CHAPTER IV

MATERIALS AND METHODS

Sandfly (Phlobotomus) is about 1.5 to 3 m.m. in length and greyish, yellowish or brownish in colour. Many species do not appear to bite man, but to feed on other mammals, bird or reptiles. If one of the local species has a predilection for the human host, its presence is usually brought to notice by its biting propensities, but in areas where such species are rare, "sandflies" are likely to be reported as scanty or absent. Their small size makes them easily being overlooked. The position of the wings, which are in most species carried erect and divergent at an angle of about 45° degrees to the body of the insect, makes their appearance characteristic. After a few specimens have been carefully observed in the live state, there is usually no possibility of confusing them with other small insects, which are found indoor.

Essential equipments for collection of sandflies:

The most essential equipment necessary for catching of sandflies are, (1) Torch (2) Aspirator of glass and rubber tubes (3) Test tubes or similar collection tubes.
MAP SHOWING THE PLACES WHERE THE PRESENT ECOLOGICAL STUDY WAS DONE.
Method for collection of sandflies:

Different methods have been adopted for collection of sandflies in the world i.e. Oil trap, Light trap, Test tube, suction tubes, animal bait; human bait etc. It has been reported by William (1965) that these methods give different results in the same area. So it is rather difficult to say which is the best.

Fairchild (1958) noted that, although light traps yielded the largest number and variety of species, they did not give a true picture of the relative abundance of sandflies.

The castor oil trap method was tried and probably due to paucity of insects, they gave negative results. In Kala-azar and non Kala-azar villages, approximately equal time was spent for collection.

The test tube method (Sinton and Barraud 1928) was adopted in the present investigation all throughout with the hope that it will give comparable results.

For ecological study of this insect, six villages viz: Kotna, Sathod, Tankari, Vasad, Kanjari and Panwad were selected from four districts viz: Baroda, Broach, Kaira and Punchmahals of Central Gujarat (map 3). In each village, ten collection stations were fixed (houses) and the same were examined every month.
The collected sandflies were kept alive by putting a drop or two of water on cotton plugs to maintain the moisture level. After this all the tubes were kept in a Barraud's box. Air inside the box was also kept moist by keeping moist sponge inside. The collection was transported by a Motor van to the Laboratory at Baroda. However, it was difficult to bring, all them alive to the laboratory after a journey of about 2-3 hours in a motor van.

**Method for collection of soil samples:**

The soil samples were taken from probable breeding places of sandflies from cattleshed and houses in search of immature stages of Phlebotomus species and for estimation of Organic Nitrogen, Soil moisture and detection of PH. The soil samples were also taken from the resting places of Phlebotomus. The soil was dug out upto a depth of 1 to 1½" inches and samples were collected in polythene bags, and mouths closed tightly by rubber ring.
Method for storage of Phlebotomus specimens:

The best method of storage had been a small sized piece of glass tubing about 1½ inches long. One end was plugged with cotton and the dead insects were introduced through the open end. This was then closed by pushing a piece of cotton into it with a match stick just sufficiently far to prevent the insect from shaking about. Carbolic acid was applied to both the plugs to protect the specimens.

A piece of paper with a pencilled note of the locality, date and any other essential details was noted on the outside of the tubes. These tubes were stored in a tin and as an additional precaution against moulds and predatory insects a piece of cotton soaked in pure carbolic acid was placed in the tin along with the tubes.

Preservation of sandflies in spirit was tried but it was found that this method of preservation was not suitable, because it was liable to cause a loss of the colour of sandflies and make them brittle so that the appendages and hairs were often found detached in spirit.
Method of mounting and preparation of slide for identification:

The specimens for identification may be either (1) Fresh or (2) Dry, depending on time and place where they were collected.

The method of mounting the specimen was temporary but slides can be stored upto 2 to 3 years without any damage.

Before mounting, a note was made about the colour of specimens, the hairs on the dorsum of the abdomen, erect or recumbent position of the hairs and their characters of diagnostic importance.

Methods for examination of fresh specimens:

The method of Sinton and Barraud (1928) was adopted. Instead of Amann's lacto phenol solution, Berlese's medium and Hoyer's medium were used.

Method for examination of Dry Specimens:

Dry specimens were wetted with alcohol and treated with 5% KOH solution. After thorough washing, preparation was made in Hoyer's medium or Berlese's medium.
IDENTIFICATION CRITERIA FOR SPECIES OF
PHLEBOTOMUS:

In the present study, Sinton's (1932) (1933) criteria for classification were used for the classification of the sandflies species. The classification of Theodor (1948) has been followed at the generic level.

