Chapter II

# MATERIALS AND METHODS

| 8 | Homoptera | Magicicada sp. | Adult | Diurnal | Terrestrial. | Phytophagus |
|---|---|---|---|---|---|---|

Table 2 A.1. Detailed account of Experimental Animals

| Sr. No. | Order | Species | Stage of develop-ment | Activity Rhythm | Habitat | Food Habit |
|---|---|---|---|---|---|---|
| 1 | Thysanura | *Lepisma saccharina* (Lepisma) fam : Lepismatidae | Adult | Diurnal | Terrestrial | Saprophagus |
| 2 | Odonata | *Aeshna sp.* (Dragonfly) fam: Aeshnidae | Naid | Diurnal | Aquatic | Predatery |
| 3 | Orthoptera | *Hieroglyphus nigrophlexus* (Boi) (Grasshopper) fam: Acrididae | Adult | Diurnal | Terrestrial | Phytophagus |
| | | *Gryllus bimaculatus* (De Greev) (House cricket) fam : Gryllidae | Adult | Nocturnal | Frequently seen in damp, dark corners of buildings. All seen in open fields | Phytophagus and general scavenger. |

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|---|---|---|---|---|---|---|
| | | Gryllotalpa fossor(Soudder).<br>(Mole cricket)<br>fam: Gryllotalpidae | Adult | Nocturnal | Fossorial | Omnivorous and also feeds on small roots and insects. |
| 4 | Dictyoptera | Periplaneta americana(L)<br>(American cockroach)<br>fam : Blattidae | Adult | Nocturnal | Terrestrial, found in dark, humid situations | Omnivorous (Scavengers) |
| 5 | Dermaptera | Labidura riparia (Pall).<br>(Earwig)<br>fam : Labiduridae | Adult | Nocturnal | Terrestrial; Prefer damp places | Omnivorous |
| 6 | Isoptera | Microtermes sp.<br>(Termite)<br>fam : Termitidae | Adult (worker) | Diurnal | Terrestrial, (social insects) | Saprophagus |
| 7 | Heteroptera | Cimex rotundatus (Sign).<br>(Bedbug)<br>fam: Cimicidae | Adult | Nocturnal | Terrestrial | Zoophagus (hematophagus) |
| | | Rhodnius prolixus (L)<br>(Rhodnius bug)<br>fam: Reduviidae | Adult | Nocturnal | Terrestrial, found in cattle shed in dark corners and debris. | Zoophagus (hematophagus) |
| | | Notonecta sp.<br>(Notonecta)<br>fam: Notonectidae | Adult | Diurnal | Aquatic | Zoophagus (predatory) |
| | | Ranatra sordidula(Dohrn)<br>(Ranatra)<br>fam: Nepidae | Adult | Diurnal | Aquatic | Zoophagus (predatory) |

| Sr. No. | Order | Species | Stage of develop-ment | Activity Rhythm | Habitat | Food Habit |
|---|---|---|---|---|---|---|
| 10 | Lepidoptera | Clania sp. (Bagworm) fam: Psychidae | Larva | Diurnal | Arborial | Phytophagus |
| | | Papilio demoleus (L). (Lemon butterfly) fam: Papilionidae | Larva | Diurnal | Terrestrial | Phytophagus |
| 11 | Diptera | Culex fatigans (Wiedemann). (Mosquito) fam: Culicidae | Adult | Nocturnal | Terrestrial | Zoophagus; (females are hematophagus) |
| | | Musca (Musca) domestica (Linn) (Housefly) fam: Muscidae | Adult | Diurnal | Terrestrial | Microphagus |
| | | Lucilia sericata (Blowfly) fam: Calliphoridae | Larva | Diurnal | Terrestrial | Zoophagus |
| 12 | Coleoptera | Platynotus belli (Fairmair) (Dung beetle) fam: Tenebrionidae | Adult | Nocturnal | Terrestrial | Saprophagus |

