuidae
Genus	:	*Mythimna*
Species	:	*separata*

3. Diagnostic Features:

A. Eggs:

The freshly laid eggs were bright yellowish in colour and spherical in shape with flattened base without any sculptures. The diameter of the egg ranged from 0.5 to 0.7 mm with an average diameter being 0.62 mm.

B. Larvae:

a) First Instar:

The newly hatched larva was tiny, cylindrical and active. The larval skin was soft and larva was pale brown coloured with black head. The length of the larva ranged from 0.9 to 1.3 mm with an average of 1.27 mm. The duration of instar ranged from 3 to 4 days with an average of 3.70 days in the rainy season and 4.0 days in winter

b) Second Instar :

The larva increased in its size, showed no remarkable change in the body colour. The length of the larva varied from 2.5 to 3.2 mm with an average of 3.0 mm. The duration of instar ranged from 4 to 5 days with an average of 4.6 days in rainy season and 5 days in winter.

c) Third Instar :

The larva increased in its size and body was elongate. Brownish longitudinal bands appeared on dorsolateral side of the body. The head of the larva was light brown coloured. The length of larva ranged from 4 to 6.5 mm with an average of 6.0 mm. The duration of instar ranged from 4 to 5.5 days with an average 5 days in rainy season and 5.3 days in winter.

d) Fourth Instar :

The colour of the larva was light green to dark green with whitish yellow mid-dorsal stripes on the body. But the colour of the instar was not constant in the same individual. The head of the larva was yellowish brown to dark brown with honey comb surface. The length of the larva ranged from 8.0 to 11.0 mm with an average of 9.6 mm.

e) Fifth Instar :

The colour of the larva varied; it was green, dark brown or black. The colour of the head capsule was dark brown which was honey comb structured. The larva showed longitudinal coloured bands laterally and whitish-yellow stripes mid-dorsally. The length of larva ranged from 18 to 20 mm with an average of 18.8 mm.

f) Sixth Instar :

The colour of the sixth instar larva varied from light brown, dark brown to black. The head capsule was dark brown with honey comb structure. The larva showed longitudinal brown coloured bands dorsolaterally. Whitish-yellow and dark brown stripes were seen mid-dorsally giving the larva dark brown colour.

The pair of prolegs on the last (tenth) abdominal segment is termed as anal prolegs. When all pairs of prolegs are present, they are borne by the third, fourth, fifth, sixth and tenth abdominal segments. The tip of the proleg is called the planta; upon it are borne hooks or claws known as crochets. The crochets are an aid in crawling and clinging to surfaces. The arrangement and form of the crochets are of much value in the identification of lepidopterous larvae. The head is usually well developed

and bears short antennae, ommatidia (simple eyes), and chewing mouthparts.

C. Pupa:

After sixth instar stage larvae become inactive and sluggish and stopped feeding. Larvae also contracted their body which is prepupal stage ranging between two to four days. The average period is 2 days in rainy and 3.7 days in winter.

The pupal stage is characterised by coppery obtect, blunt cephalic portion and tapering posterior end. The pupal length ranges from 14 to 18mm with an average of 15.8 mm. the total metamorphosis period ranged between 9 to11 days, with average of 9.5 days in rainy and 10 days in winter. Position of genital opening is a sexual dimorphic character in pupa. In both sexes 10^{th} abdominal segment has the oval opening or slit. The genital opening is situated on the ninth segment ventrally, which is smaller and closer to slit in male, where as in female genital opening is situated on the eighth abdominal segment which is away from slit.The female pupa is also differentiated from the male by presence of characteristics elevated eighth sternal plate on which the genital opening is situated.

D. Adult:

The full grown moth is pale brown in colour with beautifully designed patchy forewings which have a wavy border pattern on the fringe. The hind wings are practically white. Both sexes looked alike in antennal and wing pattern. The thorax and abdomen were full of scales

The typical noctuids are stout bodied with strong, narrow front wings. When at rest the wings are folded upon the abdomen, giving the

insect a triangular outline. Most of them are dull coloured in grays, browns or blacks, though some species, as the underwings (Catocala spp.), have brilliant coloured hind wings with bands or marked with spots and these bands and spots have been named (Robert, 1985). The labial palps are usually long, the antennae are generally hair like (sometimes brushlike in the males) and in some species there are tufts of scales on the dorsum of thorax (Comstock, 1984). The larvae of noctuids are ordinarily dull in colour, naked caterpillars usually having five pairs of prolegs. They generally feed on all sort of plants, devouring the foliage, burrowing into the stems and fruits, cutting off plants near the ground these are cutworms and destroying grass lands (Robert, 1985).