METHOD FOR DETECTION OF ABDOMINAL STATUS OF A
FEMALE SANDFLY:

The status of abdomen of female sandflies were divided into three categories according to the amount of blood meal present in the abdomen or gut. If gut was found empty or no blood meal was present in the gut then such flies were considered as unfed. If gut of a sandfly found with blood partially/full or abdomen gives bright red colour than it was placed in "Fed" group. If gut or abdomen is black or showed eggs, then it was called a gravid one.

METHOD FOR AIR TEMPERATURE AND RELATIVE HUMIDITY:

Air temperature and relative humidity were recorded by rotating whirling hygrometer for 3 minutes near the resting places of sandflies, between 9 a.m. to 11 a.m. Air temperature of rodent burrows was measured by dry bulb thermometer, at a depth of 15 cms and relative humidity by wet bulb thermometer. The readings were noted after 10 minutes.
The highest average air temperature was obtained from meteorological department of M.S. University, Baroda, as measured at Baroda.

Soil temperature:

This was measured by digging out the soil to 1 to 1½ inches of the resting places. The centigrade thermometer was then placed at a depth of 1 to 1½ inches. After five minutes the temperature was noted. The same method was adopted for rodent burrows.

ESTIMATION OF ORGANIC NITROGEN:

The soil sample was mixed thoroughly and dried in an oven at 105°C. One gram of soil was passed through 3 samples of distilled water. 10 ml. of saturated solution of Potassium alum was added to sediments of the solution and kept over night. Next day, clear liquid was poured off and only solid material was taken and mixed in 10 ml. of distilled water. 5 ml. of stock solution was taken in a Kjeldah tube and 6 ml. of 40% NaOH solution was added. This was placed for digestion. The nitrogen evolved was collected in 5 ml. of 2% Boric acid solution. This collected nitrogen was tritrated after adding a few drops of 0.1% Bromocresol methyl red indicator, with 0.01 N H₂SO₄. The end point comes through blue to white colour.
REAGENTS:

1. 2% Boric acid
2. Indicator: 1. 0.1% Bromocresol green
   2. 0.1% methyl red
   prepared in 95% alcohol.

Taken 10 ml. of 0.1% Bromocresol green and 2 ml.
of 0.1% Methyl red, mix it thoroughly and used.

3. N / 100 H₂SO₄
4. 40% NaOH.

CALCULATION:

1. N H₂SO₄ --- L 14 grams Nitrogen
0.1 N H₂SO₄ --- L 1.4 gram of Nitrogen
0.01 N H₂SO₄ --- L 0.14 gram Nitrogen
   = 14 mgm Nitrogen.

1 c.c. 0.01 N H₂SO₄ --- 0.14 mgm of Nitrogen.

c.c. H₂SO₄ required X dilution factor X 0.14
m.gm nitrogen = mgm nitrogen per gram of soil.

SOIL MOISTURE:

Soil moisture was measured by the Hot oven Method.
One gram of soil was taken in a glass petridish and the
same was transferred to the hot oven at 105°C. After
complete evaporation of soil moisture, it was left for cooling. Finally weighing was done, and soil moisture was calculated.

**CALCULATION:**

Loss in wt. = wt. of soil and dish minus wt. of soil and dish after oven.

Loss in wt. / gm. X 100 = grams percent of moisture.

**SOIL PH:**

The soil sample was dissolved in neutral distilled water. The Ph was recorded by Phmeter.

**LIGHT:**

It was noted as visualised by the author, as "Dark" (where daylight was not present and it was difficult to see the sandfly without any extra light) and "Lighted area" (where the daylight was present and there was no need for extra light for searching the insect.)

**WIND:**

Velocity of wind was noted as "Not perceptible" (When wind movement was not felt on the skin) and "Perceptible" (when it was felt on the skin)
METHOD FOR SEARCH OF IMMATURE STAGES OF PHLEBOTOMUS:

For the search of immature stages of phlebotomus the "Floatation" technique was adopted.

Method for Determination of Fecundity and Frequency of Oviposition of P.argentipes.

Laboratory bred P.argentipes flies were fed on hamster. When they were found to be fully fed, these flies were aspirated from the cage and placed in the test tubes, one in each. Each tube was provided with a strip of blotting paper, which provided a comfortable surface to sit on. To provide sufficient moisture to the flies, the cotton plug of the test tubes were soaked with distilled water. Each tubes were labelled and were kept inverted in a beaker, which had moist cotton floor. The flies were kept at a constant temperature of 30°C. and relative humidity ranged from 60 to 70% percent. The test tubes were replaced and examined every day to note when the first batch of eggs were laid as well as the numbers of eggs laid per day. The fly was transferred to a new clean test tube and labelled. The eggs were carefully collected by washing with distilled water. The number of the eggs laid by a female/day were counted. This process continued till the flies stopped
laying eggs. Finally the calculation was done for fecundity and for frequency of oviposition.