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|---|---|---|---|---|---|---|
| | | *Heliocopris sp.* (Scarabid bettle) fam: Scarabaeidae | Larva | Diurnal | Terrestrial | Saprophagus or microphagus |
| | | *Zonabris pustulata* (Blister beetle) fam: Lariidae | Adult | Diurnal | Terrestrial | Phytophagus |
| | | *Hydaticus vittatus* (Fabr.) (Diving beetle) fam: Dytiscidae | Adult | Diurnal | Aquatic | Zoophagus (predatory) |
| | | *Dineutus indicus* (Aube) (whirling beetle) fam: Gyrinidae | Adult | Diurnal | Aquatic | Zoophagus (predaceous) |
| 13 | Hymenoptera | *Componotus compressus* (Fabr) (Black ant) fam: Formicidae | Adult (Woldier) | Diurnal | Terrestrial | Omnivorous |
| | | *Apis mallifera* (Honey bee) fam : Apidae | Adult (Worker) | Diurnal | Terrestrial (social insects) | Phytophagus (exclusive pollen feeders) |

## 2.A. MATERIALS :

### 2.A.1. Experimental Animals :

The insect species were collected locally at the Shivaji University Campus and adjacent area; except the cicada and haematophagus dipteran larvae. Cicadas were collected at Panhala plateau. The dipteran larvae were obtained from the culture maintained in the laboratory. Collection was done during the period from August 1983 to February 1984. During that period the ambient temperature varied between 23°C to 28°C. The insects were sacrified within 24 hours after collection. The details of insect orders, species, stage of development, activity rhythm, habitat and food habit are presented in the table No.2.A.1.

### 2.A.2. STAINS AND CHEMICALS :

Stains and chemicals used were of BDH Analar grade.

A.2.1. The stains employed in the present investigation were prepared as follows :

i) Pappenheim's Panchrome :

The stock solution was prepared by adding the following constituents :

Methylene blue 0.4 gm.

Toluidine blue 0.2 gm

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|---|---|
| Azur A | 0.4 gm |
| Methylene violet | 0.2 gm |
| Yellow eosin | 0.3 gm |
| Methyl alcohol | 100 mls |
| Glycerine | 100 mls |
| Acetone | 20 mls. |

The working solution was prepared by taking 0.1 ml. stock solution and 0.9 ml. distilled water.

ii) Giemasa's Stain :

Constituents :

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|---|---|
| Giemsa powder | 0.5 gm |
| Methyl alcohol | 50 mls. |
| Glycerine | 50 mls |
| Acetone | 10 mls. |

The working solution was prepared by mixing 0.1 ml. stock solution in 0.9 ml. distilled water.

iii) Leishman's Stain :

Constituents :

| | |
|---|---|
| Leishman's powder | 0.5 gm |
| Methyl alcohol | 50 mls |
| Glycerine | 50 mls. |
| Acetone | 10 mls. |

Working solution at the time of use was prepared by mixing 0.1 ml stock solution in 0.9 ml distilled water.

The above stains were prepared according to the procedure described by Humson (1967).

A.2.2. <u>Chemical Fixtive</u> :

Disodium EDTA (Versene) was used as a chemical fixative. It was prepared by dissolving 2 gm of disodium EDTA crystalline powder in the Insect Ringer to make the volume 100 ml. (i.e. 2% Versene-Ringer solution).

A.2.3. <u>Insect Ringer's Solution</u> :

It was prepared by taking the following chemicals :

| | | |
|---|---|---|
| NaCl | - | 9.8 gms |
| KCl | - | 0.77 gm |
| $CaCl_2$ | - | 0.5 gm |
| $Na_2HCo_3$ | - | 0.18 gm |
| $NaH_2PO_4$ | - | 0.01 gm |
| Dextrose | - | 1.0 gm |

The above constituents were dissolved in distilled water to make the volume 1,000 ml. (pH 7 to 7.2).

A.2.4. <u>Hemolymph Dilution Fluid</u> :

2% Versene-Ringer solution with traces of methylene blue was used as a dilution fluid.

A.2.5. Adherent Medium :

One part of egg albumin mixed with four parts of 2% Versene-Ringer solution was used as an adherent medium.

2.B. Techniques Employed for the Study of Hemocytes :

In general, the techniques for light microscopy of the insect hemocytes are the modifications of those used in vertebrate hematology. None of the individual methods for studying the morphological types of hemocytes are entirely satisfactory for all insects or for all types of cells within a given insect (Jones, 1962). All the methods employed presently are empirical because of the variability in the characters of both, hemolymph and hemocytes in different species.

Considering the above shortcomings, a combination of techniques was employed during the present study. These techniques are divided into two parts : (i) Cytological techniques for morphological characterization of hemocytes; and (ii) Quantitative methods to describe the blood picture.