4. <u>Developmental Stages for Study</u>:

The eggs generally hatched within 4 to 5 days with an average 4.6 days. The larval growth was computed from the mean time of hatching (±1 hr.) to the prepupal stage. The period of larval growth lasts for 22 to 27 days with an average of 24.6 days. The pupal stage lasts for 9 to 11 days with an average 10 days, and adult stage lasts for 7 to 10days with an average 9 days. In each case of egg, larval, pupal and adult developmental stages, the studies were carried out at an interval of 24 hrs. For the study of proteins and proteases activity following stages were selected.

A. Embryonic Developmental Stages :

E_1 1 – day eggs

↓ ↓

E_5 5 - day eggs

B. Larval Developmental Stages :

L_1	1 – day larvae
↓	↓
L_{25}	25 - day larvae

C. Pupal Developmental Stages (metamorphosis) :

P_1	1 – day pupae
↓	↓
P_{10}	10 - day papae

D. Adult Developmental Stages :

A_1	1 – day adults
↓	↓
A_9	9 - day adults

5. Chemicals:

A. Chemicals for estimation of proteins:

1.	Sodium carbonate	Qualigens
2.	Copper sulphate	SDS
3.	Sodium tartarate	SDS
4.	Sodium tungstate	Qualigens
5.	Sodium molybadate	Bdh
6.	Conc. Hydrochloric acid	Qualigens
7.	Phosphoric acid	Qualigens
8.	Lithium sulphate	Qualigens
9.	Bromine water	E Merck
10.	Bovine albumin	Sigma

B. Chemicals for estimation of proteases activity :

1.	Bovine haemoglobin	Sigma
2.	Tris (hydroxymethyl methylamine)	SDS
3.	Conc. Hydrochloric acid	Qualigens
4.	Trichloroacetic acid	E Merck

II. METHODS:

1. Collection and Culture of Armyworm, *Mythimna separata* :

The kharif sorghum field was selected to collect the larvae of *M. separata* and reared in the laboratory for the maintainance of pure culture.

Newly emerged moth were kept in the big jar, containing dry cut leaf whorls and pieces of folded paper for egg laying. The males and females were kept in the ratio 2:1 in the jar. Food was also provided to the moths by keeping cotton dipped in 10% honey solution. The jar was externally covered with black papers and the open end of the jar was covered with muslin cloth fastened with rubber band to provide darkness and proper ventilation respectively.

Soon after the emergence, the moth was inactive and sluggish for some time. The moths were found to be very active during night and remain hidden during day time. They were attracted to a light the adult were very energetic and having strong power of flight strongly so to sweets like honey or decaying fruit.The mating, oviposition period and fecundity were also studied. The mating was obsereved on second to third day after emergence. The female moth started egg laying from third day onwards, occupying a pre-oviposition period of three days and continued the egg laying for about three days, thus occupying an average oviposition period of three days. The adult lived for about three to four days after their completion of egg laying, thus occupying a post-

ovipositional period of three to four days. The eggs laid every day were counted till the death of female moth. The life span of moth was lived for 7 to 10 days .The diameter are required for ovipositor females crevices with 0.5 to 1.0 mm wide. If such crevices of this size were available, female frequently interrupted ovipositor by flying. The dry cut leaf whorls and folded paper having many such crevices were provided for ovipositor. The number of eggs laid per female has been recorded to be 500 to 550. The eggs were laid in rows between the folds of paper fastened by sticky secretion

Freshly laid eggs were kept in petridishes provided with wet blotting paper at the bottom which protected the eggs from desiccation. The eggs turned to black purple colour, after three days they were transferred to a clean jar. Before hatching the colour of the eggs was changed initially to dull brown and later dark brown. In majority of the cases hatching took place during night hours. The incubation period of the eggs ranged from 4 to 5 days with an average of 4.40 days in rainy season and 4.90 days in winter.

The freshly hatched larvae were transfered into the specimen jars for the study of larval instars.The cut pieces of the fresh and tender maize leaves were provided as food. The food was changed after every 24 hours. Number of days covered by each larval instar, prepupa and pupa were recorded.

The developmental period of larve is of 22 to 27 days with an average of 24.60 days in rainy season and 26.90 days in winter. The moulting was obsereved for five times in each larva, thus six larval instars were obsereved.

The first instar larvae were tiny cylindrical and active mainly feed on outer epidermis of tender leaves, this feeding activity was later visible as elongated scars on the leaves.

The second instar larvaes are large with no change in colour and feed on outer epidermis of maize leaves. The length of this instar ranges from 2.5 to 3.2 mm.

The third instars were large and elongate. These larvae were very active and feed voraciously at night on entire leaf, cutting the leaf from edge towards midrib. During day time the larvae hide in the folds and whorls of maize leaves.The length of this instar ranges from 4 to 6.5 mm. The duration of the fourth instar ranged from 3 to 3.5 days with an average 3.2 days in rainy season and 3.3 days in winter.