METHOD FOR BREEDING OF SANDFLIES: (P. papataei and P. argentipes)

A simple method of breeding of sandfly as adopted in the Department of Entomology, School of Tropical Medicine, Calcutta was used fully except that sterilised garden soil was put instead of plaster of Paris in the earthen pots.

Six to eight fed or gravid females were collected in test tubes. A strip of blotting paper was kept inside, which provided a comfortable surface to sit on. In order to get sufficient moisture for the sandflies, the cotton plug of the test tubes were soaked with water. All these test tubes were inverted in a beaker. The cotton plug touched the floor of a beaker. The floor of which was also covered with a moist cotton. These tubes were placed for oviposition at 30°C. in an incubator continuously. After three or four days, the blood meal was digested by the sandflies which laid their eggs on the strips of blotting paper and on surface of test tubes. The eggs were carefully collected by washing with distilled water. Water with eggs was filtered through a Whatman's filter paper. Only
eggs were transferred to sterile earthen pots. The earthen pots contained 2/3rd of the sterile garden soil and 1/3rd of the rabbit fecal matter and human blood clot.

The mouths of the earthen pots were covered with fine cloth. The earthen pots were placed in a tray containing moist sand to increase humidity and to facilitate eggs hatching in the earthen pots. The complete set was then transferred to the incubator at 30°C. Periodically the sets were examined.

**Earthen pot measurements:**

- Mouth diameter: --- 35 cms.
- Body diameter: --- 36 cms.
- Base diameter: --- 11 cms.
- Height: --- 29 - 30 cms.

**ECTO PARASITE ISOLATION METHOD**

*Mite:* Examination of sandflies were made under a dissecting binocular microscope. If an ecto parasite was found, then the place of attachment, and number of parasites were noted and slides of the ecto parasites were prepared for identification and photographs were taken.
Fungus: Examination of sandflies were made under dissecting binocular microscope. If sandfly was found to be infected with a fungus, then some of the sporangia were transferred to the Sabaraoud's medium for culture and identified, while slide of the adult fly with fungual growth was prepared and photographs were taken.

Method for Artificial Feeding:

Feeding on hamster.

The hairs of abdomen of a hamster were removed by a scissor. The minute (Small) hairs were removed by Depil, a hair remover. Abdomen of this animal was rubbed with cotton. In the evening hours, this animal was introduced into a iron cage and locked. Finally it was introduced in a cage containing sandflies. Next day the animal was removed from the cage. Fed flies were also collected from the cage and kept for blood digestion.

Artificial Feeding of Male Sandfly:

A piece of cotton was soaked in 5% glucose solution and kept in a petridish. The dish was kept inside the cage and male sandflies were fed on it.
Collection of Blood samples from Sandfly for precipitin Tests:

The method adopted by National Institute of Communicable Diseases, Delhi was used. (Photograph 2).

1. Condition of Sandflies:

Sandflies (Females) showing "Red" abdomen were selected from the collection. This redness indicates fresh blood. To ensure this, collection of sandflies were made in early morning hours i.e. 7 A.M. to 9 A.M. and guts were examined. If the guts were found empty or partially black or completely black, the specimens were rejected.

2. To ensure sandflies in good conditions for dissection, the tubes containing flies were kept moist. These tubes were kept in wooden box containing moist sponge. Before transporting them to the laboratory, careful notes were made of the type of dwelling (Human/ cattleshed/sleeping room) Village (Locality) Date, etc. in a field note book.

Preparation of Blood Smear:

1. Whatman's filter paper of 5 inches diameter was divided into 8 equal sections by means of lead pencil as shown in fig.
Figures show the method of preparation of blood smear.
2. The head of sandfly was first separated out, and part of the fly containing the spermatheca was taken out.

2. The midgut with fresh blood was dissected out, and ruptured with a needle to release the blood.

4. The freshly released blood was picked up in the peripheral part of the filter paper about 1.5 cm away from the circumference. This was achieved by folding about 1.5 cm strip of the base as one of the sectors selected (out of eight) and then folding the triangle so formed at the middle of its base as shown in fig. II. The point formed at "X" should be dipped in the blood gently till a round circle of 0.5 to 1 cm. diameter was obtained. Only one sample was taken in each sector.

5. The sector was numbered with a pencil. The other details about the specimen, source of collection, date of collection, name of the village were noted on a separate sheet of paper accompanying the blood samples.

6. Discs of filter papers were separated from each other by keeping blank water proof paper disc in between them.

7. Filter paper with dried blood spots were kept in dry and cool place and they were sent to N.I.C.D. Delhi for precipitin test.
Method for detection of susceptibility of sandflies to hydrocarbon insecticides

The standard kit and method as advised by W.H.O. was adopted for detection of susceptibility of sandflies to various concentration of D.D.T. and Dieldrin. For this experiments, sandflies (Phlebotomus papatasi) of Baroda and Surat were used.