2.B.1. Cytological Techniques :

a) In Vitro Studies of Unfixed Wet Films :

Primary observations were made by placing a drop of fresh hemolymph, obtained by amputation of a suitable appendage (described later), on a clean, degreased glass

slide. The hemolymph was immediately covered with a cover-glass, ringed with petroleum jelly to exclude air. Preparations were immediately examined under light microscope. Coagulocytes were identified best in these preparations. Similarly amoeboid movements of the plasmatocytes could be studied in these preparations.

b) In Vitro Studies of Fixed Wet Films :

This procedure includes the following steps :

i) Heat Fixation : Using heat as a partial fixative, insects were kept in the hot water (52°C - 58°C) for 1-3 minutes, depending upon the size of the insect and the nature of the exoskeleton (Arnold and Hinks, 1979).

Adults of Coleoptera and larvae of Neuroptera and Scarabidae were immersed in water at 58°C for 3 minutes. Clania larvae, Gryllotalpa and Componotus were dipped in water at 56°C for 2 minutes, While Hieroglyphus, Cimex, Periplaneta, Dermaptera adults, Gryllus, Cicada, naids of Odonata, Ranatra, Rhodnius and larvae of papilio were placed in water at 54°C for 2 minutes. Musca and Apis adults were immersed at 52°C for 2 minutes, whereas the adults of Thysanura, Isoptera, Culex and larvae of Diptera and Hymenoptera were dipped for a minute at 52°C. Care was taken not to kill the insect, but only to immobilize them. Immobilized insects were then used to obtain the hemolymph.

ii) Bleeding of Insect :

Insects were bled by amputation of an appendage, thus minimizing the variations in the blood picture which could result from employing different bleeding techniques. Jones (1962), technique was adopted for the above purpose.

Adult Lepisma was bled by amputation of anal styles and gently squeezing the abdomen in order to obtain an adequate volume. Naids were bled by amputation of their legs. In adults of Orthoptera (*H. nigrophlexus*, *G. bimaculatus* and G. fossor) it was necessary to bled them by amputation of the terminal abdominal appendages. Amputation of antennae in *P. americana* gave sufficient amount of hemolymph. Dermaptera adults were bled by amputation of antenna and gently squeezing the abdomen. The adults of Isoptera were bled by amputation of all the legs and gently squeezing the thorax. For *C. rotundatus* and Notonecta sp., it was necessary to pierce the thorax at the cervical region, Ventrally and gently squeezing the thorax. The hemolymph from the adults of Rhodnius and Cicada was obtained by bleeding the antennae and legs. However, in *R. sordidula* it was obtained by amputation of the respiratory siphon. Scarabid grubs, Neuroptera and clania larvae were bled by amputation of the thoracic legs. Larvae of Diptera and Hymenoptera were bled *in situ* by piercing the abdomen, while Papilio larvae were bled by amputating, either a proleg or a true leg. Adult Coleoptera were bled by

amputation of the third walking leg. Because of the hard exoskeleton, it was necessary to squeeze the abdomen forcefully, in order to get adequate volume of hemolymph. C. fatigans, M. domestica, A. mallifera adults were bled by cutting off the wings at their bases and applying the pressure from anterior end of the thorax; whereas in C. compressus, the mandibles were pierced and gently squeezed.

iii) Monolayer Preparation (Wet Film) :

Degreased slides were kept ready with a highly calibrated drop of 2% Versene-Ringer solution. A fresh drop of hemolymph was expressed in to the drop of Versene (hemolymph exposure to the air was very brief). The wet preparations were observed under the microscope, with a condenser manipulation to improve contrast and depth of the field.

c) In Vitro Studies of Fixed Wet Film by Phase - Contrast Optics :

Wet film preparation was essentially the same as described in 2.B.1.b. With the aid of the phase optics intracellular details could be observed in the wet film without staining.

d) Monolayer Preparation and Staining :

It included the following steps :

i) Heat fixation : As described in 2.B.1.b.

ii) Bleeding of insect : As described in 2.B.1.b.

iii) Monolayer preparation (For Fixed Film)

A thin layer of adherent medium was applied to the degreased slides; on to which a highly calibrated drop of 2% Versene-Ringer solution was put. A fresh drop of hemolymph was expressed in to the solution, which acted as chemical fixative. As the hemolymph spread in to the drop of solution, the cells get adhered to the glass surface forming a monolayer. The slides were air dried.

iv) Staining Procedure : (Humason, 1967).