At the time of moulting the larvae prefer dry and rough surface, the third and fourth instar larvae migrate to oral end of jar and remain associated with muslin cloth to which the exuviae attached.The length of larvae ranges from 8.0 to 11.0cm.

The duration of fifth instar ranged from 3 to 3.5 day with an average of 3.2 days in rainy season and 3.4 days in winter. These larvae are 18 to 20 mm in length.

The length of sixth instar larva ranged from 33.0 to 39.0 mm with an average of 36.0 mm. The instar, in its duration ranged from 4 to 5 days with an average of 4.6 days in rainy season and 4.8 days in winter.

The pupa was coppery, obtect and showed blunt cephalic portion and tapering posterior end. The length of the pupa ranged from 14 to 18 mm with an average of 16.4 mm. The metamorphosis period ranged from 9 to 11 days with an average of 10 days in rainy season and 10.5 days in winter.

To study pupal diapause the sixth instar larvae were kept in big tray containing dry and loose soil. It was found that in presence of soil most of larvae prefer to pupate in soil. The sixth instar larvae leave the maize leaves and enter into the soil at the depth of about 10 to 15 cm and construct an earthen shell and pupate therein. The pupal developmental period of normal pupa in soil was of 9 to 11 days with an average of 10 days in rainy season and 10.5 days in winter. Under laboratory conditions the pupa not undergoes diapause.

The sexes were easily differentiated at the pupal stage itself based on the position of genital opening. In both sexes, tenth abdominal segment has the oval opening or slit. In case of male the genital opening was situated on the ninth segment ventrally, which was smaller and closer to the slit. But in case of female, genital opening was situated on the eighth abdominal segment which was away from the slit. The female pupa was differentiated from the male by presence of characteristic elevated eighth sternal plate on which the genital opening is situated.

Freshly formed pupae were collected and kept in the small jar lined with blotting paper and containing folded pieces of dry maize leaf whorls. On the ninth day the pupae became dark brown; such pupae were collected and kept separately in the big jar for emergence of moths. The adult developmental period is of 9 days in fed moth.

The life cycle of *M. separata* occupied an average of 48 days ranging from 46 to 49 days in rainy season and 48 to 51 days with an average of 50 days in winter.

Thus, the culture was maintained in well ventilated cage, other laboratory conditions such as average temperature and relative humidity that were prevailing during the investigation were maintained by air cooler.

2. Preparation of homogenate:

In order to perform the experimental part i.e estimation of proteins and proteases of present investigation the homogenate was prepared as below

The eggs, larvae, pupae and adults were isolated, cleaned with distilled water, weighed and homogenised in chilled buffer solution by hand in ground glassmorter and pestle. The homogenates were diluted with chilled buffer solution so as to obtain 1% concentration.

After preparing a uniform suspension of homogenates of respective developmental stages, it was later centrifuged at 10,000 rpm for 5 minutes; the supernatant collected was used for estimation of proteins and proteases activity. These samples of various developmental stages were used for further studies.

3. Estimation of proteins:

Estimation of protein was carried out by the method of Lowry *et al.* (1951)

A. Reagents :

Lowry's A - 2% Sodium carbonate in 0.1 N NaOH

Lowry's B - 0.5% Copper sulphate in 1% sodium tartarate

Lowry's C- 50ml of Lowry's A + 1 ml of Lowry's B mixed at the time of use.

Folin-ciocalteu - Phenol reagent

Sodium tungstate Na_2WO_4 $2H_2O$	100 gms
Sodium molybdate $Na_2M_0O_4$ $2H_2O$	25 gms
Distilled water	700 ml
Conc. hydrochloric acid - HCl	100 ml

85% Phosphoric acid H_3PO_4 50 ml

Reflux above mixture for 10 hrs. in glass apparatus.

To refluxed mixture add

Lithium sulphate 150 gm

Distilled water 50 ml

Bromine water 5 drops

Boil mixture to remove excess of bromine. Dilute this to IN acid for use

Standard protein solution -10mg Bovine serum albumin in 10 ml

B. Protein assay :

A sample tube was prepared by addition of 0.5 ml of sample with 1ml of distilled water in a test tube. The same amount of distilled water was used as a blank in another test tube. In both the test tubes 3 ml of freshly prepared Lowry's C solution was added and it was kept for 15 minutes in order to form a copper protein complex. After few minutes 0.5 ml of Folin's ciocalteu-phenol reagent was added to both test tubes and allowed to develop colour for one hour at room temperature. The absorbance was noted at 660 nm on calorimeter. Using standard graph of bovine serum albumin, the amount of proteins was estimated in terms of ug/ gm weight of tissue.

4. <u>Estimation of proteases activity:</u>

A. Acidic Proteases :

a) Cathepsin D like enzyme :

Biochemical assay of acidic protese Cathepsin D was carried out according to the method of Mycek (1970).

Enzyme assay :

The enzyme assay system contained 0.1 ml of 2% haemoglobin substrate buffer and 0.1ml of 1% enzyme (homogenate solution). The incubation was carried out in water bath at 37^0C for 30 min. This reaction was terminated by addition of 2 ml 10% trichloroacetic acid solution. After one hour 1.8 ml. of distilled water was added in the reaction mixture. Later it was filtered through filter paper. Blank determination was carried out in the same way except that trichloroacetic acid was added to the substrate solution before addition of the enzyme.

The absorbance was read at 300 nm on UV spectrophotometer. Using above method the enzyme activity from embryogenesis, larval stage, metamorphosis of male and female adult stages were carried out. The activity of the enzyme was expressed per mg protein per 30 min.

b) Cathepsin B like enzyme :

Biochemical assay of Cathepsin B was carried out according to the method of Mycek (1970).

Enzyme assay :

The enzyme assay system contained 0.1 ml of 2% haemoglobin substrate buffer and 0.1ml of 1% enzyme (homogenate solution). The incubation was carried out in water bath at 37^0C for 30 min. This reaction was terminated by addition of 2 ml 10% trichloroacetic acid solution. After one hour 1.8 ml. of distilled water was added in the reaction mixture. Later it was filtered through filter paper. Blank determination was carried out in the same way except that trichloroacetic acid was added to the substrate solution before addition of the enzyme.

The absorbance was read at 300 nm on UV spectrophotometer. Using above method the enzyme activity from embryogenesis, larval

stage, metamorphosis and male and female adult stages were carried out. The activity of the enzyme was expressed per mg protein per 30 min.

B. Neutral Protease :

Biochemical assay of Neutral protese was carried out according to the method of Wilkes and Prescott (1976).

Enzyme assay :

The enzyme assay system contained 0.1 ml of 5% heamoglobin substrate buffer and 0.1ml of 1% enzyme (homogenate solution). The incubation was carried out in water bath at 37^0C for 30 min. This reaction was terminated by addition of 2 ml 10% trichloroacetic acid solution. After one hour 1.8 ml. of distilled water was added in the reaction mixture. Later it was filtered through filter paper. Blank determination was carried out in the same way except that trichloroacetic acid was added to the substrate solution before addition of the enzyme.

The absorbance was read at 300 nm on UV spectrophotometer. Using above method Neutral Protease activity from embryogenesis, larval stage, metamorphosis and male and female adult stages were carried out. The activity of the enzyme was expressed per mg protein per 30 min.

C. Alkaline Proteases :

a) Chymotrypsin like enzyme :

Biochemical assay of Chymotrypsin like enzyme was carried out according to the method of Rick (1965).

Enzyme assay :

The enzyme assay system contained 0.1 ml of 2 % haemoglobin substrate buffer and 0.1 ml of 1% enzym (homogenate solution) The incubation was carried out in water bath at 37^0C for 30 min. This reaction

was terminated by addition of 2 ml 10% trichloroacetic acid solution. After one hour 1.8 ml. of distilled water was added in the reaction mixture. Later it was filtered through filter paper. Blank determination was carried out on the same way except that trichloroacetic acid was added to the substrate solution before addition of the enzyme.

The absorbance was read at 300 nm on UV spectrophotometer. Using above method the Chymotrypsin like enzyme activity from embryogenesis, larval stage, metamorphosis and male and female adult stages were carried out. The activity of the enzyme was expressed per mg protein per 30 min.

b) Trypsin like enzyme :

Enzyme assay :

Biochemical assay of Trypsin like enzyme was carried out according to the method of Rick (1965).

The enzyme assay system contained 0.1 ml of 2 % haemoglobin substrate buffer and 0.1 ml of 1% enzyme (homogenate solution) The incubation was carried out in water bath at 37^0C for 30 min. This reaction was terminated by addition of 2 ml 10% trichloroacetic acid solution. After one hour 1.8 ml. of distilled water was added in the reaction mixture. Later it was filtered through filter paper. blank determination was carried out on the same way except that trichloroacetic acid was added to the substrate solution before addition of the enzyme.

The absorbance was read at 300 nm on UV spectrophotometer. Using above method the Trypsin like enzyme activity from embryogenesis, larval stage, metamorphosis and male and female adult stages were carried out. The activity of the enzyme was expressed per mg protein per 30 min

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Plate No. 1: Field collection and maintenance of culture of *M. separata*

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