(1) Air dried monolayers were stained in couplin jars containing the working stains. Three slides were stained with each stain. Staining in Giemsa's stain and Leishman's stain required 8 to 12 minutes, whereas Pappenheim's panchrome required 15 to 20 minutes.

(2) Stained slides were thoroughly washed in running tap water.

(3) The slides then were Rinsed in distilled water and allowed to dry at room temperature.

(4) Complete dehydration was achieved with a brief bath of acetone.

(5) The stained slides were cleaned in xylene and mounted in D.P.X.

2.B.2. Quantitative Methods :

Following two techniques were employed to estimate the hemocytes quantitatively : (a) Total Hemocyte Count and (b) Differential Hemocyte Count.

(a) Total Hemocyte Count (THC) :

THC was done with the help of Neubaur's hemocytometer (Rosenberger and Jones, 1960; Wittig, 1966). The counting involved the dilution of a small volume of blood with a known volume of dilution fluid. The pipette used for diluting the blood is known as Thoma-Zeiss hemocytometer. The pipette was first rinsed with the dilution fluid. Insects were bled by amputation of any suitable appendage to obtain sufficient hemolymph. It was sucked in the pipette upto 0.5 mark or 0.1 mark depending upon the availability of the hemolymph. The tip of the pipette was wiped off carefully. Immediately, the dilution fluid was drawn up to 101 mark. The blood and dilution fluid was thoroughly mixed by rolling the bulb of the pipette with palms. The clear fluid in the capillary part of the pipette was discarded and the diluted part from the bulb was taken on the hemocytometer as follows :

Neubauer's chamber was dried clean. The coverglass was placed on it in such a way that it touches on one side of the vertical groove. The chambers were filled by gently touching the dilution pipette at $45^{o}$ to the rulled surface of the slide.

Filling in occurred by capillary action. The preparation was left for few minutes to settle down the hemocytes at the bottom of the counting chamber. Observations were then made under the microscope. Hemocytes were counted in four "Intermediate squares", each with a volume of 1/10 Cu. m.m.

While diluting following precautions were taken :

(i) The mixture was rejected, if agglutination occurred in the dilution pipette.

(ii) Counts were rejected when air bubbles appeared in the counting chamber or evenwhen the cells showed uneven distribution.

(iii) First two drops of hemolymph were used for dilution.

(iv) For each species the counts were repeated four times.

Calculations :

For calculating the total number of hemocytes following formula was adopted from Jones (1962) :

$$\frac{\text{Hemocytes in 1 mm square X dilution X depth of chamber}}{\text{Number of 1 mm squares counted}} = \text{Cell number}$$

For the sake of convenience the hemocytes from four squares were counted, hence, the following formula was actually used for the calculations.

$$\frac{X \times 200 \times 10}{4} = \text{Number of cells per cubic mm.}$$

where X is the total number of hemocytes in 4 squares.

(b) Differential Hemocyte Count (DHC) :

Preparations as described in 2.B.1.d. (fixed and stained monolayers) were used for DHC. The cells were counted using the random sampling method. For every species five slides were examined and about 100 cells were classified every time.

2.B.3. Effect of Experimental Stress on Hemocytes :

The effects of starvation and dessication were studied separately and in combination. Changes in THC and DHC were recorded. Adults of *P. americana* were used. Care was taken to see that the insects were practically of the same age group. For this purpose they were obtained from a single deme.

The experimental animals were divided in four groups:

(a) Control group : 50 roaches were kept in wire cage. They were supplied with food and water, under laboratory conditions.
(b) Test group I. Starvation : 50 roaches were kept in the similar wire cage. But they were supplied only with water.
(c) Test group II. Dessication : 50 roaches were kept in the transparent plastic containers into which several holes were made. Their top was covered with nylon mosquito netting. Food was given to this test group. The plastic containers with experimental animals were kept in dessicator containing

unhydrous calcium chloride. The dessicator was made air-tight.

(d) Test group III. Starvation and Dessication : The same procedure as for test group II was followed, except that the animals were deprived of food.

THC of all the four groups was made every 24 h for 11 days. Fixed and stained monolayers were used for DHC.

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